4-[ω-[4-Arylpiperazin-1-yl]alkoxy]phenyl)imidazo[1,2-*a*]pyridine Derivatives: Fluorescent High-Affinity Dopamine D_3 Receptor Ligands as Potential Probes for Receptor Visualization

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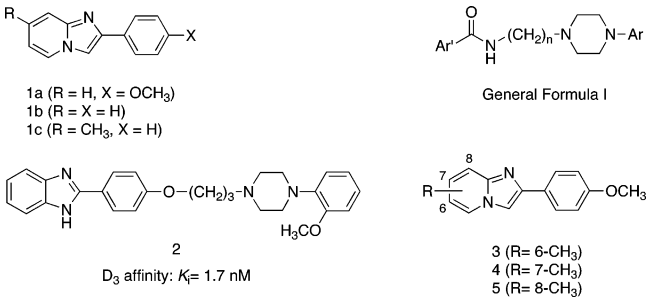
Sixteen long-chain arylpiperazines bearing the fluorescent moiety 2-phenylimidazo[1,2-*a*]pyridine were synthesized as fluorescent dopamine D_3 receptors ligands (385 nM < K_i < 0.72 nM). The most potent D_3 compounds **15a** and **19a** (K_i = 1.6 and 0.72 nM, respectively) showed good Stokes shift and high quantum yield in ethanol (Φ = 0.74 and 0.66, respectively). In the first attempt, **15a** was unable to visualize D_3 receptors expressed in CHO cells by epifluorescence microscopy.

Fluorescent high-affinity ligands represent a class of widely applicable tools. The spatial precision offered by fluorescent visualization overcomes the diverse localization and low concentration of receptor molecules. For example, fluorescent ligands have allowed the localization of α_1-adrenoceptors,[1] dopamine transporter,[2] adenosine A_1 receptors,[3] and peripheral-type benzodiazepine receptors,[4] the study of time course 5-HT_3 receptor cluster formation,[5] the real-time visual tracking of μ- and δ-opioid receptor−ligand complexes internalization and trafficking,[6] and ligand-regulated somatostatin receptor oligomerization.[7] Moreover, fluorescent ligand binding has been proposed as an alternative to radioligand binding, although some analytical issues have been encountered.[8−10] Fluorescent ligands can give also information on the biophysical characteristics of the ligand binding site because some fluorophores show quantum yield depending on the lipophilicity or pH of the environment.[11,12]

G-protein-coupled receptors (GPCRs) represent the largest family of cell-surface receptors mediating cellular communication and are a major target for drugs in current clinical use.[13] The endogenous ligands for GPCRs are a diverse range of hormones, transmitters, autocrine factors, and even photons. In each case, however, the receptor transduces the binding of ligand to an intracellular signal via activation of a heterotrimeric guanosine triphosphate binding protein (G-protein). As a consequence of this, a range of downstream intracellular signals are activated, resulting in short-term effects (e.g., changes in cellular calcium levels) and long-term effects (e.g., gene transcription). Dopamine is one such ligand for which there are five characterized GPCRs (D_{1-5} receptors).

The D_3 receptor is responsible for mediating the physiological effects of dopamine in diverse tissues such as the brain and kidney and signals via G-proteins of the Gi/o family to inhibit adenylate cyclase, modulates ion flow through potassium and calcium channels, and activates kinases, most notably mitogen-activated protein kinase. From the beginning, attention has been attracted to the restricted distribution of the D_3 receptor in the brain (islands of Calleja, ventral striatum/nucleus accumbens, dentate gyrus, and striate cortex), seemingly related to functions of dopamine associated with the limbic brain.[14] Initial pharmacological studies have investigated D_3 receptors as potential therapeutic targets for the treatment of Parkinson's disease because it was evident that the anti-Parkinsonian (motor) effects of dopaminergic agonists were due to activation of D_2 and/or D_3 receptors.[15] Recent in vitro, in vivo, and clinical observations have provided compelling evidence that dopamine D_3 receptors play a major role in the expression of the neuroprotective and neurorestorative actions of dopaminergic agonists. Therefore, the recruitment of dopamine D_3 receptors would appear to be a promising strategy for the development of more effective agents for preventing the degeneration and to be promising for the restoration of the function of dopaminergic neurons in Parkinson's disease.[16] Accumulating evidence indicates that D_3 receptor antagonists appear highly promising for attenuating cocaine reward and relapse in preclinical models of addiction,[17] for reducing alcohol craving and relapse behavior,[18] and for decreasing nicotine-seeking behavior and nicotine relapse in rodents.[19]

Chart 1. Structures of Fluorophores and Reference Dopamine D_3 Agents



During the past decade, we[20−22] and other research groups[23] have extensively studied D_3 receptor ligands with the *N*-[4-(4-arylpiperazin-1-yl)alkyl]arylcarboxamide structure (general formula **I**, Chart 1). The most relevant structural features for high D_3 receptor affinity were (i) a 2,3-dichlorophenyl or a 2-methoxyphenyl moiety linked to the N-1 of the piperazine ring, (ii) an arylcarboxamide moiety originating from an aromatic bicyclic carboxylic acid, and (iii) an intermediate alkyl chain of four methylene units. As a part of our efforts in this field, we have identified some dopamine D_3 agents as potential positron emission tomography radioligands.[24,25] Now we want to extend our interest in molecular imaging probes to fluorescent D_3 receptor ligands.

Fluorescent ligands have been prepared by tagging a ligand with a fluorophore such as fluorescein,[5] BODIPY,[6] coumarin,[9] and dansyl[26] into an area of the structure that would have minimal influence on receptor binding. However, this structural

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Table 1. Fluorescence Properties of Fluorophores **1a**−**c** and **3**−**5** in Ethanol

compd	R	X	excitation λ_{max} (nm)	emission λ_{max} (nm)	Φ
1a[a]	H	OCH_3	325	381	0.55
1b[a]	H	H	313	374	0.50
1c[a]	7-CH_3	H	315	378	0.56
3	6-CH_3	OCH_3	330	380	0.68
4	7-CH_3	OCH_3	325	385	0.43
5	8-CH_3	OCH_3	325	375	0.53

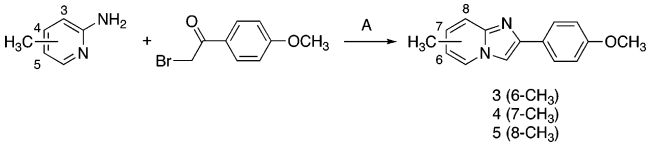
[a] Data taken from ref 29.

modification can change lipophilicity, affinity, and selectivity or even change a competitive ligand into a noncompetitive one.[27,28]

For our purpose, we envisaged the possibility of including a fluorescent core into a framework endowed with affinity for the dopamine D_3 receptor. In this way, it should be possible overcome the above-mentioned shortcomings and allow access to an entire series of fluorescent ligands with the possibility of optimizing the fluorescence properties and receptor affinity at the same time.

We have selected as fluorescent moiety compound **1a** (Chart 1), reported for the first time by Tomoda et al.,[29] which is characterized by the 2-phenylimidazo[1,2-*a*]pyridine moiety. Derivative **1a** presented an oxygen that can be easily functionalized to afford potential D_3 receptor ligands structurally related to the D_3 receptor ligands reported by Wright and co-workers, exemplified by **2** (Chart 1).[30] Moreover, Tomoda demonstrated that the presence of an electron-donating group on the imidazo[1,2-*a*]pyridine moiety enhanced the fluorescent properties of this class of compounds. In particular, the 7-methyl derivative **1c** showed higher quantum yield than the unsubstituted derivative **1b** (Table 1). Therefore, by combination of the substitution patterns of **1a** and **1c**, new fluorophores **3**−**5** were prepared that showed quantum yields similar to or higher than the quantum yield of fluorophore **1a** (Table 1). On the basis of the such observations, the potential fluorescent D_3 ligands **15a**−**18a** (Table 2) formally derived from **2** were designed by replacing the benzimidazole ring with the imidazo[1,2-*a*]pyridine substructure. The higher homologues **19a**−**22a** (Table 2) were also prepared. Finally, we have also evaluated the 1-(2,3-dichlorophenyl)piperazinyl counterparts of **15a**−**22a** (derivatives **15b**−**22b**, Table 2).

Scheme 1[a]



[a] Reagents: (A) Na_2CO_3, EtOH.

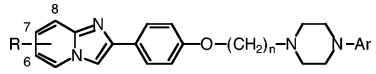
Chemistry

The new fluorophores **3**−**5** have been prepared from the reaction of the appropriate methyl-2-aminopyridine with 2-bromo-1-(4-methoxyphenyl)ethanone (Scheme 1). The target compounds were synthesized according to Scheme 2. The key intermediates **6**−**9** were prepared by condensing the appropriate 2-aminopyridine with 2-bromo-1-(4-hydroxyphenyl)ethanone. The final compounds **15a**,**b**−**18a**,**b** with a three-methylene intermediate chain were prepared from the reaction of phenols **6**−**9** with the derivatives **10a**,**b**, which were prepared by alkylating the appropriate 1-arylpiperazine with 1-bromo-3-chloropropane. To obtain the compounds with a four-methylene intermediate chain, derivatives **6**−**9** were alkylated with 1-bromo-4-chlorobutane to give **11**−**14**, which were reacted with the appropriate arylpiperazine to give the final compounds **19a**,**b**−**22a**,**b**.

Results and Discussion

The affinity values for dopamine D_3 and D_2 receptors and fluorescent properties of the target compounds are displayed in Table 2. The 1-(2-methoxyphenyl)piperazine derivatives **15a**−**18a**, which were formally derived from compound **2**, displayed moderate to high D_3 receptor affinities (K_i ranging from 176 to 1.6 nM). In particular, the replacement of the benzimidazole in ω-position of the propyl chain of **2** with imidazo[1,2-*a*]pyridine did not change the affinity for the D_3 receptor (**2**, K_i = 1.7 nM; **15a**, K_i = 1.6 nM). On the other hand, the presence of a methyl substituent on the imidazo[1,2-*a*]pyridine moiety (derivatives **16a**−**18a**) was detrimental for D_3 affinity. We next elongated the intermediate propyl chain because in other classes of D_3

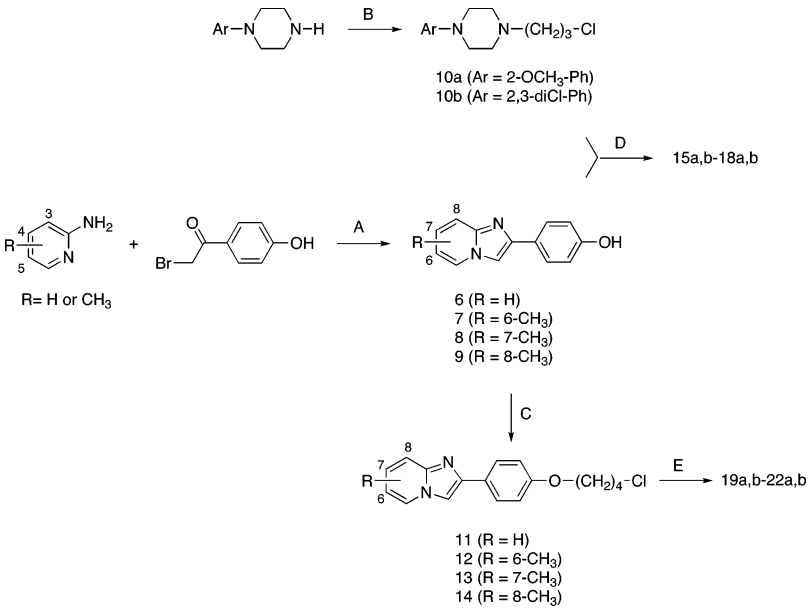
Table 2. Binding Affinities of Ligands **15a**,**b**−**22a**,**b** for Dopamine D_3 and D_2 Receptors and Their Fluorescence Properties in Ethanol



				$K_i \pm$ SEM,[a] nM				
compd	R	*n*	Ar	D_3	D_2	excitation λ_{max} (nm)	emission λ_{max} (nm)	Φ
15a	H	3	2-OCH_3-Ph	1.6 ± 0.2	49 ± 5	325	381	0.74
15b	H	3	2,3-di-Cl-Ph	156 ± 30	44 ± 2.5	330	380	0.58
16a	6-CH_3	3	2-OCH_3-Ph	176 ± 15	78 ± 5.5	323	400	0.83
16b	6-CH_3	3	2,3-di-Cl-Ph	28.9 ± 3.7	31%[b]	325	380	0.59
17a	7-CH_3	3	2-OCH_3-Ph	71 ± 8.0	78 ± 6	330	387	0.72
17b	7-CH_3	3	2,3-di-Cl-Ph	8.0 ± 0.5	83 ± 4	325	387	0.57
18a	8-CH_3	3	2-OCH_3-Ph	50 ± 3.5	49 ± 5.3	330	380	0.77
18b	8-CH_3	3	2,3-di-Cl-Ph	225 ± 12	125 ± 16	330	380	0.67
19a	H	4	2-OCH_3-Ph	0.72 ± 0.10	179 ± 20	335	380	0.66
19b	H	4	2,3-di-Cl-Ph	38 ± 5	68 ± 4	327	380	0.56
20a	6-CH_3	4	2-OCH_3-Ph	146 ± 20	47 ± 2.0	325	380	0.61
20b	6-CH_3	4	2,3-di-Cl-Ph	8.0 ± 0.25	149 ± 20	325	382	0.61
21a	7-CH_3	4	2-OCH_3-Ph	40 ± 4.5	52 ± 3.5	327	386	0.68
21b	7-CH_3	4	2,3-di-Cl-Ph	60 ± 8.0	111 ± 10	325	387	0.58
22a	8-CH_3	4	2-OCH_3-Ph	385 ± 20	159 ± 25	325	380	0.77
22b	8-CH_3	4	2,3-di-Cl-Ph	54.4 ± 1.8	560 ± 25	322	380	0.59
haloperidol				16.0 ± 2.5	2.6 ± 0.2			

[a] The values are the mean ± SEM from three independent experiments in triplicate. [b] Full K_i not obtained. Percentage of inhibition measured at 10 μM.

Scheme 2[a]



[a] Reagents: (A) Na_2CO_3, EtOH; (B) 1-bromo-3-chloropropane, Et_3N; (C); 1-bromo-4-chlorobutane, KOH; (D) 18-crown-6, KOH; (E) 1-(2-methoxyphenyl)piperazine or 1-(2,3-dichlorophenyl)piperazine.

ligands[20−22] a butyl linker was preferred (compounds **19a**−**22a**). Compounds **19a**−**21a** showed slightly higher D_3 affinity values than their lower homologues **15a**−**17a**. In particular, **19a** displayed remarkable D_3 receptor affinity (K_i = 0.72 nM). Only compound **22a** (n = 4) was less potent than **18a** (n = 3). Next, the 2-methoxyphenyl ring of derivatives **15a**−**22a** was replaced with a 2,3-dichlorophenyl ring (compounds **15b**−**22b**) because this substitution pattern is frequently shared by structures possessing a high D_3 receptor affinity. Surprisingly, this modification on the unsubstituted derivatives **15a** and **19a** caused a loss in affinity for the D_3 receptor (**15b** and **19b**, respectively). On the other hand, the same structural modification was tolerated by the methyl substituted imidazo[1,2-*a*]-pyridine derivatives. In fact, 1-(2,3-dichlorophenyl)piperazine derivatives **16b**−**18b** and **20b**−**22b** were at least as potent as their 1-(2-methoxyphenyl)piperazine counterparts except for **18b**. Among the 2,3-dichlorophenyl derivatives, **17b** and **20b** showed D_3 receptor affinity in the nanomolar range (K_i = 8 nM in both cases). Taken together, affinity data for D_3 receptors of target compounds **15a**,**b**−**22a**,**b** did not seem to be determined by only one of the structural features considered here for modification.

The D_2 receptor affinities of the target compounds do not differ greatly from those for D_3 receptor. In most cases the D_3/D_2 K_i ratios, or vice versa, were ≤10. The only relevant exceptions were represented by **15a** and **19a**, which exhibited 30- and 248-fold selectivity over the D_2 receptor.

As far as the fluorescent properties of **15a**,**b**−**22a**,**b** are concerned, we observe that the structural modifications effected on the original fluorophores **1a** and **3**−**5** were well tolerated. In fact, all final compounds were fluorescent in ethanol, showing an acceptable difference of excitation to emission maximal wavelengths (Stokes shift). The excitation wavelengths varied from 322 to 335 nm, whereas emission wavelengths varied from 380 to 400 nm. In particular, the most potent D_3 ligands **15a** and **19a** showed a Stokes shift of 56 and 45 nm, respectively. Moreover, the structural modifications performed on the original fluorophores **1a** and **3**−**5** led to an enhancement of quantum yields (Φ) in ethanol except for **16b** and **20a**,**b** (Table 2). In particular, the Φ value enhancement was more evident for the 1-(2-methoxyphenyl)piperazine derivatives. Moreover, compounds with n = 3 (**15a**,**b**−**18a**,**b**) showed Φ values equal to or higher than their homologues with n = 4 (**19a**,**b**−**22a**,**b**).

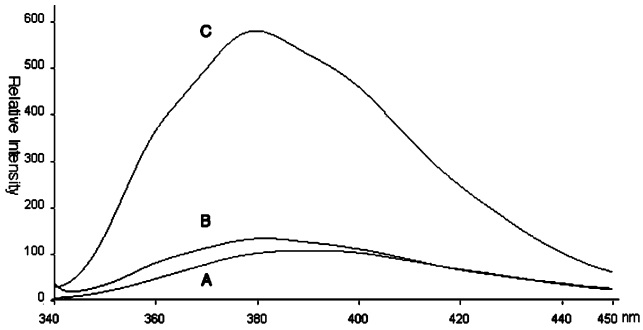


Figure 1. Emission spectra of **15a** in (A) chloroform, (B) PBS buffer, and (C) ethanol.

On the basis of Φ values in ethanol and affinity values for the D_3 receptor, **15a** was selected for a preliminary attempt to visualize D_3 receptors in CHO cells by epifluorescence microscopy. For this purpose, cells were incubated with different concentrations of **15a** in cell culture medium and were subsequently processed for epifluorescence microscopy analysis. Unfortunately, cell autofluorescence background was observed and we did not detect fluorescent labeling by **15a** at 3−1000 nM. This result prompted us to evaluate whether fluorescence of **15a** was affected by environment polarity. Actually, a loss in emission intensity of **15a** in $CHCl_3$ or PBS buffer was found (Figure 1) and this might explain, at least in part, the result from the epifluorescence microscopy experiment.

Conclusions

We have reported here the synthesis of a series of fluorescent ligands of dopamine D_3 receptor designed on the basis of the structure of the 1-(2-methoxyphenyl)piperazine derivative **2** and of the fluorescent compounds **1a** and **3**−**5**. High-affinity ligands for the human D_3 receptor were obtained. The most potent D_3 ligands **15a** and **19a** (K_i = 1.6 and 0.72 nM, respectively) showed good Stokes shift and high quantum yields in ethanol (Φ = 0.74 and 0.66, respectively). Compound **15a** was unable

to visualize D_3 receptors expressed in CHO cells in a preliminary experiment by epifluorescence microscopy. However, the fluorescent properties of the ligands presented here do not exclude their use in other fluorescence techniques, such as two-photon fluorescence microscopy. This sensitive technique allows visualization of fluorescent probes in living cells by two-near-infrared-photon excitation, thus avoiding excitation wavelengths typical of one-photon fluorescence microscopy (300−560 nm) that cause damage to the substrates and cell autofluorescence.[31]

Experimental Section

General Procedure for the Synthesis of 15a,b−18a,b. A mixture of phenol **6**−**9** (1.0 mmol), the alkylating agent **10a**,**b** (1.0 mmol), powdered KOH (10 mmol), and 18-crown-6 (1.0 mmol) in toluene (20 mL) was vigorously stirred under reflux overnight. After cooling, the reaction mixture was concentrated and the residue was partitioned between H_2O (30 mL) and EtOAc (30 mL). The organic layer was separated, washed with brine, dried over Na_2SO_4, and then concentrated in vacuo. The crude residue was chromatographed (19:1 $CHCl_3$/MeOH as eluent) to give the target compound.

(4-[3-[4-(2-Methoxyphenyl)piperazin-1-yl]propoxy]phenyl)-imidazo[1,2-*a*]pyridine (15a). Yield, 75%. ESI$^+$/MS *m*/*z* 443 (MH^+). ESI$^+$/MS/MS *m*/*z* 233 (100), 225 (20), 205 (28). ^{1}H NMR ($CDCl_3$): δ 2.02−2.11 (m, 2H), 2.65 (t, 2H, J = 7.1 Hz), 2.73 (br s, 4H), 3.14 (br s, 4H), 3.87 (s, 3H), 4.10 (t, 2H, J = 6.3 Hz), 6.76 (dt, 1H, J = 0.8, 6.6 Hz), 6.85−7.03 (m, 6H), 7.15 (app t, 1H), 7.61 (d, 1H, J = 9.0 Hz), 7.78 (s, 1H), 7.86−7.90 (m, 2H), 8.10 (d, 1H, J = 6.6 Hz). The hydrochloride salt melted at 226−228 °C (from CH_3OH/Et_2O). Anal. ($C_{27}H_{30}N_4O_2 \cdot 4HCl \cdot H_2O$) C, H, N.

General Procedure for the Synthesis of Compounds 19a,b−22a,b. A stirred mixture of alkylating agent **11**−**14** (1.0 mmol), 1-(2-methoxyphenyl)piperazine or 1-(2,3-dichlorophenyl)piperazine (1.2 mmol) and a slight excess of K_2CO_3 in CH_3CN was refluxed overnight. After cooling, the mixture was evaporated to dryness and H_2O was added to the residue. The aqueous phase was extracted with CH_2Cl_2 (2 × 20 mL). The collected organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The crude residue was chromatographed (19:1 $CHCl_3/CH_3OH$ as eluent) to afford the target compounds.

(4-[3-[4-(2-Methoxyphenyl)piperazin-1-yl]butoxy]phenyl)imidazo[1,2-*a*]pyridine (19a). Yield, 74%. ESI$^+$/MS *m*/*z* 457 (MH^+). ESI$^+$/MS/MS *m*/*z* 247 (100). ^{1}H NMR ($CDCl_3$): δ 1.76−1.90 (m, 4H), 2.53 (app t, 2H), 2.71 (br s, 4H), 3.13 (br s, 4H), 3.86 (s, 3H), 4.05 (t, 2H, J = 6.2 Hz), 6.76 (t, 1H, J = 6.6 Hz), 6.84−7.03 (m, 6H), 7.15 (app t, 1H), 7.60 (d, 1H, J = 8.8 Hz), 7.78 (s, 1H), 7.85−7.90 (m, 2H), 8.10 (d, 1H, J = 6.9 Hz). Mp 128−130 °C (from $CHCl_3$/*n*-hexane). Anal. ($C_{28}H_{32}N_4O_2$) C, H, N.

Fluorescence Spectroscopy. Emission and excitation spectra of **15a**,**b**−**22a**,**b** were recorded as detailed in Supporting Information. Fluorescence quantum yields were calculated in reference to that of 2-aminopyridine in ethanol as a standard (excitation wavelength, 285 nm; Φ = 0.37),[29] according to Demas et al.[32]

Fluorescent Labeling of CHO Cells. Fluorescence microscopy observation in CHO cells overexpressing human D_3 receptors was performed as previously described (see experimental details in Supporting Information).[4]

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Supporting Information Available: Procedure for the syntheses and spectral data of **3**−**9**, **10b**, **11**−**14**; spectral data of **15b**, **16a**,**b**−**18a**,**b**, **19b**, **20a**,**b**−**22a**,**b**; biological methods and statistical analysis; excitation and emission spectra of **15a** and **19a** in ethanol; experimental procedures for fluorescence spectroscopy and fluorescent labeling of CHO cells; elemental analysis data of **15a**,**b**−**22a**,**b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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