Modulating photostability and mitochondria selectivity in far-red/NIR emitting coumarin fluorophores through replacement of pyridinium by pyrimidinium.

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ABSTRACT

Mitochondrial dysfunction has been associated with several human pathologies, including cancer, aging and neurodegenerative diseases. Thus, the availability of selective fluorescent probes for mitochondria could play an important role in the future for monitoring cellular function and disease progression. In this work, we have studied how the photophysical properties and subcellular accumulation of nonconventional coumarin-based COUPY fluorophores can be fine-tuned through replacement of the para-pyridinium moiety with several heterocycles. Among them, ortho,para-pyrimidinium substitution provided novel fluorophores with suitable photophysical properties for bioimaging applications, including emission in the far-red to NIR region, large Stokes’ shifts and high photostability. Furthermore, the compounds exhibited excellent cell membrane permeability in living cells and a higher selectivity for mitochondria compared with the parent COUPY fluorophores. Overall, these results provide useful insights for the development of novel mitochondria-targeted fluorescent probes based on small organic molecules, since higher selectivity for this organelle can be achieved through the replacement of conventional N-alkylated pyridinium moieties by the corresponding N-alkylated-ortho,para-pyrimidinium counterparts.
INTRODUCTION

Fluorescence imaging in combination with organic fluorophores has become a powerful tool for understanding of biological events at a molecular level. In this context, the use of fluorescent probes with operability in the less energetic far-red and near-infrared (NIR) region of the electromagnetic spectrum offers several appealing features for in vivo imaging applications, such as increased tissue penetration depth and minimal autofluorescence interference from natively occurring biomolecules. For this reason, recent efforts have been devoted to the development of novel organic chromophores operating in the phototherapeutic window with optimal photophysical (e.g., long-wavelength absorption and emission, brightness, large Stokes’ shifts and photostability) and physicochemical (e.g., aqueous solubility, good cell permeability and target specificity) properties. Ideally, such fluorescent compounds should be based on chemically stable, low molecular weight scaffolds amenable to smart and simple structural modifications to fine-tune the abovementioned properties as well as to facilitate conjugation to targeting ligands. Besides biocompatibility, an ideal fluorophore should permit the interrogation of intracellular architectures and dynamics without disturbing and compromising the integrity of the cellular target.

Mitochondria are involved in many key cellular processes, including ATP synthesis, calcium signaling and redox homeostasis. Mitochondrial dysfunction has been associated with a wide range of human pathologies, such as cancer disease, aging and metabolic and neurodegenerative diseases. Thus, the availability of fluorescent dyes that can stain selectively mitochondria opens the door to monitoring cellular function and disease progression by studying mitochondrial morphology and mitophagy. Among fluorescent mitochondrial probes described to date, some of them intrinsically target this organelle such as some cyanine derivatives (e.g., IR-780 and MHI-148) and some rhodamines (Rhodamine 123). Another approach to confer mitochondria selectivity consists of incorporating lipophilic positively charged moieties such as triphenylphosphonium or pyridinium groups, which exploit the negative potential across outer and inner mitochondria membrane. In this context, anticancer mitochondria-targeted fluorescent molecules are considered attractive theranostic agents. Similarly, mitochondria-targeted photocages based on organic chromophores provide a powerful method for releasing bioactive compounds within this organelle.

In our group, we have recently described a new class of coumarin-based chromophores in which the carbonyl group of the electron-withdrawing lactone in conventional coumarin
was replaced by cyano(4-pyridine)methylene moiety (e.g., compounds 2 and 3; see Scheme 1) with the aim of increasing the push-pull character of the π-delocalized system.\textsuperscript{11} N-Alkylation of the pyridine heterocycle provides low molecular weight fluorophores, nicknamed COUPYs, with several attractive characteristics, including emission in the far-red/NIR region, large Stokes’ shifts and good brightness.\textsuperscript{11} Moreover, COUPY dyes (e.g., see compounds 4 and 5 in Scheme 1 as representative examples) exhibited moderate to good aqueous solubility and excellent cell membrane permeability, and accumulate preferentially in two specific cellular compartments, mitochondria and nucleoli. After the discovery of this promising fluorescent platform, we have initiated a systematic study to unveil the principles governing the structure-photophysical property relationships (SPPR) of COUPY dyes, which are necessary to design new fluorescent probes according to need. Compared with conventional coumarins (e.g., 1), the molecular framework of COUPY scaffolds offers several advantages for carrying out a systematic SPPR study. Indeed, we have found that absorption and emission maxima can be red-shifted through the incorporation of strong electron-withdrawing groups like CF\textsubscript{3} either at the 4-position or via N-alkylation of the pyridine heterocycle,\textsuperscript{11} while photostability can be increased through replacement of the electron-donating N,N-dialkyl groups (e.g., NMe\textsubscript{2} or NEt\textsubscript{2}) at position 7 with azetidine.\textsuperscript{12} In addition, conjugatable versions of COUPY dyes can be easily obtained by incorporation of suitable functional groups (e.g., carboxylic acid, amino, azide or alkyne) via N-alkylation of the pyridine moiety. Such COUPY derivatives allowed us to label receptor-binding peptides on solid-phase by using efficient click chemistry methodologies,\textsuperscript{13} and to develop novel photosensitizers for photodynamic anticancer therapy via conjugation to cyclometalated Ir(III) complexes.\textsuperscript{14}

On the basis of all these precedents, in this work we focused on investigating how the modification of the pyridine moiety in COUPY chromophores could influence the photophysical properties and subcellular accumulation of the compounds. With this idea in mind, herein we describe the synthesis and characterization of four analogues (compounds 10-13, Scheme 1) of our original COUPY dyes (4-5). Surprisingly, the replacement of para pyridine heterocycle by ortho pyridine (10) or ortho,ortho pyrimidine (11) had a negative effect on the spectroscopic properties of the compounds since both absorption and emission were blue-shifted. By contrast, the incorporation of ortho,para pyrimidine (12-13) led to highly photostable fluorophores with improved photophysical properties compared with the parent compounds, including emission in the far-red to NIR region and large Stokes’s shifts. Furthermore, high cell permeability was retained in both ortho,para-pyrimidine-containing
dyes and a higher selectivity for mitochondria of HeLa cells was achieved. Overall, these results provide new insights for the design and optimization of mitochondria-targeted fluorescent probes.

Scheme 1. Rational design of novel COUPY fluorophores.
RESULTS AND DISCUSSION

Design, synthesis and characterization of COUPY dyes.

COUPY dyes 10-13 were synthesized by following our previously described methodology for the parent dyes (4-5), which is based on the reaction of a thiocoumarin precursor with suitable heteroarylacetonitrile derivatives followed by N-methylation of the pyridine or pyrimidine moieties (Scheme 2). First, thiocoumarin 18 was reacted with 2-(pyridin-2-yl)acetonitrile (14), 2-(pyrimidin-2-yl)acetonitrile (15) or 2-(pyrimidin-4-yl)acetonitrile (16) in the presence of NaH followed by AgNO3 treatment, which afforded COUPY scaffolds 6, 7 and 8, respectively, after purification by silica column chromatography with moderate to good yields (51-82%). Similarly, coumarin 9 was obtained by condensation of thiocoumarin 19 with 16 (yield 75%). All the compounds were fully characterized by reversed-phase HPLC (Figure S1), HR ESI-MS and 1H, 19F (only in the case of 9) and 13C NMR.

Scheme 2. Synthesis of COUPY scaffolds (6-9) and of the corresponding N-methylated dyes (10-13).

It is worth noting that 1H-1H NOESY experiments (Figures 1 and S2-S5) account for the existence of two species in equilibrium in solution because of the rotation around the exocyclic C=C bond, which reproduces the behavior previously found in the parent COUPY scaffolds (compounds 2 and 3, Scheme 1).11 As shown in Figure 1, chemical exchange cross-peaks between the resonances of E and Z rotamers were observed in the 2D NOESY spectrum of coumarin 8. In the case of coumarins 6, 8 and 9, the two rotamers were present in a ~60:40
ratio according to the integration of the $^1$H NMR spectrum, being the $E$ rotamer the major species. This is in contrast with $para$-pyridine-containing coumarins 2 and 3 in which the $E/Z$ ratio was $\sim$90:10. Surprisingly, the $Z$ rotamer was the major species in solution (95%) in the COUPY scaffold containing the $ortho,ortho$ pyrimidine heterocycle (7), which was confirmed by the existence of a NOE cross-peak between the proton at position 3 of the coumarin moiety and the proton at the $ortho$ position of the pyrimidine ring (Figure S3). It is worth noting that in all of the COUPY scaffolds the chemical shift of H3 appears at higher $\delta$ in the $Z$ isomer (e.g., 8.18 ppm in 7 and 8.26 ppm in 8) than in the $E$ isomer (e.g., 6.70 ppm in 7 and 6.75 ppm in 8).

**Figure 1.** (A) Structures of the $E$ and $Z$ rotamers of coumarin 8. (B) Expansion of the 2D NOESY spectrum ($t_w = 500$ ms, 25 °C) of 8 in DMSO-$d_6$ showing NOE cross-peaks and exchange cross-peaks between rotamer resonances of the same sign as the diagonal.
Next, we synthesized the corresponding N-methylated pyridinium (10) and pyrimidinium (11-13) dyes by reaction with methyl trifluoromethanesulfonate in DCM at room temperature. The four new coumarin derivatives were isolated after silica column chromatography as reddish-orange (10-11), purple (12) and dark blue (13) solids (yields 64-80 %), and their purity was assessed by reversed-phase HPLC (Figure S1). Characterization was carried out by HR ESI-MS and 1D and 2D NMR spectroscopy. As expected, methylation occurred at the less sterically-hindered nitrogen in the ortho,para-pyrimidine derivatives (12 and 13) according to NOESY NMR characterization, and in all cases the E rotamer was the major species in solution (Figures S6-S9).

Photophysical characterization of COUPY fluorophores.

Having at hand COUPY dyes 10-13, we investigated the effect of replacing the para-pyridine moiety in the parent fluorophores (4-5) by ortho-pyridine or by ortho,ortho- or ortho,para-pyrimidine on the spectroscopic and photophysical properties of the compounds with the aim of establishing new SPPR. Although water or phosphate-buffered saline (PBS) are often used to evaluate the usefulness of a new fluorophore within a spectroscopy cuvette, the heterogeneity and complexity of the cellular environment cannot be accurately described by using a simple aqueous buffer. For this reason, we decided to register the UV-Vis absorption and emission spectra in four solvents of different polarity (PBS buffer pH 7.4, EtOH, ACN and DCM; Figures 2 and S10-S17) to get some insights on the photophysical behaviour of the compounds in polar and less-polar environments. As shown in Table 1, the photophysical properties of coumarins 10-13 were compared with those of the parent compounds 4-5 to facilitate the establishment of SPPR.

All the new compounds exhibited an intense absorption band in the visible region of the electromagnetic spectrum, with absorption maxima ranging from 502 nm (10) to 600 nm (13) in aqueous solution (PBS pH 7.4), and from 543 nm (10) to 624 nm (13) in DCM. To our surprise, the absorption maximum of coumarin 10 was significantly blue-shifted with respect the reference compound 4 (e.g., compare λ_{abs} = 515 and 543 nm for 10 and λ_{abs} = 552 and 569 nm for 4 in EtOH and DCM, respectively). Although not as pronounced as in the case of 10, a similar trend was found in ortho,ortho-pyrimidine-containing coumarin (11) since its absorption maximum was also blue-shifted (18-24 nm depending on the solvent) with respect 4. By contrast, a slight red-shift was found in the absorption maximum of the ortho,para-pyrimidine-containing coumarin 12 (12 nm in PBS and 6 nm in DCM). This red-shift was considerably larger in the 4-CF_{3} analogue (e.g., compare λ_{abs} = 600 nm for 13 and λ_{abs} = 568
nm for 5 in PBS). As previously found with the first generation of COUPY dyes,\textsuperscript{11,12} \textbf{10-13} showed negative solvatochromism since the absorption maxima was blue-shifted with increasing polarity of the solvent (e.g., compare the $\lambda_{\text{abs}}$ of \textbf{10-13} in DCM with the corresponding values in non-protic (ACN) and protic (EtOH) polar solvents). Moreover, the molar absorption coefficients of the two derivatives containing the \textit{ortho,para}-pyrimidine moiety were similar even higher than to those of their respective parent dyes, especially in the 4-CF$_3$ analogue (e.g., compare $\varepsilon = 58$ mM$^{-1}$cm$^{-1}$ for \textbf{13} and $\varepsilon = 20$ mM$^{-1}$cm$^{-1}$ for \textbf{5} in EtOH).

The emission maxima of coumarins \textbf{10} and \textbf{11} were also blue-shifted both in polar and less-polar solvents with respect coumarin \textbf{4} (e.g., $\lambda_{\text{em}} = 564$ nm for \textbf{11} and $\lambda_{\text{em}} = 603$ nm for \textbf{4} in EtOH), which reproduces the effect of replacing \textit{para}-pyridine by \textit{ortho}-pyridine or \textit{ortho,ortho}-pyrimidine moieties on the compounds’ absorption maxima. By contrast, the emission maxima of COUPY dyes containing the \textit{ortho,para}-pyrimidine moiety was red-shifted with respect their parent compounds (11-24 nm in \textbf{12} and 6-19 nm in \textbf{13}), especially in polar solvents. As a consequence, COUPY dyes \textbf{12} and \textbf{13} showed emission in the far-red to NIR region (Figures 2 and S10-S17), being the emission maximum in polar media particularly appealing in the case of the 4-CF$_3$ fluorophore ($\lambda_{\text{em}} = 673$ nm for \textbf{13} in PBS). Interestingly, coumarin \textbf{12} exhibited larger Stokes’ shifts than its parent dye \textbf{4} in all the solvents evaluated while the replacement of \textit{para}-pyridine by \textit{ortho,para}-pyrimidine in \textbf{5} led to slightly smaller values. Nevertheless, the Stokes’ shifts of \textbf{12} and \textbf{13} in polar solvents are sufficiently large (e.g., 71 and 73 nm in PBS, respectively) to avoid the light reabsorption problems typically found in bioimaging applications.\textsuperscript{1}

As shown in Table 1, compounds \textbf{10} and \textbf{11} exhibited very weak fluorescence in all the solvents investigated. By contrast, fluorescence quantum yields for coumarin \textbf{13} were much higher than those of the parent compound \textbf{5}, especially in less-polar solvents (e.g., 0.41 and 0.05 in DCM, respectively). This tendency was reversed in the case of the 4-CH$_3$ analogue since the $\Phi_F$ for \textbf{12} was smaller compared with \textbf{4} (e.g., 0.11 and 0.70 in DCM, respectively). Both \textit{ortho,para}-pyrimidine-containing compounds exhibited moderate fluorescence quantum yields in polar protic solvents ($\Phi_F = 0.13$ for \textbf{12} and $\Phi_F = 0.08$ for \textbf{13} in EtOH).
Table 1. Photophysical data of the coumarin derivatives 4-5 and 10-13 in different solvents.

| Compd. | X, Y, Z | Solvent | $\lambda_{\text{abs}}$ (nm)$^a$ | $\varepsilon$ (M$^{-1}$cm$^{-1})^b$ | $\lambda_{\text{em}}$ (nm)$^c$ | Stokes Shift (nm)$^d$ | $\Phi_F$ $^e$ |
|--------|--------|---------|-----------------|-----------------|-----------------|-----------------|----------|
| 4      | CH, CH, N | PBS     | 545             | 34000           | 604             | 59              | 0.12     |
|        |        | EtOH    | 552             | 59000           | 603             | 51              | 0.45     |
|        |        | ACN     | 548             | 75000           | 609             | 61              | 0.18     |
|        |        | DCM     | 569             | 67000           | 607             | 38              | 0.70     |
| 10     | N, CH, CH | PBS     | 502             | 25000           | 583             | 81              | 0.01     |
|        |        | EtOH    | 515             | 31000           | 592             | 77              | 0.02     |
|        |        | ACN     | 505             | 26000           | 589             | 84              | 0.02     |
|        |        | DCM     | 543             | 36000           | 594             | 51              | 0.20     |
| 11     | N, N, CH | PBS     | 525             | 37000           | 561             | 36              | 0.01     |
|        |        | EtOH    | 528             | 59000           | 564             | 36              | 0.11     |
|        |        | ACN     | 526             | 23000           | 575             | 49              | 0.02     |
|        |        | DCM     | 551             | 45000           | 565             | 14              | 0.02     |
| 12     | N, CH, N | PBS     | 557             | 56000           | 628             | 71              | 0.01     |
|        |        | EtOH    | 558             | 58000           | 615             | 57              | 0.13     |
|        |        | ACN     | 557             | 50000           | 621             | 64              | 0.10     |
|        |        | DCM     | 575             | 73000           | 618             | 43              | 0.11     |
| 5      | CH, CH, N | PBS     | 568             | 14000           | 660             | 92              | 0.02     |
|        |        | EtOH    | 568             | 20000           | 648             | 80              | 0.05     |
|        |        | ACN     | 569             | 47000           | 668             | 99              | 0.02     |
|        |        | DCM     | 600             | 34000           | 657             | 57              | 0.05     |
| 13     | N, CH, N | PBS     | 600             | 35000           | 673             | 73              | 0.02     |
|        |        | EtOH    | 598             | 58000           | 667             | 69              | 0.08     |
|        |        | ACN     | 597             | 42000           | 674             | 77              | 0.10     |
|        |        | DCM     | 624             | 60000           | 663             | 39              | 0.41     |

$a$ Wavelength of the absorption maximum; $b$ Molar absorption coefficient at $\lambda_{\text{max}}$; $c$ Wavelength of the emission maximum upon excitation at a wavelength 20 nm below $\lambda_{\text{max}}$; $d$ Stokes’ shift; $e$ Fluorescence quantum yields ($\Phi_F$) were measured by comparative method using Cresyl Violet in ethanol ($\Phi_{F,\text{Ref}} = 0.54$) as reference for compounds 4-5 and 12-13. Fluorescein dissolved in aqueous sodium hydroxide (0.1 M; $\Phi_{F,\text{Ref}} = 0.92$) was used as reference in the case of compounds 10-11.17
Finally, the photostability of the most promising coumarins (12 and 13) was investigated in PBS buffer (pH 7.4) under green LED irradiation (Figures 3 and S18). As shown in Figure 3, the replacement of pyridine in coumarin 4 with ortho,para-pyrimidine had a positive effect on the photostability of the resulting fluorophore (12). By contrast, coumarin 13 was found less photostable than its parent compound 5 and coumarin 12, which indicates that replacement of the CH$_3$ group at the 4-position with CF$_3$ in ortho,para-pyrimidine-containing COUPY dyes does not lead to an improvement of the overall photostability of the compounds. Nevertheless, it is worth noting that the two new pyrimidine-containing COUPY dyes were found photostable up to light fluences larger than 1000 J/cm$^2$ (12) and 200 J/cm$^2$ (13), which are more than 50- and 10-fold, respectively, higher than those typically used in imaging experiments with living cells.
Figure 3. Fluorescence bleaching of COUPY dyes (4-5, 12-13) in PBS buffer pH 7.4 (5 μM) irradiated with green light (505 nm, 100 mW/cm²).

In summary, all these observations allowed us to establish some structure-photophysical property relationships. First, replacement of para-pyridine in COUPY dyes with ortho-pyridine or ortho,ortho-pyrimidine moieties had a negative effect on the spectroscopic properties of the fluorophores since both absorption and emission maxima were blue-shifted. Moreover, compounds 10 and 11 were found weakly fluorescent and their extinction coefficients were smaller than those of the parent coumarin 4. By contrast, the photophysical properties of COUPY dyes were clearly improved when para-pyridine was replaced with ortho,para-pyrimidine. On the one hand, both absorption and emission maxima were red-shifted with respect the parent compounds, especially in polar protic solvents. Indeed, the incorporation of the second nitrogen atom in the pyridine moiety in 5 caused a red-shift of the absorption (32 nm) and emission (12 nm) bands in PBS pH 7.4 in the resulting coumarin 13 when compared with the parent compound. Very interestingly, the emission of 13 was extended beyond 750 nm (Figure 2), which would facilitate in vivo imaging. Although the incorporation of the ortho,para-pyrimidine moiety caused a slight decrease in the fluorescent quantum yields of the compounds (e.g., compare \( \Phi_F \) of 4 and 12), the combination of this heterocycle with the CF₃ group at position 4 seems to compensate this phenomenon, leading to moderate \( \Phi_F \) values in protic and non-protic polar solvents (0.08-0.13) in the case of compound 13.
Fluorescence imaging of COUPY dyes in living cells

Taking into account the large photostability and the photophysical properties of the two *ortho,para*-pyrimidine-containing COUPY dyes (12 and 13), we decided to evaluate their usefulness as fluorescent probes in a more realistic situation (e.g., in living cells). As previously stated, the heterogeneous environment of an organic fluorophore within a cell or within a specific cellular organelle might be considerably different than the homogeneity of a solution in a spectroscopy cuvette. In fact, the presence of biomolecules such as proteins and lipids or the interaction with the components of cellular membranes might provide the fluorescent probe with an environment less hydrophilic than expected, thereby modifying key parameters for bioimaging applications such as brightness.

The cellular uptake of 12 and 13 was first investigated in living HeLa cells (2 μM, 30 min incubation) by using confocal microscopy and compared with that of the *para*-pyridine-containing coumarins (compounds 4 and 5, respectively). Irradiation was carried out with a yellow light laser (λex = 561 nm) in the case of 4-CH3 coumarins (4 and 12), while the higher red-shift absorption of the 4-CF3 compounds (5 and 13) allowed the use of a red one (λex = 633 nm). As shown in Figure 4, in all cases the fluorescence signal was clearly observed inside the cell, which confirms that the excellent cellular uptake of the parent COUPY dyes was retained after replacement of pyridine with pyrimidine. In addition, it is worth noting that no cell toxicity was observed during these studies. The overall pattern of staining (but not the relative fluorescence intensity between organelles; see below for a discussion) of pyrimidine-containing coumarins (12 and 13) was similar to that found with the parent fluorophores (4 and 5), suggesting accumulation in mitochondria, nucleoli and, to a lesser extent, in intracellular vesicles, mostly lysosomes. Co-localization experiments with two specific markers for labeling mitochondria (MitoTracker Green FM, MTG), and lysosomes (Lysotracker Green FM, LTG) confirmed the subcellular localization of the compounds.
**Figure 4.** Cellular uptake of COUPY fluorophores 4 (A), 5 (B), 12 (C) and 13 (D). Single confocal planes of HeLa cells incubated with the compounds (2 μM, 30 min, 37ºC). White arrows point out mitochondria, white arrowheads nucleoli and yellow arrowheads vesicles staining. Scale bar: 20 μm. All images are at the same scale as A and colour coded using the Fire lookup table from Fiji (intensity calibration bar is showed in B).

As shown in Figure 5, the distribution of the fluorescence emission of the compounds was similar to that of Mitotracker Green FM, which confirmed accumulation into the mitochondria. Pearson’s and Manders’ (M1 and M2) coefficients were used to measure the degree of co-localization.\(^{11-12,18}\) On the one hand, Pearson’s coefficients of 0.86 (12) and 0.87 (13) confirmed a clear correlation between the coumarins’ signals and MTG (Pearson’s coefficients range from \(-1\) to +1, being +1 the indicator of a perfect match). Such coefficients were higher than those obtained for the parent COUPY dyes (0.65 for 4 and 0.73 for 5), indicating a better correlation between pyrimidine-containing coumarins and MTG. On the other hand, the Manders’ coefficients (which range from 0 to 1 and determine the intensities of one channel co-localizing with the other) also confirmed that 12 and 13 were mainly placed in the mitochondria. The degree of co-localization of 12 over MTG (M1 coefficient) was 0.40, whereas that of MTG over 12 (M2 coefficient) was 0.73. These values indicate that there is more MTG signal co-localizing with 12 than 12 that co-localizing with MTG. The localization of the coumarin probe in other organelles such as nucleoli and intracellular
vesicles accounts for the differences in both Manders coefficients. It is worth noting that smaller values for M1 (0.28) and M2 (0.68) were obtained in the case of the parent coumarin 4, which indicates that the signal from the fluorophore that co-localizes with MTG is higher in the case of pyrimidine-containing coumarin (12) than in the pyridine analogue (4), thereby suggesting a higher preference for mitochondria. Although not as pronounced, a similar trend was found for coumarins 13 (M1 = 0.19 and M2 = 0.86) and 5 (M1 = 0.18 and M2 = 0.76).

Figure 5. Co-localization studies with COUPY dyes 12 (top) and 13 (bottom) and Mitotracker Green FM. Single confocal plane of HeLa cells incubated with 12 or 13 (2 μM, red) and Mitotracker Green FM (0.1 μM, green). A), D) Overlay of the two staining. B), E) coumarin 12 and 13 signal, respectively. C), F) Mitotracker Green FM signal. Scale bar: 10 μm. All images are at the same scale as A.

As previously found with the parent fluorophores,12 co-localization experiments with LTG (Figure 6) confirmed that most of fluorescence observed in intracellular vesicles along the cytoplasm was associated with lysosome accumulation (Pearson’s coefficients being equal to 0.52 (12) and 0.49 (13) on average). The smaller Mander’s coefficients for the co-localization of the compounds over LTG (M1 = 0.02 for 12 and 13) and of LTG over the coumarins (M2 = 0.45 for 12 and 0.62 for 13) in the co-localization experiment with LTG compared with MTG.
might be explained by a reduced affinity of COUPY dyes for lysosome and/or by lower abundance of this organelle in cells compared with mitochondria.

Very interestingly, the nucleoli inside the nuclei was much less intensely stained in compounds 12 and 13 than in the parent coumarins: compare in Figure 4 panels C (12) and D (13) with panels A (4) and B (5), respectively. As shown in Figure 7, measurement of the mean fluorescence intensity both in mitochondria and nucleoli confirmed this observation. Indeed, the mean intensity for mitochondria and nucleoli was quite similar in the parent coumarins 4 and 5 whereas the mean intensity for nucleoli staining was considerably reduced in the case of the pyrimidine analogues 12 and 13. These results are in line with the higher M1 coefficients measured in co-localization experiments with MTG and indicate that replacement of para-pyridine by ortho,para-pyrimidine in COUPY dyes caused an increase in the selectivity of the compounds towards mitochondria.

**Figure 6.** Co-localization studies with COUPY dyes and Lysotracker Green FM. Single confocal plane of HeLa cells incubated with coumarins 12 and 13 (2 μM, red) and Lysotracker Green FM (0.2 μM, green). A), D) Overlay of the two staining. B), E) coumarins 12 and 13 signal, respectively. C), F) Lysotracker Green FM staining. White arrowheads point out COUPY dyes vesicles colocalizing with Lysotracker staining. Scale bar: 10 μm. All images are at the same scale as A.
Finally, the photostability of coumarins 12 and 13 was evaluated in a more realistic situation and compared with that of the parent compounds (4 and 5, respectively). For this purpose, living HeLa cells were incubated at 37 °C with the compounds and irradiated continuously with the laser beam of the confocal microscope (\(\lambda_{ex}= 561\) nm at 4.32 \(\mu\text{W}\) for 4 and 12, and \(\lambda_{ex}= 633\) nm at 10.2 \(\mu\text{W}\) for 5 and 13), and images were acquired with an interval time of 0.42 sec for 4.5 min. As shown in Figures S19 and S20, the overall fluorescence signal was significantly decreased in all the cases. In good agreement with photobleaching studies (Figure 3), the ortho,para-containing coumarin 12 was found slightly more photostable than its parent compound (4), while this trend was found opposite in the case of the 4-CF\(_3\) containing compounds. Nevertheless, it is worth noting that the laser power used in the photobleaching experiments was much higher than the one used during conventional observation (0.3 \(\mu\text{W}\) and 1.7 \(\mu\text{W}\) with 561 nm and 633 nm lasers, respectively) which confirms a good photostability and applicability of these molecules for imaging living cells by using fluorescence microscopy.
CONCLUSIONS

In summary, in this manuscript we have investigated how the photophysical properties and subcellular accumulation of coumarin-based COUPY fluorophores can be fine-tuned through replacement of the para-pyridine moiety with different heterocycles (ortho-pyridine and ortho,ortho- or ortho,para-pyrimidine), with the aim of establishing new structure-photophysical property relationships. COUPY dyes 10-13 were easily obtained from cheap, commercially available compounds in three linear synthetic steps, the reaction of thiocoumarins with suitable heteroarylacetanitride derivatives followed by N-methylation of the pyridine or pyrimidine moieties being the key steps. Very interestingly, the photophysical properties of the new fluorophores were strongly influenced by these modifications. On the one hand, the replacement of para-pyridine moiety with ortho-pyridine (10) or ortho,ortho-pyrimidine (11) had a negative effect on the spectroscopic properties of the dyes since both absorption and emission were blue-shifted, leading to weakly fluorescent compounds. On the other hand, the absorption and emission maxima of ortho,para-pyrimidine-containing fluorophores (12-13) were red-shifted with respect the parent compounds, particularly in polar solvents. Besides emission in the far-red to NIR region, the newly synthesized fluorophores exhibited other appropriate characteristics for bioimaging applications such as large Stokes’ shifts and high photostability. Furthermore, coumarins 12 and 13 retained the excellent cell permeability of COUPY dyes but exhibited a higher preference for mitochondria. Overall, these results provide useful indications for the development of novel mitochondria-targeted fluorescent probes based on small organic compounds since we have demonstrated that the selectivity for this organelle can be improved through the replacement of N-alkylated pyridinium moieties by the corresponding N-alkylated-ortho,para-pyrimidinium counterparts.
EXPERIMENTAL SECTION

Materials and Methods

Unless otherwise stated, common chemicals and solvents (HPLC grade or reagent grade quality) were purchased from commercial sources and used without further purification. Aluminium plates coated with a 0.2 mm thick layer of silica gel 60 F254 were used for thin-layer chromatography analyses (TLC), whereas flash column chromatography purification was carried out using silica gel 60 (230-400 mesh). Reversed-phase high-performance liquid chromatography (HPLC) analyses were carried out on a Jupiter Proteo C18 column (250x4.6 mm, 90 Å 4 µm, flow rate: 1 mL/min) using linear gradients of 0.1% formic acid in H2O (A) and 0.1% formic acid in ACN (B). NMR spectra were recorded at 25 °C in a 400 MHz spectrometer using the deuterated solvent as an internal deuterium lock. The residual protic signal of chloroform or DMSO was used as a reference in 1H and 13C NMR spectra recorded in CDCl3 or DMSO-d6, respectively. Chemical shifts are reported in part per million (ppm) in the δ scale, coupling constants in Hz and multiplicity as follows: s (singlet), d (doublet), t (triplet), q (quartet), qt (quintuplet), m (multiplet), dd (doublet of doublets), dq (doublet of quartets), br (broad signal), etc. The proton signals of the E and Z rotamers were identified by simple inspection of the 1H spectrum and the rotamer ratio was calculated by peak integration. 2D-NOESY spectra were acquired in DMSO-d6 with a mixing time of 500 ms, either at 298k or 350 K. Electrospray ionization mass spectra (ESI-MS) were recorded on an instrument equipped with single quadrupole detector coupled to an HPLC, and high-resolution (HR) ESI-MS on a LC/MS-TOF instrument.

Synthesis of pyrimidin-acetonitrile derivatives

2-(Pyrimidin-2-yl)acetonitrile (15)

A solution of KCN (0.63 g, 9.70 mmol) in DMSO (5 mL) was slowly added to a solution of 2-(chloromethyl)pyrimidine (0.5 g, 3.89 mmol) in DMSO (4 mL). The reaction mixture was stirred overnight at 35 °C with a hot plate magnetic stirrer. After addition of 50 mL of K2CO3 (10%), the aqueous phase was extracted with diethyl ether (10 x 30 mL). The combined organic fractions were dried over anhydrous Na2SO4, filtered and evaporated under reduced pressure. The crude product was purified by column chromatography (silica gel, 0-15% ethyl acetate in DCM) to give 84 mg of a colourless oil (yield 19%). TLC: Rf (ethyl acetate/DCM 1:1) 0.44. 1H NMR (400 MHz, CDCl3, δ (ppm)): 8.74 (2H, d, J = 5.1 Hz), 7.29 (1H, t, J = 5.1 Hz), 4.11 (s, 2H). 13C{1H} NMR (101 MHz, CDCl3, δ (ppm)): 161.4, 158.0, 120.3, 116.1, 28.6. LRMS (ESI-TOF) (m/z): [M+H]+ Calcd for C6H6N3, 120.06; found, 119.90.
2-(Pyrimidin-4-yl)acetonitrile (16)

A modified method was followed to synthesize compound 16.\(^\text{19}^\)

\((E)-N,N\)-Dimethyl-2-(pyrimidin-4-yl)ethen-1-amine (17)

4-Methylpyrimidine (10 g, 106 mmol) and 1,1-dimethoxy-\(N,N\)-dimethylethanamine (38 g, 319 mmol) were dissolved in 50 mL of anhydrous DMF. The solution was heated at 140°C with a hot plate magnetic stirrer for 24 h. The crude product was evaporated under reduced pressure to provide 17 as a brown oil, which was used without further purification. TLC: \(R_f\) (DCM/MeOH 9:1) 0.50. \(^1\)H NMR (400 MHz, CDCl\(_3\), \(\delta\) (ppm)): 8.73 (1H, s), 8.21 (1H, d, \(J = 5.6\) Hz), 7.76 (1H, d, \(J = 13\) Hz), 6.71 (1H, dd, \(J = 5.6, 1.6\) Hz), 4.99 (1H, d, \(J = 13\) Hz), 2.95 (6H, s).

2-(Pyrimidin-4-yl)acetonitrile (16)

A solution of hydroxylamine-O-sulfonic acid (7.31 g, 57.5 mmol) in Milli-Q water (100 mL) was added to the crude product 17, and the reaction mixture was heated at 50 °C with a hot plate magnetic stirrer for 45 min. Then, the mixture was cooled at 0 °C and saturated NaHCO\(_3\) was added until basic pH, and the aqueous phase extracted with ethyl acetate (6 x 200 mL). The combined organic phases were dried over anhydrous Na\(_2\)SO\(_4\), filtered and evaporated under reduced pressure. The brown oil was purified by column chromatography (silica gel, 0-25% ethyl acetate in DCM) to give 1.46 g of a yellow solid (yield 11%). TLC: \(R_f\) (ethyl acetate/DCM 1:1) 0.34. \(^1\)H NMR (400 MHz, CDCl\(_3\), \(\delta\) (ppm)): 9.20 (1H, s), 8.80 (1H, d, \(J = 5.2\) Hz), 7.51 (1H, d, \(J = 5.2\) Hz), 3.94 (2H, s). \(^{13}\)C\{\(^1\)H\} NMR (101 MHz, CDCl\(_3\), \(\delta\) (ppm)): 159.3, 159.2, 158.3, 119.7, 115.5, 26.5. LRMS (ESI-TOF) (m/z): [M+H]\(^+\) Calcd for C\(_6\)H\(_6\)N\(_3\), 120.06; found, 119.90.

Synthesis of COUPY scaffolds (6-9)

2-(Cyano(2-pyridine)methylene)-7-(\(N,N\)-diethylamino)-4-methyl-coumarin (6)

7-(\(N,N\)-Diethylamino)-4-methyl-2-thiocoumarin 18 (0.25 g, 1.01 mmol), 2-(pyridin-2-yl)acetonitrile 14 (0.24 g, 2.02 mmol) and NaH (60% dispersion in mineral oil, 0.36 g, 9 mmol) were dissolved in anhydrous acetonitrile (15 mL) and the mixture was stirred for 2 h under an argon atmosphere at room temperature and protected from light. Then, AgNO\(_3\) (0.34 g, 2.02 mmol) was added and the resulting mixture was stirred for 2 h under Ar at room temperature. The crude product was evaporated under reduced pressure and purified by column chromatography (silica gel, 0-25% ethyl acetate in DCM) to give 240 mg of an orange solid (yield 72%). TLC: \(R_f\) (ethyl acetate/DCM 1:1) 0.90. \(^1\)H NMR (400 MHz, DMSO-\(d_6\), \(\delta\) (ppm)): (E + Z rotamers) 8.56 (1H, m), 8.13 (0.6H, dt, \(J = 8.2, 0.8\) Hz), 7.82
(1.4H, m), 7.46 (1.4H, m), 7.18 (1H, m), 6.70 (1H, dd, J = 9.0, 2.4 Hz), 6.65 (1H, m), 6.42 (0.4H, d, J = 2.4 Hz), 3.46 (4H, q, J = 7.2 Hz), 2.35 (1.8H, d, J = 0.8 Hz), 2.32 (1.2H, m, J = 0.8 Hz), 1.14 (6H, m). 13C (1H) NMR (101 MHz, DMSO-d6, δ (ppm)): (E + Z rotamers) 163.2, 161.8, 154.0, 153.8, 152.7, 152.0, 150.6, 150.5, 149.0, 148.8, 144.6, 144.0, 137.1, 136.8, 126.0, 125.9, 121.8, 121.2, 120.7, 120.2, 119.6, 119.3, 110.8, 110.4, 109.8, 109.2, 109.1, 96.7, 96.1, 85.3, 81.0, 44.0, 43.8, 18.5, 18.0, 12.4. HRMS (ESI-TOF) (m/z): [M+H]+ Calcd for C21H22N3O, 332.1763; found, 332.1759. Analytical HPLC (30–100% B in 30 min, formic acid additive): Rf = 15.2 min.

2-(Cyano(2-pyrimidine)methylene)-7-(N,N-diethylamino)-4-methyl-coumarin (7)

7-(N,N-Diethylamino)-4-methyl-2-thiocoumarin 18 (0.25 g, 1.01 mmol), 2-(pyrimidin-2-yl)acetonitrile 15 (0.24 g, 2.02 mmol) and NaH (60% dispersion in mineral oil, 0.36 g, 9 mmol) were dissolved in anhydrous acetonitrile (15 mL) and the mixture was stirred for 2 h under an argon atmosphere at room temperature and protected from light. Then, AgNO3 (0.34 g, 2.02 mmol) was added and the resulting mixture was stirred for 2 h under Ar at room temperature. The crude product was evaporated under reduced pressure and purified by column chromatography (silica gel, 0-10% ethyl acetate in DCM) to give 171 mg of an orange solid (yield 51%). TLC: Rf (ethyl acetate/DCM 1:1) 0.63. 1H NMR (400 MHz, DMSO-d6, δ (ppm)): (major rotamer) 8.74 (2H, d, J = 4.8 Hz), 8.18 (1H, s), 7.53 (1H, d, J = 9.0 Hz), 7.17 (1H, t, J = 4.8 Hz), 6.76 (1H, dd, J = 9.0, 2.4 Hz), 6.46 (1H, d, J = 2.4 Hz), 3.47 (4H, q, J = 7.2 Hz), 2.39 (3H, s), 1.15 (6H, t, J = 6.9 Hz). 13C (1H) NMR (101 MHz, DMSO-d6, δ (ppm)): (major rotamer) 166.6, 162.9, 157.0, 154.1, 150.9, 146.6, 126.3, 118.4, 116.6, 110.5, 109.9, 109.7, 96.0, 82.5, 44.1, 18.7, 12.4. HRMS (ESI-TOF) (m/z): [M+H]+ Calcd for C20H21N4O, 333.1715; found, 333.1710. Analytical HPLC (30–100% B in 30 min, formic acid additive): Rf = 21.2 min.

2-(Cyano(4-pyrimidine)methylene)-7-(N,N-diethylamino)-4-methyl-coumarin (8)

7-(N,N-Diethylamino)-4-methyl-2-thiocoumarin 18 (0.25 g, 1.01 mmol), 2-(pyrimidin-4-yl)acetonitrile 16 (0.24 g, 2.02 mmol) and NaH (60% dispersion in mineral oil, 0.36 g, 9 mmol) were dissolved in anhydrous acetonitrile (15 mL) and the mixture was stirred for 2 h under an argon atmosphere at room temperature and protected from light. Then, AgNO3 (0.34 g, 2.02 mmol) was added and the resulting mixture was stirred for 2 h under Ar at room temperature. The crude product was evaporated under reduced pressure and purified by column chromatography (silica gel, 0-25% ethyl acetate in DCM) to give 275 mg of an orange solid (yield 82%). TLC: Rf (ethyl acetate/DCM 1:1) 0.50. 1H NMR (400 MHz,
DMSO-$d_6$, $\delta$ (ppm): ($E + Z$ rotamers) 9.04 (1H, m), 8.69 (0.6H, d, $J = 5.6$ Hz), 8.62 (0.4H, d, $J = 5.6$ Hz), 8.26 (0.4H, d, $J = 0.8$ Hz), 8.18 (0.6H, dd, $J = 5.6$, 1.6 Hz), 7.59 (0.4H, d, $J = 9.2$ Hz), 7.56 (0.6H, d, $J = 8.8$ Hz), 7.42 (0.4H, dd, $J = 5.6$, 1.6 Hz), 6.81 (1.6H, m), 6.75 (0.6H, d, $J = 0.8$ Hz), 6.51 (0.4H, d, $J = 2.8$ Hz), 3.49 (4H, m), 2.43 (1.2H, d, $J = 1.2$ Hz), 1.16 (6H, t, $J = 7.0$ Hz).

$^{13}$C{$^1$H} NMR (101 MHz, DMSO-$d_6$, $\delta$ (ppm)): ($E + Z$ rotamers) 165.5, 165.2, 160.3, 159.1, 158.1, 157.6, 156.9, 156.7, 154.2, 151.2, 151.0, 148.5, 147.6, 126.5, 126.4, 118.6, 118.4, 117.5, 116.9, 116.7, 110.6, 110.4, 110.3, 110.2, 109.2, 96.6, 95.8, 82.8, 78.5, 44.1, 43.9 18.8, 18.2, 12.4, 12.3. HRMS (ESI-TOF) ($m/z$): [M+H]$^+$ Calcd for C$_{20}$H$_{21}$N$_4$O, 333.1715; found, 333.1709. Analytical HPLC (30–100% B in 30 min, formic acid additive): $R_t = 17.5$ min.

2-(Cyano(4-pyrimidine)methylene)-7-(N,N-dimethylamino)-4-trifluoromethyl-coumarin (9)

7-(N,N-Dimethylamino)-4-trifluoromethyl-2-thiocoumarin 19 (0.25 g, 0.92 mmol), 2-(pyrimidin -4-yl)acetonitrile 16 (0.22 g, 1.83 mmol) and NaH (60% dispersion in mineral oil, 0.33 g, 8.25 mmol) were dissolved in anhydrous acetonitrile (15 mL) and the mixture was stirred for 2 h under an argon atmosphere at room temperature and protected from light. Then, AgNO$_3$ (0.31 g, 1.83 mmol) was added and the resulting mixture was stirred for 2 h under Ar at room temperature. The crude product was evaporated under reduced pressure and purified by column chromatography (silica gel, 0-10% ethyl acetate in DCM) to give 248 mg of a red solid (yield 75%). TLC: $R_f$ (ethyl acetate/DCM 1:1) 0.30 and 0.64. $^1$H NMR (400 MHz, DMSO-$d_6$, $\delta$ (ppm)): ($E + Z$ rotamers) 9.17 (0.55H, d, $J = 1.2$ Hz), 9.15 (0.45H, d, $J = 1.2$ Hz), 8.83 (0.55H, d, $J = 5.6$ Hz), 8.78 (0.45H, d, $J = 5.6$ Hz), 8.74 (0.45H, s), 8.28 (0.55H, dd, $J = 5.6$, 1.6 Hz), 7.56 (0.45H, dd, $J = 5.6$, 1.6 Hz), 7.44 (1H, m), 7.03 (0.55H, s), 7.00 (0.55H, d, $J = 2.8$ Hz), 6.87 (1H, m), 6.64 (0.45H, d, $J = 2.4$ Hz), 3.13 (3.3H, s), 3.11 (2.7H, s).

$^{13}$C{$^1$H} NMR (101 MHz, DMSO-$d_6$, $\delta$ (ppm)): ($E + Z$ rotamers) 163.3, 162.6, 158.7, 158.3, 157.9, 157.7, 157.6, 154.6, 154.5, 153.7, 153.6, 125.3, 118.8, 117.9, 117.4, 116.8, 111.0, 110.9, 110.8, 110.7, 110.5, 110.4, 103.5, 102.5, 98.1, 97.4, 89.7, 89.5, 85.2, 39.7. $^{19}$F NMR (376 MHz, DMSO-$d_6$) $\delta$ (ppm): ($E + Z$ rotamers) -63.0 (1.35F, s), -63. (1.65F, s). HRMS (ESI-TOF) ($m/z$): [M+H]$^+$ Calcd for C$_{18}$H$_{14}$F$_3$N$_4$O, 359.1114; found, 359.1112. Analytical HPLC (30–100% B in 30 min, formic acid additive): $R_t = 22.8$ min.

**Synthesis of COUPY fluorophores (10-13)**

2-(Cyano(1-methyl(2-pyridin-1-ium))methylene)-7-(N,N-diethylamino)-4-methyl-coumarin triflate (10)
Methyl trifluoromethanesulfonate (47.5 μL, 0.42 mmol) was added to a solution of 2-(cyano(2-pyridine)methylene)-7-(N,N-diethylamino)-4-methyl-coumarin 6 (70 mg, 0.21 mmol) in DCM (30 mL) under an argon atmosphere. The mixture was stirred for 4 h at room temperature and protected from light. The reaction mixture was evaporated under reduced pressure and purified by column chromatography (silica gel, 0-3% MeOH in DCM) to give 77 mg of a red solid (yield 74%). TLC: Rf (DCM/MeOH 9:1) 0.35. 1H NMR (400 MHz, 77 ºC, DMSO-d6, δ (ppm)): 9.02 (1H, d, J = 6.4 Hz), 8.53 (1H, td, J = 7.6, 1.2 Hz), 8.26 (1H, d, J = 8.0 Hz), 8.00 (1H, td, J = 7.6, 1.2 Hz), 7.59 (1H, d, J = 9.2 Hz), 6.83 (1H, dd, J = 9.2, 2.4 Hz), 6.62 (1H, br s), 6.51 (1H, br s), 4.28 (3H, s), 3.46 (4H, q, J = 7.2 Hz), 2.43 (3H, s), 1.15 (6H, t, J = 7.2 Hz).

13C{1H} NMR (101 MHz, 77 ºC, DMSO-d6, δ (ppm)): 165.6, 153.9, 151.2, 149.3, 147.8, 147.1, 144.6, 130.5, 126.2, 125.3, 120.5 (q, J = 323 Hz, Tf), 116.5, 110.3, 109.4, 108.0, 96.2, 71.0, 46.0, 43.7, 17.6, 12.0. 19F NMR (376 MHz, DMSO-d6) δ (ppm): -77.8 (3F, s). HRMS (ESI-TOF) (m/z): [M]+ Calcd for C22H24N3O, 346.1914; found, 346.1911. Analytical HPLC (30–100% B in 30 min, formic acid additive): Rt = 5.5 min.

2-(Cyano(1-methyl(2-pyrimidin-1-ium))methylene)-7-(N,N-diethylamino)-4-methyl-coumarin triflate (11)

Methyl trifluoromethanesulfonate (47.5 μL, 0.42 mmol) was added to a solution of 2-(cyano(2-pyrimidine)methylene)-7-(N,N-diethylamino)-4-methyl-coumarin 7 (70 mg, 0.21 mmol) in DCM (30 mL) under an argon atmosphere. The mixture was stirred for 4 h at room temperature and protected from light. Then, an additional amount of methyl trifluoromethanesulfonate (47.5 μL, 0.42 mmol) was added to the reaction mixture and was stirred during 2 h. The reaction mixture was evaporated under reduced pressure and purified by column chromatography (silica gel, 0-4% MeOH in DCM) to give 66 mg of an orange solid (yield 64%). TLC: Rf (DCM/MeOH 9:1) 0.31. 1H NMR (400 MHz, DMSO-d6, δ (ppm)): 9.28 (1H, dd, J = 4.2, 2.0 Hz), 9.16 (1H, dd, J = 6.6, 1.8 Hz), 7.91 (1H, dd, J = 6.4, 5.6 Hz, 1H), 7.70 (1H, d, J = 9.2 Hz), 7.06 (1H, s), 6.94 (1H, dd, J = 9.2, 2.8 Hz), 6.70 (1H, d, J = 2.8 Hz), 4.14 (3H, s), 3.50 (4H, q, J = 7.2 Hz), 2.49 (3H, s), 1.15 (6H, t, J = 7.2 Hz).

13C{1H} NMR (101 MHz, DMSO-d6, δ (ppm)): 162.9 157.6, 154.7, 154.6, 152.5, 151.8, 127.1, 120.7 (q, J = 323 Hz, Tf), 118.7, 116.8, 111.6, 110.4, 109.2, 96.0, 73.4, 46.5, 44.2, 18.5, 12.3. 19F NMR (376 MHz, DMSO-d6) δ (ppm): -77.8 (3F, s). HRMS (ESI-TOF) (m/z): [M]+ Calcd for C21H23N4O, 347.1866; found, 347.1865. Analytical HPLC (30–100% B in 30 min, formic acid additive): Rt = 5.1 min.
2-(Cyano(1-methyl(4-pyrimidin-1-ium))methylene)-7-(N,N-diethylamino)-4-methyl-coumarin triflate (12)

Methyl trifluoromethanesulfonate (47.5 μL, 0.42 mmol) was added to a solution of 2-(cyano(4-pyrimidine)methylene)-7-(N,N-diethylamino)-4-methyl-coumarin 8 (70 mg, 0.21 mmol) in DCM (30 mL) under an argon atmosphere. The mixture was stirred for 4 h at room temperature and protected from light. The reaction mixture was evaporated under reduced pressure and purified by column chromatography (silica gel, 0-2% MeOH in DCM) to give 83 mg of a violet solid (yield 80%). TLC: Rf (DCM/MeOH 9:1) 0.28. 1H NMR (400 MHz, 77 ºC, DMSO-d6, δ (ppm)): 9.11 (1H, m), 8.53 (1H, dd, J = 7.6, 2.0 Hz), 8.00 (1H, d, J = 7.6 Hz), 7.84 (1H, d, J = 9.2 Hz), 7.60 (1H, br s), 7.09 (1H, dd, J = 9.2, 2.4 Hz), 6.89 (1H, d, J = 2.4 Hz), 4.02 (3H, s), 3.60 (4H, q, J = 7.2 Hz), 2.63 (3H, s), 1.23 (6H, t, J = 7.2 Hz). 13C{1H} NMR (101 MHz, DMSO-d6, δ (ppm)): 167.3, 163.4, 155.6, 155.5, 152.7, 152.2, 147.5, 127.5, 120.7 (q, J = 323 Hz, Tf), 117.4, 114.8, 113.1, 111.5, 110.7, 95.9, 44.4, 42.5, 18.9, 12.4. 19F NMR (376 MHz, DMSO-d6) δ (ppm): -77.8 (3F, s). HRMS (ESI-TOF) (m/z): [M]+ Calcd for C21H23N4O347.1866; found, 347.1866. Analytical HPLC (30–100% B in 30 min, formic acid additive): Rt = 5.4 min.

2-(Cyano(1-methyl(4-pyrimidin-1-ium))methylene)-7-(N,N-dimethylamino)-4-trifluoromethyl-coumarin triflate (13)

Methyl trifluoromethanesulfonate (47.3 μL, 0.40 mmol) was added to a solution of 2-(cyano(4-pyrimidine)methylene)-7-(N,N-dimethylamino)-4-trifluoromethyl-coumarin 9 (70 mg, 0.20 mmol) in DCM (30 mL) under an argon atmosphere. The mixture was stirred for 4 h at room temperature and protected from light. The reaction mixture was evaporated under reduced pressure and purified by column chromatography (silica gel, 0-2% MeOH in DCM) to give 75 mg of a blue solid (yield 72%). TLC: Rf (DCM/MeOH 9:1) 0.48. 1H NMR (400 MHz, 77 ºC, DMSO-d6, δ (ppm)): 9.40 (1H, s), 8.81 (1H, dd, J = 7.4, 1.8 Hz), 8.21 (1H, br s), 7.65 (1H, dq, J = 9.2, 2.0 Hz), 7.13 (1H, dd, J = 9.2, 2.6 Hz), 7.01 (1H, br s), 4.13 (3H, s), 3.22 (6H, s). 13C{1H} NMR (101 MHz, 77 ºC DMSO-d6, δ (ppm)): 166.8, 163.2, 158.8, 156.0, 155.4, 154.5, 153.1, 152.4, 136.7 (q, J = 32 Hz), 125.4, 124.9, 121.5 (q, J = 277 Hz), 120.5 (q, J = 323 Hz), 116.6, 115.6, 113.3, 109.7, 108.4, 107.7, 101.5, 97.7, 97.2, 43.0, 40.0. 19F NMR (376 MHz, DMSO-d6) δ (ppm): -63.1 (3F, s), -77.8 (3F, s). HRMS (ESI-TOF) (m/z): [M]+ Calcd for C19H16F3N4O 373.1271; found, 373.1274. Analytical HPLC (30–100% B in 30 min, formic acid additive): Rt = 5.1 min.
Photophysical characterization of the compounds.

Absorption spectra were recorded in a Jasco V-730 spectrophotometer at room temperature. Molar absorption coefficients (ε) were determined by direct application of the Beer-Lambert law, using solutions of the compounds in each solvent with concentrations ranging from 10^{-6} to 10^{-5} M. Emission spectra were registered in a Photon Technology International (PTI) fluorimeter. Fluorescence quantum yields (Φ_F) were measured by comparative method using Cresyl Violet in ethanol (Φ_{F:Ref} = 0.54) as reference for compounds 4-5 and 12-13. Fluorescein dissolved in aqueous sodium hydroxide (0.1 M; Φ_{F:Ref}=0.92) was used as reference in the case of compounds 10-11. Then, optically-matched solutions of the compounds and CV were excited and the fluorescence spectra was recorded. The absorbance of sample and reference solutions was set below 0.1 at the excitation wavelength and Φ_F were calculated using the following equation (1):

$$\Phi_{F:Sample} = \frac{\text{Area}_{Sample}}{\text{Area}_{Ref}} \times \left(\frac{\eta_{Sample}}{\eta_{Ref}}\right)^2 \times \Phi_{F:ref} \quad (1)$$

where Area_{Sample} and Area_{Ref} are the integrated fluorescence for the sample and the reference and η_{Sample} and η_{Ref} are the refractive index of sample and reference solutions respectively. The uncertainty in the experimental value of Φ_F has been estimated to be approximately 10%.

Photostability studies were performed by monitoring fluorescence bleaching of a 5 µM PBS solution (pH 7.4) of the compounds at 37 °C irradiated with a high power 505 nm LED (100 mW/cm²). Fluorescence intensity values were recorded at t = 0 (F₀) and after different irradiation times (F).

Cell Culture and Treatments. HeLa cells were maintained in DMEM (Dulbecco Modified Eagle Medium) containing high glucose (4.5 g/L) and supplemented with 10% fetal calf serum (FCS) and 50 U/mL penicillin-streptomycin. For cellular uptake experiments and posterior observation under the microscope, cells were seeded on glass-bottom dishes (P35G-1.5-14-C, Mattek). Twenty-four hours after cell seeding, cells were incubated for 30 min at 37 °C with COUPY dyes (4, 5, 12 or 13, 2 µM) in supplemented DMEM. Then cells were washed three times with DPBS (Dulbecco’s phosphate-buffered saline, pH 7.0-7.3) to remove the excess of the fluorophores and kept in low glucose DMEM without phenol red for fluorescence imaging.

For colocalization experiments with Mitotracker Green FM, HeLa cells were treated with COUPY dyes (2 µM) and MitoTracker Green FM (0.1 µM) for 30 min at 37 °C in
nonsupplemented DMEM. After removal of the medium and washing three times with DPBS, cells were kept in low glucose DMEM without phenol red for fluorescence imaging. For colocalization experiments with Lysotracker Green FM and Hoechst 33342, HeLa cells were treated with COUPY dyes (2 μM) and LysoTracker Green FM (0.2 μM) for 30 min at 37 °C in nonsupplemented DMEM. After removal of the medium and washing three times with DPBS, cells were incubated for 10 min at 37 °C with Hoechst 33342 (1 μg/mL) in supplemented DMEM. Finally, cells were washed and kept in low glucose DMEM without phenol red for fluorescence imaging.

**Fluorescence Imaging.** All microscopy observations were performed using a Zeiss LSM 880 confocal microscope equipped with a 405 nm laser diode, an argon-ion laser, a 561 nm laser, and a 633 nm laser. The microscope was also equipped with a Heating Insert P S (Pecon) and a 5% CO₂ providing system. Cells were observed at 37°C using a 63× 1.2 glycerol immersion objective. Coumarins 4 and 12 were excited using the 561 nm laser and detected from 570 to 670 nm. Coumarins 5 and 13 were excited using the 633 nm laser and detected from 643 to 758 nm. In colocalization studies, Mitotracker Green FM and Lysotracker Green were observed using the 488 nm laser line of the argon-ion laser, whereas the 405 nm laser diode was used for observing Hoechst 33342.

Bleaching experiments were performed at 37 °C by continuous image acquisition with an interval time of 0.42 sec for 4.5 min. Coumarins 4 and 12 were bleached using the 561 nm laser at 4.32 μW and coumarins 5 and 13 were bleached with the 633 nm laser at 10.2 μW. Image processing and analysis was performed using Fiji.\(^{20}\)

**Intensity measurement:** the nuclei and coumarin channels were processed by median filtering (radius = 2), Gaussian filtering (sigma = 2) and background subtraction (rolling ball radius = 300). Mean intensity in the nucleoli was measured in the maximum intensity projection of the processed coumarin image after manually draw ROIs around each nucleoli. Intensity measurement in mitochondria needed of further processing. First, the nuclei were segmented using Intermodes algorithm,\(^{21}\) and the resulting binary image was processed by filling holes and opening operations. Then the binary image of the nuclei was subtracted to the processed coumarin image to get rid of any nuclear signal. After the subtraction, the coumarin staining channel was projected by the maxim intensity and the mitochondria were segmented using the Phansalkar algorithm (radius = 5).\(^{22}\) The binary image of the mitochondria was used as a mask to obtain the coumarin signal in the mitochondria and then measure its mean intensity.
**Bleaching analysis:** Images were first processed by median filtering (radius = 1) and background subtraction (rolling ball radius = 50). Then a ROI was manually drawn around each cell and in the background to measure the mean intensity along time. Intensity normalization was performed using the following equation (2):

\[
\text{Normalized intensity} = \frac{\text{Cell}(t) - \text{Backg}(t)}{\text{Cell}(0) - \text{Backg}(0)} \quad (2)
\]

were Cell(t) is the mean intensity in a cell at a t time, Cell(0) is the mean intensity in that cell at the beginning of the experiment, Backg(t) is the mean intensity in the background at a t time and Backg(0) is the mean intensity in the background at the beginning of the experiment. After intensity normalization, the time at which the intensity dropped to half of the initial one (t_{50}) was obtained. Differences between the different t_{50} of coumarin 4 and 12 were tested with a T-Student.

**Co-localizations coefficients.** The mitotracker or lysotracker and coumarin channels were processed by median filtering (radius = 1), Gaussian filtering (sigma = 1) and background subtraction (rolling ball radius = 300). Colocalization coefficients were measured using the JaCoP plugin\(^1\) on the different stacks of images (n = 4) with each stack containing 3–5 cells. The threshold for the coumarin channel was set to include the signal in the mitochondria, nucleoli and vesicles. The threshold for the mitotracker or lysotracker channels was set to select specifically mitochondria and lysosomes respectively.

**ASSOCIATED CONTENT**

**Supporting Information**
Copies of HPLC traces and UV–vis absorption and fluorescence emission spectra of the compounds; additional fluorescence imaging studies; 1D NMR (\(^1\)H, \(^{13}\)C, and \(^{19}\)F), MS, and selected 2D NMR spectra.
This material is available free of charge via the Internet at [http://pubs.acs.org](http://pubs.acs.org).

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