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Marie Auvray, Frédéric Bolze, Delphine Naud-Martin, Matthieu Poulain, Margaux Bossuat, et al.. On the road for more efficient biocompatible two-photon excitable fluorophores. *Chemistry - A European Journal*, 2022, <10.1002/chem.202104378>. <hal-03768554>

HAL Id: hal-03768554

<https://hal.science/hal-03768554v1>

Submitted on 4 Sep 2022

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On the road for more efficient biocompatible two-photon excitable fluorophores

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Supporting information for this article is given via a link at the end of the document.

Abstract: Red to NIR absorption and emission wavelengths are key requirements for intravital bioimaging. One of the way to reach such excitation wavelengths is to use two-photon excitation. Unfortunately, there is still a lack of two-photon excitable fluorophores that are both efficient and biocompatible. Thus, we design a series of biocompatible quadrupolar dyes in order to study their ability to be used for live-cell imaging, and in particular for two-photon microscopy. Hence, we report the synthesis of 5 probes based on different donor cores (phenoxazine, acridane, phenazasiline and phenothiazine) and the study of their linear and non-linear photophysical properties. TD-DFT calculations were performed and were able to highlight the structure-property relationship of this series. All these studies highlight the great potential of three of these biocompatible dyes for two-photon microscopy, as they both exhibit high two-photon cross-sections (up to 3 650 GM) and emit orange to red light. This potential was confirmed through live-cell two-photon microscopy experiments, leading to images with very high brightness and contrast.

Introduction

Two-photon absorption is a widely used non-linear process, from 3D microfabrication to bioimaging.^[1,2] In this case, two-photon absorption allows to reach easily excitation wavelengths in the red or near IR window. It is a key parameter for biological experiments, as red light can go deeper in the tissue and prevent photodamages. Unfortunately, most molecules do not possess the ability to strongly absorb simultaneously two photons. For example, fluorophores that are commonly used for bioimaging like cyanines, BODIPY or rhodamine are poor to moderate two-photon absorbers, with cross-sections around 10 to 100 GM for the most efficient.^[3-5] It is therefore necessary to design specific molecules for this type of absorption. Since the 1990s, several groups have been working on the development of such structures,

especially by establishing relationships between the structure of a molecule and its two-photon absorption properties.^[6-11] Thus, their work underlines the great potential of quadrupolar (D- π A- π -D or A- π D- π A) and octupolar structures for two-photon absorption.^[12,13] This has led to the development of excellent two-photon absorbers, which possess two-photon cross-sections of several thousand GM.^[14,15] However, efficient two-photon absorbers have most of the time a high molecular weight, that make them very hydrophobic, and as a consequence are not biocompatible. Indeed, the two-photon cross-section of a compound depends strongly on the extension of the π -system as it is directly correlated to the distance displacement of the charge during the transition. Thus, a real gap appears between molecules with cross-sections of several thousand GM and those that can actually be used for biological applications.^[16,17] A relevant criteria for evaluating the efficiency of a two-photon excitable fluorophore is the ratio of its cross-section to its molecular weight. Two-photon fluorophores currently used for biological applications often possess cross-section to molecular ratio below 3,^[16,17] whereas most efficient two-photon absorbers possess ratio around 20.^[15,18] It means that biocompatible two-photon absorbers are smaller but as a consequence are also far less efficient. The development of new two-photon excitable fluorophores adapted to bioimaging, *i.e.* "efficient and compact", and which are therefore biocompatible, remains a major challenge. Our lab has been involved in the development of biocompatible two-photon dyes. In this context, we developed divinylpyridiniums dyes based on a quadrupolar design with cross-section to molecular weight ratio up to 5.0 (Figure 1).^[19-21] Unfortunately, they emit in the green range. In this work, we proposed to study a new family of quadrupolar dyes by enriching previous cores, to increase their two-photon absorption properties. Recently, phenoxazine, acridane, phenazasiline and phenothiazine have been used as very efficient donor cores in material chemistry.^[22-26] However, these cores have never been

extensively studied for their non linear properties and we decided to explore their potential to be used as donor cores for two-photon absorption (Figure 1).

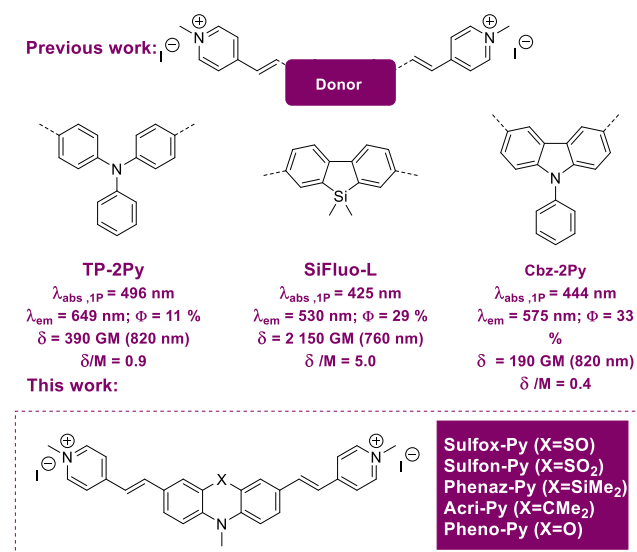


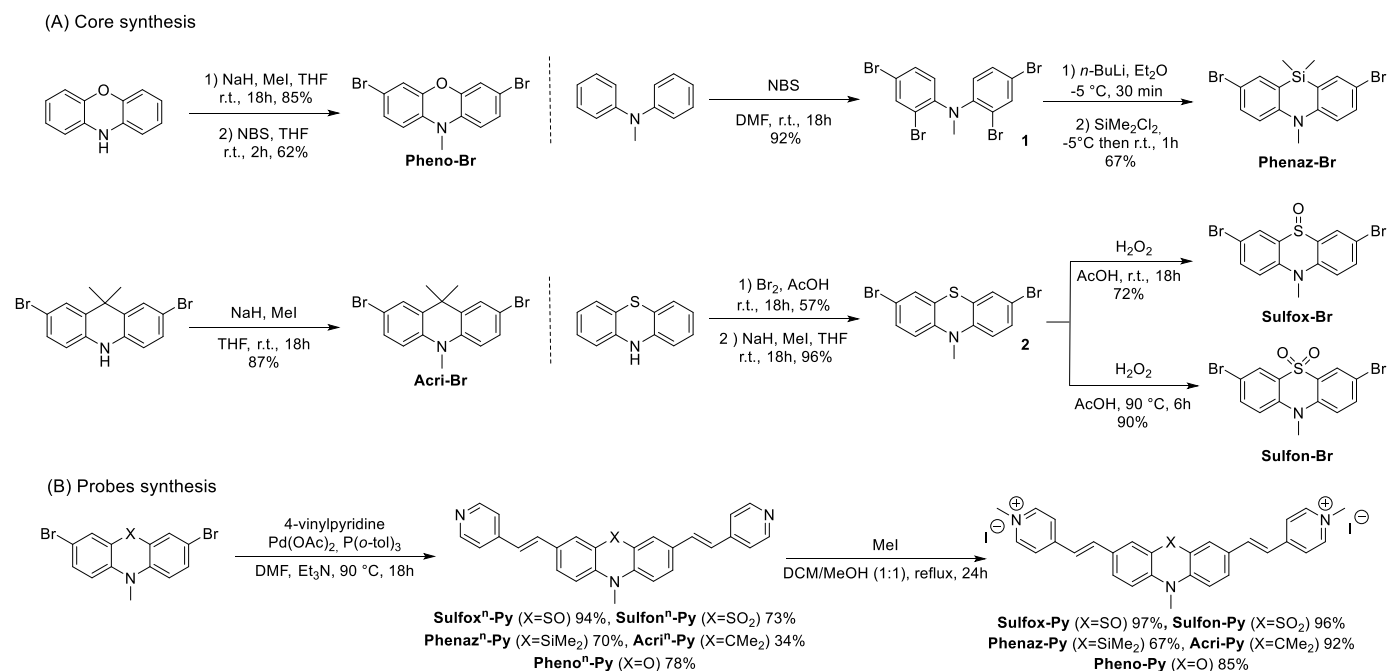
Figure 1. Previous work and goal of this work – photophysical properties are described in glycerol

Results and Discussion

Synthesis

All the probes can easily be synthesized in three to five steps from commercially available precursors. Their synthesis is described in Scheme 1. First, the five cores (Acri-Br, Pheno-Br,

Phenaz-Br, Sulfox-Br and Sulfon-Br) were synthesized through different pathways depending on the available starting materials. Acri-Br was obtained in one methylation step from 2,7-dibromo-9,9-dimethyl-9,10-dihydroacridine with a 87% yield. Pheno-Br was obtained in two steps starting with phenoxazine. Methylation of phenoxazine afforded the corresponding *N*-methylated compound, which was then brominated with NBS in THF with a yield of 62%. Phenaz-Br was obtained through a synthetic pathway previously described in the literature for similar compounds.^[27-29] First, compound 1 was obtained by bromination of *N*-methyl-diphenylamine using NBS in DMF with a very good yield of 92%. A selective halogen-metal exchange on this derivative was performed with *n*-BuLi at -5°C in Et₂O. The addition of dichlorodimethylsilane on the resulting dilithiated intermediate led to Phenaz-Br in a 67% yield. Sulfox-Br and Sulfon-Br were obtained in three steps from phenothiazine. First, compound 2 was obtained in a two-step sequence consisting of a bromination using dibromine in acetic acid followed by a methylation. Depending on the conditions, oxidation of compound 2 using hydrogen peroxide led either to the sulfoxide Sulfox-Br (overnight at r.t.) or to the sulfone Sulfon-Br (by heating at 90 °C for 6h) respectively in 72% and 90% yields. A Heck reaction was then performed on all brominated derivatives, leading to the corresponding vinylpyridines with yields between 34% for Acriⁿ-Py to 94% for Sulfoxⁿ-Py. Methylation of the two pyridine moieties by methyl iodide afforded the desired probes Sulfox-Py, Sulfon-Py, Phenaz-Py, Acri-Py and Pheno-Py with yields between 67% for Acri-Py to 97 % for Sulfox-Py. To conclude, all the probes were obtained in three to five steps with global yields from 27% for Acri-Py to 36% for Sulfox-Py.



Scheme 1. Synthetic pathways of Sulfox-Py, Sulfon-Py, Phenaz-Py, Acri-Py and Pheno-Py

Table 1. Photophysical properties of Sulfon-Py, Sulfox-Py, Phenaz-Py, Acri-Py and Pheno-Py in glycerol and in water

Compound	Solvent	ϵ ($M^{-1}.cm^{-1}$)	$\lambda_{abs,MAX}$ (nm)	$\lambda_{em,MAX}$ (nm)	Φ_F (%)	Brightness ($M^{-1}.cm^{-1}$)
Sulfon-Py	Water	80 900	421	556	1	810
	Glycerol	56 200	432	547	24	13 500
Sulfox-Py	Water	69 800	431	571	1	700
	Glycerol	52 500	443	556	23	12 100
Phenaz-Py	Water	55 400	472	620	< 0.1	< 55
	Glycerol	49 600	490	610	11	5 500
Acrid-Py	Water	56 800	483	665	< 0.1	< 57
	Glycerol	51 300	505	627	9	4 600
Pheno-Py	Water	39 500	526	688	< 0.1	< 40
	Glycerol	34 300	544	664	1.5	520

Linear optical properties

The optical properties of all dyes have been investigated in water ($\mu = 1.002 \times 10^{-3}$ Pa.s at 20 °C), and in glycerol ($\mu = 1.420$ Pa.s at 20 °C) at room temperature. These data are summarized in Table 1. All the dyes are water soluble as they obey Beer Lambert's law in water. They show a maximal absorption between 421 and 526 nm and strong molar absorption coefficients (from 39 500 for Pheno-Py to 80 900 $M^{-1}.cm^{-1}$ for Sulfon-Py). Apart from the oxidized sulfur-containing derivatives, they are all quite non fluorescent in water ($\Phi_F < 0.1\%$) with emission wavelengths ranging from green to far-red for Pheno-Py ($\lambda_{em,MAX} = 688$ nm). Even though Sulfo-Py and Sulfox-Py fluoresce poorly in water ($\Phi_F = 1\%$), they show a non-negligible brightness (810 $M^{-1}.cm^{-1}$ for Sulfon-Py and 700 $M^{-1}.cm^{-1}$ for Sulfox-Py), due to their high molar absorption coefficients in this solvent (80 900 $M^{-1}.cm^{-1}$ for Sulfon-Py and 69 800 $M^{-1}.cm^{-1}$ for Sulfox-Py). We studied the photophysical properties of the dyes in glycerol as, thanks to its high viscosity, it could mimic a constrained environment, for example the immobilization of the probe in its biological target. Normalized absorption and emission spectra in glycerol are shown in Figure 2. As in water, their range in absorption and emission wavelengths are different according to their core. Sulfon-Py and Sulfox-Py absorb around 435 nm and emit green light with high brightnesses (12 000 and 13 500 $M^{-1}.cm^{-1}$) thanks to their good molar absorption coefficients and good quantum yields. Even though they are fluorescent in water, they have very good turn-on ratios between water and glycerol. Hence, they should fluoresce strongly once their biological target is reached, with a good signal-to-noise ratio. However, their low emission wavelengths in the green region could be a drawback for bioimaging. Phenaz-Py and Acrid-Py absorb at slightly higher wavelengths (around 500 nm) and emit orange-to-red light with good fluorescence quantum yield (11 and 9% respectively). Silicon and carbon atoms confer similar features to the central core of the dyes. However, Acrid-Py shows a slight bathochromic shift for absorption and emission compared to Phenaz-Py (490 nm and 610 nm for Phenaz-Py and 505 nm and 627 nm for Acrid-Py). This is logically accompanied by a decrease in quantum yield and therefore in brightness. Both compounds are promising for bioimaging with very good brightness in glycerol (5 500 $M^{-1}.cm^{-1}$ for Phenaz-Py and 4 600 $M^{-1}.cm^{-1}$ for Acrid-Py) and low brightness in water, as well as red emission (620 nm for Phenaz-Py and 665 nm Acrid-Py in water). The only limitation is that they absorb in the blue. However, this can be overcome by a biphotonic absorption process. Finally, Pheno-Py is the most red-shifted compound both

in absorption ($\lambda_{abs,MAX} = 526$ nm) and emission ($\lambda_{em,MAX} = 664$ nm). Although Pheno-Py emits in the far red, its very low quantum yield in glycerol (1.5%) and its moderate molar absorption coefficient (around 30% lower than the others) make it an unattractive probe. Its brightness only reaches 520 $M^{-1}.cm^{-1}$ in glycerol.

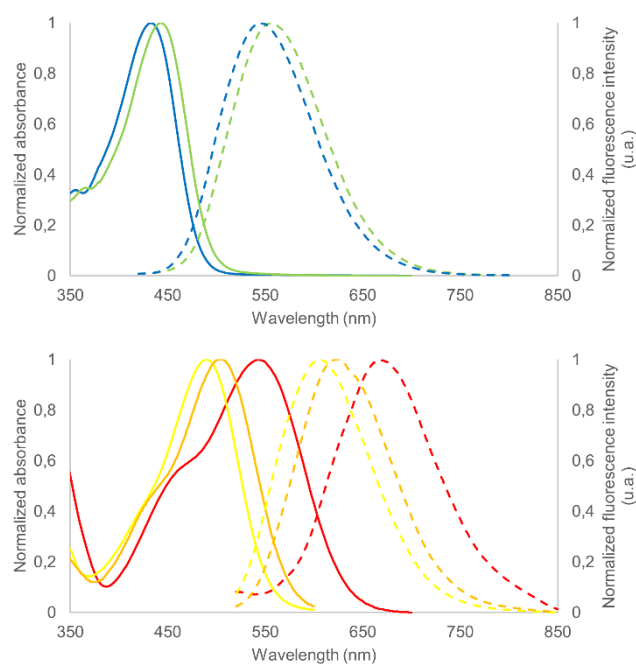


Figure 2. Normalized absorption (solid lines) and emission spectra (dotted lines) in glycerol of Sulfon-Py (Blue), Sulfox-Py (Green), Phenaz-Py (Yellow), Acrid-Py (Orange) and Pheno-Py (Red).

Unlike previous studies on xanthene core,^[30] the modification of the central atom by several heteroatoms does not lead to impressive red-shift. We even observed the contrary effect as the sulfone analog displays the lowest absorption/emission wavelengths whereas the sulforosamine core emits in the far red.^[30]

Two-photon absorption properties

The two-photon excitation spectra of all probes were recorded in glycerol at room temperature, as for the linear photophysical properties, and performed in duplicate (Figure 3) by the TPEF method (Two-photon excited fluorescence). The experiments cannot be performed in water, as the TPEF method

requires fluorescent compounds. First of all, we confirmed the quadratic dependence of the process by measuring the power dependence of the emission (Supporting Information, Figure S1 and Table S1). All data are summarized in Table 2.

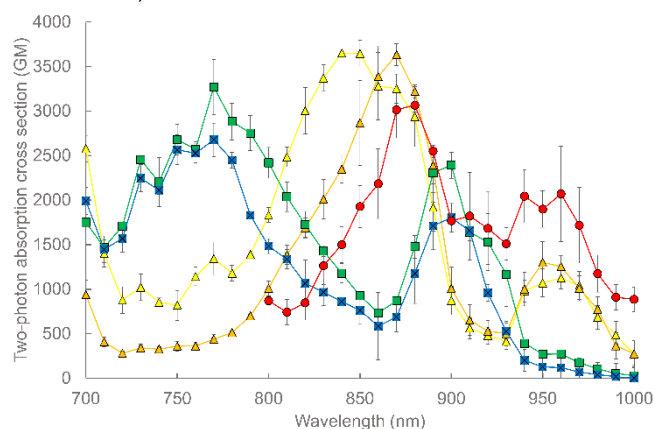


Figure 3. Two-photon excitation spectra of Sulfon-Py (Blue), Sulfox-Py (Green), Phenaz-Py (Yellow), Acri-Py (Orange) and Pheno-Py (Red).

Table 2. Two-photon absorption properties of Sulfon-Py, Sulfox-Py, Phenaz-Py, Acri-Py and Pheno-Py in glycerol

Compound	$\lambda_{\max,2PA}$ abs (nm)	δ_{\max} (GM)	δ_{\max}/M (GM.mol.g ⁻¹)	Φ (%)	TP Brightness $\delta_{\max} \cdot \Phi$ (GM)
Sulfon-Py	770	2 680	5.6	24	640
	900	1 800	3.7		430
Sulfox-Py	770	3 270	7.0	23	750
	900	2 400	5.2		550
Phenaz-Py	840	3 650	7.7	11	400
	970	1 260	2.7		140
Acri-Py	870	3 630	8.0	9	330
	950	1 300	2.7		120
Pheno-Py	880	3 070	7.1	1.5	50
	960	2 070	4.8		30

All compounds show very high absorption cross sections in glycerol: they all have a maximum above 2 500 GM.

All dyes display two absorption bands: an intense one around 770-880 nm and a lower one around 900-960 nm. This is typical of V-shaped non centrosymmetric quadrupolar dyes.^[31-32] The lower energy band corresponds to the transition of a strongly one-photon (and weakly two-photon) allowed excited state (Supporting Information, Figure S4). This reflects the dipolar contribution which is weak in the case of two-photon absorption. Conversely, the higher energy band corresponds to a transition to a strongly two-photon (and weakly one-photon) allowed excited state. This band represents the predominant quadrupolar contribution to two-photon absorption.

Sulfon-Py and Sulfox-Py have two absorption bands: one with a maximum at 770 nm (2 680 GM for Sulfon-Py and 3 270 GM for Sulfox-Py), and a second with a maximum at 900 nm (1 800 GM for Sulfon-Py and 2 400 GM for Sulfox-Py). Sulfon-Py has slightly lower cross-sections, which is consistent with the poorer donor nature of the core (sulfone versus sulfoxide). Both compounds also display very high two-photon brightnesses in glycerol (640

GM for Sulfon-Py and 750 GM for Sulfox-Py at 770 nm). Even if they exhibit the highest brightnesses of this series, their main drawback remains their green emission.

Phenaz-Py, Acri-Py and Pheno-Py have a first band around 860 nm and a second around 950 nm. These compounds have high two-photon brightnesses (330 GM at 870 nm for Acri-Py and 400 GM at 840 nm for Phenaz-Py) except for Pheno-Py because of a low fluorescence quantum yield. The two-photon properties and orange-to-red emission of Phenaz-Py and Acri-Py make them good candidates for two-photon fluorescence microscopy.

The dyes can be ranked thanks to their two-photon cross-section as follows: $\delta(\text{Sulfon-Py}) < \delta(\text{Pheno-Py}) < \delta(\text{Sulfox-Py}) < \delta(\text{Phenaz-Py}) \approx \delta(\text{Acri-Py})$. Following the intuitive expectations, we found the two-photon absorption cross-section is larger for electron-rich cores. For Pheno-Py, the ranking is hardest to rationalize as the mesomeric effect and the inductive effect of the oxygen atom are opposite.

The δ/M ratios of all the dyes are more than twice as high as those reported for biocompatible dyes. This good balance between impressive two-photon cross-sections and moderate molecular weights is an additional asset for their use in cellular imaging.

Molecular modeling

In order to better understand the photophysical properties of this serie of dyes, we performed TD-DFT calculations (M06-2X/6-311+G(d,p) level of theory) including solvent effect (water). For all the probes, the calculated absorption wavelengths are in agreement with the experimental data: relative deviations between these 2 values range from 1% for Sulfox-Py to 5% for Acri-Py (Table 3). It allows to validate the choice of the computational method. For all probes, the major contribution to the transition $S_0 \rightarrow S_1$ is HOMO \rightarrow LUMO (SI, Table S2). In all cases the electronic density of the HOMO is mostly located on the donor core whereas the electronic density of the LUMO is mostly located on the two branches (Figure 4). It is consistent with the quadrupolar design A- π -D- π -A of the probes and their typical charge transfer transitions. The energy of the LUMO of these probes are only slightly different. Thus, the differences in $\Delta E(\text{HOMO-LUMO})$ for all probes can be mainly explained by the energy of the HOMO. As all HOMOs have a similar shape, this difference could be explained by the inductive effect of the central atom. In fact, the attracting groups (SO and SO₂) will tend to lower the energy of the HOMO while the donor groups (SiMe₂ and CMe₂) will tend to increase its energy. The HOMO of Pheno-Py is higher in energy because of an additional anti-bonding interaction close to the oxygen atom. Concerning the two-photon absorption properties, for quadrupolar compounds, they can be correlated with transition dipole moments from S_0 to S_1 and from S_1 to S_2 , and the energetic gap $\Delta = (E(S_1) - E(S_2))/2$ according to formula (1).^[33]

$$\delta_{\max} \propto \frac{\mu_{S_0 \rightarrow S_1}^2 \times \mu_{S_1 \rightarrow S_2}^2}{\Delta^2} \quad (1)$$

Thus, we calculated these transition dipole moments for all probes. Results are shown in Table 3. For Sulfon-Py, Sulfox-Py, Phenaz-Py and Acri-Py, calculated results are consistent with experimental data. The ranking of δ_{\max} agrees with calculations for all molecules, except for Pheno-Py. Neither the change in basis set nor the change in functional led to a better agreement with experimental values for this probe (Supporting Information, Table S3 and Table S4).

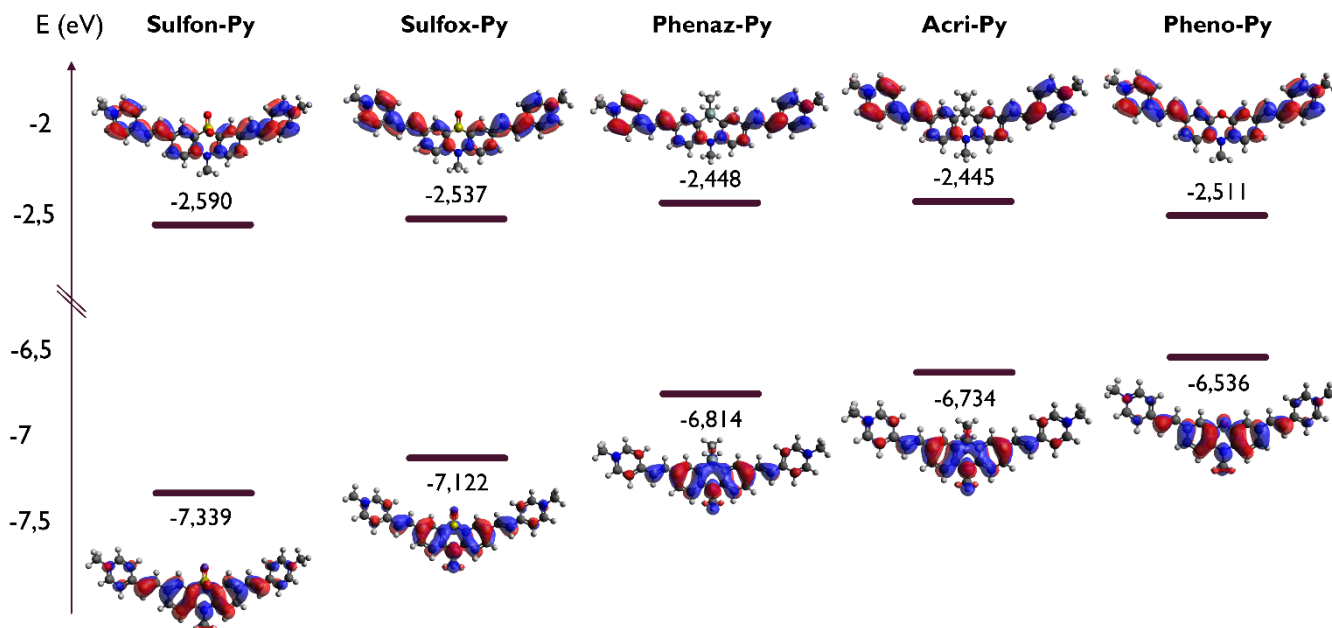


Figure 4. Frontiers molecular orbitals of Sulfon-Py, Sulfox-Py, Phenaz-Py, Acrid-Py and Pheno-Py (6-311+G(d,p)/M06-2X)

Table 3. Comparison of experimental and calculated photophysical properties (6-311+G(d,p)/M06-2X)

Compound	$\lambda_{\text{abs, MAX, water exp}}$ (nm)	$\lambda_{\text{abs, MAX, water th}}$ (nm)	$\mu_{S_0 \rightarrow S_1}$	$\mu_{S_1 \rightarrow S_2}$	Δ (eV)	$\frac{\mu_{S_0 \rightarrow S_1}^2 \times \mu_{S_1 \rightarrow S_2}^2}{\Delta^2}$	$\delta_{\text{MAX, exp}}$ (GM)
Sulfon-Py	407	421	5.56	5.37	0.47	4 036	2 680
Sulfox-Py	425	431	5.28	5.96	0.47	4 483	3 270
Phenaz-Py	450	472	5.81	6.75	0.47	6 962	3 650
Acrid-Py	460	483	5.58	6.72	0.48	6 103	3 630
Pheno-Py	505	526	4.99	7.91	0.55	5 150	3 070

Live-cell imaging

The ability of these probes to be used for live-cell imaging was assessed through one-photon and two-photon experiments on A549 live cells (lung carcinoma epithelial cells). To do so, we chose Acrid-Py because this probe offers the best compromise between emission wavelength (665 nm in water) and biphotonic brightness (330 GM at 870 nm). First, its biocompatibility was evaluated by a MTT assay (Supporting Information, Figure S2). This probe has no significant effect on cell viability, as its IC_{80} is higher than $10 \mu\text{M}$. A549 live cells were incubated with $2 \mu\text{M}$ of Acrid-Py for 2h. Images were then acquired after one (at 496 nm) or two-photon excitation (850 or 950 nm). In all cases, images with high brightness and contrast were obtained (Figure 5). First, it indicated that Acrid-Py was able to cross the membrane and enter the cell without any permeabilization step. It is also consistent with the high one-photon ($4\,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 505 nm) and two-photon brightnesses (330 GM at 870 nm) measured *in vitro* in a model solvent. In addition, this compound is photostable, as more than 80% of the signal remains after a 10-min acquisition under two-photon excitation, during which an image was recorded each 30 sec (Supporting Information, Figure S3).

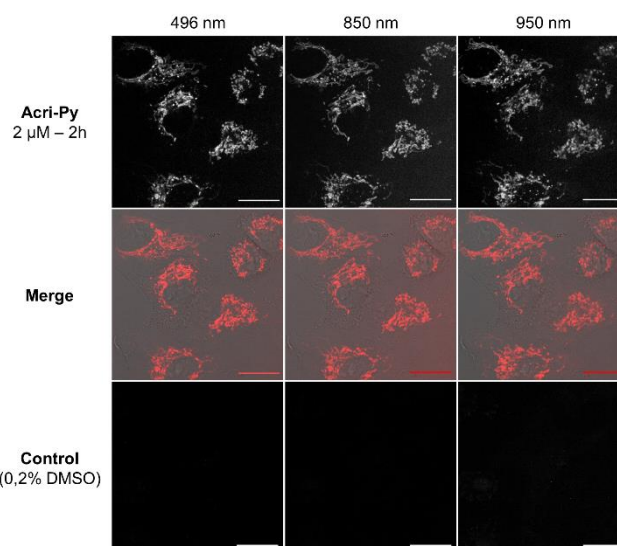


Figure 5. Confocal microscopy imaging of live A549 cells incubated with Acrid-Py at $2 \mu\text{M}$ during 2h under one (496 nm) or two-photon excitation (850 nm or 950 nm). Emission slit settings: 500-700 nm. Merge corresponds to the superimposition of the confocal fluorescence colored in red and the brightfield images. Scale bar: $20 \mu\text{m}$.

Conclusion

We developed 5 quadrupolar probes: Sulfon-Py, Sulfox-Py, Phenaz-Py, Acrid-Py and Pheno-Py (Figure 6).

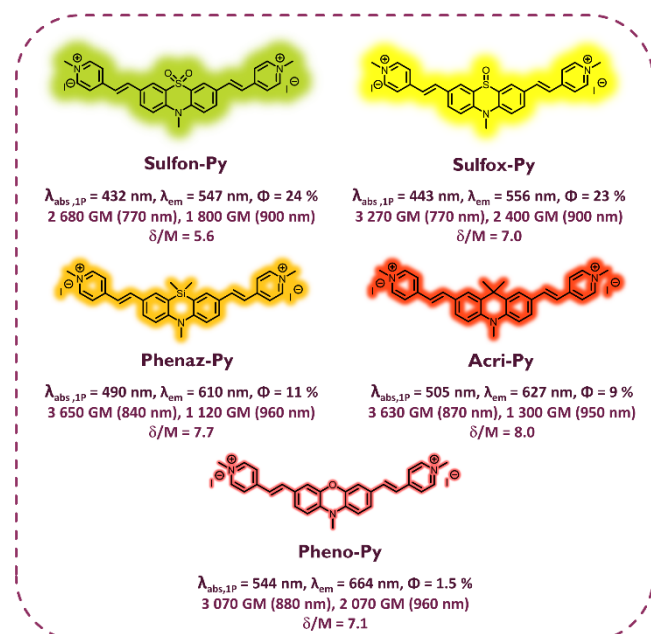


Figure 6. Structures of all the probes and their photophysical properties in glycerol

Their syntheses were easily carried out within three to five steps with global yields between from 27% for Acrid-Py to 36% for Sulfox-Py. The photophysical studies highlight the great potential of these probes to be used for two-photon live-cell imaging. Indeed, these water-soluble dyes exhibit high two-photon cross-sections (from 2 680 GM at 770 nm for Sulfon-Py to 3 650 GM at 840 nm for Phenaz-Py) and therefore high two-photon brightnesses. These dyes possess impressive δ/M ratio (up to 8.0) compared to commonly used biocompatible dyes. In addition, three of them emit orange to red light. Among these probes, we chose to further investigate the properties of Acrid-Py, as this probe offers the best compromise between emission wavelength (665 nm in water) and biphotonic brightness (330 GM at 870 nm). Its biocompatibility was assessed through an MTT Assay and this probe shows no significant effect on cell-viability. Thus, we were then able to perform live-cell imaging under one or two-photon excitations, either at 850 nm or 950 nm, obtaining images with high brightness and contrast. All these studies also pointed out the behaviour of Pheno-Py, showing a high two-photon absorption cross-section of 3 070 GM, associated with a very low fluorescence quantum yield. This makes us wonder about other possible applications such as two-photon photodynamic therapy, as its low quantum yield suggest an ability to access to the triplet state, as for its sulphur analogue, phenothiazine.^[34]

Acknowledgements

M. A. thanks the Ministère de l'Enseignement Supérieur et de la Recherche for her doctoral fellowship. The authors greatly acknowledge the Multimodal Imaging Center-Light Microscopy Facility (Institut Curie, CNRS UMS2016, Inserm US43, Université

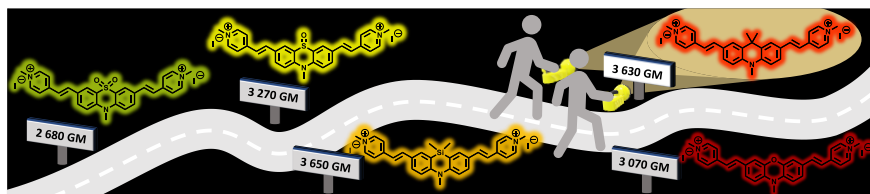
Paris-Saclay) particularly Laetitia Besse and Dr. Marie-Noëlle Soler for their technical assistance in the use of the Leica confocal SP5 system. This work has benefited from the facilities and expertise of the Small Molecule Mass Spectrometry platform of ICSN (Centre de Recherche de Gif - www.icsn.cnrs-gif.fr). Theoretical calculations were performed using HPC resources from the "Mésocentre" computing center of CentraleSupélec and École Normale Supérieure Paris-Saclay supported by CNRS and Région Île-de-France (<http://mesocentre.centralesupelec.fr/>).

Keywords: Fluorescent probes • Live-cell imaging • Molecular Modeling • Quadrupolar structures • Two-photon absorption

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Entry for the Table of Contents



Two-photon absorption is a widely used process for bioimaging, as it allows to go deeper in tissue than conventional absorption and also limit photodamages. Unfortunately, classical dyes possess poor two-photon cross-sections. In this work, we developed a series of quadrupolar dyes and showed that these biocompatible probes exhibit high two-photon cross-sections (up to 3 650 GM) and are therefore good candidates for two-photon bioimaging.