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FUNCTIONALIZATION OF A VISCOSITY-SENSITIVE FLUOROPHORE FOR PROBING OF BIOLOGICAL SYSTEMS

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Abstract: Functionalization of a viscosity-sensitive visible wavelength fluorophore 2-(1,1-dicyanopropenyl-2)-6-dimethylaminonaphthalene (DDNP), with the intent to incorporate its favorable optical properties into a probe for structural and functional imaging by fluorescence microscopy, is described. Spiperone, a highly potent ligand for the dopamine D_2 receptors, was conjugated via an ethylpiperazine moiety to the fluorophore giving fluorescent probes that can be excited in the UV and Vis range.

Introduction: In the search for novel fluorescent probes for use with fluorescence microscopy we have prepared 2-(1,1-dicyanopropenyl-2)-6-dimethylaminonaphthalene (DDNP),[1] which exhibits unique optical properties. DDNP can be efficiently excited in the visible part of the spectrum between 440 and 490 nm, which is compatible with currently commerçially available laser sources and microscope optics. At these wavelengths autofluorescence, resulting from endogenous fluorescent compounds in cells, is minimum, thus contributing little to the background signal. Moreover, DDNP is environmentally sensitive, i. e., it is poorly fluorescent in water, but its fluorescence increases in viscous environments and when bound to cell membranes or proteins. This property dramatically increases the signal to noise ratio of the recorded image and allows for the omission of the extensive washing of the preparation after staining.

Most commercially available fluorescent probes can be excited in the UV range but only a few can be excited in the Vis range and are environmentally sensitive.[2] Based on DDNP, we have developed compounds that can have ligands of choice attached to their structure thus making them selective fluorescent probes for labeling a specific cell function and/or organelles. Because DDNP does not contain a reactive functional group that could be used to attach a ligand without perturbing the electronic and geometric structure of the fluorophore, we envisaged its functionalization by substituting the dimethylamino group with a substituted amine, bearing a side chain which could be used for the attachment of a ligand of choice. To test the idea, piperazine was chosen as the amine and the neuroleptic spiperone, a dopamine-D_2 receptor antagonist, as the ligand. We initially assumed that the electronic effects of various disubstituted naphthyl amino derivatives would not differ significantly

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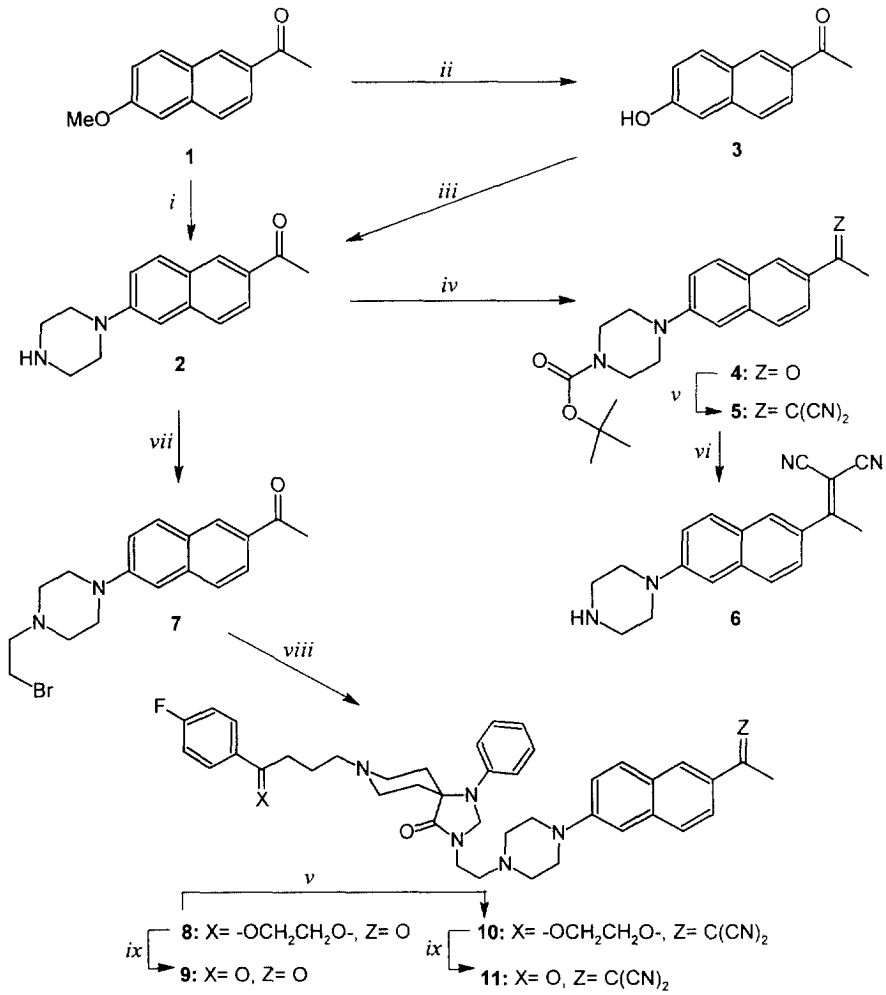
from the effects of the dimethylamino group, but cognizant of the fact that not only electronic, but also steric effects play an important role in fluorescence processes in similar molecules.[3]

Chemistry: An analogous approach to the one used in preparation of DDNP, i. e. methoxy group substitution in 1-(6-methoxy-2-naphthyl)-1-ethanone (**1**) by the lithium salt of piperazine in anhydrous toluene - hexamethylphosphoric triamide (HMPT) mixture was successful only after thorough drying of the commercial anhydrous piperazine. It has been found that HMPA decomposed under the reaction condition giving dimethylamine.[4] The latter can compete with the intended amine in the reaction with compound **1**, giving the corresponding dimethylamino compound. In our case the dimethylamino derivative was isolated as a side product, although it was, due to it's lower polarity, easy to separate it from compound **2**. This prompted us to seek other possibilities for the preparation of substituted aminonaphthalenes. The transformations of an aminonaphthalene derivative with β,β-dihalo-diethylamine[5] to form the piperazine ring, although possible, was not attempted because of the lack of regioselective reactions that would result in the appropriate 2,6-disubstituted naphthalenes. Moreover, some of the intended intermediates, like 2-naphthylamine, are carcinogenic. Another alternative was the Bucherer reaction starting from the corresponding naphthol and piperazine.[6] The Bucherer reaction is proven to be versatile in the preparation of such compounds[7] and highly regioselective. First, we developed an improved method for the preparation of 1-(6-hydroxy-2-naphthyl)-1-ethanone (**2**).[8] Acid hydrolysis of the methoxy compound **1** in a large excess of boiling HCl gave a high yield of compound **2**.[9] Reaction of compound **3** with an excess of piperazine hydrate in the presence of sodium sulphate(IV) gave **2** in moderate yield.

When we attempted the Knoevenagel reaction of 1-(6-piperazino-2-naphthyl)-1-ethanone (**3**) with malononitrile in pyridine, we isolated the desired compound **6** in a very low yield. We assumed that the free NH group in the piperazine ring catalyzed the decomposition of **6**. Protection of the free NH group with a *tert*-butyloxycarbonyl group produced a compound (**4**), that proceeded to react smoothly with malononitrile in pyridine to give compound **5** in 77 % yield. Treatment with TFA at room temperature for 5 minutes removed the protecting group and afforded compound **6** as a single product. Compound **6** was an appropriately substituted amine for further functionalization; unfortunately it proved to be unstable at room temperature. Nevertheless, we tried to react freshly prepared **6** with bromoethylspiperone ketal, but only an unidentified tarry mixture was obtained.

Because of the instability of the intermediate **6**, we decided to attach the fluorophore in its acetyl form (**2**) to the ligand, followed by the Knoevenagel reaction. To achieve this we reacted compound **2** with 1,2-dibromoethane to yield **7**, which reacted smoothly with the spiperone ketal[10] to give compound **8**. Deketalyzation produced a new fluorescent probe **9** with an excitation maximum at approximately 340 nm. We characterized the spiperone moiety containing compounds by their respective NMR and HRMS. Transformation of **8** into **9** was demonstrated by changes in the ^{1}H NMR spectrum. The lack of the two multiplets at approximately 3.75 and 4.00 ppm, corresponding to the ethylene protons of the ketal protecting group, and the downfield shift of the signals, corresponding to fluorophenyl group protons in ortho position to the newly formed carbonyl group in

the spectrum of **9**, supported the proposed structure. After the Knoevenagel reaction of compound **8** with malononitrile in pyridine, a fluorescent compound **10**, that can be excited at approximately 400 nm was obtained. The removal of the ketal protective group finally gave a yellow-orange probe **11**, which was stable under the reaction conditions, but slowly decomposed if kept neat or in a dichloromethane solution at room temperature. If the dichloromethane solutions contained 1-5 % methanol the sample could be kept in a freezer for months without significant decomposition.



i: anh. piperazine, Li, HMPT/toluene; *ii*: refl. conc. HCl[9]; *iii*: piperazine hydrate, $NaHSO_3$; *iv*: NaOH, *n*-Bu_4NHSO_4, di-*tert*-butyl dicarbonate, H_20/toluene; *v*: malononitrile, pyridine; *vi*: TFA, r.t.; *vii*: NaOH, *n*-Bu_4NHSO_4, 1,2-dibromoethane, H_20, r.t.; *viii*: NaOH, *n*-Bu_4NHSO_4, spiperone ketal, H_20/toluene; *ix*: HCl, MeOH

Scheme 1.

Fluorescence: In **Table 1** optical properties of selected compounds are compared with those of DDNP and ADMAN. As observed in the case of ADMAN and DDNP,[1] absorption, excitation and emission maxima shifted to lower energies with acetyl- to 1,1-dicyanopropenyl-group conversion.

We have already shown that in the solid state the dimethylamino group in DDNP was practically coplanar with the naphthalene ring, while the 1,1-dicyanopropenyl-group was twisted out of plane.[1] In solution it is assumed that the probability of emission is related to the population of molecules of similar geometry. This is due to low rotation rates of the malononitrile and dialkylamino groups in analogous (*p-N,N*-dialkylaminobenzylidene)-malononitriles in the ground state (5×10^4 and 1×10^7 s^{-1}, respectively) in comparison to the relaxation times of these fluorophores.[11] Presently described compounds differ from ADMAN or DDNP only in the substituent in position 6 of the naphthalene ring. Our results indicate that exchange of the dimethylamino group in DDNP with a substituted piperazino group perturbed in several solvents the electron distribution of the fluorophore as a consequence of size or decreased flexibility, resulting in hypsochromic shifts and diminished solvent polarity dependence of the maxima.

Table 1. Absorption (λ_{max}), Fluorescence Excitation (λ_{ex}) and Emission Maxima (λ_{em},[nm]), Extinction Coefficients (ε, [l cm^{-1} mol^{-1}]) and Quantum Yields (ϕ).

Solvent		DDNP	2	4	5	8	10	11	ADMAN
Hexane	λ_{max} (ε)[a]	412 (25)	331 (16.3)	325 (17.6)	384 (18.6)			391[b]	343 (21)[c]
	λ_{ex}	410				340	395		345[j]
	λ_{em} (ϕ)[d]	468 (2)			505 (3)[e,f]	405[f,g]	474 (2.4)[f,h]		394 (30)[c]
CH_3CN	λ_{max} (ε)[a]	428 (19)	343 (17.4)	338 (14.4)	395 (16.6)			402 (16.4)	352 (18)
	λ_{ex}	428			396	343	402		355
	λ_{em} (ϕ)[d]	600 (53)			598 (46)[e,f]	454[f,g]	598 (25)[f,h]		492 (700)
MeOH	λ_{max} (ε)[a]	426 (20)	344 (15.0)	341 (14.3)	395 (16.9)			401 (14.8)	366 (18)
	λ_{ex}	428			396	346	400		365
	λ_{em} (ϕ)[d]	610 (13)			602 (21)[e,f]	505[f,g]	606 (18)[f,h]		506 (480)
H_2O	λ_{max} (ε)[a]	385 ([b])	332 (9.1)[i]	327 (1.6)[i]	436 (11.5)			423 (14)[i]	355 ([b])
	λ_{ex}	393					405; 428		360
	λ_{em} (ϕ)[d]	529 (15)			586 (22)[e,f]	461[f,g]	587 (27)[f,h]		528 (220)

[a] $\varepsilon\times10^{-3}$; [b] concentration not determined; [c] in cyclohexane; [d] $\phi\times10^3$, Quantum yields determined against Rhodamine 101 (ϕ=1)[12]; [e] 1 % CH_3CN; [f] λ_{ex} = 420 nm; [g] 0.2 % CH_3CN; [h] 0.25 % CH_3CN; [i] 0.5 % MeOH; [j] 0.2 % MeOH;

Binding assays:[13] IC_{50} assays of compound **11** against ^{3}H-spiperone (**Figure 1**) revealed its quite high affinity, at approximately 1 order of magnitude lower than spiperone.[14] This confirms previous findings that modification of spiperone with alkylation at the *N*-3 position produces minimal effects on its binding properties.[15] Since **2** structurally resembles quipazine, a high affinity ligand for serotonergic $5HT_2$ receptors,[16] and there is a significant overlap between $5HT_2$ and D_2 ligand structure,[17,18] we assayed compound **2** for D_2 competition against ^{3}H-spiperone. No significant binding was observed up to 1×10^{-4} molar concentration.

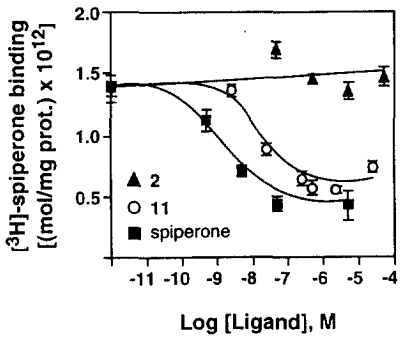


Figure 1. Radioligand binding determination for **2** and **11** against [^{3}H]-spiperone.

Conclusions: We have demonstrated that ligands of known biological activity can be attached regiospecifically to highly sensitive fluorophores (*i.e.* ADMAN, DDNP) to probe specific biological systems. This approach was tested by attaching spiperone, a highly potent ligand for dopaminergic D_2 receptors, to the described ADMAN and DDNP analogues. We have shown that such functionalization did not significantly diminish binding properties of the ligand. Also we have shown that piperazinylnaphthyl substitution can be modulated to induce changes in optical properties, namely, absorption, excitation and emission maxima as a result of steric effects producing likely distortion in the amine moiety, affecting the electronic distribution in the fluorophore. In **11** this resulted in hypsochromic shift of the maxima and less pronounced dependence of the fluorescent properties on the polarity/viscosity of the environment in comparison to DDNP. In addition, the unique fluorescent quantum yield properties of the DDNP fluorophore in viscous environments and in the presence of proteins[1] makes **11** uniquely suitable for its use as a fluorescent probe for the dopamine D_2 receptor. Scope and limitations of this approach utilizing other cyclic and acyclic amines are being investigated.

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13. Binding Assays: LZR- cells[19] were grown in Costar 125 cm^2 cell culture flasks in DMEM, high glucose, 5% Fetal bovine serum, 5% enriched calf serum, 200 mM glutamine, at 37 °C, under 5-7% CO_2 until confluent. Cells were harvested by removal of growth media, briefly washed with Versene, followed by incubation in cold harvest buffer for 5 minutes. The cells were mechanically dissociated and homogenized in a teflon glass Potter-Elvehjem tissue grinder and made to a final volume of 25 mL with harvest buffer, and spun at 100,000×G for 1:15 in a Sorvall 50B Ultracentrifuge to pellet cell membranes. Pellets were frozen in 1mL assay buffer and stored at -80 °C for future use. On the day of the assay, LZR- membranes were diluted to 100 mL volume in assay buffer. Membrane aliquots (1.6 mL) were added to glass 13×100 tubes for competitive binding assays of **2** or **11**, spiperone or buffer blanks against 2.16 nM [3H]-spiperone. Drug concentrations used were: spiperone, 500 fM, 5 nM, 50 nM, 500 nM, 5 μM; compounds **2** or **11**, 5 nM, 50 nM, 500 nM, 5 μM, 50 μM. Assays were conducted at 40 °C in final volume of 2 mL. Tubes were vortexed between and after additions, and incubated for one and a half hours. All tubes were filtered at -40 kPa to -60 kPa vacuum on a Skatron 7000 Cell Harvester through Schleicher & Schuell #32 Glass filters presoaked in 10% poly(ethylenenimine) in deionized water. All tubes were rinsed through the filters with 2× ~2 mL buffer. The filters were kept under vacuum until partially dry and then placed in 5 mL scintillation minivials. Filters were wetted with toluene, followed by addition of 2 mL Insta-Gel scintillation cocktail and counted in a Packard Instruments 1900CA scintillation counter. Protein assays were conducted according to the BCA procedure[20] against bovine serum albumin standard curve in assay buffer.
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