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Enantio- and Diastereocontrolled Dopamine D1, D2, D3 and D4 Receptor Binding of *N*-(3-Pyrrolidinylmethyl)benzamides Synthesized from Aspartic Acid

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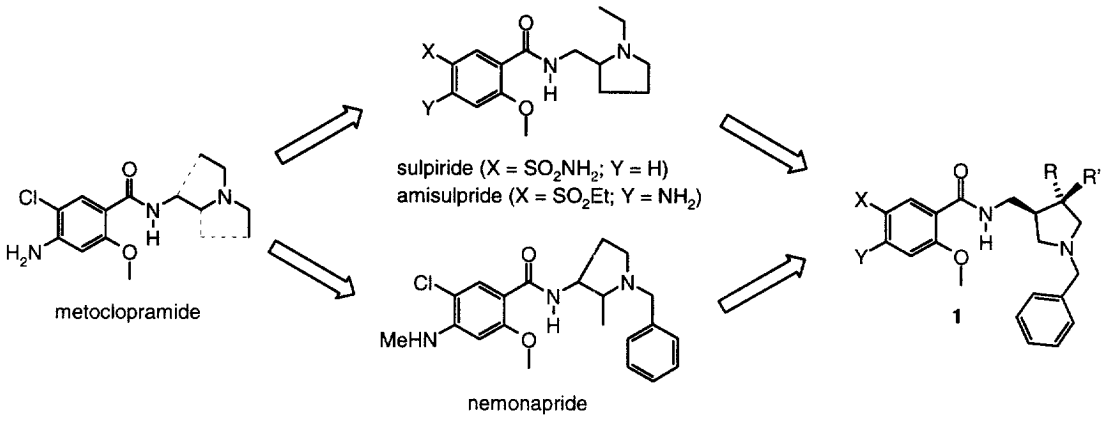
Abstract: Subreceptor selectivity tuning of *N*-(3-pyrrolidinyl)benzamides leading to the selective dopamine D3 ligand **ent1h** and the derivatives **1g** and **1e/ent1e** which preferably recognize human D2 or D4 receptors, respectively, is described. Binding profiles were controlled by both, absolute and relative configuration. The enantiopure target compounds were synthesized from aspartic acid.

The 2-methoxybenzamides represent a very important family of drugs used as antiemetics, gastric motility stimulants and antipsychotics.[1] Structurally, the compounds may be divided into benzamides of *N,N*-disubstituted ethylenediamines including metoclopramide [2] and conformationally restricted 2-aminomethylpyrrolidine and 3-aminopyrrolidine analogs. Highly interesting representatives of the 2-aminomethylpyrrolidine series, such as sulpiride or amisulpride show an atypical neuroleptic profile which is obviously due to D2/D3 antagonistic effects with both presynaptic and limbic selectivity.[3] On the other hand, benzamides of 3-aminopyrrolidines including the antipsychotic nemonapride (*cis*-isomer, racemic) are known for a very strong affinity to the dopamine receptors D2, D3 and D4.[4] Structural hybridation of both types leads to benzamides of 3-aminomethylpyrrolidines including 4-alkyl substituted derivatives (**1**). Here, we report the first investigations on stereoselective synthesis and dopamine receptor binding of this class of compounds.[5]



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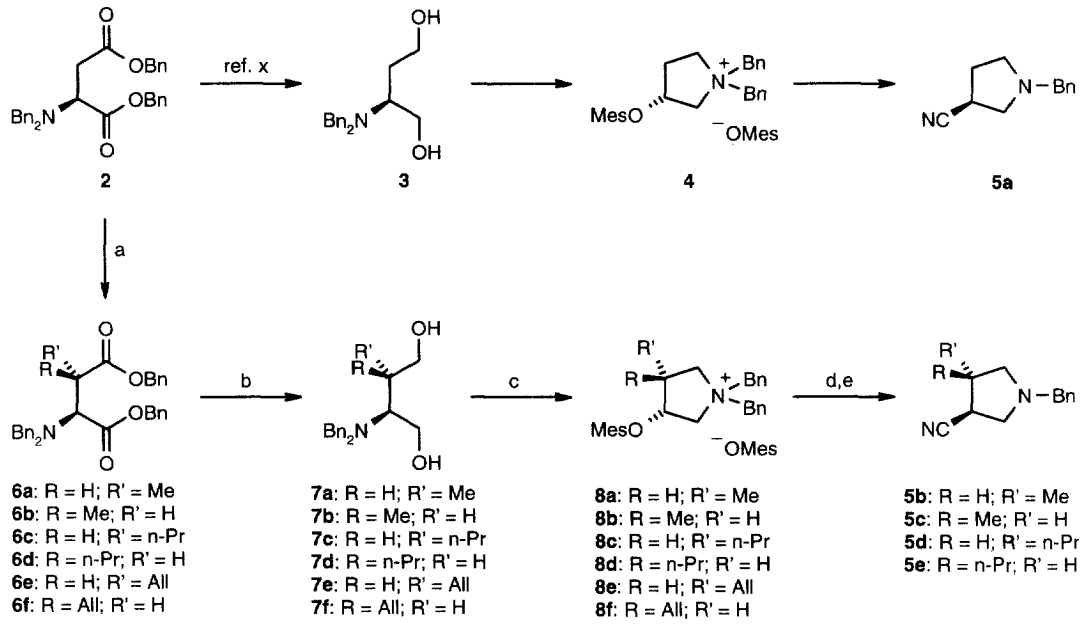
The preparation of the target compounds was envisioned by coupling of isomerically pure 3-aminomethylpyrrolidines, including 4-alkyl derivatives, with typical 2-methoxybenzoic acids proved as valuable building blocks for dopamine antagonists. For an efficient EPC synthesis of the 3-aminomethylpyrrolidine **9a** and its optical antipode **ent9a** we took advantage of our recently described ß-amino acid methodology employing the *N*-benzylpyrrolidine carbonitriles **5a** and **ent5a** as the key intermediates.[6,7] Thus, the *(S)*-ß-proline precursor **5a** was obtained from the *N,N*-dibenzylaspartate **2** [8] through the aminobutanediol **3** and the pyrrolidinium mesylate **4** in 92% overall yield. Analogously, **ent5a** was prepared from unnatural *(R)*-aspartic acid.

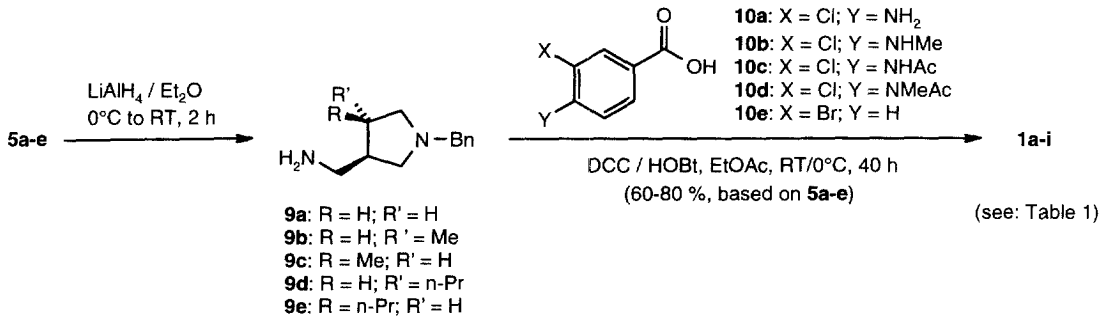


a: $LiN(SiMe_3)_2$, THF, MeI, -50°C to -20°C, 2.5 h (**6a,b**: 98%); n-PrI, -20°C to -6°C, 18 h (**6c,d**: 52%); AllI, -65°C to -25°C, 1.5 h (**6e,f**: 82%); b: $LiAlH_4$, THF, -40°C to -20°C, 4 h (**7a,b**: 95%; **7c,d**: 45%; **7e,f**: 58%); c: MesCl, Et_3N, THF, -40°C to -20°C, 1h, low temperature flash chromatography; d: $Pd(OH)_2/C$, H_2, MeOH, RT, 2-15 min (63-97%, based on **7**); Bu_4NCN, NaCN, DMSO, 60°C, 20-48 h (**5b**: 21%, **5c**: 73%, **5d**: 26%, **5e**: 80%).

Besides the substitution pattern of the 2-methoxybenzamide moiety and the absolute configuration in position 3 of the pyrrolidine framework, the incorporation of alkyl substituents into the pyrrolidine 4-position should be used as a tool for tuning the dopamine subreceptor selectivity. Following a protocol, we previously used for a *C*-benzylation,[9] deprotonation of the protected aspartate **2** by $LiN(SiMe_3)_2$ and subsequent trapping of the ester enolate by methyl iodide or propyl iodide resulted in formation of the 3-substituted derivatives **6a,b** and **6c,d**, respectively. Whereas the methylation products **6a,b** were obtained in a 7:4 *threo/erythro* ratio the propylation gave a 1:1 mixture of **6c** and **6d**. The reduced electrophilicity of propyl iodide, when compared to methyl iodide, required more drastic reaction conditions resulting in a product yield of only 52%. This problem could be circumvented by using allyl iodide as a *C3* equivalent which was expected to be hydrogenated catalytically to a propyl group on a later step of the synthesis. In fact, the allylation reaction proceeded at low temperature (-65 to -25°C) and afforded 82% of the diastereomers **6e,f** as a 1:1 mixture of diastereomers. Subsequent ester reduction

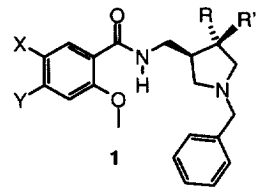
by $LiAlH_4$ gave the diols **7a-f**. Chromatographic separation of the diastereomeric esters **6a,b**, **6c,d** and **6e,f** was possible. However, reduction of the diastereomeric mixtures and subsequent flash chromatographic purification of the respective aminobutanediols proved to be the more convenient and efficient alternative. Transformation of **7a-f** into the pyrrolidinium mesylates **8a-f** involving migration of the dibenzylamino group was induced by treatment with methanesulfonyl chloride. Subsequent catalytic hydrogenation and cyanide displacement of the mesyloxy group afforded **5b,c** from **8a,b**. Synthesis of the propyl substituted nitriles **5d,e** could be performed from the respective allyl or propyl precursors when the allyl-route gave a higher overall yield. Using the same reactions, the enantiomers **ent5b-e** were synthesized from *(R)*-aspartic acid.

The reduction of the carbonitriles **5a-e** and **ent5a-e** was best performed by $LiAlH_4$ in Et_2O, when the aminomethylpyrrolidines **9a-e** and **ent9a-e** were formed quantitatively. Subsequent DCC coupling of **9a** and **ent-9a** to the aromatic building blocks **10a-e** gave the 2-methoxybenzamides **1a-e** and **ent1a-e**, respectively. The 4-alkylpyrrolidines **9b-e** and **ent9b-e** were reacted with the aminobenzoic acid **10b** to afford the benzamides **1f-i** and **ent1f-i**, respectively.[10]



The methoxybenzamides **1a-i** and the enantiomers **ent1a-i** were evaluated *in vitro* for their abilities to displace [^{3}H]spiperone from the cloned human dopamine receptors $D2_{long}$, $D2_{short}$,[11] D3 [12] and D4.4 [13] being stably expressed in CHO cells. The D1 affinities were determined by employing bovine striatal membrane preparations and the D1 selective antagonist [^{3}H]SCH 23390 as the radioligand.[14] As a reference drug, the more active *(S)*-enantiomer of sulpiride was utilized. Our SAR studies were initiated by investigating the binding properties of the *(R)*-configured pyrrolidinylmethylbenzamides **1a-e** depending on the substitution pattern at the 2-methoxybenzamide moiety (Table 1). We observed that the 4-amino-5-chloro derivative **1a**, incorporating substituents identical to metoclopramide, shows moderate binding to the dopamine receptors D1 - D4 with preference to D4 (Ki = 140 nM). Corresponding to the strong affinities of nemonapride reported in the literature, dopamine receptor binding to all the subreceptors investigated was strongly enhanced when the *N*-methylaniline **1b** gave Ki values of 23 nM for D4, 360 nM for D3 as well as 110 nM and 86 nM for $D2_{long}$ and $D2_{short}$, respectively. The D1 binding remained modest (Ki = 9700 nM). According to recent studies indicating that sterically demanding residues localized at the aromatic aminofunction of nemonapride analogs increases D4 selectivity [15] we investigated the binding properties of the acetamides **1c** and **1d**. This structural variation, however, strongly reduced the affinities to D1, D2, D3 and D4. On the other hand, the 5-bromo-2-methoxybenzamide **1e** showed selective D4 receptor binding with Ki values of 46, 5200, 2000, 1500 and 2100 for D4, D1, $D2_{long}$, $D2_{short}$ and D3, respectively.[16]

Table 1: Binding data (Ki values [nM]) of *N*-(3-pyrrolidinyl)benzamides employing human dopamine $D2_{long}$, $D2_{short}$, D3 and D4.4 as well as bovine D1 receptors.[17]



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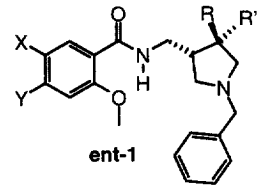

ent-1

Compound	X	Y	R	R^1	D1	$D2_{long}$	$D2_{short}$	D3	D4.4
1a	Cl	NH_2	H	H	11 000	820	540	2 200	140
ent1a	Cl	NH_2	H	H	11 000	1 400	640	570	180
1b	Cl	NHMe	H	H	9 700	110	86	360	23
ent1b	Cl	NHMe	H	H	5 100	110	58	47	19
1c	Cl	NHAc	H	H	41 000	27 000	25 000	8 700	340
ent1c	Cl	NHAc	H	H	35 000	19 000	21 000	2 700	250
1d	Cl	NMeAc	H	H	87 000	38 000	28 000	11 000	7 000
ent1d	Cl	NMeAc	H	H	75 000	30 000	19 000	7 200	4 200
1e	Br	H	H	H	5 200	2 000	1 500	2 100	46
ent1e	Br	H	H	H	3 700	3 100	1 600	1 100	48
1f (cis)	Cl	NHMe	H	Me	12 000	51	21	570	240
ent1f (cis)	Cl	NHMe	H	Me	11 000	150	79	110	47
1g (trans)	Cl	NHMe	Me	H	14 000	28	10	210	220
ent1g (trans)	Cl	NHMe	Me	H	20 000	330	120	320	150
1h (cis)	Cl	NHMe	H	n-Pr	240	45	28	310	46
ent1h (cis)	Cl	NHMe	H	n-Pr	2 800	190	190	31	200
1i (trans)	Cl	NHMe	n-Pr	H	3 300	54	24	270	130
ent1i (trans)	Cl	NHMe	n-Pr	H	4 100	980	540	350	990
(S)-sulpiride					50 000	120	51	88	2 100

Binding data are the means of two to three experiments performed in triplicate at eight concentrations (0.01-100000 nM). Ki values [nM] were obtained from nonlinear regression analysis using the programme PRISM and subsequent application of the equation of Cheng and Prusoff.

The binding profiles of the *(S)*-configured benzamides **ent1a** and **ent1c-e** were similar to those of their enantiomers. However, the methylamine **ent1b** showed enantioselective D3 binding resulting in a Ki value of 47nM. Thus, **ent1b** displays D1, D2 and D3 affinities comparable to the atypical antipsychotic drug *(S)*-sulpiride

except an approximately 100 fold stronger D4 receptor binding. Stereoselective and variable binding profiles were determined for 4-methyl- and 4-propylpyrrolidine derivatives **1f-i** and its enantiomers **ent1f-i**. It turned out, that the *(3R)*-configuration disfavors D3 binding and induces remarkable D2 receptor affinity which is also subreceptor selective for **1f,g,e**. For the *cis*-configured test compound **1h** it is accompanied by substantial D4 activity. Interestingly, **1h** shows also moderate D1 binding (Ki = 240 nM). Within the *(3S)*-series the cis-isomers **ent1f** and **ent1h** displayed D3 affinities superior to those of the trans-isomers **ent1g** and **ent1i**. Comparison of the Ki values indicates strong and selective D3 binding of the propyl substituted derivative **ent1h** with a Ki value of 31nM and a selectivity > 6 when compared to D1, D2 and D4.

In conclusion, benzamides of 3-aminomethylpyrrolidines including 4-alkyl derivatives exhibited interesting dopamine receptor binding profiles. The subreceptor selectivity depended on absolute and relative configurations. Thus, *(3S)*-configuration resulted in remarkable D3 affinity which turned out D3-selective for *cis*-configured 4-alkyl derivatives. On the other hand, *(3R)*-configuration induced strong D2 binding.

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References and Notes:

1. For examples, see: Norman, M.H.; Kelley, J.L.; Hollingsworth, E.B. *J. Med. Chem.* **1993**, 36, 3417, and references cited therein.
2. Harrington, R.A.; Hamilton, C.W.; Brogden, R.N.; Linkewich, J.A.; Romankiewicz, J.A.; Heel, R.C. *Drugs* **1983**, 25, 451.
3. O'Connor, S.E.; Brown, R.A. *Gen. Pharmacol.* **1982**, 13, 185. Schoemaker, H.; Claustre, Y.; Fage, D.; Rouquier, L.; Chergui, K.; Curet, O.; Oblin, A.; Gonon, F.; Carter, C.; Benavides, J.; Scatton, B. *J. Pharmacol. Exp. Ther.* **1997**, 280, 83.
4. Iwanami, S.; Takashima, M.; Hirata, Y.; Hasegawa, O.; Usuda, S. *J. Med. Chem.* **1981**, 24, 1224.
5. Racemic derivatives have been briefly mentioned in previous studies, see: Harada, H.; Morie, T.; Hirokawa, Y.; Yoshida, N.; Kato, S. *Chem. Pharm. Bull.* **1995**, 43, 1364. Thominet, M.; Acher, J.; Monier, J.C. *Ger. Offen. 2,901,170*, 26 Jul 1979 (CA **1980**, 92: 6406h).
6. Gmeiner, P.; Orecher, F.; Thomas, C.; Weber, K. *Tetrahedron Lett.* **1995**, 36, 381.
7. Thomas, C.; Orecher, F.; Gmeiner, P. *Synthesis* **1998**, 1491.
8. Gmeiner, P.; Junge, D.; Kärtner, A. *J. Org. Chem.* **1994**, 59, 6766.
9. Thomas, C.; Ohnmacht, U.; Niger, M.; Gmeiner, P. *Bioorg. Med. Chem. Letters* **1998**, 8, 2885.
10. Typical experimental procedure and characterization data: To a suspension of $LiAlH_4$ (51 mg, 1.3 mmol) in Et_2O (1.5 ml) at 0°C was added a solution of **5b (5c)** (61.5 mg, 0.31 mmol) in Et_2O. After 1 h the suspension was allowed to warm up to RT and stirred for 1h. After dropwise addition of aqueous $NaHCO_3$ the mixture was filtered through $MgSO_4$/Celite. The filtrate was evaporated to leave analytically pure **9b (9c)**. For the subsequent coupling **9b (9c)** was dissolved in EtOAc (1 ml) and added to a solution of **10b** (66.8 mg, 0.31 mmol), HOBt (46.0 mg, 0.34 mmol) and DCC (70.3 mg, 0.34 mmol) in EtOAc (1 ml). After stirring for 16h at RT, the mixture was stored for 24h at 0°C, filtered through Celite and evaporated. Flash chromatographic purification (silica gel, CH_2Cl_2 / EtOH saturated with NH_3 10:1) provided **1f** (76.9 mg, 62%) (**1g**, 74%). **9b**: ^{1}H NMR ($CDCl_3$, 360 MHz): δ = 1.05 (d, J=6.9 Hz, 3H, C$\underline{H}_3$), 1.71 (ddddd, J=7.9, 7.9, 6.5, 5.8, 5.5 Hz, 1H, H-3), 1.86 (dddq, J=7.3, 7.0, 6.5, 6.9 Hz, 1H, H-4), 2.12 (dd, J= 9.0, 7.0

Hz, 1H, H-5b), 2.42 (dd, J=9.5, 5.5 Hz, 1H, H-2a), 2.64 (dd, J=12.3, 7.9 Hz, 1H, C$\underline{H}_2NH_2$), 2.66 (dd, J=9.5, 7.9 Hz, 1H, H-2b), 2.76 (dd, J=12.3, 5.8 Hz, 1H, C$\underline{H}_2NH_2$), 2.80 (dd, J= 9.0, 7.3 Hz, 1H, H-5a), 3.52 (d, J=12.9 Hz, 1H, C$\underline{H}_2$Ph), 3.62 (d, J=12.9 Hz, 1H, C$\underline{H}_2$Ph), 7.20-7.35 (m, 5H, arom.). ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ = 19.7 ($\underline{C}H_3$), 36.6 (C-4), 46.3 ($\underline{C}H_2NH_2$), 49.6 (C-3), 58.4 (C-2), 60.6 ($\underline{C}H_2$Ph), 62.3 (C-5), 126.8, 128.1, 128.7, 139.3 (arom.). NOE: Irradiation of H-3 gave a strong positive enhancement at C$\underline{H}_3$ and H-2b and a weak positive enhancement at H-5b. Irradiation of H-4 gave a strong positive enhancement at H-5a, a medium positive enhancement at C$\underline{H}_2NH_2$ and a weak positive enhancement at H-2a. Irradiation of C$\underline{H}_3$ gave a strong positive enhancement to H-3 and H-5b. HRMS (EI) calcd. for $C_{13}H_{20}N_2$ (M^+): 204.1626; Found: 204.1625. α_D^{21}= +34.6° (0.5 $CHCl_3$). **9c**: ^{1}H NMR ($CDCl_3$, 360 MHz): δ = 0.94 (d, J=7.2 Hz, 3H, C$\underline{H}_3$), 2.03 (dd, J= 9.2, 7.2 Hz, 1H, H-5a), 2.14 (dd, J=8.8, 8.3 Hz, 1H, H-2a), 2.21 (dddd, J=8.6, 8.6, 8.3, 6.5, 5.8 Hz, 1H, H-3), 2.37 (dddq, J=8.6, 7.2, 7.0, 7.2 Hz, 1H, H-4), 2.58 (dd, J=12.2, 8.6 Hz, 1H, C$\underline{H}_2NH_2$), 2.82 (dd, J=12.2, 5.8 Hz, 1H, C$\underline{H}_2NH_2$), 2.98 (dd, J= 9.2, 7.0 Hz, 1H, H-5b) 3.00 (dd, J=8.8, 6.5 Hz, 1H, H-2b), 3.60 (s, 2H, C$\underline{H}_2$Ph), 7.20-7.35 (m, 5H, arom). ^{13}C-NMR ($CDCl_3$, 63 MHz): δ = 14.3 ($\underline{C}H_3$), 33.6 (C-4), 42.5 ($\underline{C}H_2NH_2$), 43.8 (C-3), 58.6 (C-2), 60.9 ($\underline{C}H_2$Ph), 62.4 (C-5), 126.8, 128.2, 128.7, 139.4 (arom.). NOE: Irradiation of H-3, H-2a gave a strong positive enhancement at H-4 and H-5b. Irradiation of H-4 gave a strong positive enhancement at H-3, H5-b, a weak positive enhancement at H-2b. Irradiation of C$\underline{H}_3$ gave a strong positive enhancement at C$\underline{H}_2NH_2$, H-5a and a weak positive enhancement at H-2a. HRMS (EI) calcd. for $C_{13}H_{20}N_2$ (M^+): 204.1626; Found: 204.1644. α_D^{20}= +11.0° (0.81 $CHCl_3$). **1f**: ^{1}H NMR ($CDCl_3$, 360 MHz): δ = 1.03 (d, J=6.7 Hz, 3H, CHC$\underline{H}_3$), 2.14 (dd, J=9.0, 7.0 Hz, 1H, NC$\underline{H}_2$), 2.28 (dd, J=9.2, 6.8 Hz, 1H, NC$\underline{H}_2$), 2.35-2.53 (m, 2H, C$\underline{H}$C$\underline{H}$CH$_3$), 2.94 (dd, J=9.0, 7.5 Hz, 1H, NC$\underline{H}_2$), 2.95 (d, J=5.2 Hz, 3H, NHC$\underline{H}_3$), 2.98 (dd, J=9.2, 7.5 Hz, 1H, NC$\underline{H}_2$), 3.41 (ddd, J=13.4, 7.9, 5.2 Hz, 1H, NHC$\underline{H}_2$), 3.52 (ddd, J=13.4, 6.1, 5.0 Hz, 1H, NHC$\underline{H}_2$), 3.59 (d, J=12.9 Hz, 1H, NC$\underline{H}_2$Ph), 3.64 (d, J=12.9 Hz, 1H, NC$\underline{H}_2$Ph), 3.86 (s, 3H, OC$\underline{H}_3$), 4.70 (q, J=5.2 Hz, 1H, N$\underline{H}$CH$_3$), 6.09 (s, 1H, C$\underline{H}$COCH$_3$), 7.20-7.35 (m, 5H, arom.), 7.73 (dd, J=5.2, 5.0 Hz, 1H, N$\underline{H}$CH$_2$), 8.08 (s, 1H, C$\underline{H}$CCl). Anal. calcd. for $C_{22}H_{28}N_3O_2Cl$ (401.9): C 65.74 H 7.02 N 10.45; Found: C 65.55 H 7.20 N 10.45. α_D^{21}=-15.9° (1.0, $CHCl_3$). **1g**: ^{1}H NMR ($CDCl_3$, 360 MHz): δ = 1.08 (d, J=6.5 Hz, 3H, CHC$\underline{H}_3$), 1.94-2.05 (m, 2H, C$\underline{H}$C$\underline{H}$CH$_3$), 2.11 (dd, J=9.0, 6.9 Hz, 1H, NC$\underline{H}_2$), 2.25 (dd, J=9.5, 4.9 Hz, 1H, NC$\underline{H}_2$), 2.64 (dd, J=9.5, 7.5 Hz, 1H, NC$\underline{H}_2$), 2.88 (dd, J=9.0, 6.9 Hz, 1H, NC$\underline{H}_2$), 2.94 (d, J=5.1 Hz, 3H, NHC$\underline{H}_3$), 3.42 (ddd, J=13.3, 6.9, 5.4 Hz, 1H, NHC$\underline{H}_2$), 3.49 (ddd, J=13.3, 5.8, 5.5 Hz, 1H, NHC$\underline{H}_2$), 3.53 (d, J=13.0 Hz, 1H, NC$\underline{H}_2$Ph), 3.65 (d, J=13.0 Hz, 1H, NC$\underline{H}_2$Ph), 3.88 (s, 3H, OC$\underline{H}_3$), 4.70 (q, J=5.1 Hz, 1H, N$\underline{H}$CH$_3$), 6.09 (s, 1H, C$\underline{H}$COCH$_3$), 7.18-7.35 (m, 5H, arom.), 7.84 (dd, J=5.5, 5.4 Hz, 1H, N$\underline{H}$CH$_2$), 8.09 (s, 1H, C$\underline{H}$CCl). Anal. calcd. for $C_{22}H_{28}N_3O_2Cl$ (401.9): C 65.74 H 7.02 N 10.45; Found: C 65.79 H 7.13 N 10.52. α_D^{21}= -5.6° (1.0, $CHCl_3$).

11. Hayes, G.; Biden, T.J.; Selbie, L.A.; Shine, J. *Mol. Endocrinol.* **1992**, 6, 920.
12. Sokoloff, P.; Andrieux, M.; Besancon, R.; Pilon, C.; Martres, M.-P.; Giros, B.; Schwartz, J.-C. *Eur. J. Pharmacol.* **1992**, 225, 331.
13. Asghari, V.; Sanyal, S.; Buchwaldt, S.; Paterson, A.; Jovanovic, V.; Van Tol, H.H.M. *J. Neurochem.* **1995**, 65, 1157.
14. Ohnmacht, U.; Tränkle, C.; Mohr, K.; Gmeiner, P. *Pharmazie* **1999**, in press, and references cited therein.
15. Ohmori, J.; Maeno, K.; Hidaka, K.; Nakato, K.; Matsumoto, M.; Tada, S.; Hattori, H.; Sakamoto, S.; Tsukamoto, S.; Usuda, S.; Mase, T. *J. Med. Chem.* **1996**, 39, 2764.
16. For very recent SAR studies on D4 selective ligands, see: Löber, S.; Hübner, H.; Gmeiner, P. *Bioorg. Med. Chem. Letters* **1999**, 9, 97.
17. Binding assays were done in 50 mM TrisHCl pH 7.4 buffer supplemented with 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM DL-dithiothreitol, 100 μg/ml bacitracin and 5 μg/ml soybean trypsin inhibitor in a final volume of 200 μl containing the radioligand [^{3}H]SCH 23390 (0.3 nM) for bovine D1 receptor or [^{3}H]spiperone (0.5 nM) for human $D2_{long}$, $D2_{short}$, D3 or D4.4 receptors, respectively and the compounds tested at concentrations from 0.01-100 000 nM. Nonspecific binding was measured in the presence of butaclamol for D1 (1 μM) or haloperidol for $D2_{long}$, $D2_{short}$, D3 and D4.4 (10 μM). Incubation started by adding membranes with a protein concentration of 40-130 μg/ml was run at 30 °C for 60 min and stopped by rapid filtration through GF/C filters coated in 0.3 % polyethylenimine, using an automated cell harvester (Inotech). Filters were washed 5 times with ice-cold 50 mM Tris-HCl pH 7.4 buffer containing 120 mM NaCl and counted in a MicroBeta TriLux (Wallac ADL).