

RESEARCH ARTICLE OPEN ACCESS

π -Extended COUPY Fluorophores for Targeted Near-Infrared Fluorescence and Lifetime Imaging in Live Cells

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Received: 23 December 2025 | **Revised:** 23 May 2026 | **Accepted:** 9 June 2026

Keywords: coumarin | imaging agents | NIR dyes | peptide conjugation | polymethines

ABSTRACT

Fluorescent probes operating in the near-infrared (NIR) region offer deep-tissue visualization with minimal background interference, reduced phototoxicity, and enhanced signal-to-noise ratios. However, most conventional NIR fluorophores exhibit poor photostability, low fluorescence quantum yields, and limited chemical versatility, which constrain their use in demanding biological environments. Here, we report a new family of π -extended COUPY dyes (3–7), rationally engineered to deliver efficient far-red/NIR performance through vinylogation of the exocyclic double bond of the coumarin scaffold. This single structural modification induces a bathochromic shift exceeding 100 nm in both absorption and emission spectra while preserving the compact architecture and synthetic accessibility of the parent coumarin. The resulting dyes combine high molar absorptivity, moderate-to-high quantum yields, and exceptional photostability under biologically-relevant conditions. Their performance was validated in live-cell imaging using both intensity-based and fluorescence lifetime imaging microscopy (FLIM), demonstrating compatibility with advanced imaging techniques. Furthermore, site-specific bioconjugation to a clinically relevant peptide confirmed their suitability for targeted imaging. Collectively, π -extended COUPY dyes represent a promising class of synthetically modular, chemically robust, and biologically compatible far-red/NIR fluorophores for high-resolution imaging, targeted strategies, and advanced bioimaging modalities, and provide a foundation for future studies aimed at evaluating their performance in more complex biological models.

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1 | Introduction

Fluorescent probes operating in the far-red and near-infrared (NIR) region of the electromagnetic spectrum [1, 2] are indispensable tools for advanced bioimaging. The NIR region is commonly divided into the first (NIR-I, ~700–1000 nm) and second (NIR-II, ~1000–1700 nm) near-infrared windows [3]. Longer excitation wavelengths minimize scattering and absorption by biological tissues, enabling deep penetration while reducing autofluorescence and phototoxicity [4, 5]. These properties make NIR fluorophores uniquely suited for in vivo imaging, real-time biological monitoring, and phototherapeutic applications [6, 7]. While NIR-II fluorophores have attracted increasing attention for deep-tissue and whole-body in vivo imaging, the far-red/NIR-I region remains highly relevant for microscopy-based applications, including live-cell imaging and fluorescence lifetime imaging microscopy (FLIM), where efficient detection, high spatial resolution, and compatibility with standard optical instrumentation are critical. Current NIR dyes rely on a limited set of scaffolds, including porphyrins [8], cyanines [9], hemicyanines [10], phthalocyanines [11], and modified rhodamines and BODIPYs [12, 13]. Among these, cyanine dyes dominate due to their tunable absorption and emission profiles and high molar absorptivity [14]. Indocyanine Green (ICG), FDA-approved in 1956, exemplifies their long-standing clinical relevance [15]. However, cyanines suffer from nonradiative decay associated with structural flexibility, low fluorescence quantum yields [16], poor photostability [17], short Stokes' shifts, and susceptibility to nucleophilic or oxidative degradation [18]. Beyond photophysical drawbacks, many NIR fluorophore platforms are associated with increased structural complexity and demanding synthetic routes, which can limit their accessibility, modular functionalization, and straightforward conjugation to biomolecules. Together, these challenges underscore the urgent need to expand the chemical space of far-red/NIR-active dyes through innovative yet synthetically efficient designs, enabling improved robustness for advanced bioimaging and targeted photomedicine [19–21].

The clinical impact of NIR dyes is evident in fluorescence-guided surgery (FGS), which enables real-time visualization of tumors and critical structures [22, 23]. Conjugation to targeting ligands improves specificity, enhancing surgical precision and reducing off-target damage. Beyond intensity-based approaches, FLIM provides unique contrast by measuring fluorescence decay rather than emission intensity. This parameter is inherently independent of fluorophore concentration, excitation power, and photobleaching, making FLIM particularly suited for probing dynamic biological processes and microenvironmental parameters in live cells and tissues with high spatial and temporal resolution [24–26]. FLIM requires dyes with nanosecond lifetimes, high photostability, and low cytotoxicity [27, 28].

Coumarin derivatives have emerged as versatile platforms for bioimaging because their modular architecture allows systematic tuning of photophysical properties through rational modifications [29–31]. A well-established approach to improve coumarin performance involves introducing electron-donating groups (EDGs) at position 7, which, together with the electron-withdrawing lactone moiety, generate a push–pull electronic system across the π -conjugated framework. However, modifications of the lactone moiety remain underexplored, with few

examples such as thionation or dicyanomethylene substitution (Figure 1) [32].

In 2018, our group introduced COUPYs—coumarin-based fluorophores incorporating a cyano(1-alkyl-4-pyridin-1-ium)methylene fragment at C2— [33] achieving tunable absorption/emission from green to far-red, large Stokes' shifts, excellent cellular uptake, and preferential mitochondrial localization. Building upon the original COUPY dye **1** scaffold (Figure 1), subsequent structure–property studies explored variations such as replacing the 7-*N,N*-dialkylamino group with azetidine and substituting the pyridinium moiety with pyrimidinium heterocycles [34, 35]. Notably, introduction of a julolidine ring combined with a CF₃ at C4 yielded COUPY dye **2** (Figure 1), which exhibits the most red-shifted absorption and emission within the series [36]. Beyond imaging, COUPY dyes demonstrated strong phototherapeutic potential, both as free molecules [37] and when nanoencapsulated [38] or combined with transition metal complexes [39–42].

Here, we report a new generation of COUPY dyes specifically engineered for efficient NIR operation through vinylogous extension of the exocyclic double bond at C2—a design strategy recently applied to develop Ru-COUPY complexes as photosensitizers for in vivo one-photon NIR photodynamic therapy [43]. In this work, we present five new π -extended COUPY derivatives (**3–7**), structurally derived from compounds **1** and **2** (Figure 1). These dyes exhibit significantly enhanced spectroscopic properties, with absorption and emission maxima red-shifted by over 100 nm compared to their predecessors, compatibility for FLIM in live cells, and straightforward conjugation to targeting peptides via click chemistry.

2 | Results and Discussion

2.1 | Design, Synthesis, and Characterization of π -extended COUPY Dyes **3–7**

As shown in Figure 2A, COUPY dyes **3** and **4** are analogues of COUPY **1**, featuring a vinylogous extension between the coumarin core and the cyano(1-alkyl-4-pyridin-1-ium)methylene fragment; dye **4** additionally includes a carbon-carbon bridge originating at position C3 to restrict rotation. Dyes **5** and **6** derive from COUPY **2** with the same π -extension, while dye **6** replaces CF₃ with a pentafluoroethyl group. COUPY **7** achieves the largest conjugation by adding two extra double bonds relative to COUPY **2**. Representative synthetic routes for dyes **5** and **7** are shown in Figure 2B and Scheme S1. Starting from Coumarin-153, sequential reactions—acetonitrile carbanion addition, DIBAL-H reduction, Knoevenagel condensation, and methylation—afforded dye **5** in high yield. Dye **7** was obtained via Wittig extension of aldehyde **9**, followed by reduction, condensation, and methylation. Routes for dyes **3**, **4**, and **6** are detailed in Schemes S2–S4. All π -extended COUPY dyes (**3–7**) and intermediates were fully characterized by 1D/2D NMR and HRMS, and purity confirmed by HPLC-MS (Figure S74).

The stereochemistry of the polymethine chain in COUPY dyes **3–7** was elucidated by 2D ¹H,¹H NOESY NMR experiments in CD₃OD (Figures S76–S81). For compounds **3**, **4**, and **7**, only a

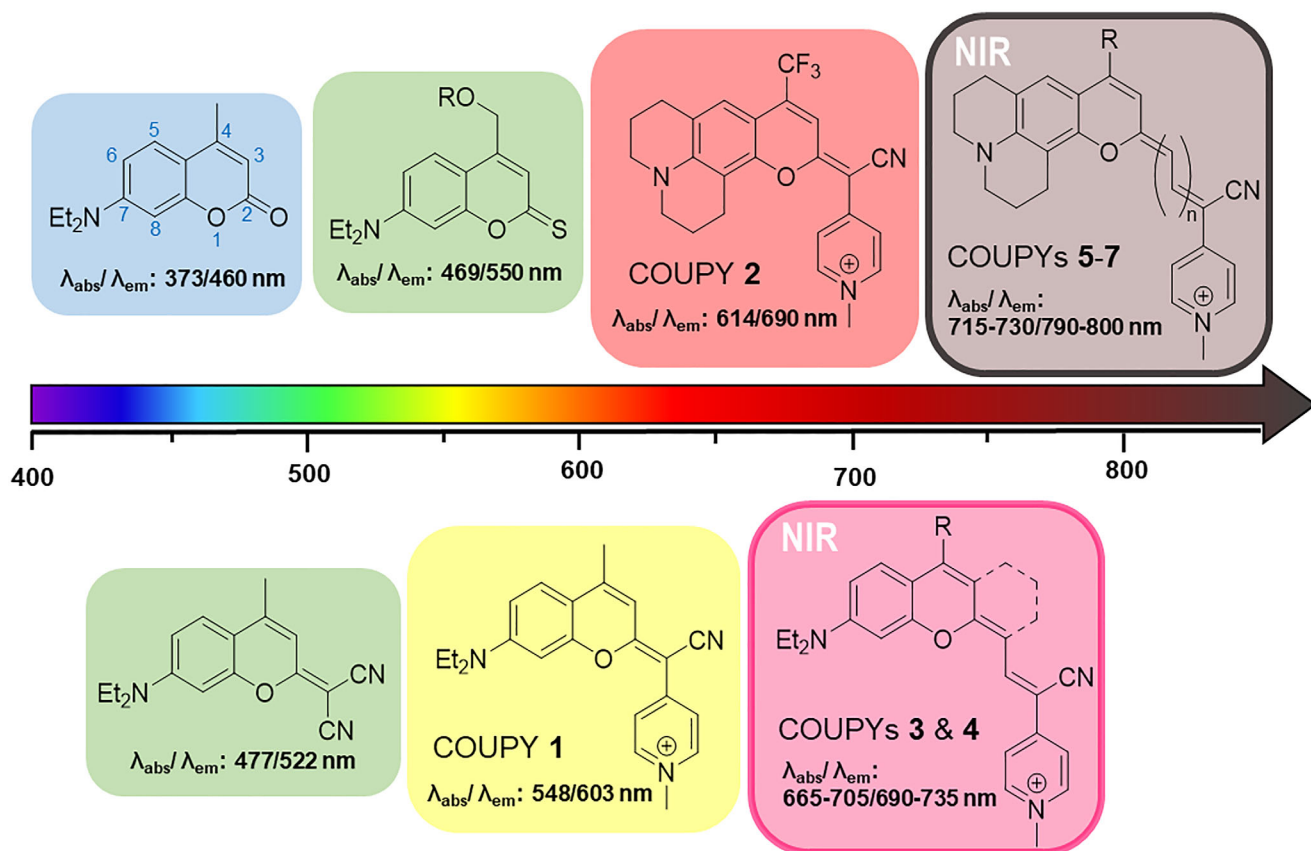


FIGURE 1 | Structural evolution of the coumarin scaffold toward NIR-active π -extended COUPY dyes. Modifications at C2 progress from parent 7-*N,N*-diethylamino-4-methyl coumarin through thiocoumarin and dicyanocoumarin derivatives to first-generation of COUPY fluorophores (1–2) and second-generation π -extended COUPY dyes (3–7) developed in this work. For each compound, absorption and emission maxima in ACN ($\lambda_{\text{abs}}/\lambda_{\text{em}}$) are shown. The gradual extension of the π -conjugated system through strategic modifications produces pronounced bathochromic shifts, ultimately enabling absorption and emission in the far-red/NIR-I region. The color gradient illustrates the spectral progression across the series.

single isomer was detected in solution. The configuration of the double bonds was assigned as *Z,Z* for compounds **3** and **4**, and *Z,E,Z* for compound **7**, based on the presence of diagnostic NOE cross-peaks. In contrast, detailed analysis of the ^1H and ^{19}F NMR spectra of COUPY dyes **5** and **6** revealed the coexistence of two distinct species in solution, as evidenced by duplicated proton and fluorine signals. This equilibrium was particularly pronounced for COUPY **5**, with a ratio of 1:0.6 between the two forms, while COUPY **6** exhibited a ratio of 1:0.25. The presence of a rotameric equilibrium was confirmed by exchange cross-peaks in the NOESY spectra. The major isomer in both cases was *Z,Z*.

The 3D structures and conformational preferences of COUPY dyes **3–7** were studied through theoretical calculations using the multiconfigurational complete-active-space self-consistent field (CASSCF) [44, 45] and the density functional theory (DFT/PBE0) method [46, 47], confirming fully planar conformations for all dyes in their dominant rotamer (Figure S82). The isomeric distributions observed in the NMR measurements were analyzed from both thermodynamic and kinetic perspectives. The thermodynamics for all possible double-bond configurations were calculated for compounds **3** (one observed isomer) and **5** and **6** (two observed isomers) as representative systems. The seven possible rotamers were labelled **a** to **g** for each molecule, as illustrated in Figures 3A and S83–S84. ΔG values shown in

Figure 3B reveal that the isomer **a** is the most stable arrangement in all three systems ($\Delta G = 0$). Rotamer **d** consistently appears as the second most stable configuration. The ΔG values for the **d** arrangement decrease in the order **3**>**5**>**6**, indicating that its predicted abundance is lowest for **3**, whereas in **5** and **6**, rotamers **a** and **d** structures are close in energy and therefore coexist in equilibrium. Indeed, NMR analysis confirmed that the isomers present in equilibrium for **5** and **6** correspond to dispositions **a** and **d**, while only **a** was detected for **3**, reinforcing the agreement between experimental observations and theoretical predictions.

Thermodynamic control of isomer distribution requires kinetically accessible isomerizations at room temperature. Energy profiles for the **a**↔**d** interconversion in dyes **3**, **5**, and **6** around the O–C1–C2–H dihedral angle (Figure 3C) evidence activation energies below 20 kcal/mol (Figure 3D), far lower than the ~80 kcal/mol for C=C rotation in (*2Z*)-but-2-en-2-ol (Figure S85), used as a pure C=C model, and significantly higher than the torsion around the C–C bond of butan-2-ol, used as a C–C model (Figure S85). These values explain the occurrence of **a**↔**d** isomerizations at room temperature within seconds, enabling thermodynamic equilibration of the rotamers. Similar kinetics were observed for other C–C rotations, such as **a**↔**c** (Figure S86). The relatively low torsional barriers indicate weakened C=C linkages compared to typical double bonds (Figure 3D). Bond

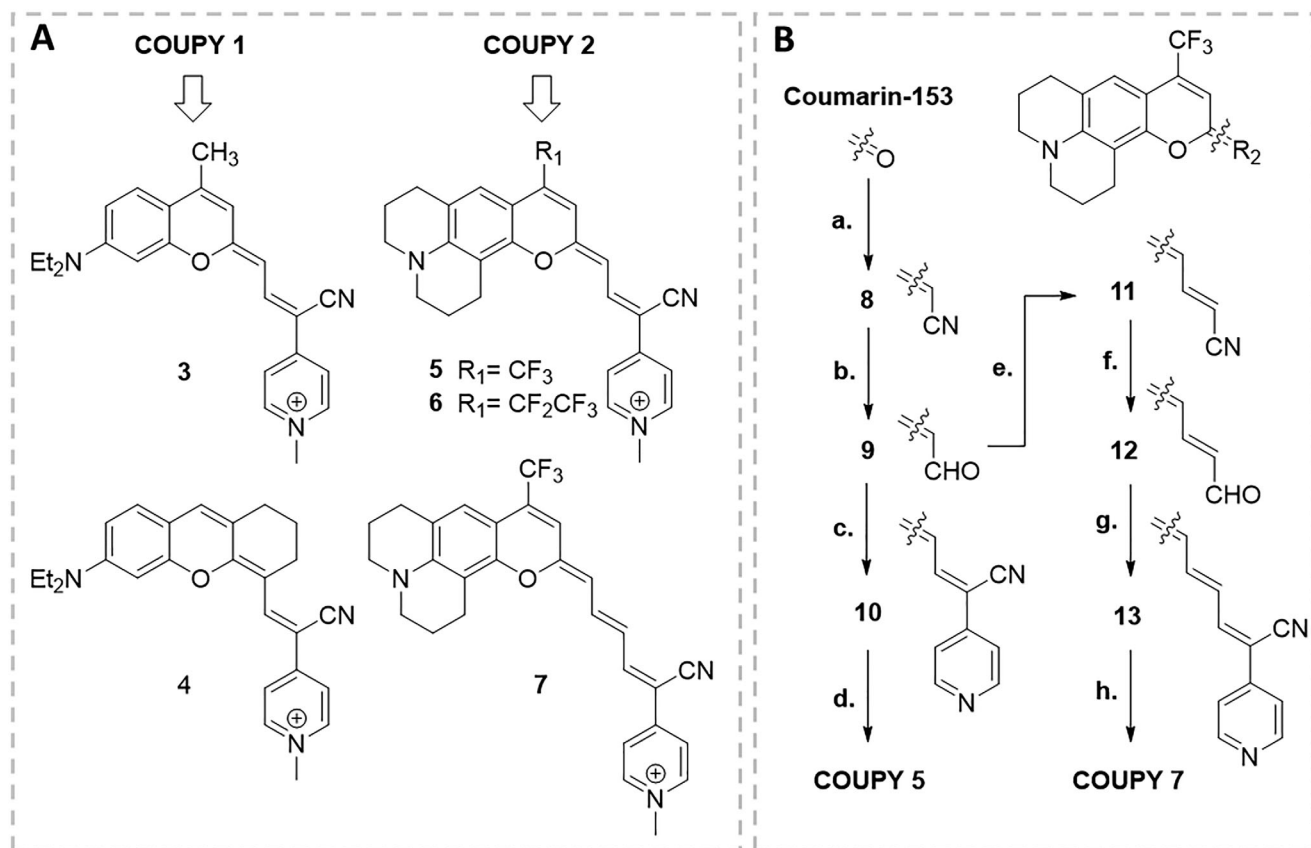


FIGURE 2 | Structures and synthetic routes of NIR-active π -extended COUPY dyes. (A) Chemical structure of compounds 3–7. (B) Representative synthetic routes for dyes 5 and 7. Reagents and conditions: (a) i. CH_3CN , n -BuLi, THF, Ar, $-78^\circ C$, 30 min, ii. HCl aq., rt, 3 h, 70%; (b) i. DIBAL-H, toluene, rt, 30 min, ii. potassium sodium tartrate, rt, 30 min, 50%; (c) 4-pyridylacetonitrile-HCl, piperidine, EtOH, $80^\circ C$, 16 h, 66%; (d) MeOTf, DCM, rt, 4 h, 98%; (e) Ph_3PCHCN , toluene, $60^\circ C$, 7 days, 84%; (f) i. DIBAL-H, toluene, rt, 30 min, ii. potassium sodium tartrate, rt, 30 min, 63%; (g) 4-pyridylacetonitrile-HCl, piperidine, EtOH, $80^\circ C$, 16 h, 74%; (h) MeOTf, DCM, rt, 4 h, 73%.

orders along the polymethine chain, obtained from CASSCF wave functions (Figure 3E), range from 1.25 to 1.45—far from the ideal value of 2 typical of a double bond—consistent with the $C=C$ torsion profiles. This reduction arises from the strong push-pull character of the COUPY scaffold as well as to the presence of electron-withdrawing groups (CF_3 , CN, and pyridinium) that deplete electron density from the polymethine chain.

In COUPY dyes 5–7, CF_3 and CN/pyridinium compete to attract electron density from the conjugated system, with the C_2-C_3 bond acting as a boundary between the two attractive effects. The CF_3 group strengthens C_1-C_2 , while extended conjugation in 7 increases the bond orders of C_4-C_5 and C_5-C_6 ; notably, the C_3-C_4 bond order remains comparable to that in 5. Across 5–7, the C_2-C_3 bond is weaker than the C_3-C_4 , likely due to competing effects of CF_3 and pyridinium (Figure 3E). In contrast, dyes 3 and 4 show uniform $C-C$ bond strengths, with bond orders between 1.30 and 1.35. Although these trends clarify the influence of EWGs on the polymethine chain, rotational energy profiles and $C=C$ bonds are similar across all dyes (Figures 3D and S86). Therefore, we conclude that the reduced bond order strongly accelerates isomerization kinetics across the entire COUPY family, with a similar impact for all members. As a direct consequence of these fast isomerization kinetics, the relative populations of the associated rotamers are governed by thermodynamic equilibrium

at room temperature and constitute intrinsic molecular properties of the COUPY scaffold. These populations are therefore independent of the synthetic batch, and identical rotamer ratios are observed for independently prepared samples of COUPYs 5 and 6, provided that solvent, temperature, and purity are comparable. The presence of rotamers thus reflects an inherent conformational feature of the COUPY family rather than a source of structural heterogeneity or batch-to-batch inconsistency.

2.2 | Photophysical Characterization: Experimental and Computational Studies

The photophysical properties of π -extended COUPY dyes 3–7 was evaluated in three organic solvents with different polarity and hydrogen bonding capabilities (MeOH, ACN, and DCM), and compared to those of the parent COUPY dyes 1 and 2 (Tables 1 and S1). Compounds 3–7 exhibit intense far-red/NIR absorption (Figures 4A and S87), with maxima ranging from 663 nm (3) to 724 nm (6) in MeOH, and moderate-to-high molar absorption coefficients (34 – $56 \text{ mM}^{-1}\text{cm}^{-1}$ in MeOH). Dyes incorporating both a julolidine moiety and strong EWGs (CF_3 or CF_2CF_3) at C4 display broad absorption bands centered at 708–724 nm, extending up to 800 nm (5–6) or 900 nm (7). As previously observed for dyes 1 and 2, the new COUPY derivatives also display

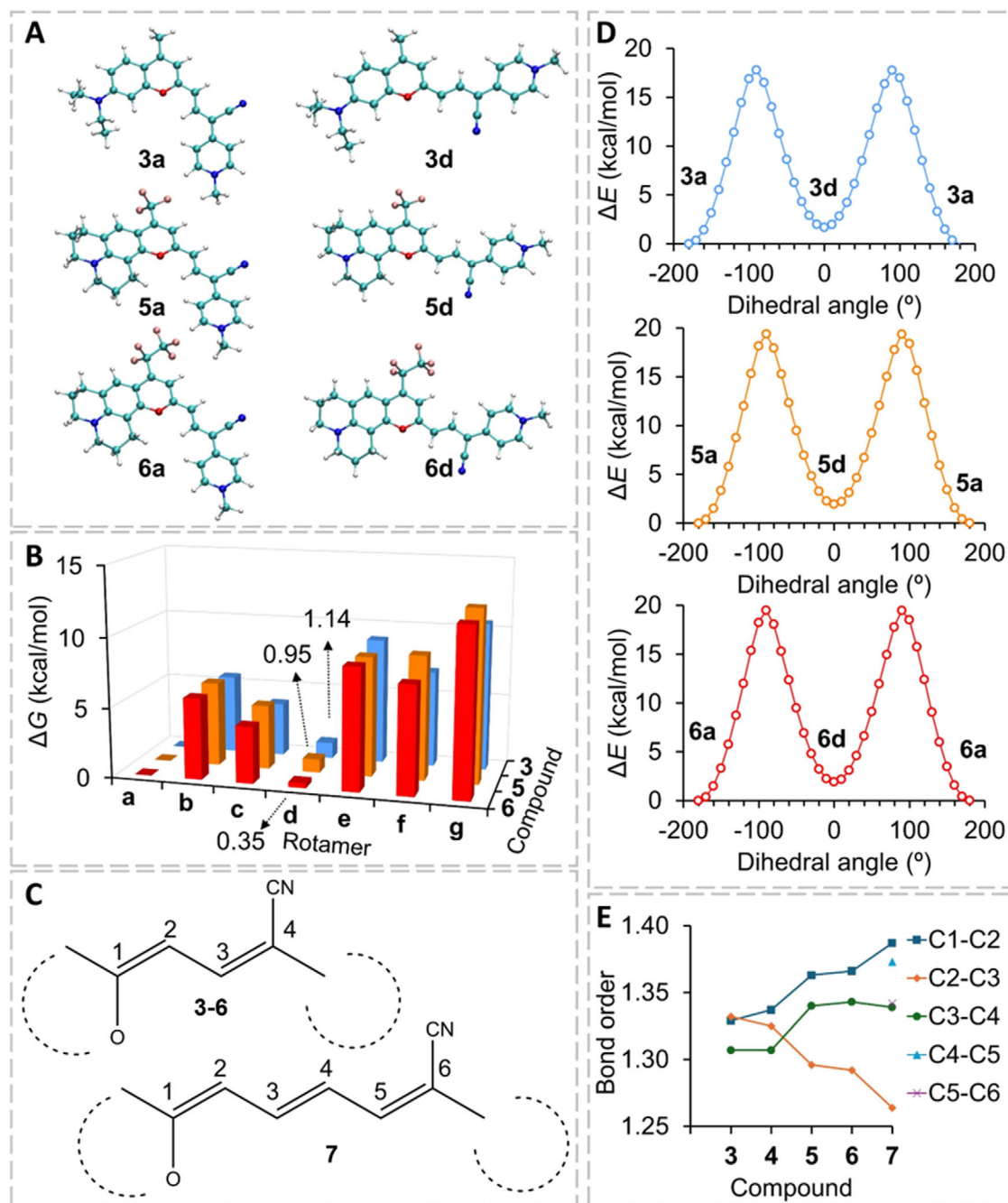


FIGURE 3 | (A) DFT-optimized structures of molecular models **3**, **5**, and **6** in their lowest-energy rotamers (**a** and **d**). (B) Gibbs energy differences (ΔG) [47, 48] of rotamers **b–g** relative to **a**, computed using the PBE0/6-31G* method in MeOH [49]. (C) Polymethine chain numbering. (D) Ground-state relaxed scans of O–C1–C2–H dihedral angles for **3a**→**3d**, **5a**→**5d**, and **6a**→**6d** isomerizations (PBE0/6-31G*, MeOH). (E) Bond order trends in the ground state for C–C bonds (C) across the COUPY family studied in this work computed with the CASSCF(12,12)/ANO-RCC-VDZP method in vacuo [44, 45].

negative solvatochromism, exemplified by **5** shifting from $\lambda_{\text{abs}} = 760$ nm in DCM to $\lambda_{\text{abs}} = 708$ nm in MeOH, accompanied by a pronounced hyperchromic effect in nonpolar solvents.

Overall, structural modifications in dyes **3–7** markedly alter absorption compared to parent COUPY dyes. Vinylogation of the exocyclic double bond induces a ~ 100 nm redshift in the absorption maxima, accompanied by a hyperchromic effect. Notably, conformational restriction in dye **4** adds a further 44 nm redshift relative to compound **3** (158 nm vs. **1**). Interestingly,

introducing a second double bond in COUPY dye **7** does not further shift the absorption maximum but generates an additional NIR band exhibiting halogen-sensitive solvatochromism in DCM (Figure S87). This behavior was further confirmed in other halogenated solvents (e.g., hexafluoroisopropanol, trifluoroethanol, 1,2-dichlorobenzene), suggesting potential applications in chemical sensing (Figure S88) [53].

Emission properties follow similar trends in π -extended COUPY dyes **3–7**. Vinylogation of the exocyclic double bond produces

TABLE 1 | Photophysical data of COUPY dyes 1–7 in MeOH.

COUPY	$\lambda_{\text{abs}}^{\text{a}}$	$\epsilon(\text{mM}^{-1}\text{cm}^{-1})^{\text{b}}$	$\lambda_{\text{em}}^{\text{c}}$	Stokes' Shift ^d	$\Phi_{\text{F}}^{\text{e}}$	Brightness ($\text{M}^{-1}\text{cm}^{-1}$) ^f
1	549	51.2	602	53	0.23	11,776
2	613	23.1	685	72	0.03	693
3	663	55.6	688	25	0.72	40,032
4	707	56.4	736	29	0.28	15,792
5	708	37.9	789	81	0.15	5,685
6	724	36.9	791	67	0.15	5,535
7	715	34.0	790	75	0.06	2,040

^aWavelength of the absorption maximum (λ_{abs});^bMolar absorption coefficients at λ_{abs} ;^cWavelength of the emission maximum upon excitation 5 nm below λ_{abs} ;^dStokes' shift (nm);^eFluorescence quantum yield (Φ_{F}), determined using Cresyl Violet [50] (for 1–2), Rhodamine 800 [51] (for 3), and ICG [52] (for 4–7) as references;^fBrightness ($\epsilon \times \Phi_{\text{F}}$). Data for compounds 1–2 taken from ref. 36.

>100 nm redshifts in fluorescence maxima relative to the parent dyes 1 and 2. In MeOH, dyes 3–7 display intense fluorescence bands with maxima ranging from 688 nm (3) to 791 nm (6) (Figures 4B and S89). Despite similar absorption coefficients ($\epsilon = 56 \text{ mM}^{-1}\text{cm}^{-1}$), COUPY dyes 3 and 4 differ markedly in fluorescence quantum yield ($\Phi_{\text{F}} = 0.72$ for 3 vs. 0.04 for 4 in MeOH). No significant differences were observed among COUPY dyes 5–7 in terms of emission maxima and quantum yields.

To evaluate the behavior of π -extended COUPY dyes under biologically relevant conditions, additional photophysical measurements were performed in aqueous phosphate-buffered saline (PBS). The results (Figures S87–S88 and Table S1) show that the dyes preserve suitable absorption, emission and brightness properties in aqueous media, supporting their compatibility with live-cell imaging studies (*vide infra*).

To gain deeper insight into the electronic transitions, multi-configurational complete-active-space second-order perturbation theory (CASPT2) [54, 55] calculations on top of the DFT and time-dependent (TD)-DFT optimized structures for S_0 and S_1 , respectively. This computational protocol, which predicts absorption spectra of organic molecules with high accuracy [56–59], was used to determine the absorption and emission energies for COUPY dyes 3–7 in ACN, MeOH, and DCM [49]. Results are shown in Table S2 and correspond to $S_0 \rightarrow S_1$ excitations of univocal $^1(\pi, \pi^*)$ character (Figure 4C). Computed values reproduce not only the absorption and emission band maxima but also the observed solvatochromism. Overall, the excellent agreement between theory and experiment validates the photophysical trends discussed above.

2.3 | Dark and Light Stability of π -Extended COUPY Dyes in Biological Media

The stability of π -extended COUPY dyes was evaluated in cell culture medium (DMEM supplemented with 10% FBS), under dark conditions and deep-red light irradiation (650 nm; 95 $\text{mW}\cdot\text{cm}^{-2}$). HPLC-MS analysis confirmed that all compounds

remained intact after 24 h in the dark at 37°C (Figures 4C and S91–S95). Under continuous irradiation, dyes 4–6 exhibited excellent photostability: compounds 4 and 6 retained >93% integrity after a cumulative light dose of 170 $\text{J}\cdot\text{cm}^{-2}$, while compound 5 showed no photodegradation (Figures 4D and S96–S100). Dye 3 was slightly less stable, yet >70% remained after 30 min of exposure. Dye 7 also showed a modest decrease in photostability, consistent with the well-known trend in cyanine dyes where longer polymethine chains reduce photostability. Nevertheless, >80% of dye 7 remained intact after exposure to 170 $\text{J}\cdot\text{cm}^{-2}$. Importantly, COUPY dyes 3 and 7 maintained high photostability at fluences up to 25 $\text{J}\cdot\text{cm}^{-2}$, over an order of magnitude above typical fluorescence microscopy doses [60]. This robustness under biologically relevant conditions highlights the potential of π -extended COUPY dyes for long-term imaging and photonic applications in live-cell environments.

2.4 | Live-Cell Imaging With π -Extended COUPY Dyes 3–7

Encouraged by the favorable photophysical properties of COUPY dyes 3–7, we next evaluated their performance in live-cell fluorescence imaging under NIR excitation. HeLa cells were incubated with each dye (5 μM , 30 min at 37°C) and imaged using widefield microscopy under red ($\lambda_{\text{ex}} = 635 \text{ nm}$) and NIR ($\lambda_{\text{ex}} = 747 \text{ nm}$) excitation. All dyes produced strong intracellular fluorescence (Figure 5) upon excitation at both wavelengths, confirming their suitability for NIR imaging. The staining pattern was predominantly filamentous, indicating mitochondrial localization, with a secondary, less intense vesicular signal. No signs of cytotoxicity or phototoxicity were detected under the imaging conditions employed.

To further elucidate subcellular localization, colocalization experiments were performed using confocal microscopy, which provides enhanced spatial resolution and improved signal-to-noise ratio by eliminating out-of-focus fluorescence. Confocal colocalization with MitoTracker Orange (MTO) and LysoTracker Green (LTG) confirmed mitochondrial accumulation for dyes 3–6

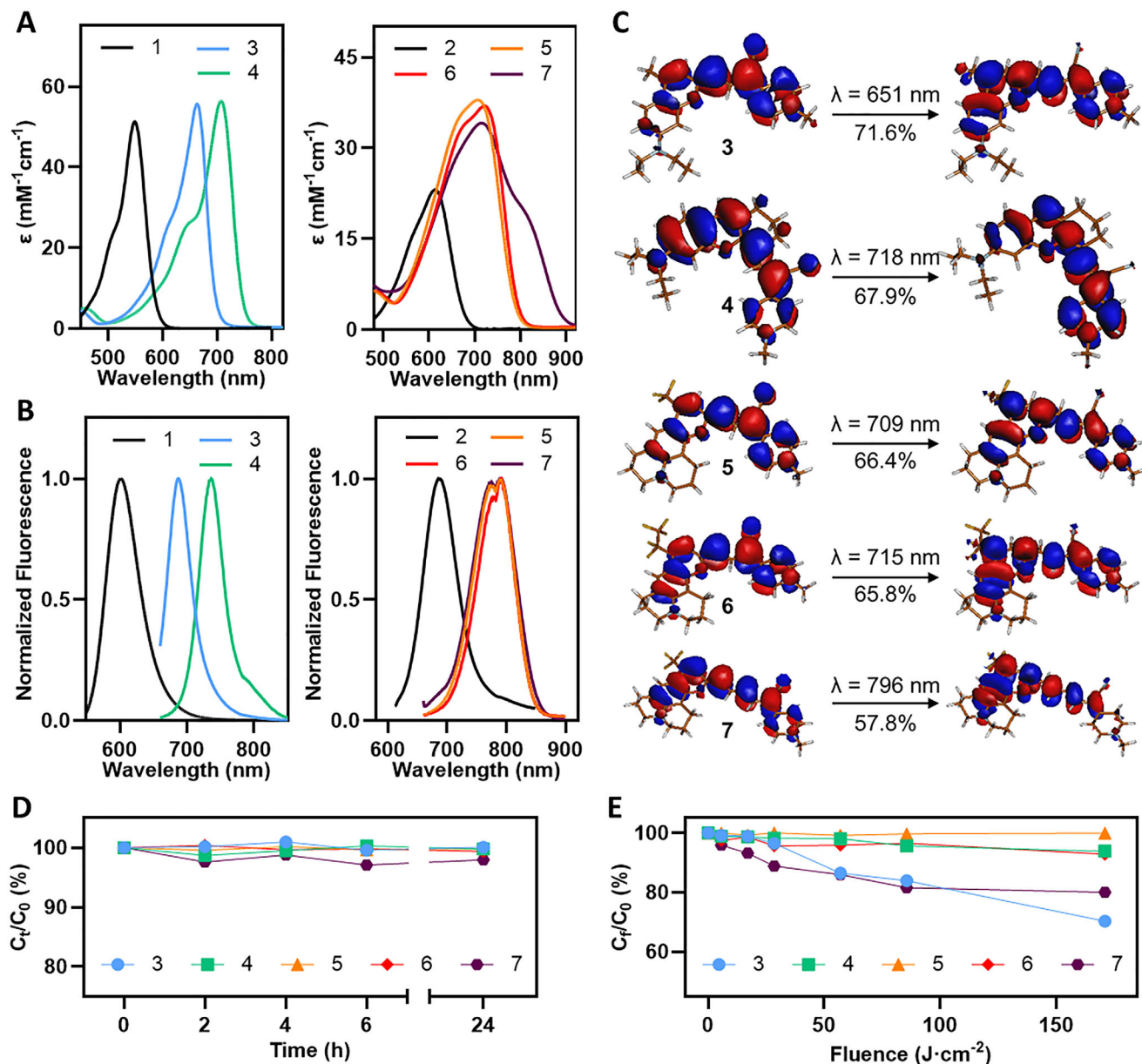


FIGURE 4 | Photophysical and stability characterization of π -extended COUPY dyes 1–7. (A, B) Absorption and emission spectra in MeOH, respectively. (C) State-average (SA)-CASSCF(12,12) natural orbitals for dominant one electron transition that describe $S_0 \rightarrow S_1$ absorptions in ACN for dyes 3–7. Transition weights are shown as percentages. (D, E) Chemical stability in DMEM + 10% FBS under dark conditions (D) and after deep-red LED irradiation (650 nm, 95 mW cm⁻²; total dose: 170 J·cm⁻²) (E).

(Figure S101). Dye 7 showed weak emission signal under confocal conditions ($\lambda_{\text{ex}} = 633$ nm). However, widefield imaging at 747 nm, enabled visualization of its subcellular distribution. Colocalization analysis using Pearson's correlation coefficient (PCC) (Table S3) revealed that COUPY 3 exhibited the highest mitochondrial selectivity (0.74) with negligible lysosomal overlap (0.16). Dyes 4 and 7 showed moderate mitochondrial correlation with MTO (0.59 and 0.50) and low lysosomal correlation with LTG (0.18 and 0.20), whereas no clear organelle preference was observed for dyes 5 and 6 (MTO: 0.40–0.43; LTG: 0.29–0.35), consistent with their more diffuse intracellular staining patterns. Overall, COUPY 3 emerged as the most selective mitochondrial probe, while other derivatives exhibited broader or mixed intracellular distribution.

2.5 | Fluorescence Lifetime Imaging Microscopy in Live Cells

To evaluate the FLIM performance of the π -extended COUPY dyes, fluorescence lifetimes were first determined in PBS using a Leica Stellaris confocal microscope equipped with high-sensitivity HyD detectors. Initial measurements were conducted at a concentration of 5 μ M, using 550 nm excitation for the parent COUPY 1 and 638 nm for the π -extended derivatives 3–7, thereby establishing a reference dataset for subsequent cellular studies. In aqueous solution, the average fluorescence lifetimes spanned a broad range across the series, from approximately 0.2 ns to 4.15 ns (Table S5).

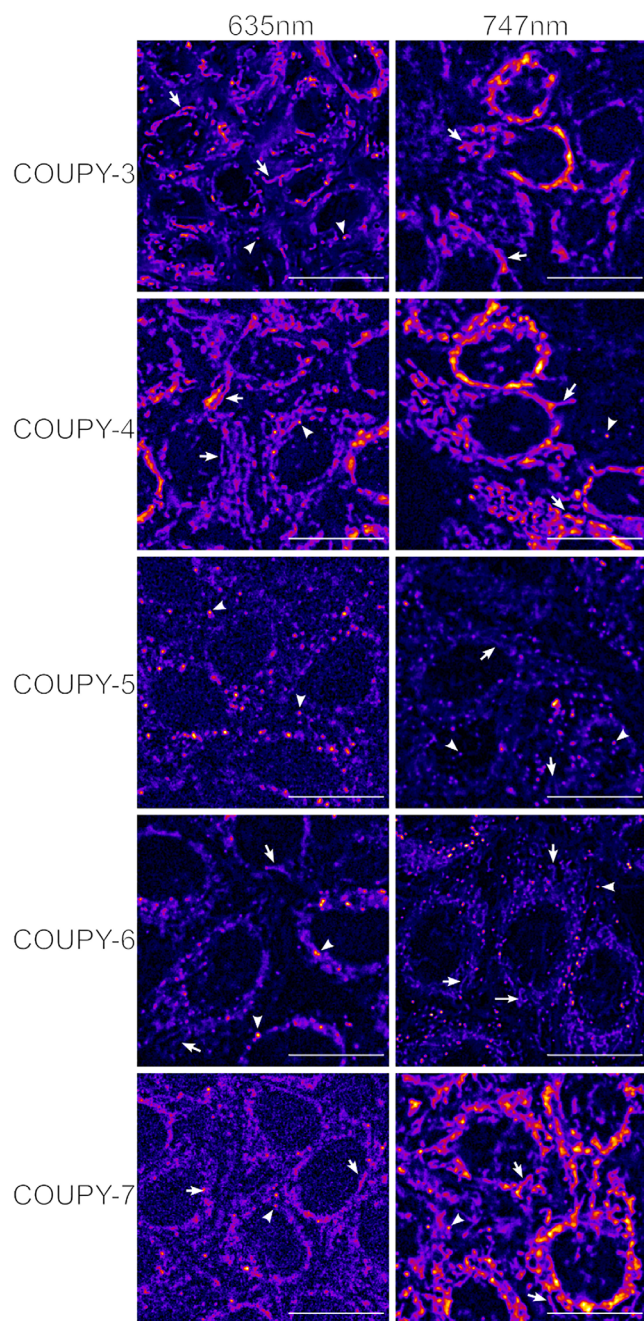


FIGURE 5 | Cellular uptake of π -extended COUPY dyes 3–7 in live HeLa cells. Representative fluorescence images after incubation (5 μ M, 30 min at 37°C). Excitation was performed using 635 nm and 747 nm LEDs, and emission was collected in the 666–724 nm and 770–850 ranges, respectively. White arrows point out filamentous mitochondrial staining; white arrowheads denote vesicular localization. Scale bar: 20 μ m. LUT: Fire.

For live-cell imaging, HeLa cells were incubated with the dyes following a standardized protocol (30 min at 37°C) (Figure 6). Although initial experiments were performed at 5 μ M, the probes were subsequently evaluated at a lower concentration of 0.5 μ M to assess their suitability under minimally perturbative and biologically relevant conditions. Upon cellular internalization, all compounds exhibit a systematic increase in fluorescence lifetime relative to their PBS values (Table S5). This consistent

lifetime extension, amounting to nearly a two-fold increase for most derivatives, is indicative of reduced non-radiative decay rates (k_{nr}) and highlights the sensitivity of the excited-state dynamics to the intracellular microenvironment, likely associated with mitochondrial localization and interactions with cellular membranes or proteins (*vide infra*).

FLIM measurements performed at 0.5 μ M using excitation wavelengths of 638 nm and 700 nm revealed a predictable and broadly tunable lifetime window in cells, ranging from 4.15 ns for COUPY 1 to 0.44 ns for COUPY 6. A modest dependence of the measured lifetime on excitation wavelength was observed, with longer excitation wavelengths yielding slightly increased lifetimes. In the case of COUPY 7, the photon budget achieved at a 0.5 μ M imaging concentration was insufficient to enable reliable lifetime determination or robust phasor analysis.

Analysis of COUPY dyes 3–6 at low concentration (0.5 μ M) revealed a high degree of lifetime uniformity across intracellular regions, as evidenced by narrow phasor distributions and homogeneous lifetime maps. This uniformity demonstrates that, under the conditions employed, the dyes behave as single-molecule probes within the cellular environment, effectively avoiding complications arising from local aggregation or pronounced microenvironmental heterogeneity, which are often encountered at higher probe concentrations. Although two rotameric species are detected by NMR spectroscopy for COUPY 5 as a consequence of partial C=C bond-order reduction, comparison between experimental absorption spectra and high-level theoretical calculations performed for the dominant rotamer shows that their optical properties are essentially indistinguishable. Consequently, rapid rotamer interconversion under equilibrium conditions does not lead to distinct spectroscopic signatures of lifetime. The FLIM response of COUPY 5 can therefore be attributed to the ensemble-averaged excited-state behavior of the rapidly equilibrating rotamers, fully consistent with the observed lifetime uniformity in live cells.

The dynamic performance of the probes was further corroborated by live-cell time-lapse imaging experiments (Movies S1 and S2 in the Supporting Information), acquired with second-scale temporal resolution using 638 nm excitation and very low dye concentrations (0.2 μ M for COUPY 3 and 0.5 μ M for COUPY 5). These experiments yielded high-contrast images without detectable phototoxicity over the 5-minute acquisition period.

Fluorescence lifetime quantification was carried out using the FLIM/FALCON module implemented in LAS X software. Mean fluorescence lifetimes were extracted on per cell basis by defining regions of interest (ROIs) corresponding to individual cells and analyzing the associated phasor distributions. Multiple cells were evaluated for each condition to ensure statistical robustness. Overall, these results establish the π -extended COUPY dyes as a robust platform of far-red/NIR-I fluorescent probes offering stable, tunable, and microenvironment-responsive fluorescence lifetimes well suited for FLIM in live-cell imaging.

To gain a deeper insight into the non-radiative decay channels governing S_1 de-excitation and to rationalize how spatial constraints in the intracellular microenvironment influence fluorescence lifetimes, the excited-state dynamics of compounds

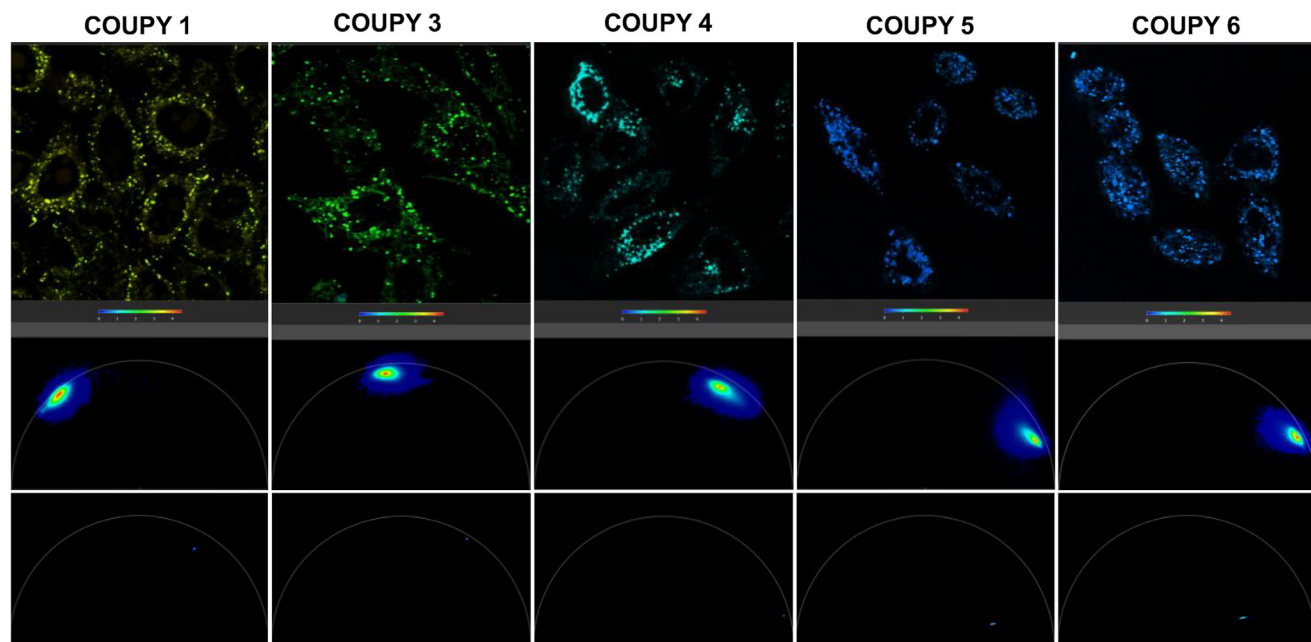


FIGURE 6 | FLIM analysis of COUPY dyes **1** and **3–6**. Top: Representative confocal images of HeLa cells incubated with the compounds (0.5 μM , 30 min at 37°C), color-coded by fluorescence lifetime. The corresponding color scale bar is shown below each image. Middle: Phasor plots of intracellular fluorescence signals for the compounds. Bottom: Phasor plots of the dyes imaged in PBS.

3 and **4** were investigated using quantum-chemistry methods at $T = 298\text{ K}$ (Figure S102). In both systems, regions of near-degeneracy between S_1/T_1 and S_1/S_0 were accessed within several hundreds of femtoseconds ($\Delta E < 0.5\text{ eV}$). However, intersystem crossing was found to be negligible due to very small S_1/T_1 spin-orbit couplings (SOCs), indicating that non-radiative deactivation proceeds predominantly via internal conversion through S_1/S_0 crossing regions. Accordingly, S_1/S_0 crossing structures of **3** and **4** were characterized using extended-multi-state (XMS)-CASPT2 calculations [61], as implemented in OpenMolcas [62]. XMS-CASPT2 analytical gradients [63] and S_1/S_0 non-adiabatic couplings [64] were computed at each optimization step. TDDFT frames with low S_1/S_0 energy differences ($<0.5\text{ eV}$) were used as starting guesses. As shown in Figure 7, the two relevant S_1/S_0 crossing points (CI1 and CI2) lie significantly closer in energy to the S_1 minimum for **4** ($\Delta E = 0.32$ and 0.34 eV , respectively) than for **3** ($\Delta E = 0.57$ and 0.49 eV , respectively). While all crossing energies remain sufficiently high to allow fluorescence with nanosecond-scale lifetimes, the lower values in **4** render non-radiative channels more competitive, in agreement with its reduced fluorescence quantum yield and shorter lifetime observed experimentally (Tables 1, S1 and S5).

Analysis of the optimized crossing geometries reveals that the dominant nuclear motions driving S_1 deactivation of **3** and **4** involve out-of-plane distortions, primarily torsions around C–C bonds. In the case of the $(S_1/S_0)_{\text{CI1}}$ pathway, the $\text{C}_3\text{—C}_4$ bond order decreases from approximately 1.3 to below 1.1, whereas along the $(S_1/S_0)_{\text{CI2}}$ pathway the quaternary nitrogen atom also undergoes pyramidalization. Such molecular motions are expected to be partially hindered in more compact environments, such as the intracellular environment, providing a mechanistic rationale for the longer fluorescence lifetime of **3** measured in live HeLa cells ($\sim 2\text{ ns}$) compared to PBS solution ($\sim 1\text{ ns}$).

2.6 | COUPY-Octreotide Conjugates for Ligand-Targeted Live-Cell Imaging

Somatostatin receptors (SSTRs), particularly subtype 2 (SSTR2), are G protein-coupled receptors widely expressed in the central nervous system and peripheral tissues and frequently overexpressed in several cancers [65], making them attractive targets for imaging and therapy [66, 67]. Octreotide (Figure 8A, $R_1 = \text{H}$), an FDA-approved cyclooctapeptide, binds SSTR2 with high affinity and selectivity and is clinically used in cancer diagnosis (e.g., OctreoScan) [68]. To harness the potential of NIR-active π -extended COUPY dyes for ligand-targeted imaging, we designed two octreotide conjugates, **COUPY-OCT-1** and **COUPY-OCT-2**, by functionalizing COUPY **5** at the pyridinium moiety with azide or alkyne groups (Figure 8B) and coupling to octreotide via Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC). This modular click approach enabled evaluation of both orientations, ensuring chemical versatility for bioimaging applications (Figure 8C),

COUPY derivatives bearing alkynyl (**24**) and azido (**26**) groups were obtained by alkylation of precursor **10** with the corresponding 2-bromoacetamide derivatives [69] (Figure 8B), while octreotide sequences with short PEG spacers and azide (**28**) or alkyne (**29**) functionalities at the N-terminal end of the peptide sequence were assembled by Fmoc/tBu solid-phase peptide synthesis (SPPS). Cleavage and deprotection were performed with TFA/ H_2O /EDT/TIS, followed by disulfide cyclization in aqueous buffer. Final conjugates were obtained via CuAAC in MeOH/ H_2O using $[\text{Cu}(\text{ACN})_4]\text{PF}_6$ as a catalyst (Figure 8C) and purified by RP-HPLC.

Spectroscopic analysis confirmed that conjugation did not alter the photophysical properties of COUPY **5** (Figure 8D). Live-cell imaging in HeLa cells, which express SSTR2 [70], revealed strong

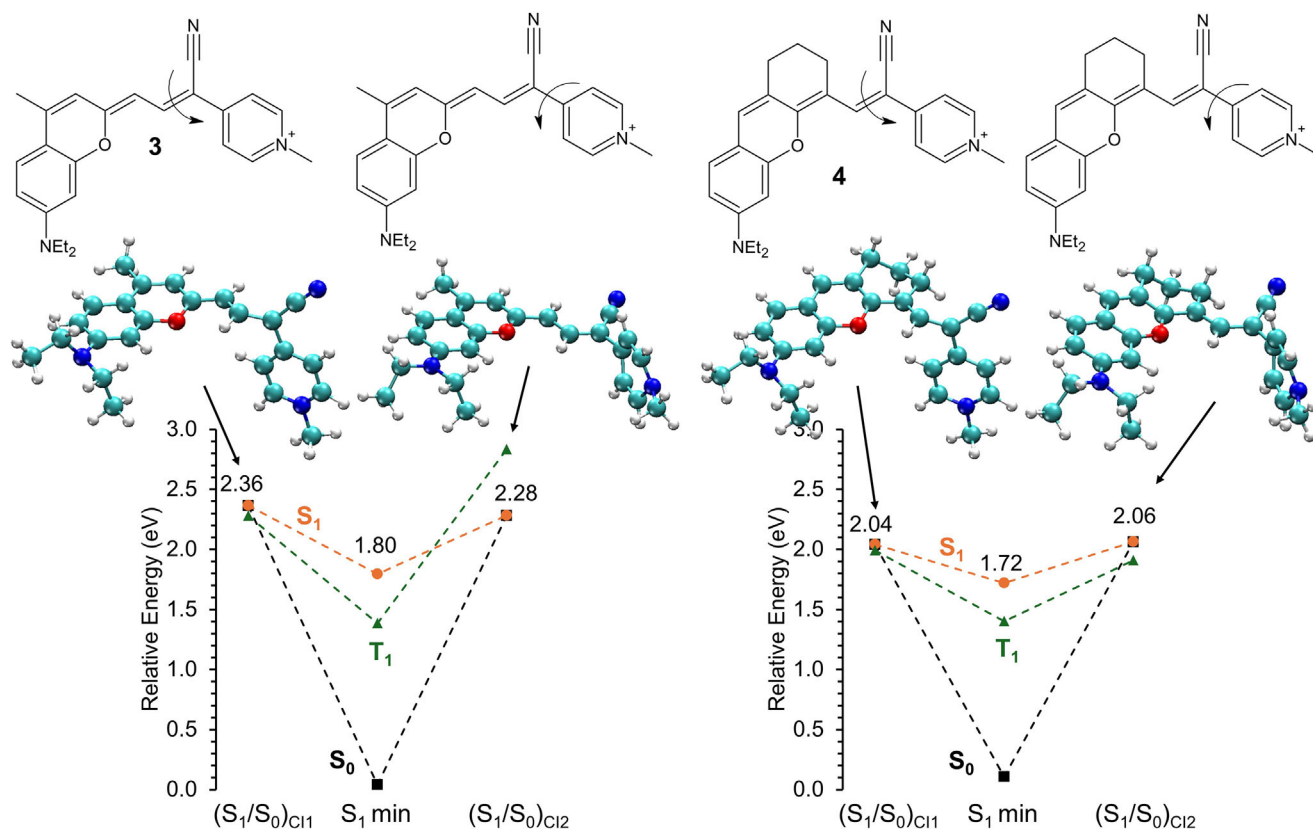


FIGURE 7 | Energy diagram representing the S_1 minimum, $(S_1/S_0)_{C11}$, and $(S_1/S_0)_{C12}$ energies that correspond to two non-radiative decay channels for COUPY dyes **3** and **4**. All geometries have been optimized with the extended-multi-state (XMS)-CASPT2 method with a SA-CASSCF(12,12) wave function reference. All energies are relative to the S_0 state at the corresponding S_0 minimum.

intracellular fluorescence under 747 nm excitation, with emission detected in the 770–850 nm range (Figure 9A), indicating efficient cellular uptake. Unlike free COUPY **5**, both conjugates showed predominant vesicular staining, suggesting endosomal entrapment. Confocal colocalization with membrane marker Wheat Germ Agglutinin (WGA) and LTG yielded moderate overlap ($PCC = 0.35\text{--}0.42$) and Manders' coefficients indicated partial association with membrane and lysosomal compartments, consistent with endosomal-lysosomal trafficking (Figure 9B, Table S4). To determine whether internalization was energy-dependent, the same experiments were repeated at 4°C in the presence of WGA. Under these conditions, both conjugates remained at the cell membrane without vesicular uptake, suggesting receptor-mediated endocytosis (Figure 9A). FLIM analysis of **COUPY-OCT-2** showed a fully vesicular pattern and a broad phasor distribution, with mean lifetimes comparable to COUPY **5** in PBS or intracellularly. The increased dispersion in the phasor cluster reflects heterogeneous microenvironments within labeled vesicles (endosomes, lysosomes), which modulate photophysical behavior and broaden lifetime profiles (Figure S103).

To evaluate the potential functional effect of COUPY–Octreotide conjugates, the pancreatic neuroendocrine tumor (PanNET) cell line QGP-1 was used as a model system. Owing to their high expression of somatostatin receptors, particularly SSTR2, PanNETs are among the main tumor targets for the use of first-generation somatostatin analogs (SSA) such as Octreotide and Lanreotide, both for diagnostic imaging, and for treatment with

“cold” SSA or with peptide receptor radioligand therapy (PRRT). In this regard, the QGP-1 cell line is characterized by strong SSTR2 expression, making it an excellent model for testing the conjugate [71]. Fluorescence analysis following treatment of these cells with COUPY **5** and **COUPY-OCT-2** (Figures 10A and B) demonstrated clear cellular uptake of both compounds, thus reproducing the results observed in HeLa cells. However, co-treatment with the previously described SSTR2 receptor antagonist BIM-23627 [72] reduced the uptake of the **COUPY-OCT-2** conjugate, but not that of COUPY **5**, demonstrating the involvement of SSTR2 in the cellular internalization of the conjugate.

These findings are further supported by immunocytochemistry images of SSTR2 (Figure 10C and D), which is known to undergo internalization upon activation by its ligands [73]. As illustrated by the images, treatment with the **COUPY-OCT-2** conjugate induced marked receptor internalization, an effect that was blocked by the antagonist BIM-23627.

Finally, we evaluated the functional effect of the conjugate in QGP-1 cells as a relevant SSTR2-positive tumor model. Cells were treated for 10 days to allow tumorsphere formation, a common indicator of tumor aggressiveness. As shown in Figure 10E and F, treatment with the **COUPY-OCT-2** conjugate reduced tumorsphere size. However, co-treatment with the SSTR2 antagonist BIM-23627, which alone had no effect, did not fully reverse this reduction. This observation suggests that partial receptor activation by the octreotide moiety of the conjugate is sufficient

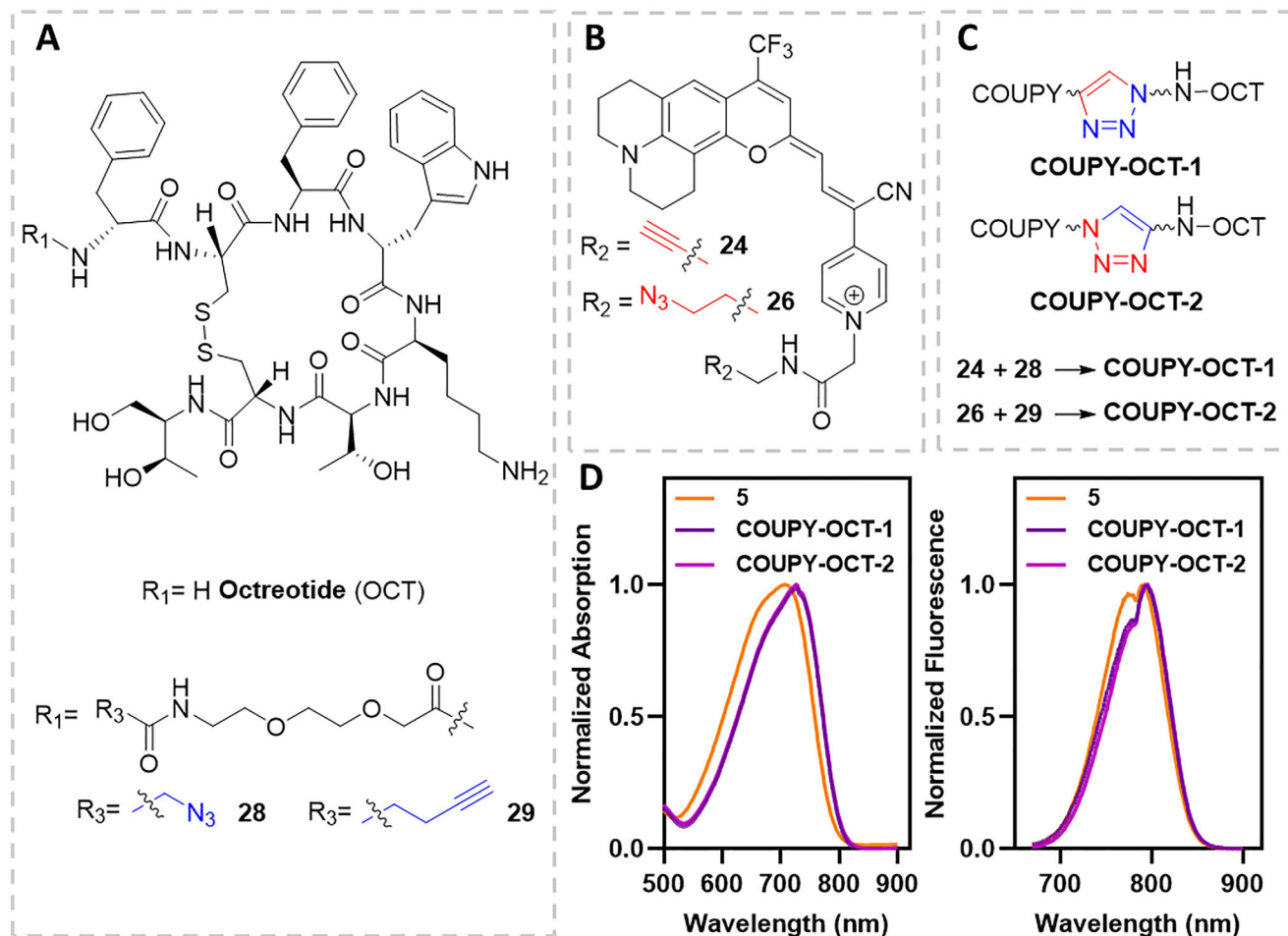


FIGURE 8 | Design and characterization of COUPY-Octreotide conjugates. (A) Structures of Octreotide and its azide and alkynyl derivatives (**28** and **29**, respectively). (B) Conjugatable π -extended COUPY dyes **24** and **26** bearing alkynyl and azide groups, respectively. (C) Condensed structures of **COUPY-OCT-1** and **COUPY-OCT-2** conjugates obtained via CuAAC. (D) Absorption and emission spectra of COUPY **5** and COUPY-Octreotide conjugates in MeOH, respectively.

to induce downstream biological responses. These effects are consistent with the known activity of somatostatin analogs and highlight that receptor engagement by the conjugate is functionally preserved.

3 | Conclusion

We have developed a new series of π -extended COUPY dyes (**3–7**) by introducing vinylogation at the exocyclic double bond of the coumarin-based COUPY scaffold. This modification significantly alters the electronic structure, producing pronounced bathochromic shifts that extend absorption and emission into the far-red/NIR region. Interestingly, this effect is not strictly incremental: while the first extension induces a major redshift (~ 100 nm), further conjugation leads to more complex behavior, including the emergence of secondary NIR bands rather than continuous displacement of the main maximum. For instance, COUPY dye **6** reaches 724 nm (absorption) and 791 nm (emission) in MeOH, whereas dye **7** introduces an additional NIR band without further shifting the primary absorption maximum, illustrating the interplay between conjugation length and conformational constraints in tuning optical properties. Compared to

parent dyes **1** and **2**, π -extended COUPY derivatives exhibit higher molar absorptivity while retaining moderate-to-high fluorescence quantum yields, a rare combination for far-red/NIR fluorophores. This balance of brightness and spectral positioning is advantageous for imaging applications requiring deep tissue penetration and minimal background interference.

Live-cell imaging studies confirmed excellent membrane permeability and robust intracellular fluorescence under red and NIR excitation. COUPY dye **3** showed strong mitochondrial selectivity, whereas dyes **5** and **6** provided diffuse staining, enabling complementary imaging strategies. FLIM measurements revealed that dye **3** combines high quantum yield with nanosecond lifetimes, supporting its use in lifetime-based imaging, while dye **5** exhibited lifetime heterogeneity across organelles, indicating sensitivity to microenvironmental changes.

Taken together, these results position the π -extended COUPY dyes within the broader landscape of non-cyanine far-red/NIR-I fluorophores, where achieving a balanced combination of brightness, excited-state lifetime, photostability, and performance in aqueous biological media remains challenging. Many rigidified donor-acceptor systems and other polymethine-free small

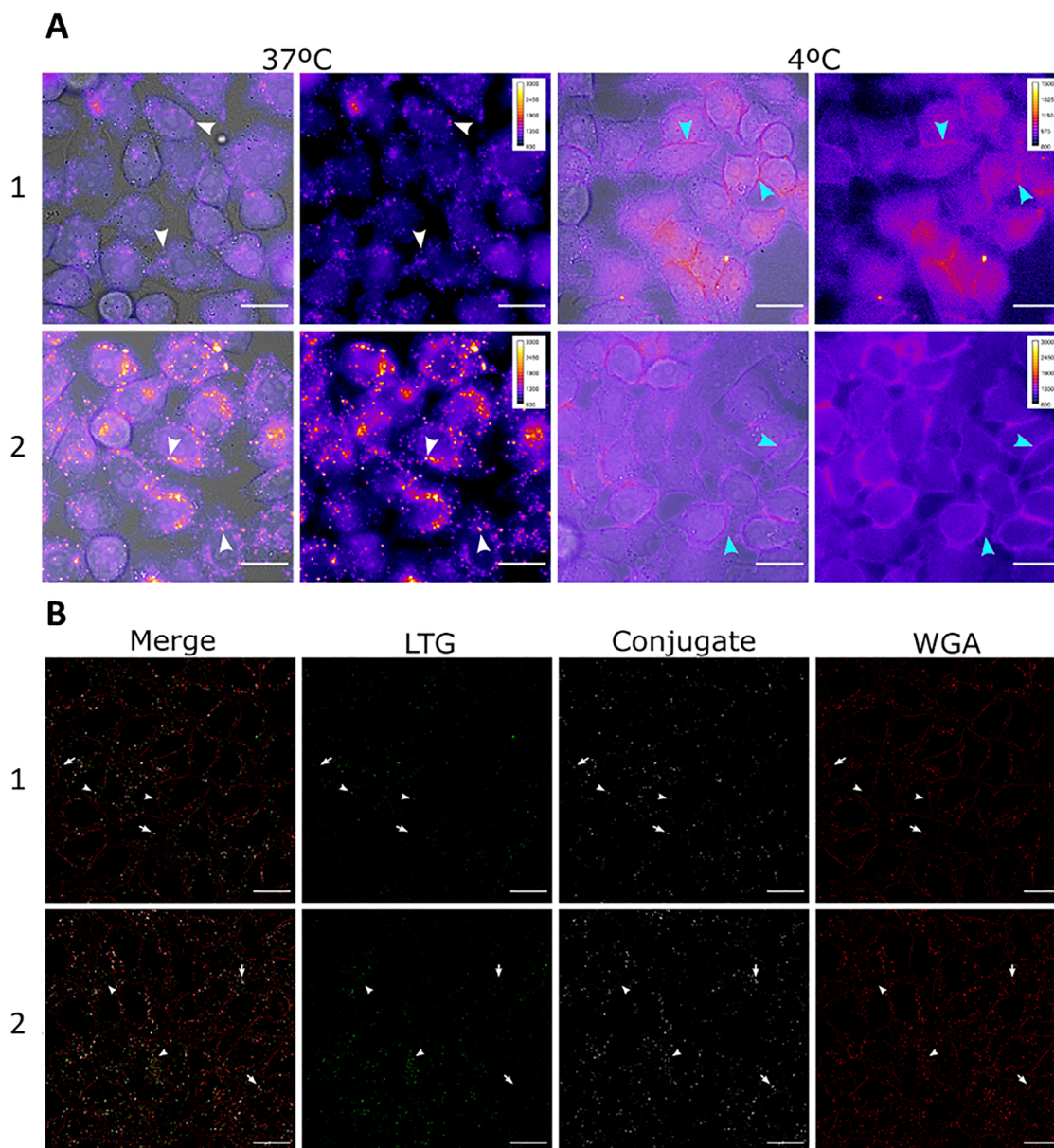


FIGURE 9 | Cellular imaging and colocalization of COUPY–Octreotide conjugates in live HeLa cells. (A) Fluorescence imaging after incubation with **COUPY-OCT-1** and **COUPY-OCT-2** (10 μ M, 30 min) in the presence of WGA at 37°C or 4°C; excitation: 747 nm LED. Arrowheads indicate vesicular staining (white) and membrane-associated fluorescence (cyan). Scale bar: 20 μ m. LUT: Fire. (B) Colocalization with WGA (emission at 555 nm) and LTG in HeLa cells after sequential incubation (10 μ M) conjugates + WGA 30 min; LTG, 5 min, 37°C). Images correspond to single confocal microscopy planes acquired upon excitation at 633 nm. White arrows and arrowheads point out colocalization with endosomes and lysosomes, respectively. Scale bar: 20 μ m.

molecules display moderate fluorescence quantum yields and short lifetimes under biological conditions, as exemplified by aza-BODIPY derivatives, despite their high molar absorptivities and photostability, often which require bulky structural modifications to preserve emission efficiency in water [3, 74, 75]. In this context, the π -extended COUPY platform effectively combines bathochromically shifted absorption and emission in the 700–800 nm range with moderate-to-high quantum yields, nanosecond fluorescence lifetimes compatible with FLIM, and high photostability under prolonged irradiation.

Finally, site-specific conjugation of COUPY dye **5** to octreotide via click chemistry preserved favorable photophysical properties

and enabled receptor-mediated uptake in HeLa cells, with vesicular localization consistent with endosomal trafficking. FLIM analysis revealed heterogeneous lifetimes within labeled vesicles, reinforcing the suitability of these dyes for dynamic and targeted imaging strategies. Importantly, studies in the SSTR2-positive pancreatic neuroendocrine tumor cell line QGP-1 further confirmed receptor-dependent internalization and functional engagement, as demonstrated by antagonist competition assays and receptor internalization.

Overall, π -extended COUPY dyes constitute a versatile platform for far-red/NIR-active probes, combining synthetic modularity, chemical robustness and biological compatibility. Their

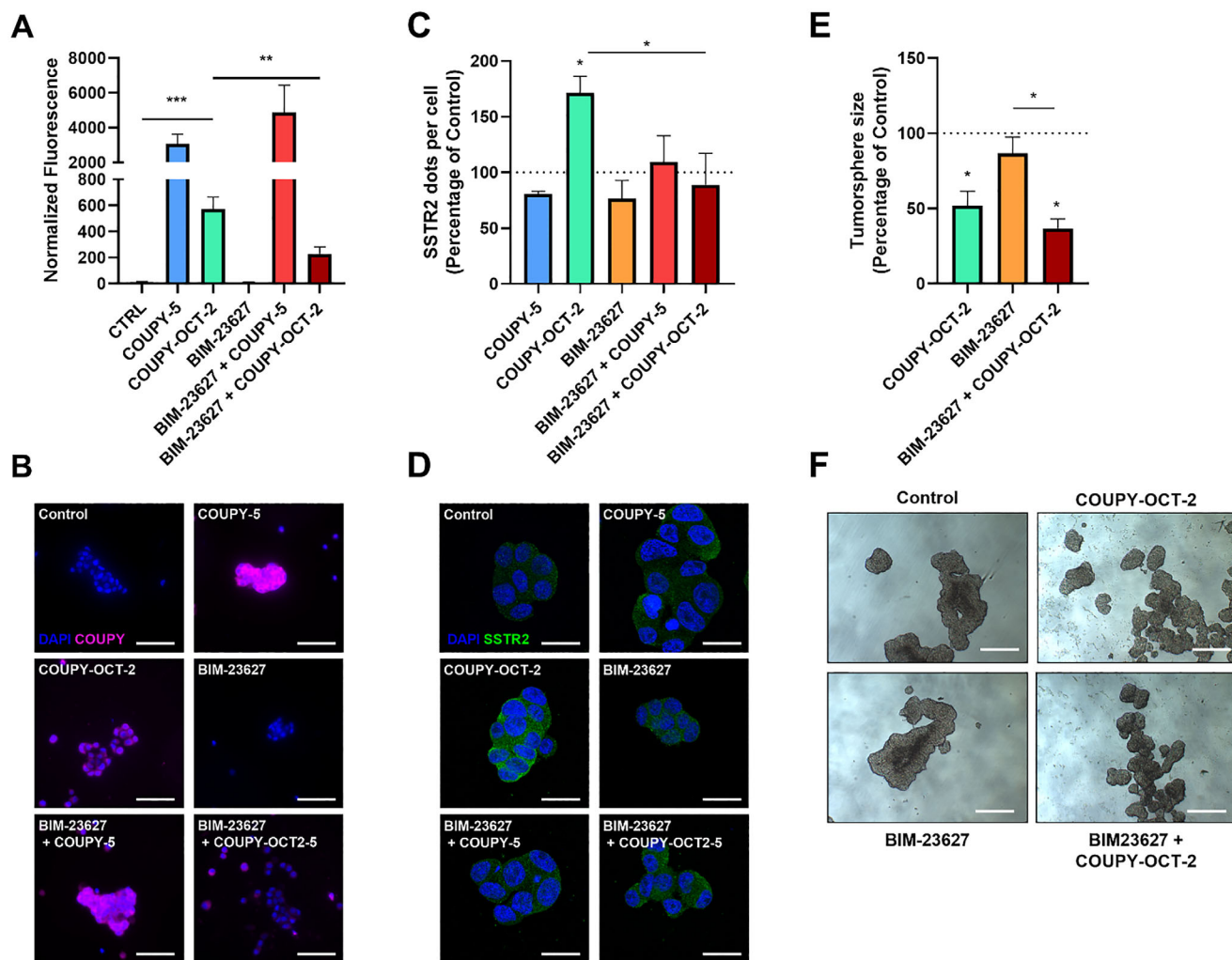


FIGURE 10 | In vitro effects of **COUPY-OCT-2** conjugate in QGP-1 cells. (A) Fluorescence emitted by **COUPY 5** or **COUPY-OCT-2** (5 μ M, 30 min) in the presence or absence of the SSTR2 antagonist BIM-23627. Representative images are shown in (B) where nuclei are shown in blue and fluorescent COUPY compounds in pink. Scale bar: 200 μ m. (C) Quantification of SSTR2 receptor internalization following treatment with **COUPY 5**, **COUPY-OCT-2**, and/or BIM-23627 (5 μ M, 30 min). Internalization was quantified by counting intracellular puncta. Untreated control cells were set at 100% and indicated by a dashed line. Representative images are shown in (D), where nuclei are shown in blue and the SSTR2 receptor in green. Scale bar: 50 μ m. (E) Tumorsphere formation capacity of QGP-1 cells in response to **COUPY-OCT-2** and/or BIM-23627 (5 μ M, 10 days) compared to untreated cells set at 100% and marked by a dashed line. Representative images are shown in F. Scale bar: 500 μ m. Asterisks indicate statistically significant differences (* p < 0.05; ** p < 0.01) determined by one-way ANOVA.

performance in both intensity- and lifetime-based imaging, together with successful bioconjugation, positions them as a versatile platform for advanced cellular bioimaging and targeted probe development, and provides a foundation for future theranostic studies.

Author Contributions

Diego Abad-Montero: investigation, conceptualization, writing – original draft, methodology, formal analysis, data curation, writing – review and editing. **Manel Bosch:** investigation, methodology, formal analysis, data curation. **Rodrigo Garcés-Castellanos:** investigation, data curation. **Michael Q. Schottenhamel:** investigation, data curation. **Sergio Pedraza-Arevalo:** investigation, methodology, formal analysis, data curation, writing – review and editing. **Alejandro Ibáñez-Costa:** investigation, methodology, writing – review and editing, formal analysis, data

curation. **Justo P. Castaño:** resources, supervision, funding acquisition. **Tania García-Becerra:** investigation, methodology, software, formal analysis, data curation. **Elena Rebollo:** investigation, methodology, software, formal analysis, data curation, resources, funding acquisition. **Marta E. Alberto:** investigation, methodology, formal analysis, writing – review and editing, funding acquisition. **Antonio Francés-Monerris:** investigation, methodology, formal analysis, data curation, writing – original draft, writing – review and editing, resources, funding acquisition. **Vicente Marchán:** conceptualization, funding acquisition, writing – original draft, writing – review and editing, project administration, supervision, resources.

Acknowledgments

This work was supported by funds from the Spanish Ministerio de Ciencia, Innovación e Universidades-Agencia Estatal de Investigación (MICIU/AEI/10.13039/501100011033) (PID2023-146161OB-I00 to V.M.; CNS2024-154908 to A.F.-M.; PID2022-136227OB-I00 to J.P.C.), and by “ERDF A way of making Europe.” (PID2023-146161OB-I00 to V.M.), Grupo

Español de Tumores Neuroendocrinos y Endocrinos (Junior grants 2023 to SPA; 2022 to AIC), Junta de Andalucía (BIO0139) and CIBERObn Fisiopatología de la Obesidad y Nutrición (Instituto de Salud Carlos III). M. E. A. acknowledges financial support under the PRIN D.D. 104/2022 PNRR M4.C2.1.1–Project Code: 2022AN47CACUP: H53D23003820001, funded by the European UnionNextGenerationEU– Project Title “La-G4-DACA”–Grant Assignment DD 862, 16/06/2023. M. E. A. is also grateful for the CINECA award under the ISCRA initiative, for the availability of high-performance computing resources and support (IsCc4_PAC-DACA project). D.A.-M. was a recipient fellow of the University of Barcelona (APIF). A.F.-M. is grateful for the support of the Generalitat Valenciana (GV) through the project CIAICO/2022/121. The Stellaris system at the Molecular Imaging Platform was supported by grant EQC2021-007200-P, funded by MCIN/AEI/10.13039/501100011033 and EU NextGenerationEU/PRTR; the authors also acknowledge the continued support from Leica Microsystems. The valuable collaboration of the technicians from the NMR, HRMS, and confocal microscopy of the Scientific and Technological Centers (CCiTUB) of the University of Barcelona is also appreciated.

Funding

This work was supported by Ministerio de Ciencia, Innovación y Universidades, Agencia Estatal de Investigación, Generalitat Valenciana, CINECA, European Union Next Generation, Instituto de Salud Carlos III, Junta de Andalucía, Grupo Español de Tumores Neuroendocrinos y Endocrinos, CIBERObn Fisiopatología de la Obesidad y Nutrición.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: anie73238-sup-0001-SuppMat.pdf.

Supporting File 2: anie73238-sup-0002-MovieS1.mp4.

Supporting File 3: anie73238-sup-0003-MovieS2.mp4.