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Synthesis of novel 4- and 5-substituted benzyl ether derivatives of vesamicol and in vitro evaluation of their binding properties to the vesicular acetylcholine transporter site

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Abstract—Detection of the central cholinergic deficits, a consistent feature of Alzheimer's disease, is essential to allow preventive measures and/or symptomatic treatment already at a very early stage of the disease. The vesicular acetylcholine transporter (VAChT) represents an appropriate target to establish PET radiotracer that are adequate for brain imaging the loss of cholinergic terminals. Here we describe the synthesis and binding characteristics of novel derivatives of vesamicol, known to represent a specific antagonist of VAChT sites. Novel benzyl ether derivatives of vesamicol either 4- or 5-substituted at the cyclohexylring have been synthesized by different regioselective ring opening reactions of a same epoxide precursor. The affinity and selectivity of the novel compounds to VAChT sites were analyzed by competitive radioligand binding studies in rat brain and liver membrane preparations using tritium labeled radioligands. The 4-substituted fluorobenzylether of vesamicol **10b** was shown to exhibit a high affinity to VAChT sites (K_i-value$_{10b}$ = 10.7 ± 1.7 nM), but demonstrated also binding capacities to sigma receptors (K_i-value$_{10b}$ = 18.5 ± 6.9 nM, [^{3}H]DTG; K_i-value$_{10b}$ = 30.6 ± 9.6 nM, [^{3}H]haloperidol). The data suggest the potential of vesamicol derivatives to design appropriate radiotracer for PET imaging of central cholinergic deficits.

1. Introduction

(−)-Vesamicol (**1**) acts as a potent inhibitor (IC_{50} = 20 nM) of vesicular acetylcholine (ACh) uptake and transport.[1] It binds to an allosteric site on the specific vesicular acetylcholine transporter complex (VAChT).[2] This transporter protein, responsible for the transfer of ACh into synaptic vesicles, is located exclusively in cholinergic neurons. Vesamicol and several of its analogues are therefore potentially suitable substances for the study of presynaptic cholinergic mechanisms. The degeneration of cholinergic neurons in brain is one significant neuropathological feature in Alzheimer's Disease (AD).[3] An appropriate radiotracer based on the vesamicol structure that binds selectively to the VAChT could offer a tool for diagnosis and follow-up of neurodegenerative diseases by in vivo imaging using positron emission tomography (PET).

Accordingly, several efforts have been made to synthesize ^{18}F labeled vesamicol derivatives (Fig. 1) with high affine and selective binding to VAChT and with pharmacodynamic properties optimal for in vivo imaging like moderate to high extraction into the brain, fast elimination from unspecific binding sites and a high metabolic stability. However, vesamicol and most of its derivatives were reported to exhibit an additional high affinity binding, especially to σ-1 and σ-2 receptors,[4,5] that are found in different parts of the central nervous system. Therefore new compounds related to the chemical structure of vesamicol and developed for potential use in cholinergic in vivo imaging have to be proved as to their selectivity in binding to the VAChT.

In vivo studies in rodents[6,8] and nonhuman primates revealed ^{18}F labeled NEFA,[6] FBT[7] and FEOBV[8] (Fig. 1) as suitable radiotracer for specific in vivo imaging of

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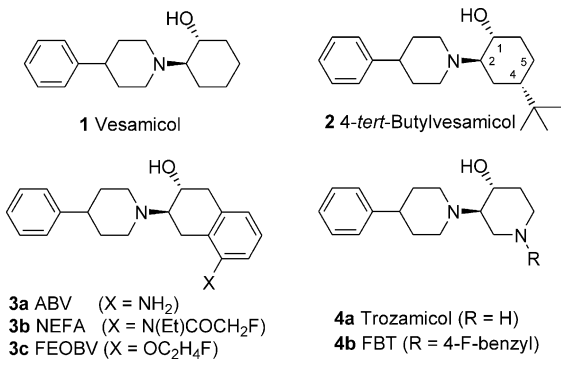


Figure 1. Ligands for the vesicular acetylcholine transporter (VAChT).

VAChT.[7] However, representative studies in human are missing so far. This may partly be due to the unfavourable pharmacokinetic properties of the high lipophilic compounds by which they may easily penetrate through the blood–brain barrier, but on the other hand, also produces a high radiation dose in the gastrointestinal tract during the elimination of the substance from the body.

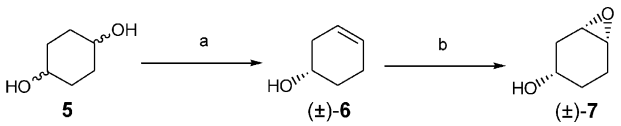
In this paper, we report the synthesis and the binding behaviour of novel benzylether analogues of vesamicol with substitution on C-4 or C-5 of the cyclohexyl moiety. It was the aim to find new compounds being more hydrophilic and having higher binding affinity and selectivity to the VAChT in comparison to those hitherto known.

2. Results and discussion

Extensive structure–activity relationship (SAR) studies by Rogers et al.[1] indicated that 4-*tert*-butylvesamicol (Fig. 1, **2**) and the 5-amino derivative of benzovesamicol (ABV, Fig. 1, **3a**) may represent a suitable chemical basis for the synthesis of promising VAChT ligands, which finally resulted in the development of new fluorinated derivatives of benzovesamicol and aminobenzovesamicol (Fig. 1, **3b** and **c**) as well as in new amino analogues of the 4-*tert*-butylvesamicol (Fig. 1, **4a** and **b**). However, there is a lack of systematic investigations with the aim of altering **2** and keeping the cyclohexyl moiety intact, most likely due to the inherent problems to cope with the stereochemistry of such molecules.[9]

We synthesized congeners of 4-*tert*-butylvesamicol containing an ether linkage to the cyclohexyl moiety. In detail, both 4- and 5-substituted vesamicol analogues were prepared[1,9] in order to test for the impact of the ether oxygen to modify the binding kinetics to the VAChT. Additionally, it was of interest to know whether the hitherto unknown 5-substituted derivatives with the substituent in *cis*-orientation in relation to the hydroxy group would contribute to improve the binding characteristics to the VAChT.

2.1. Chemistry



Scheme 1. Synthesis of epoxy alcohol **7**. Reagents and conditions: (a) H_2SO_4 (cat.), 245 °C; (b) *tert*-butylhydroperoxide, $Mo(CO)_6$ (cat.), benzene, reflux.

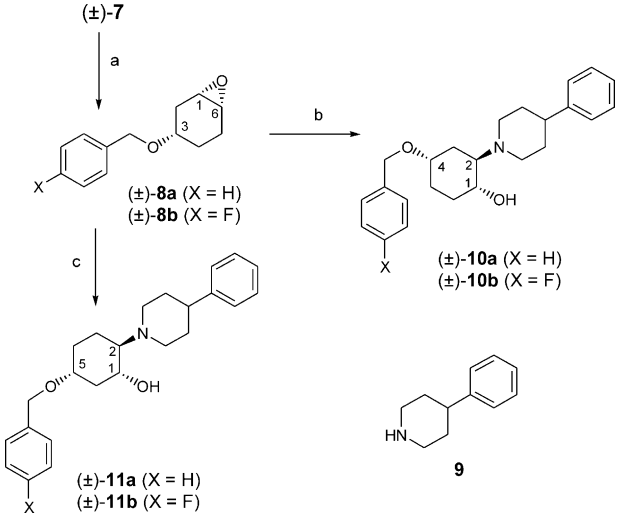
Applying two different regioselective nucleophilic ring opening reactions of the same epoxide precursor[10] a convenient approach to 4- and 5-substituted diastereomerically pure ligands on the basis of vesamicol was elaborated. In the key step of the reaction sequence we took advantage of the high regiochemical control under which *cis*-epoxide **8** does normally react with amino nucleophiles.[11]

Starting with a partial elimination of water from cyclohexane-1,4-diol (**5**) we obtained **6**[12,13] which was epoxidized in a diastereoselective manner by means of *tert*-butyl hydroperoxide and $Mo(CO)_6$ to afford the racemic epoxy alcohol **7** (Scheme 1).[14a]

Subsequent *O*-benzylation led to (±)-*cis*-1-benzyloxy-3,4-epoxy cyclohexane (**8a**). The reaction of 4-phenylpiperidine (**9**) with **8a** in ethanol at 60 °C for 3 d provided crystalline **10a** being a result of an axial attack on C-1 in **8a**.

However, in the case of an initial metal chelation, promoted by $LiClO_4$ in acetonitrile, we observed a reversal of selectivity in accordance to the literature.[11] The amino nucleophil now attacks the oxirane on C-6 leading to 5-substituted **11a** in 89% yield (Scheme 2).

The fluorinated analogue of epoxide **8a**, prepared from **7** and 4-fluorobenzyl bromide, reacted with **9** under conditions as described for the conversion of **8a**. The



Scheme 2. Synthesis of 4-substituted (**10**) and 5-substituted vesamicol analogues (**11**). Reagents and conditions: (a) NaH/DMF, benzylbromide or 4-fluorobenzylbromide (**8a**, 73%; **8b**, 61%); (b) **9**, EtOH, 55–60 °C, 3 d (**10a**, 73%; **10b**, 59%); (c) **9**, MeCN, $LiClO_4$, 20 °C, 16 h (**11a**, 89%; **11b**, 86%).

diastereoisomeric purities of all compounds obtained from the ring opening of **8a** and **b** were determined for the crystallised reaction products by 400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectroscopy. Protons of the cyclohexyl moiety both in 4- and 5-substituted vesamicol derivatives were assigned with the aid of ^{1}H COSY spectra of selected compounds.

2.2. In vitro competition studies

Binding affinities to the VAChT of the benzylether derivatives of vesamicol (Scheme 2, compounds **10a**, **b**, **11a** and **b**) were tested in competitive binding assays in rat brain membranes using (−)-[^{3}H]vesamicol as high affinity VAChT radioligand ($K_d = 23$ nM[15,16,4]). The binding affinities of the benzylether derivatives to sigma receptors were analyzed using rat liver membrane homogenates, because rat liver is rich in σ_1 and σ_2 binding sites. Competition experiments were performed using [^{3}H]DTG as radioligand known to be a potent and selective sigma receptor antagonist (σ_1 and σ_2, $K_d = 17.9$ nM for rat liver[17]). The selectivity of the new vesamicol derivatives was further tested in rat liver tissue using [^{3}H]haloperidol as radiotracer to label σ_1 receptor sites, but which demonstrates also a binding capacity to dopamine D_2 receptors. To further estimate the binding affinities of the benzylether derivatives to D_2 receptors, displacement experiments were performed in rat brain tissue using the selective and high affine D_2 antagonist [^{3}H]raclopride as radioligand.

The potency of the new compounds synthesized to succesfully compete for VAChT sites with (−)-[^{3}H]vesamicol is expressed by K_i-values[18] calculated for **10a**, **b**, **11a** and **b**. For comparison, competition experiments were also performed using unlabeled (−)-vesamicol (Table 1).

The K_i-values of 4-benzylether derivatives of vesamicol (**10a** and **b**) amounted to the same order of magnitude as those for (−)-vesamicol ($K_{i(Vesamicol)} = 7.4 \pm 1.0$ nM; $K_{i(10a)} = 8.5 \pm 1.6$ nM; $K_{i(10b)} = 10.7 \pm 1.7$ nM (Table 1 and Fig. 2a and b).

The structural changes induced by the benzylether linkage to the cyclohexyl moiety of vesamicol did not impair the binding affinity to VAChT when compared to vesamicol as far as the 4-*O*-benzyl structure is concerned (Table 1, second column).

In contrast, 5-*O*-benzylether derivatives **11a** and **b** showed a poor competition for the VAChT sites, as the K_i-values calculated were 10 to 100 times higher as compared to those for the 4-*O*-benzylether derivatives ($K_{i(\mathbf{11a})} = 49.7 \pm 2.2$ nM; $K_{i(\mathbf{11b})} = 330 \pm 90$ nM; Table 1 and Figs 2c and d). These results are in agreement with those published by Rogers et al.[6] who described the coupling site of a given substituent to be in position 4 as a prerequisite for high affinity binding to the VAChT (Fig. 1, **2**).

The binding selectivity of both the 4- and 5-*O*-benzylether derivatives of vesamicol to the VAChT revealed to be not better than that of vesamicol, as they also demonstrated binding capacities to sigma receptors estimated from competition experiments using either [^{3}H]DTG (σ-1 and σ-2 ligand) or [^{3}H]haloperidol (σ-1 and D2-ligand) as radioligands. The resulting K_i-values of the fluorinated benzylether derivatives of vesamicol (**10b** and **11b**) were in the nanomolar range as it was the case for vesamicol (tested with [^{3}H]DTG: K_i-value$_{vesamicol} = 43.8 \pm 14$ nM, K_i-value$_{(\mathbf{10b})} = 18.5 \pm 6.9$ nM, K_i-value$_{(\mathbf{11b})} = 19.2 \pm 7.0$ nM; tested with [^{3}H]haloperidol: K_i-value$_{vesamicol} = 53.2 \pm 6.0$ nM, K_i-value$_{(\mathbf{10b})} = 30.6 \pm 9.6$ nM, K_i-value$_{(\mathbf{11b})} = 15.8 \pm 3.2$ nM; Table 1). The binding affinities of the fluorinated compounds **10b** and **11b** to dopamine D_2 receptors were comparatively low when measured in rat brain membranes with [^{3}H]raclopride (2.5 nM) as radioligand. The IC_{50} values of **10b** (2.7 ± 0.5 μM) and of **11b** (34.6 ± 5.0 μM) demonstrated

Table 1. Summary of inhibition constants (K_i) of the benzylether **10** and **11** for VAChT sites and for σ-1 and σ-2 receptors estimated from competion experiments in rat brain and liver membranes, respectively

	VAChT ([^{3}H]vesamicol)[a]		σ_1, σ_2 ([^{3}H]DTG)[b]		σ_1 ([^{3}H]haloperidol)[c]	
Compd	K_i (nM)	N[d]	K_i (nM)	N[d]	K_i (nM)	N[d]
10a	8.5±1.6	6	11.0±2.0	3	ND[e]	—
10b	10.7±1.7	6	18.5±6.9	3	30.6±9.6	4
11a	49.7±2.2	6	19.2±7.0	2	ND	—
11b	330±90	6	15.8±3.2	3	21.3±11.0	3
Vesamicol	7.4±1.0	7	43.8±14	3	53.2±6.0	4
DTG	ND	—	31.2±9.0	5	ND	—
Haloperidol	ND	—	ND	—	50.2±19	5

K_i-values were determined in competitive binding studies using different ^{3}H labeled radioligands in the presence of compounds given at increasing concentrations from 10^{-11} M to 10^{-5} M. K_i values are expressed as mean±SD.

[a] Binding on rat brain membranes, [^{3}H]vesamicol [6.1±1.3 nM]; $K_{d\ rat\ brain} = 23$ nM (refs 15,16). Nonspecific binding was defined as the binding of [^{3}H]vesamicol in the presence of 10^{-5} M unlabeled (−)-vesamicol HCl.

[b] Binding on rat liver membranes, [^{3}H]DTG [4.9±10.5 nM]; $K_{d\ rat\ liver} = 17.9$ nM (ref 17). Nonspecific binding was defined as the binding of [^{3}H]DTG in the presence of 10^{-5} M DTG.

[c] Binding on rat liver membranes, [^{3}H]haloperidol [3.1±1.1 nM]; $K_{d\ rat\ liver} = 11.4$ nM (own determination). Nonspecific binding was defined as the binding of [^{3}H]haloperidol in presence of 10^{-5} M DTG.

[d] N, number of experiments performed.

[e] ND, not determined.

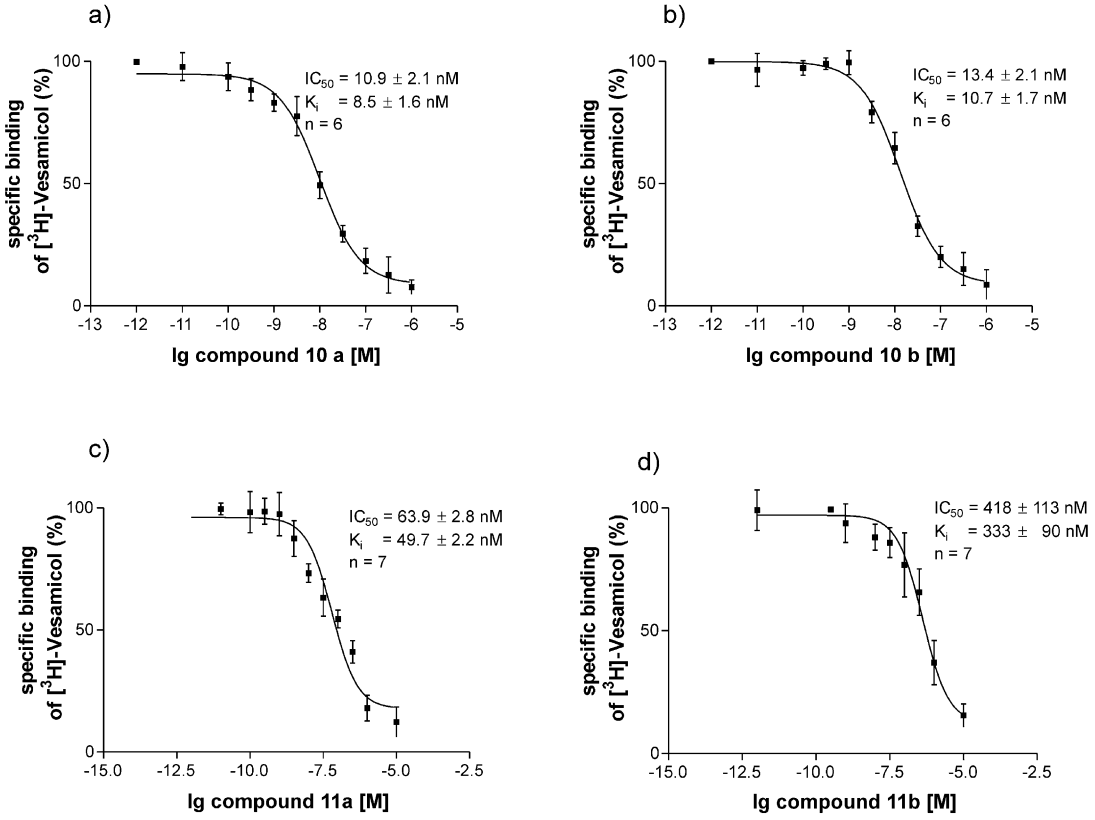


Figure 2. Concentration-dependent displacement of [^{3}H]vesamicol binding in rat brain membranes by 4-*O*-benzylether **10a** (a) and **10b** (b) and 5-*O*-benzylether **11a** (c) and **11b** (d). Using nonlinear regression analysis data were best fitted to a one-site-binding model in all cases.

that the synthesized compounds did not interact significantly with D_2 receptors. Therefore it can be concluded that in case of [^{3}H]haloperidol (σ_1 and D_2 ligand) the displacement of the radioligand by the fluorinated benzylether derivatives is reflecting their competition with [^{3}H]haloperidol on its σ-1 receptor binding sites.

3. Conclusion

In conclusion, a convenient synthesis of both 4-substituted (**10a** and **b**) and 5-substituted benzylether (**11a** and **b**) analogues of vesamicol has been accomplished. **10b** shows a high affinity to the VAChT as was demonstrated by an effective displacement of [^{3}H] vesamicol from its binding sites in rat brain homogenates ($K_i = 10.7 \pm 1.7$ nM). However, **10b** also binds with high affinity to σ_1 and σ_2 receptors as was measured in competition experiments in rat liver membrane preparations using ^{3}H-labeled sigma ligands. Therefore it can be concluded that this study was successful in the application of regiochemically controlled reaction steps for the synthesis of both 4- and 5-substituted vesamicol analogues. However, the selectivity of the new vesamicol derivatives has to be further improved by chemical modifications aiming at their future use for in vivo imaging of cholinergic terminals with PET.

4. Experimental

4.1. Chemistry

Melting points were determinated with a Linström apparatus and are uncorrected. NMR spectra (^{1}H, ^{13}C, ^{1}H COSY) were recorded at 400 or 100 MHz with tetramethylsilane as an internal standard using a Varian Unity Inova spectrometer. Splitting patterns have been designated as follows: s = singlet; bs = broad singlet; d = doublet; t = triplet; dd = double doublet; m = multiplet. Mass spectra were recorded on a Mariner Biospectrometry Workstation (Applied Biosystems) (MS-TOF) using direct inlet system and Electron Spray Ionisation (ESI). Analytical TLC was performed on silica gel coated sheets (Merck Kieselgel 60 F254, 0.25 mm thickness). Visualisation of the spots was effected by irradiation with a UV lamp or by charring with a solution of 5% sulfuric acid in EtOH. Silica gel 60 (70–230 mesh) was used for column chromatography. Elemental analyses were performed with a CHN-*O*-Rapid analyser (Foss Heraeus). Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Following abbreviations are used: MeOH, methanol; EtOH, ethanol; MeCN, acetonitrile; MTBE, methyl-*tert*-butylether; DMF, dimethylformamide; TBHP, *tert*-butyl hydroperoxide.

(1*SR*) Cyclohex-3-enol (**6**) and (1*SR*,3*SR*,6*RS*) 7-oxabicyclo[4.1.0]heptan-3-ol (**7**) were prepared[12,14a]

and characterized[13,14b] according to the described procedures.

4.1.1. (1*SR*,3*SR*,6*RS*) 3-Benzyloxy-7-oxa-bicyclo[4.1.0]-heptane (8a). Following an already reported procedure;[19] to a solution of **7** (1.69 g, 14.8 mmol) in DMF (12 mL) NaH (0.72 g of 60% suspension in mineral oil, 18 mmol) was added at 0 °C and stirred for 30 min. Benzylbromide (2.2 mL, 17.8 mmol) was added at 0 °C via a syringe. The reaction mixture was allowed to warm to 22 °C and stirring was continued for 18 h. H_2O (20 mL) was added and the resulting mixture was extracted with MTBE (3×15 mL). The combined extracts were washed (satd NaCl, 10 mL) and dried ($MgSO_4$). After filtration and concentration, the oily residue was purified via column chromatography (silicagel: 16 g, column: 150×20 mm, solvent: hexane–MTBE, 5:1) to yield 2.19 g (73%) of a yellowish oil. R_f 0.31 (hexane/MTBE, 5:1). ^{1}H NMR ($CDCl_3$): δ 1.46 (ddd, J = 24.0, 12.0, 4.4 Hz, 1H, 4-H_a), 1.70 (m, 1H, 4-H_b), 1.77 (m, 1H, 5-H_a), 1.85 (dd, J = 15.0, 10.1 Hz, 1H, 2-H_a), 2.22 (m, 1H, 5-H_b), 2.34 (m, 1H, 2-H_b), 3.09 (m, 2H, oxirane: 1-H and 6-H), 3.32 (m, 1H, 3-H), 4.51 (AB, 2H, $PhCH_2O$), 7.25–7.33 (m, 5H, ArH). ^{13}C NMR ($CDCl_3$): δ 23.79 (2C, not resolved), 30.83, 50.92, 51.85, 69.83, 73.06, 127.50 (3C, not resolved), 128.36, 138.64.

4.1.2. (1*SR*,3*SR*,6*RS*) 3-(4-Fluoro-benzyloxy)-7-oxa-bicyclo[4.1.0]heptane (8b). It was obtained from **2** (2.1 g, 18.4 mmol), NaH (0.88 g, 60%, 22.1 mmol) and 4-fluorobenzylbromide (2.72 mL, 22.1 mmol) in the same way as described above for benzylether **8a** and purified via column chromatography (silicagel: 16 g, column: 150×20 mm, solvent: hexane-MTBE, 5:1) to yield 61% of a yellowish oil. R_f 0.25 (hexane/MTBE, 5:1). ^{1}H NMR ($CDCl_3$): δ 1.46 (ddd, J = 24.4, 12.4, 4.4 Hz, 1H, 4-H_a), 1.69 (m, 1H, 4-H_b), 1.77 (m, 1H, 5-H_a), 1.85 (dd, J = 14.8, 10.0 Hz, 1H, 2-H_a), 2.21 (m, 1H, 5-H_b), 2.34 (m, 1H, 2-H_b), 3.10 (m, 2H, oxirane: 1-H and 6-H), 3.31 (dddd, J = 11.6, 10.0, 6.4, 3.2 Hz, 1H, 3-H), 4.51 (AB, 2H, $PhCH_2O$), 7.25–7.33 (m, 5H, ArH). ^{13}C NMR ($CDCl_3$): δ 23.72 (2C, not resolved), 30.78, 50.83, 51.79, 69.14, 73.15, 115.17 (d, J = 21.4 Hz, 2C, 3-C), 129.16 (d, J = 8.4 Hz, 2C, 2-C), 134.36 (d, J = 3.0 Hz, 1C, 1-C), 162.21 (d, J = 245.7 Hz, 1C, 4-C).

4.1.3. (1*RS*,2*RS*,4*SR*) 4-Benzyloxy-2-(4-phenyl-piperidin-1-yl)-cyclohexanol (10a). A mixture of **8a** (4 g, 19.6 mmol) and **9** (2.96 g, 19.8 mmol) in EtOH (abs., 14 mL) was stirred at 55–60 °C for 3 days. After concentration the red residue was dissolved in $CHCl_3$ (30 mL), washed with H_2O (20 mL), dried (Na_2CO_3) and subsequently treated with silica gel (2 g) until the colour disappeared. After filtration and evaporation, the solid residue was recrystallized from MeOH–EtOH (10 mL/25 mL) to yield 5.2 g (73%) white crystals. Mp 132.5–136 °C. ^{1}H NMR ($CDCl_3$): δ 1.26–1.41 (m, 2H, 5-H_2), 1.62–1.87 (3m, 5H, 3′-H_2, 5′-H_2, 6-H_A), 1.93-2.04 (m, 2H, 6-H_B, 3-H_B), 2.08–2.14 (m, 1H, 3-H_A), 2.15–2.22 (m, 1H, 2′-H_A), 2.48 (tt-like, J = 12.0, 3.8 Hz, 1H, 4′-H), 2.64–2.79 (2m, 3H, 6′-H_2, 2-H), 2.91–2.95 (m, 1H, 2′-H_B), 3.46 (m, 1H, 1-H), 3.84 (m, 1H, 4-H), 4.06 (bs, 1H, OH), 4.46 (AB, 2H, J = 12.2 Hz), 7.17–7.35 (m, 10H, ArH). ^{13}C NMR ($CDCl_3$): δ 26.07, 27.62 (2C, not resolved), 33.90, 34.24, 42.97, 45.22, 53.24, 64.53, 68.52, 69.82, 73.30, 126.13, 126.80, 127.28, 127.35, 128.32, 128.38, 138.98, 146.24. MS (ESI) m/z 366.3 (M + 1). Anal. calcd for $C_{24}H_{31}NO_2$: C, 78.86; H, 8.55; N, 3.83. Found: C, 78.61; H, 8.63; N, 4.05.

4.1.4. (1*RS*,2*RS*,4*SR*) 4-(4-Fluoro-benzyloxy)-2-(4-phenyl-piperidin-1-yl)-cyclohexanol (10b). It was obtained from **8b** (0.444 g, 2 mmol) and **9** (0.3 g, 2 mmol) in the same way as described above for compound **10a** and recrystallized from MeOH (10 mL) to yield 0.452 g (59%), white crystals. Mp 128–130 °C. ^{1}H NMR ($CDCl_3$): δ 1.27–1.41 (m, 2H, 5-H_2), 1.63–1.76 (m, 2H, 3′-H_a, 5′-H_a), 1.78–1.88 (2m, 3H, 6-H_a, 3′-H_b, 5′-H_b), 1.95–2.02 (m, 2H, 6-H_b, 3-H_b), 2.08–2.12 (m, 1H, 3-H_a), 2.17–2.22 (m, 1H, 2′-H_a), 2.48 (tt-like, J = 12.1, 3.7 Hz, 1H, 4′-H), 2.65–2.79 (2m, 3H, 6′-H_2, 2-H), 2.92–2.95 (m, 1H, 2′-H_b), 3.46 (m, 1H, 1-H), 3.83 (m, 1H, 4-H), 4.04 (bs, 1H, OH), 4.46 (AB, 2H, J = 11.8 Hz), 7.00–7.33 (3m, 9H, ArH). ^{13}C NMR ($CDCl_3$): δ 26.09, 27.54, 27.60, 33.90, 34.24, 42.96, 45.23, 53.27, 64.54, 68.47, 69.14, 73.37, 115.14 (d, J = 21.3 Hz, 2C, 3-C), 126.15, 126.79, 128.39, 128.93 (d, J = 8.4 Hz, 2C, 2-C), 134.8 (not resolved, 1C, 1-C), 146.21, 162.18 (d, J = 238 Hz, 1C, 4-C). MS (ESI) m/z 384.3 (M + 1). Anal. calcd for $C_{24}H_{30}FNO_2$: C, 75.16; H, 7.88; N, 3.65. Found: C, 74.87; H, 7.97; N, 3.92.

4.1.5. (1*RS*,2*RS*,5*RS*) 5-Benzyloxy-2-(4-phenyl-piperidin-1-yl)-cyclohexanol (11a). To a solution of $LiClO_4$ (0.37 g, 3.5 mmol) and **8a** (0.41 g, 2 mmol) in MeCN (3 mL) was added **9** (0.37 g, 2.5 mmol) and stirred for 16 h at 22 °C. The suspension of a white solid obtained was stirred with $CHCl_3$ (20 mL) and H_2O (15 mL) for 5 min. The organic phase was separated, concentrated, and the solid residue was recrystallized from MeOH–EtOH (8 mL/12 mL) to yield 0.653 g (89%) white crystals. Mp 174–175.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.13–1.26 (m, 1H, 3-H_A), 1.28–1.42 (m, 2H, 4-H_A, 6-H_A), 1.60–1.72 (m, 1H, 3′-H_A), 1.74–1.92 (m, 4H, 3-H_B, 3′-H_B, 5′-H_2), 2.13–2.33 (m, 3H, 6′-H_A, 4-H_B, 6-H_B), 2.49 (tt-like, J = 12, 4 Hz, 1H, 4′-H), 2.59 (m, 1H, 2-H), 2.70–2.90 (m, 3H, 6′-H_B, 2′-H_2), 3.33–3.45 (m, 2H, 5-H, 1-H), 3.94 (bs, 1H, OH), 4.57 (AB, 2H, $PhCH_2O$), 7.17–7.36 (m, 10, ArH). ^{13}C NMR ($CDCl_3$): δ 18.58, 31.43, 33.88, 34.29, 38.75, 42.91, 45.37, 53.67, 66.26, 69.92, 70.19, 74.79, 126.17, 126.78, 127.53, 127.57, 128.39, 128.41, 138.66, 146.13. MS (ESI) m/z 366.3 (M + 1). Anal. calcd for $C_{24}H_{31}NO_2$: C, 78.86; H, 8.55; N, 3.83. Found: C, 78.55; H, 8.69; N, 4.01.

4.1.6. (1*RS*,2*RS*,5*RS*) 5-(4-Fluoro-benzyloxy)-2-(4-phenyl-piperidin-1-yl)-cyclohexanol (11b). It was obtained from **8b** (0.679 g, 3.05 mmol), $LiClO_4$ (0.57 g, 5.34 mmol) and **9** (0.46 g, 3.05 mmol) in the same way as described above for compound **11a** and recrystallized from EtOH (30 mL) to yield 1.005 g (86%), white crystals. Mp 178.5–180 °C. ^{1}H NMR ($CDCl_3$): δ 1.14–1.25 (m, 1H, 3-H_A), 1.28–1.41 (m, 2H, 4-H_A, 6-H_A), 1.60–1.71 (m, 1H, 3′-H_A), 1.74–1.92 (m, 4H, 3-H_B, 3′-H_B, 5′-H_2), 2.12–2.32 (m, 3H, 6′-H_A, 4-H_B, 6-H_B), 2.49 (tt-like, J = 12, 4 Hz, 1H, 4′-H), 2.57 (m, 1H, 2-H), 2.70–2.90 (m, 3H, 6′-H_B,

2′-H_2), 3.32–3.45 (m, 2H, 5-H, 1-H), 3.94 (bs, 1H, OH), 4.52 (AB, 2H, 4-F-PhCH_2O), 7.17–7.36 (m, 10, ArH). ^{13}C NMR ($CDCl_3$): δ 18.58, 31.43, 33.86, 34.28, 38.73, 42.89, 45.37, 53.67, 66.23, 69.53, 69.89, 74.92, 115.22 (d, $J = 21.3$ Hz, 2C, 3-C), 126.18, 126.78, 128.41, 129.28 (d, $J = 8.3$ Hz, 2C, 2-C), 134.37 (d, $J = 3.0$ Hz, 1C, 1-C), 146.11, 162.18 (d, $J = 246.4$ Hz, 1C, 4-C). MS (ESI) m/z 384.3 (M + 1). Anal. calcd for $C_{24}H_{30}FNO_2$: C, 75.16; H, 7.88; N, 3.65. Found: C, 74.98; H, 8.03; N, 3.94.

4.2. Radioligand binding and competition experiments

4.2.1. Radioligands and drugs. [^{3}H]Vesamicol ([^{3}H]phenylpiperidinylcyclohexanol, AH5183) (specific radioactivity 1258 GBq/mmol resp. 34 Ci/mmol), [^{3}H]DTG ([^{3}H]1,3-di-*o*-tolylguanidine) (specific radioactivity 1110 GBq/mmol resp. 30 Ci/mmol), [^{3}H]haloperidol (specific radioactivity: 444 GBq/mmol resp. 22 Ci/mmol), [^{3}H]raclopride (specific radioactivity 2812 GBq/mmol resp. 76 Ci/mmol) were obtained from Perkin Elmer Life Sciences, Boston, MA, USA). L-(−)-Vesamicol hydrochloride, haloperidol hydrochloride, 1,3-di(2-tolyl)-guanidine (DTG) and S(−)-sulpiride were purchased from TOCRIS, BIOTREND Chemikalien GmbH, Köln).

4.2.2. Tissue preparation. Female Sprague–Dawley rats (150–200 g) were anaesthetized with ether and decapitated. Their brains were rapidly removed from the skull. The brain tissue minus cerebellum was homogenized in 10 volumes (w/v) of ice cold 0.25 M sucrose solution using a Teflon-glass homogenizer (50 strokes). Aliquotes were stored at −20 °C until use. Crude membranes for drug competition assays were obtained by centrifugation of the brain homogenate at 15 000 × g for 15 min at 4 °C, washed twice (once with 0.25 M sucrose solution, once with distilled water) and resuspended to a concentration of 200 mg/mL wet tissue in assay buffer (50 mM Tris pH 7.4 with 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$).

Rat liver (for analyzing sigma receptor binding) was prepared the same way. The liver lobes were minced before homogenization.

Various concentrations (10^{-11} to 10^{-5} M) of the synthesized compounds were incubated in duplicates with membranes (20 mg wet weight) for 2 h at 25 °C during agitation (200 Umin^{-1}) in a total volume of 1 mL of assay buffer in the presence of one of the different radiotracers: [^{3}H]Vesamicol (AH5183) [6.1 ± 1.3 nM]; [^{3}H]DTG (σ-1 and σ-2 ligand) [4.9 ± 0.5 nM], [^{3}H]haloperidol (σ-1 ligand, D_2 ligand) [3.1 ± 1.1 nM] and [^{3}H]raclopride (specific D_2 ligand) [2.3 ± 0.5 nM], respectively. (−)-Vesamicol, DTG and haloperidol were tested for comparison.

Nonspecific binding was determined using the corresponding unlabeled tracer compound at concentrations of 10^{-5} M [i.e., (−)-vesamicol, haloperidol, or DTG]. After incubation membrane bound radiotracer was separated from free radioligand by rapid vacuum filtration using a Brandel cell harvester (Gaithersburg, MD). Each sample was diluted with 4 mL of cold PBS buffer and filtered under reduced pressure through Whatman GF/B glas-fiber filters presoaked for 3 h in 0.5% polyethyleneimine. The filters were washed 3 times with 4 mL ice-cold buffer. Filters were dried, soaked in 10 mL Rotaszint eco plus cocktail overnight and counted in a TriCarb Liquid Scintillation Counter (TriCarb2900TR, Packard, Meriden, USA) at 70% counting efficiency.

4.2.3. Data analysis. Inhibitory concentration at 50% specific binding of the radioligand (IC_{50}-value) was determined by a nonlinear curve fitting method, using Graphpad Prism, vers. 3 (GraphPad Software, Inc., San Diego, USA). Specific binding of radioligands was defined as total binding minus nonspecific binding. Nonspecific binding was determined in parallel experiments in the presence of 10^{-5} M of the corresponding unlabeled ligand.

K_i-values were derived from IC_{50}-values according to the equation: $K_i = IC_{50}/(1 + C/K_d)$,[18] whereby C is the concentration of the radioligand and K_d is the dissociation constant of the (radio)ligand. K_d-values were taken from the literature as indicated or—in the case of [^{3}H]haloperidol–estimated from saturation experiments as described above.

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References and notes

1. Rogers, G. A.; Parsons, S. M.; Anderson, D. C.; Nilsson, L. M.; Bahr, B. A.; Kornreich, W. D.; Kaufman, R.; Jacobs, R. S.; Kirtman, B. *J. Med. Chem.* **1989**, *32*, 1217.
2. Bahr, B. A.; Clarkson, E. D.; Rogers, G. A.; Noremberg, K.; Parsons, S. M. *Biochemistry* **1992**, *31*, 5752.
3. Perry, E. K. *Br. Med. Bull.* **1986**, *42*, 63.
4. Custers, F. G. J.; Leysen, J. E.; Stoof, J. C.; Herscheid, J. D. M. *Eur. J. Pharmacol.* **1997**, *338*, 177.
5. Efange, S. M. N.; Mach, R. H.; Smith, C. R.; Khare, A. B.; Foulon, C.; Akella, S. K.; Childers, S. R.; Parsons, S. M. *Biochem. Pharmacol.* **1995**, *49*, 791.
6. Rogers, G. A.; Stone-Elander, S.; Ingvar, M.; Eriksson, L.; Parsons, S. M.; Widen, L. *Nucl. Med. Biol.* **1994**, *21*, 219.
7. Mach, R. H.; Voytko, M. L.; Ehrenkaufer, R. L. E.; Nader, M. A.; Tobin, J. R.; Efange, S. M. N.; Parsons, S. M.; Gage, H. D.; Smith, C. R.; Morton, T. E. *Synapse* **1997**, *25*, 368.
8. Mulholland, G. K.; Wieland, D. M.; Kilbourn, M. R.; Frey, K. A.; Sherman, P. S.; Carey, J. E.; Kuhl, D. E. *Synapse* **1998**, *30*, 263.
9. (a) Efange, S. M. N.; Michelson, R. H.; Khare, A. B.; Thomas, J. R. *J. Med. Chem.* **1993**, *36*, 1754. (b) Efange, S. M. N.; Khare, A.; Parsons, S. M.; Bau, R.; Metzenthin, T. *J. Med. Chem.* **1993**, *36*, 985.
10. Chini, M.; Crotti, P.; Favero, L.; Macchia, F.; Pineschi, M. *Tetrahedron Lett.* **1994**, *35*, 433.
11. Chini, M.; Crotti, P.; Flippin, L. A.; Macchia, F. *J. Org. Chem.* **1991**, *56*, 7043.

12. Godek, C. J.; Moir, R. Y.; Purves, C. B. *Can. J. Chem.* **1951**, *29*, 946.
13. Schulz, J.; Gani, D. J. *Chem. Soc., Perkin Trans. 1* **1997**, 657.
14. (a) Sharpless, K. B.; Michaelson, R. C. *J. Am. Chem. Soc.* **1973**, *95*, 6136. (b) Takai, K.; Oshima, K.; Nozaki, H. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 3791.
15. Marien, M. R.; Parsons, S. M.; Altar, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 876.
16. Ruberg, M.; Mayo, W.; Brice, A.; Duyckaerts, C.; Hauw, J. J.; Simon, H.; LeMoal, M.; Agid, Y. *Neuroscience* **1990**, *35*, 327.
17. Huang, Y.; Hammond, P. S.; Wu, L.; Mach, R. H. *J. Med. Chem.* **2001**, *44*, 4404.
18. Cheng, Y. C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
19. The synthesis of **8a** starting from **7** has been described without experimental details: Baker, R.; Leeson, P. D.; Liverton, N. J.; Kulagowski, J. L. *J. Chem. Soc. Chem. Commun.* **1990**, 462.