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# Coumarin- and rhodamine-fused deep red fluorescent dyes: synthesis, photophysical properties and bioimaging *in vitro*

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A series of deep red fluorescent dyes (CR1 to CR3) was developed via introducing a coumarin moiety into the rhodamine molecular skeleton. The novel dyes possessed the individual advantages of coumarin

and rhodamine derivatives, and the emission wavelength was extended to deep red region (>650 nm) due to the extension of fused-ring conjugate structure simultaneously. To illustrate its value, we designed and conveniently synthesized a series of novel deep red bioimaging dyes (CR1E to CR3E) by esterification of CR1 to CR3, which could selectively stain mitochondria. They were superior to the MitoTrackers for mitochondrial staining in terms of large Stokes shift, excellent contrast for imaging, high photostability, and low cytotoxicity. Furthermore, the fluorescence of the coumarin moiety and rhodamine-like fluorophore could be switched like classical rhodamine. Thus, they could be used as an effective platform in constructing fluorescence sensors. Based on this fact, we constructed a novel ratiometric sensor (CR1S) for  $Hg^{2+}$  with good selectivity which could be successfully applied to the imaging of  $Hg^{2+}$  in living A549 cells. This design strategy is straightforward and adaptable to various deep red dyes and sensing platforms by simply introducing different fluorophores.

#### Introduction

Due to the virtue of its highly sensitive and high-speed spatial analysis of cells, fluorescence bioimaging has become a facile and powerful tool for visualizing morphological details and monitoring biomolecules in living systems.<sup>1</sup> This imaging technique is highly dependent on the photophysical and photochemical properties of the applied fluorescent dyes. Among the commonly used fluorescent dyes, rhodamine derivatives are highly favored in bioimaging and biosensor because of their excellent photophysical properties, such as high molar extinction coefficients, excellent fluorescence quantum yields, and great photostability.<sup>2</sup> For example, Rhodamine 123 has been described to selectively sequester in active mitochondria and be used as a marker for mitochondrial membrane potentials (MMP).<sup>3</sup> In addition, rhodamine derivatives can undergo equilibrium between spirocyclic and open-ring forms. The spirocyclic forms of these dyes are colorless and nonfluorescent due to the small  $\pi$ -conjugated systems, while the

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open-ring forms show intensive spectroscopic signals in the absorption and fluorescence spectra by generating the large  $\pi$ -conjutated systems.<sup>4</sup> Thus, they can be used as an effective platform based on spirocyclic/open-ring switching mechanism in constructing fluorescence turn-on sensors for heavy metal ions and biomolecules.<sup>1a, 2, 4-5</sup>

However, most rhodamine-based sensors only show a single emission, which is perturbed by environmental factors, such as sensor molecular concentration, pH, temperature, solvent polarity, and so on.<sup>6</sup> To eliminate these influences, ratio measurements are favorable, because the simultaneous observation of the absorption or emission intensities at two different wavelengths can increase the selectivity and sensitivity. Thus a lot of rhodamine sensors based on fluorescence resonance energy transfer (FRET) have been designed by introducing additional fluorophore as a donor, such as naphthalimine, dansyl, coumarin, fluorescein, or BODIPY derivatives.<sup>6</sup> The FRET process will occur only when there is a substantial overlap between the donor emission spectrum and the acceptor absorption spectrum. In addition, the emission of this energy transfer system is still confined to the characteristic emission band of the acceptor (rhodamine; <600 nm).

In contrast to visible and above all ultraviolet, fluorescence imaging with deep red light is advantageous because of its minimum photodamage to biological samples, deep tissue penetration, and negligible interference from the background autofluorescence of biomolecules in the organism.<sup>7</sup> Recently, several studies have aimed to obtain long wavelength dyes.<sup>1a</sup> These efforts include the extension of the conjugate system<sup>2b, 7a, 8</sup> and the introduction of donor and acceptor groups into the chromophore,<sup>9</sup> as well as the replacement of the oxygen bridge atom of rhodamine derivatives by other elements.<sup>2b, 10</sup> However, there are some disadvantages of these methods including their difficult synthesis, chemical instability, and/or high sensitivity to photobleaching. Moreover, rhodamine derivatives exhibit very small Stokes shift (Rhodamine B: 19 nm vs 600 cm<sup>-1</sup>)<sup>7a</sup>. The large Stokes shift could reduce self-absorption as well as obtain ACS Paragon Plus Environment

high resolution and low detection limits. Therefore, it remains highly desirable to explore new design strategies for the development of deep red fluorescent dye with great photostability and large Stokes shift while being used as ratiometric sensing platforms.

Scheme 1. The structural changes of CR1 to CR3 between spirocyclic and open-ring forms



In this work, we constructed a series of novel deep red fluorescent dyes by hybridizing coumarin and rhodamine moieties (CR1, CR2, and CR3, Scheme 1). The new dyes not only retained the advantages of coumarin and rhodamine derivatives themselves, but also demonstrated following expected advantages: (i) the emission wavelength was extended to the deep red region (>650 nm in H<sub>2</sub>O) when the fused-ring conjugate structure was extended during its facile synthesis; (ii) the fluorescence of the coumarin moiety and rhodamine-like fluorophore was switched by the spirocyclic/open-ring process of the carboxylic acid functional group (-COOH) at the 2'-position. Considering their good photophysical properties, these deep red fluorescent dyes were promising candidates for biological applications, such as biosensor, biomarker, and bioimaging. Based on these new dyes, we first developed deep red mitochondria-specific fluorescent dyes (CR1E, CR2E, and CR3E). Cell-imaging experiments indicated that these dyes were superior over MitoTrackers because of their deep red emission with large Stokes shift, excellent contrast for imaging, high photostability, and low cytotoxicity. To illustrate its value as a ratiometric platform, we further constructed a ratiometric sensor for Hg<sup>2+</sup> (CR1S) with good selectivity and "turn-on" response in the deep red region which was different from all previously reported rhodamine-based FRET systems.

#### **Results and Discussion**

**Design and Synthesis.** The absorption and emission wavelengths of traditional coumarin dyes were highly dependent on the substituents of the amino group because of intramolecular charge transfer (ICT) character. Thus, the coumarin- and rhodamine-fused compounds **CR1** to **CR3** were designed using different amino substituents. The detailed synthetic routes and methods were shown in Scheme 2. Compounds **CR1** to **CR3** were synthesized from 3-alkylaminophenol in two steps. Compounds **1**, **2**, **3**, **8**, and **9** were commercially available. Compound **2** was added to 85% phosphoric acid and heated to reflux for 24 h to obtain compound **5**. The condensation of compounds **1** and **8** in toluene for 4 h afforded the intermediate **4**, which was heated to reflux with compound **9** in 1,1,2,2-tetrachloroethane with  $P_2O_5$  for 4 h to produce **CR1**. Similar synthetic pathways were used to synthesize **CR2** and **CR3**.

To demonstrate its properties as fluorescent bioimaging agents, we designed a series of deep red fluorescent dyes (**CR1E** to **CR3E**) by esterification of **CR1** to **CR3** in ethanol with concentrated H<sub>2</sub>SO<sub>4</sub> for 2 h, which can selectively stain mitochondria. Mitochondria function as the "powerhouses" of cells by generating ATP through oxidative phosphorylation.<sup>11</sup> These organelles are related to several diseases, ranging from metabolic disorders to cancer.<sup>11c, 12</sup> The mitochondria of cancer cells are structurally and functionally different from their normal counterparts. To understand the cellular processes and molecular mechanisms of mitochondrial function, several fluorescent dyes have been developed to selectively stain mitochondria by electrostatic interactions due to its highly negative MMP (about -180 mV).<sup>13</sup> For example, lipophilic and positively charged dyes, such as Rhodamine 123<sup>3</sup> and MitoTracker Red CMXRos (MTR),<sup>14</sup> can selectively accumulate in mitochondria. However, most of these dyes are limited to wavelengths below 600 nm and have small Stokes shift or poor photostability. Compound **CR1E** to **CR3E** demonstrated the long wavelength emissions in the deep red region and large Stokes shift due to the ICT of the coumarin moiety.

In these new dyes, the fluorescence of the coumarin moiety and rhodamine-like fluorophore could be switched like classical rhodamine. Different from all previously reported rhodamine-based sensing systems, these dyes **CR1** to **CR3** have the advantage of deep red emission and very large Stokes shift. To validate our molecular design strategy, we developed a ratiometric sensor (**CR1S**) for Hg<sup>2+</sup> based on the well-known desulfurization reaction, which can be used in fluorescence imaging of Hg<sup>2+</sup> in the living A549 (human lung adenocarcinoma apithelial) cells. Compound **CR1S** was synthesized from **CR1** which was heated to reflux with POCl<sub>3</sub> in 1,2-dichloroethane for 4 h and then reacted with saturated Na<sub>2</sub>S.<sup>15</sup> Details of the synthesis were given in the Experimental Section. The structures of the intermediate compounds (4-7) and their respective products (**CR1** to **CR3**, **CR1E** to **CR3E** and **CR1S**) were characterized by <sup>1</sup>H NMR, <sup>13</sup>C NMR, and HRMS.

Scheme 2. Synthesis of deep red dyes CR1 to CR3, CR1E to CR3E and the ratiometric sensor CR1S



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Conditions: (a) toluene, reflux, 4 h; (b)  $P_2O_5$ , 1,1,2,2-tetrachloroethane, reflux, 4 h; (c)  $C_2H_5OH$ ,  $H_2SO_4$ , reflux, 2 h; (d) 85% phosphoric acid, reflux, 24 h; (e) POCl<sub>3</sub>, ClCH<sub>2</sub>CH<sub>2</sub>Cl, reflux, 4 h; (f) excess Na<sub>2</sub>S saturated aqueous solution.



**Figure 1.** Intermolecular hydrogen bonds (IHB) of compounds **CR2** and **CR3** with ellipsoids shown at the 50% probability level (other hydrogen atoms are omitted for clarity).

X-ray Structures. The molecular structures of CR2, CR3, CR2E, CR3E and CR1S were elucidated by X-ray crystallography, which confirmed a coumarin- and rhodamine-fused skeleton. Single crystals of compounds CR2, CR3, CR2E, CR3E, and CR1S suitable for X-ray crystallographic analysis were obtained by slow evaporation of CH<sub>3</sub>OH and H<sub>2</sub>O solutions of the compounds at ambient temperature. Details of X-ray experimental conditions, cell data, and refinement data of CR2, CR3, CR2E, CR3E, and CR1S were summarized in Table S1. These compounds were all racemic mixtures, given the helix structure and the presence of both enantiomers in unit cells. Compounds CR2, CR3, CR2E, and CR3E formed a monoclinic unit cell and the P2(1)/n space group, whereas CR1S formed a triclinic unit cell and the P2(1)/n space group, whereas CR1S formed a triclinic unit cell and the rystal lattice with IHB (Figure 1). The water molecule was located between the dye and perchlorate. The lengths and angles of the IHB were presented in Table 1. The IHB between the dye and water molecule was stronger than that between the water molecule and perchlorate. For example, the distances of H<sub>1</sub>···O<sub>10</sub> and H<sub>106</sub>···O<sub>9</sub> for CR2 were 1.780 Å and 2.086 Å, respectively.

 Table 1. IHB Data of Compounds CR2 and CR3

		$O_a$ - $H_a$	$H_a {\cdots} O_b$	$O_a$ - $O_b$	O <sub>a</sub> -H <sub>a</sub> …	
	O <sub>a</sub> -H <sub>a</sub> …O <sub>b</sub>	(Å)	(Å)	(Å)	O <sub>b</sub> (deg)	
CR2	$O_1$ - $H_1$ ··· $O_{10}$	0.841	1.780	2.586	159.97	
	O <sub>10</sub> -H <sub>10b</sub> …O <sub>9</sub>	0.840	2.086	2.926	178.62	
CR3	O <sub>6</sub> -H <sub>6</sub> …O <sub>10</sub>	0.840	1.815	2.618	159.35	
	$O_{10}$ - $H_{10a}$ ···· $O_1$	0.841	2.073	2.892	164.69	

The representative C-N bond lengths were listed in Table 2 (for atomic labels, see Figure 2). The X-ray crystallographic data showed that the representative C-N bond length *r* values of **CR2** (1.348 Å and 1.351 Å), **CR3** (1.348 Å and 1.353 Å), and **CR2E** (1.352 Å and 1.346 Å) were almost equal. Therefore,  $\pi$ -conjugate interactions existed between the two C-N bonds. The large discrepancy for **CR3E** (1.342 Å and 1.371 Å) may have been caused by intermolecular interactions of its crystalline state. These conclusions were consistent with the results of density functional theory (DFT) calculations (error  $\leq 0.015$  Å; see Table 2). All benzoyl moieties were nearly vertical to the tetracyclic molecular skeletons by their rotation around the bridged single bond. The torsion angles  $\theta$  of **CR2**, **CR3**, **CR2E**, and **CR3E** were listed in Table 2 (for atomic labels, see Figure 2). The  $\theta$  values of **CR2** and **CR3** were closer to a vertical angle than those of **CR2E** and **CR3E** because of the small steric hindrance of benzoyl moieties, which were further confirmed by DFT calculations except for **CR3E**. The C–N bond of **CR1S** (1.374 and 1.369) was a single bond, which was longer than that of **CR2** and **CR3**. The benzo[*c*]thiophen-1(3*H*)-one moiety was nearly vertical to the tetracyclic molecular skeleton as shown in Figure 2.



**Figure 2.** Crystal structures of compounds **CR2**, **CR3**, **CR2E**, **CR3E** and **CR1S** with ellipsoids shown at the 50% probability level (hydrogen atoms are omitted for clarity).

Table 2. Crystallographic Data and Theoretical Calculation of Compounds CR2, CR3, CR2E, CR3E and

CR1S

	$r^{a}/r^{b}$ (Å)		$\theta^a/\theta^b$ (deg)
CR2	1.348/1.353	1.351/1.356	88.31/82.69
CN2	$N_1$ - $C_7$	N <sub>2</sub> -C <sub>30</sub>	$C_{19}$ - $C_{11}$ - $C_{12}$ - $C_{18}$
CDA	1.348/1.353	1.353/1.357	85.34/83.05
UKJ	$N_1$ - $C_7$	N <sub>2</sub> -C <sub>33</sub>	C <sub>19</sub> -C <sub>11</sub> -C <sub>12</sub> -C <sub>17</sub>
	1.352/1.356	1.346/1.358	98.20/105.14
CK2E	$N_1$ - $C_{16}$	N <sub>2</sub> -C <sub>27</sub>	$C_{11}$ - $C_{12}$ - $C_9$ - $C_8$
CR3E	1.342/1.357	1.371/1.359	104.65/90.41
	$N_1$ - $C_{14}$	N <sub>2</sub> -C <sub>27</sub>	$C_{22}$ - $C_{20}$ - $C_{9}$ - $C_{8}$
CR1S	1.374/1.396	1.369/1.388	
	$N_1 - C_{24}$	$N_2 - C_{13}$	-

<sup>*a*</sup>The bond data determined from crystal structure. <sup>*b*</sup>The bond data determined from theoretical calculation.

Theoretical Basis. To investigate the geometric and electronic properties of novel deep red dyes, quantum calculations using the Gaussian 03 program<sup>16</sup> were performed. The calculated typical C-N bond lengths and torsion angles were summarized in Table 2. As expected, the results of geometry optimizations were consistent with the experimental data from single crystal structures. The calculated data for CR3 (1.353 Å, 1.357 Å, and 83.05°) agreed with the experimental data (1.348 Å, 1.353 Å, and 85.34°). Conversely, the calculated data for CR3E (1.357 Å, 1.359 Å, and 90.41°) slightly deviated from the corresponding experimental data (1.342 Å, 1.371 Å, and 104.65°). The discrepancy between calculation results and experimental data may have been caused by intermolecular interactions in the actual experiments, such as  $\pi$ - $\pi$  stacking or the interaction with counterion. The calculated HOMOs and LUMOs of compounds CR1 to CR3 and CR1E to CR3E were shown in Figure 3. The electron density of these dyes in terms of the HOMOs and LUMOs was predominantly localized in the tetracyclic structures. These dves exhibited ICT characteristics of the coumarin moiety. The benzovl moieties had little influence on photophysical properties because only a small portion of the electron density was localized in their structures. This result was further confirmed by the photophysical properties of the dyes.



**Figure 3.** Spatial distributions of calculated HOMOs and LUMOs of compounds **CR1** to **CR3** and **CR1E** to **CR3E**.

**Photophysics.** To study the structure-property relationships, we investigated the photophysical properties of **CR1** to **CR3** and **CR1E** to **CR3E**. Compounds **CR1** to **CR3** were sensitive to environmental pH because of the -COOH group at the 2'-position. We first examined the absorption profiles of **CR1** to **CR3** and **CR1E** to **CR3E** at different pH levels (Figure 4). The absorption of **CR1** to **CR3** significantly decreased within the pH range from 2 to 4, whereas their absorption was stable at pH > 4 because of the occurrence of their spirocyclic forms. Consequently, the spirocyclic and open-ring forms of **CR1** to **CR3** were in equilibrium, which was similar to the properties of other rhodamine derivatives. To ensure the formation of open-ring forms, the photophysics of **CR1** to **CR3** was investigated in different solvents with 0.5% trifluoroacetic acid. The absorption of **CR1E** to **CR3E** was independent of the solution pH (from 2 to 8) because these compounds lack the unique spirocyclic/open-ring process of rhodamines. However, the absorption of **CR1E** to **CR3E** was remarkably decreased at the pH range of 8 to 10 because of the open

ring of coumarin moieties in basic solutions. Thus, compounds **CR1E** to **CR3E** were suitable deep red fluorescence imaging reagents under the physiological condition.



Figure 4. pH-dependence of the absorption intensity of compounds CR1 to CR3 and CR1E to CR3E (c =  $5 \mu$ M).

The absorption and fluorescence spectra of CR1 to CR3 and CR1E to CR3E were investigated in CHCl<sub>3</sub>, C<sub>2</sub>H<sub>5</sub>OH, and H<sub>2</sub>O (Figures S1, 5, and S2, respectively). The corresponding photophysical data was summarized in Table 3. The absorption and fluorescence peaks in C<sub>2</sub>H<sub>5</sub>OH were red-shifted in both the CR1 $\rightarrow$ CR2 $\rightarrow$ CR3 and CR1E $\rightarrow$ CR2E $\rightarrow$ CR3E series, with the increasing donor property of the amino group in the coumarin moiety. Similar results were obtained in CHCl<sub>3</sub> and H<sub>2</sub>O because of the increased ICT characteristics of the coumarin moiety. Compounds CR1 to CR3 and CR1E to CR3E showed a larger Stokes shift than the classic rhodamine dyes. For example, CR3E exhibited a Stokes shift about 1549 cm<sup>-1</sup> (65 nm) in C<sub>2</sub>H<sub>5</sub>OH, which was more than twice that of Rhodamine B (600 cm<sup>-1</sup>, 19 nm).<sup>7a</sup> The molar extinction coefficients of **CR1** to **CR3** in CHCl<sub>3</sub> and C<sub>2</sub>H<sub>5</sub>OH were ~70,000 M<sup>-1</sup> cm<sup>-1</sup>, whereas those of **CR1E** to **CR3E** in CHCl<sub>3</sub>, C<sub>2</sub>H<sub>5</sub>OH, and H<sub>2</sub>O ranged from ~64,000 M<sup>-1</sup> cm<sup>-1</sup> to ~108,000  $M^{-1}$  cm<sup>-1</sup>. The fluorescence quantum yields of CR1 and CR1E in CHCl<sub>3</sub> were 0.507 and 0.494, respectively, which are significant for deep red fluorescent dyes. However, the fluorescence quantum yields of CR1E to CR3E in H<sub>2</sub>O only ranged from 0.002 to 0.014. Thus, biological imaging using CR1E to **CR3E** could provide strong contrast, and almost no background fluorescence would be observed. The

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fluorescence quantum yields decreased in both the CR1 $\rightarrow$ CR2 $\rightarrow$ CR3 and CR1E $\rightarrow$ CR2E $\rightarrow$ CR3E series as the energy gap was decreased.<sup>17</sup> This result is consistent with the literature.<sup>7a, 18</sup> The -COOH and -COOC<sub>2</sub>H<sub>5</sub> groups had little influence on the photophysical properties, as compared with CR1 and CR1E in different solvents. These results were previously predicted by the DFT calculations.



Figure 5. Absorption and fluorescence spectra of a) compounds CR1 to CR3 in C<sub>2</sub>H<sub>5</sub>OH with 0.5% trifluoroacetic acid and b) compounds CR1E to CR3E in C<sub>2</sub>H<sub>5</sub>OH (c = 5  $\mu$ M).

Compound	Solvent	$\lambda_{\max}$ (nm)	$\lambda_{\rm em}  ({\rm nm})$	Stokes shift $(nm/cm^{-1})$	$\varepsilon^{b} (\mathrm{M}^{-1}\mathrm{cm}^{-1})$	$\Phi^c$	τ(ns)
	CHCl <sub>3</sub>	602	636	34/888	77400	0.51	4.16
$\mathbf{CR1}^{a}$	C <sub>2</sub> H <sub>5</sub> OH	591	637	46/1222	70800	0.21	2.29
	H <sub>2</sub> O	594	646	52/1355	26800	0.01	0.31
	CHCl <sub>3</sub>	610	647	37/937	76600	0.27	3.79
$\mathbf{CR2}^{a}$	C <sub>2</sub> H <sub>5</sub> OH	598	651	53/1361	64800	0.05	0.98
	H <sub>2</sub> O	601	658	57/1441	44800	< 0.01	0.15
	CHCl <sub>3</sub>	623	664	41/991	75000	0.09	2.87
$\mathbf{CR3}^{a}$	C <sub>2</sub> H <sub>5</sub> OH	612	674	62/1503	69200	0.01	0.47
	H <sub>2</sub> O	612	684	72/1720	38600	< 0.01	0.16
	CHCl <sub>3</sub>	601	640	39/1014	99000	0.49	4.31
CR1E	C <sub>2</sub> H <sub>5</sub> OH	595	642	47/1230	64000	0.20	2.12
	H <sub>2</sub> O	596	652	56/1441	71600	0.01	0.27
CR2E	CHCl <sub>3</sub>	608	647	39/991	108000	0.33	4.07

 Table 3. Photophysical Properties of Compounds CR1 to CR3 and CR1E to CR3E

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	C <sub>2</sub> H <sub>5</sub> OH	602	658	56/1414	77800	0.04	0.86
	$H_2O$	602	666	64/1596	76200	< 0.01	0.16
	CHCl <sub>3</sub>	625	669	44/1052	107800	0.11	3.44
CR3E	C <sub>2</sub> H <sub>5</sub> OH	616	681	65/1549	87600	0.01	0.43
	H <sub>2</sub> O	616	687	71/1678	66200	< 0.01	0.13

<sup>*a*</sup>The photophysics are investigated in different solvents with 0.5% trifluoroacetic acid. <sup>*b*</sup>The molar extinction coefficients at maximum absorption. <sup>*c*</sup>Using cresyl violet in methanol as the reference ( $\Phi = 0.54$ ).<sup>19</sup>

Intracellular imaging for mitochondria (CR1E to CR3E). The molecular structures of CR1E to **CR3E** contain a positive charge and are highly similar to those of the mitochondria-specific fluorescent dves MTR and Rhodamine 123.<sup>3</sup> Thus, these positively charged deep red dves may preferentially accumulate in mitochondria by electrostatic interactions. The electrostatic interactions can accumulate and increase by a 100-fold to 500-fold, as described by the Nernst equation.<sup>13a</sup> To demonstrate this possibility, novel deep red fluorescent dyes **CR1E** to **CR3E** were incubated with living A549 cells (Figures 6). The localization of dyes' accumulation could be confirmed by dual-channel laser scanning confocal microscopy in the co-staining experiments. The extensive overlapping with MitoTracker Green FM (MTG) indicated that these deep red dyes were able to efficiently penetrate the cell membrane and specifically label mitochondria. These results revealed the potential imaging and therapeutic applications of these novel deep red dyes. To confirm their mechanism of accumulation in mitochondria, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used to change MMP before the staining procedure. Upon treatment with CCCP, the MMP was lost because of the rapid acidification of mitochondria and the dysfunction of ATP synthase.<sup>20</sup> After treatment with 30 µM CCCP, fluorescence intensity in mitochondria became faint, because the decreasing MMP affected the accumulation of the cationic dye (CR1E to **CR3E**) in mitochondria (Figure S3).

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Figure 6. Confocal fluorescence microscopy images of A549 incubated with MTG (10 nM) and compounds CR1E to CR3E (50 nM).



**Figure 7.** Photostability of compounds **CR1E** to **CR3E** and MitoTrackers (5  $\mu$ M) in PBS buffer (pH = 7.4).

As is well known, photostability is very important for fluorescent dyes due to the temporal monitoring of dynamic events inside cells. To evaluate the practical utility of the novel deep red dyes, their photostability in PBS buffer was performed by continuous irradiation of compounds **CR1E** to **CR3E** (compared with MitoTrackers) using a 500 W Xe lamp as the light source. After 30 min continuous irradiation, the absorption intensity of **CR1E** to **CR3E** decreased to 76% to 84%, whereas that of MTR dropped to 64% as shown in Figure 7. It is significant that the photostability of **CR1E** to **CR3E** was much higher than that of cyanine dyes, such as MTG (7%) and MitoTracker Deep Red FM (MTDR, 12%). The

cytotoxicity CR1E in cells of to CR3E A549 was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. The cytotoxicity of MTG, MTR, and MTDR were tested as the controls. Compounds CR1E to CR3E exhibited low cytotoxicity (cell viability > 90%) at concentrations up to 100 nM after incubated with A549 cells for 24 h (Figure 8). However, MTG and MTDR reduced the cell viability below 90% even at 10 nM.



Figure 8. The cytotoxicity of compounds CR1E to CR3E and MitoTrackers in A549 cells.



**Figure 9.** Confocal fluorescence microscopy images of A549 cells incubated with Hoechst 33342 (1 mg/mL) and compounds **CR1E** to **CR3E** (50 nM).

These deep red emissive properties helped reduce the interference of autofluorescence from biomolecules in the organism and obtained a higher signal-to-noise ratio. To evaluate the potential

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imaging and diagnostic applications of novel deep red dyes, **CR1E** to **CR3E** were incubated with different cell lines (cancer cells, A549 and HeLa; normal cells, L929). As shown in Figure S4-S6, the strong intracellular fluorescence may be attributed to the distribution of dyes in the inner mitochondrial membrane, where a relatively organic-like mitochondrial matrix of cancer and normal cells exists. Although the fluorescence quantum yields of **CR1E** to **CR3E** were relatively low (0.002 to 0.014) in a polar solvent (H<sub>2</sub>O), the fluorescence intensity in mitochondria was sufficient to provide strong contrast for imaging due to its enrichment inside mitochondria. The morphologies of mitochondria in cancer and normal cells can be clearly visualized. Furthermore, almost no background fluorescence could be observed, especially in the nucleus. To demonstrate the compatibility of **CR1E** to **CR3E** with other dyes such as Hoechst 33342, co-staining experiments were performed. We were able to clearly visualize the blue nuclear staining in the DAPI channel and the red mitochondrial staining by the novel dyes in the Cy5 channel (Figure 9). The superimposition patterns between **CR1E** to **CR3E** and Hoechst 33342 allowed for the simultaneous imaging of mitochondria and DNA.

**Ratiometric bioimaing for Hg^{2+} (CR1S).** Based on the well-known desulfurization reaction, we developed a new ratiometric sensor (CR1S, Scheme 1) for  $Hg^{2+}$  in this work. With compound CR1S in hand, we firstly investigated its absorption response to  $Hg^{2+}$  at different pH levels (Figure S7). The intensities of compound CR1S solutions in a citric acid (0.1 M)-phosphate (0.2 M) buffer (H<sub>2</sub>O/CH<sub>3</sub>OH = 1:1, v/v) within pH 2 to 8 were found to be stable. However, the addition of  $Hg^{2+}$  resulted in a distinct increasing in the absorption intensity at 596 nm with increasing acidity (from pH 8 to 2), which was similar with the reported  $Hg^{2+}$  sensor.<sup>15</sup> According to reference, we chose pH = 4 as the measuring conditions.



**Figure 10.** Absorption (a) and fluorescence spectra (b) of **CR1S** (5  $\mu$ M) with different Hg<sup>2+</sup> concentrations in a citric acid (0.1 M)-phosphate (0.2 M) buffer (pH = 4, H<sub>2</sub>O/CH<sub>3</sub>OH = 1:1, v/v).  $\lambda_{ex}$ = 380 nm. The insets show the color changes and fluorescence changes in the absence and presence of Hg<sup>2+</sup>.

Figure 10 showed the spectroscopic properties of **CR1S** at various  $Hg^{2+}$  concentrations in a citric acid (0.1 M)-phosphate (0.2 M) buffer (pH = 4, H<sub>2</sub>O/CH<sub>3</sub>OH = 1:1, v/v). The UV/Vis spectra of **CR1S** only showed the absorption profile from the coumarin moiety of the spirocyclic form with a maximum at 381 nm (Figure 10a). The addition of  $Hg^{2+}$  immediately decreased the absorption peak at 381 nm with red-shifted and a new absorption band at 596 nm appeared, which corresponded to the absorption of rhodamine-like moiety. The spectral shifts were about 215 nm, and two isosbestic points at 340 nm and 400 nm were formed. They exhibited a clear colour change from colourless to blue and colorimetric performance could be easily monitored by naked-eye for instantaneous visual sensing (inset, Figure 10a). Similar ratio changes can also be observed from the fluorescence spectra (Figure 10b). The intensity at 430 nm from the coumarin moiety decreased and that at 653 nm from rhodamine-like moiety gradually

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increased with increasing  $Hg^{2+}$  concentration. Indeed, this coumarin- and rhodamine-fused  $\pi$ -conjugated system had very large spectral shifts of up to 223nm, which were much larger than those of TBET or FRET-based rhodamine systems.<sup>21</sup> In addition, such fluorescence performance could be detected by naked-eye as depicted in the inset of Figure 10b, where photos of solutions of compound **CR1S** before and after addition of  $Hg^{2+}$  under UV light irradiation were displayed.

As shown in Figure S8, the linear fitting can be obtained between  $Hg^{2+}$  concentrations ranging from 0  $\mu$ M to 100  $\mu$ M and the absorption ratio ( $A_{596}/A_{381}$ ) as well as the square root of fluorescence intensity ratio ( $I^{1/2}_{653}/I^{1/2}_{430}$ ) of **CR1S**, respectively. The response to  $Hg^{2+}$  of **CR1S** was also very fast and spectral data were recorded within 10 seconds after the addition of  $Hg^{2+}$ . The selectivity of **CR1S** for different metal ions was also investigated. As shown in Figure S9, the fluorescence response of **CR1S** displayed excellent selectivity to  $Hg^{2+}$  over all other tested ions. We proposed that the high thiophilicity of  $Hg^{2+}$  resulted in the formation of the open-ring complex, and then hydrolyzed to **CR1**. This result can be confirmed by the ESI-MS signals at m/z 511.2 (corresponding to **CR1**) in the solution of **CR1S** treated with excess  $Hg^{2+}$  (Figure S10).



**Figure 11.** Confocal fluorescence imaging of  $Hg^{2+}$  in A549 cells with (a) **CR1S** (2 µm) for 10 min and (b) **CR1S** (2 µm) for 10 min and then  $Hg^{2+}$  (20 µm) for 10 min.

Taking into account of the advantages of ratiometric fluorescence changes, compound **CR1S** was used for in vitro  $Hg^{2+}$  detection in living A549 cells. As shown in Figure 11a, A549 cells incubated with **CR1S** (2  $\mu$ M) for 10 min showed a clear blue fluorescence, whereas weak red fluorescence was observed in the **ACS Paragon Plus Environment**  red channel, which indicated that **CR1S** mainly existed in the spirolactam form. After  $Hg^{2+}$  (20 µm) treatment for 10 min, the blue fluorescence intensity decreased and the red fluorescence intensity significantly increased (Figure 11b). As shown in the ratio imaging (Red/Blue) in Figure 11, distinct changes in ratio fluorescence responses in A549 cells were observed. This indicated the potential of biological imaging application of the new ratiometric deep red sensor.

#### Conclusions

We have introduced a new strategy to design deep red fluorescent dyes (CR1 to CR3) containing a coumarin- and rhodamine-fused conjugated skeleton. The novel deep red fluorescent dyes have excellent photophysical properties, such as high molar extinction coefficients, excellent fluorescence quantum yields in CHCl<sub>3</sub>, great photostability and large Stokes shift. Importantly, these dyes CR1 to CR3 could undergo equilibrium between the coumarin moiety and rhodamine-like fluorophore switched by the spirocyclic/open-ring process of the -COOH, which can be used as versatile sensing platform for designing ratiometric fluorescent sensors. Considering their good photophysical properties, we designed a series of deep red fluorescent bioimaging agents (CR1E to CR3E) by esterification of CR1 to CR3, which can selectively stain mitochondria. Cell-imaging experiments indicated that these dyes were superior over MitoTrackers because of their deep red emission with large Stokes shift, excellent contrast for imaging, high photostability, and low cytotoxicity. Furthermore, using CR1 as a platform, we developed a novel ratiometric sensor (CR1S) for mercury ions with the decreased fluorescence of coumarin moiety and large turn-on deep red fluorescence of rhodamine-like moiety. CR1S could be successfully applied to the ratiometric imaging of  $Hg^{2+}$  in living A549 cells. This design strategy may be extended for development of new deep red fluorescent dyes by simply introducing different fluorophores, and dyes CR1 to CR3 will be good candidates for biosensor, biomarker, and bioimaging.

**Experimental Section** 

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Materials and Methods. All commercial chemicals were used without further purification. Compound 2 (7-amino-1-methyl-1,2,3,4-tetrahydroquinoline hydrochloride) was purchased from HangZhou Trylead Chemical Technology Co., Ltd. MTG, MTR, MTDR and Hoechst were purchased from Invitrogen (USA). CCCP was purchased from Alfa Aesar. Crystallographic data of CR2, CR3 and CR2E were done on a Saturn724+ CCD with Mo K $\alpha$  radiation ( $\lambda = 0.71073$  Å) at 173 K. Crystallographic data of **CR3E** was done on a Rigaku raxis Rapid IP Area Detector with Cu K $\alpha$  radiation ( $\lambda = 1.54178$  Å) at 173 K. Crystallographic data of **CR1S** was done on an Image Plate X-Ray Diffractameter with Mo Kα radiation  $(\lambda = 0.71073 \text{ Å})$  at 293 K. The quantum chemical calculations were optimized using the restricted B3LYP functions with a 6-31 G (d) basis set at the DFT level. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded at 400 and 100 MHz respectively. The HRMS were measured with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS). Absorption and fluorescence spectra of all compounds were obtained in an absorption spectrometer and a fluorescence spectrometer at room temperature, respectively. The fluorescence lifetimes were measured in FLS920 Combined Steady State & Time Resolved Fluorescence Spectrometer. A549, HeLa and L929 cells (gifted from the center of cells, Peking Union Medical College) were cultured in confocal dishes in culture media (McCoy's 5A, DMEM or RPM1640 respectively, supplemented with 10% FBS, 50 unit/mL penicillin, and 50 mg/mL of streptomycin) under 5% carbon dioxide/air at 37 °C in a humidified incubator for 24 h. The cells were washed with PBS for three times before fluorescent imaging. Confocal microscopy and image analysis was acquired with a laser scanning confocal microscopy (for Hoechst 33342, excited at 405 nm, DAPI channel; for MTG, excited at 488 nm, FITC channel; for CR1E to CR3E, excited at 640 nm, Cy5 channel). The microscope was equipped with a live-cell incubation chamber maintaining a humidified atmosphere of 37 °C and 5% CO<sub>2</sub>. Images were processed by NIS-Elements Viewer 3.20.

Synthesis of 7-diethylamino-4-hydroxycoumarin (4). 3-Diethylaminophenol 1 (12.8 g, 77.5 mmol) and diphenyl malonate 8 (18.8 g, 73.4 mmol) were added into anhydrous toluene (50 mL). The mixture was heated to reflux for 4 h and compound 4 was obtained by filtration as grey solid (5.2 g, 30%).<sup>22</sup> mp 233-235 °C; <sup>1</sup>H NMR (400 MHz, DMSO)  $\delta$  (ppm): 11.98 (s, 1H), 7.55-7.53 (d, 1H, *J* = 9.0 Hz), 6.67-6.64 (d, 1H, *J* = 2.2 Hz, *J*<sub>2</sub> = 9.0 Hz), 6.45-6.44 (d, 1H, *J* = 2.2 Hz), 5.23 (s, 1H), 3.43-3.38 (q, 4H, *J* = 7.0 Hz), 1.13-1.09 (t, 6H, *J* = 7.0 Hz); <sup>13</sup>C NMR (100 MHz, DMSO)  $\delta$  (ppm): 166.7, 162.8, 156.1, 150.8, 124.1, 108.1, 103.6, 96.4, 86.1, 44.0, 12.3; ESI-HRMS m/z calcd for [C<sub>13</sub>H<sub>15</sub>NO<sub>3</sub>+H]<sup>+</sup> 234.11247, found 234.11205.

Synthesis of 7-hydroxy-1-methyl-1,2,3,4-tetrahydroquinolin (5). Compound 2 (3.2 g, 1.6 mmol) was added into 85% phosphoric acid (15 mL) and heated to reflux for 24 h.<sup>23</sup> After cooling to room temperature, the reaction solution was neutralized with 200 mL 40% NaOH aqueous solution. Plenty of Na<sub>3</sub>PO<sub>4</sub> was precipitated and filtrated. The filtrate was extracted with CH<sub>2</sub>Cl<sub>2</sub> (total 60 mL for three times). The organic phase was concentrated under vacuum and purified by column chromatography to afford compound **5** as white solid using v(CH<sub>3</sub>COOC<sub>2</sub>H<sub>5</sub>)/v(petroleum ether) = 1:3 as the eluent (1.3g, 48%). mp 103-104 °C; <sup>1</sup>H NMR (400 MHz, DMSO)  $\delta$  (ppm): 8.74 (s, 1H), 6.64-6.62 (d, 1H, *J* = 7.9 Hz), 5.98-5.97 (d, 1H, *J* = 2.1 Hz), 5.95-5.93 (dd, 1H, *J* = 2.2 Hz, *J*<sub>2</sub> = 7.9 Hz), 3.13-3.10 (t, 2H, *J* = 5.6 Hz), 2.75 (s, 3H), 2.57-2.54 (t, 2H, *J* = 6.4 Hz), 1.86-1.80 (m, 2H). <sup>13</sup>C NMR (100MHz, DMSO)  $\delta$  (ppm): 156.4, 147.3, 128.9, 112.9, 102.9, 98.2, 50.5, 38.7, 26.5, 22.3. ESI-HRMS m/z calcd for [C<sub>10</sub>H<sub>13</sub>NO+H]<sup>+</sup> 164.10699, found 164.10657.

Synthesis of 4-hydroxy-9-methyl-6,7,8,9-tetrahydropyrano[3,2-g]quinolin-2-one (6). Compound 5 (1.0 g, 3.9 mmol) and diphenyl malonate 8 (0.64 g, 3.9 mmol) were added into anhydrous toluene (8 mL). The mixture was heated to reflux for 6 h and compound 6 was obtained by filtration as grey solid (0.8 g, 88%).<sup>22</sup> mp 255-257 °C; <sup>1</sup>H NMR (400 MHz, DMSO) δ (ppm): 11.83 (s, 1H), 7.26 (s, 1H), 6.35 (s, 1H), ACS Paragon Plus Environment

5.23 (s, 1H), 3.33-3.31 (t, 2H, J = 5.6 Hz), 2.92 (s, 3H), 2.73-2.70 (t, 2H, J = 6.1 Hz), 1.87-1.84 (t, 2H).
<sup>13</sup>C NMR (100 MHz, DMSO) δ (ppm): 166.4, 162.7, 154.7, 149.7, 121.7, 118.8, 103.2, 95.5, 86.0, 50.0,
38.5, 26.6, 21.2. ESI-HRMS m/z calcd for [C<sub>13</sub>H<sub>13</sub>NO<sub>3</sub>+H]<sup>+</sup> 232.09682, found 232.09648.

Synthesis of 4-hydroxypyrano[3,2-g]julolidine-2-one (7). 8-Hydroxyjulolidine 3 (3.7 g, 19.3 mmol) and diphenyl malonate 8 (4.1 g, 15.9 mmol) were added into anhydrous toluene (10 mL). The mixture was heated to reflux for 4 h and compound 7 was obtained by filtration as straw yellow solid (3.6 g, 88%).<sup>22</sup> mp 268-270 °C; <sup>1</sup>H NMR (400 MHz, DMSO)  $\delta$  (ppm): 11.73 (s, 1H), 7.15 (s, 1H), 5.21 (s, 1H), 3.24-3.20 (m, 4H), 2.70 (s, 4H), 1.91-1.83 (m, 4H). <sup>13</sup>C NMR (100 MHz, DMSO)  $\delta$  (ppm): 166.5, 162.8, 151.0, 146.0, 119.8, 117.3, 105.3, 103.1, 85.8, 49.2, 48.7, 26.9, 21.0, 20.1, 20.0. ESI-HRMS m/z calcd for [C<sub>15</sub>H<sub>15</sub>NO<sub>3</sub>+H]<sup>+</sup> 258.11247, found 258.11210.

**Synthesis** of compound **CR1**. Compound (440 1.9 mmol) and mg, 2-(4-diethylamino-2-hydroxy)-benzoylbenzoic acid 9 (629 mg, 2.0 mmol) were heated to reflux in 15 mL 1,1,2,2-tetrachloroethane until the substances have dissolved completely. A total of 2.8 g phosphorus pentoxide was then added in portions and the reaction mixture was heated to reflux for 4 h.<sup>24</sup> After cooling, the mixture was dissolved using water (25 mL) and chloroform (25 mL), and the water phase was extracted with chloroform (total 100 mL for three times). The combined organic phases were concentrated under vacuum and purified by column chromatography using  $v(CH_2Cl_2)/v(CH_3OH) = 100:1$  as the eluent. The dye fractions are concentrated under vacuum, and the residue is dissolved in 2 mL ethanol. After addition of 3 mL perchloric acid (note: the operation should be executed very carefully due to the instability of perchlorate salts), the solution was precipitated by dropwise addition of water (100 mL). Compound CR1 was obtained by filtration as dark green solid (682 mg, 59%). mp 287-289 °C; <sup>1</sup>H NMR (400 MHz, DMSO) δ (ppm): 8.02-7.94 (m, 2H), 7.71-7.60 (m, 2H), 7.31-7.29 (d, 1H), 6.90-6.87 (m, 2H), 6.68 (s, 1H), 6.60-6.55 (m, 2H), 3.52-3.44 (m, 8H), 1.16-1.11 (m, 12H). <sup>13</sup>C NMR (100 MHz, **ACS Paragon Plus Environment** 

CD<sub>3</sub>OD/CD<sub>2</sub>Cl<sub>2</sub>)  $\delta$  (ppm): 166.9, 163.6, 163.4, 158.7, 158.4, 158.0, 157.9, 156.6, 136.8, 133.7, 132.9, 131.4, 130.4, 129.6, 128.8, 127.8, 116.9, 116.7, 112.7, 104.6, 101.3, 98.0, 97.9, 52.9, 47.2, 46.4, 12.7. ESI-HRMS m/z calcd for  $[C_{31}H_{31}N_2O_5]^+$  511.22275, found 511.22229. Elem. anal. calcd for  $C_{31}H_{31}ClN_2O_9$ ·1/2H<sub>2</sub>O: C, 60.05; H, 5.20; N, 4.52, found: C, 60.11; H, 5.14; N, 4.33.

**Synthesis** of compound **CR2**. Compound (659 2.9 mmol) and mg, 2-(4-diethylamino-2-hydroxy)-benzoylbenzoic acid 9 (1.1 g, 3.5 mmol) were heated to reflux in 20 mL 1,1,2,2-tetrachloroethane until the substances have dissolved completely. A total of 4.0 g phosphorus pentoxide was then added in portions and the reaction mixture was heated to reflux for 4 h.<sup>24</sup> After cooling, the mixture was dissolved using water (25 mL) and chloroform (25 mL), and the water phase was extracted with chloroform (total 100 mL for three times). The combined organic phases were concentrated under vacuum and purified by column chromatography using  $v(CH_2Cl_2)/v(CH_3OH) = 100:1$  as the eluent. The dye fractions are concentrated under vacuum, and the residue is dissolved in 3 mL ethanol. After addition of 5 mL perchloric acid (note: the operation should be executed very carefully due to the instability of perchlorate salts), the solution was precipitated by dropwise addition of water (100 mL). Compound **CR2** was obtained by filtration as dark green solid (720 mg, 42%). mp 289-290 °C; <sup>1</sup>H NMR (400 MHz, DMSO) δ (ppm): 7.97-7.96 (m, 1H), 7.74-7.60 (m, 3H), 7.29-7.27 (m, 1H), 6.87 (s, 1H), 6.69 (s, 1H), 6.57 (s, 1H), 6.49 (s, 1H), 3.44 (s, 6H), 3.01 (s, 3H), 2.84-2.81 (t, 2H, J = 5.8 Hz), 1.92 (s, 2H), 1.15-1.12 (t, 6H, J = 6.8 Hz). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD/CD<sub>2</sub>Cl<sub>2</sub>)  $\delta$  (ppm): 168.2, 162.9, 158.2, 157.9, 157.5, 155.7, 137.5, 133.5, 132.8, 131.7, 130.4, 130.3, 128.5, 124.3, 124.2, 116.4, 104.4, 101.2, 98.0, 96.7, 52.4, 47.2, 40.0, 27.8, 21.8, 12.8. ESI-HRMS m/z calcd for  $[C_{31}H_{29}N_2O_5]^+$  509.20710, found 509.20627. Elem. anal. calcd for C<sub>31</sub>H<sub>29</sub>ClN<sub>2</sub>O<sub>9</sub>·3/2H<sub>2</sub>O: C, 58.54; H, 5.07; N, 4.40, found: C, 58.33; H, 4.80; N, 4.49. **Synthesis** of compound **CR3**. Compound (1.2)4.7 mmol) and g, 2-(4-diethylamino-2-hydroxy)-benzoylbenzoic acid 9 (1.8 g, 5.7 mmol) were heated to reflux in 50 mL **ACS Paragon Plus Environment** 

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1,1,2,2-tetrachloroethane until the substances have dissolved completely. A total of 7.8 g phosphorus pentoxide was then added in portions and the reaction mixture was heated to reflux for 4 h.<sup>24</sup> After cooling, the mixture was dissolved using water (25 mL) and chloroform (25 mL), and the water phase was extracted with chloroform (total 100 mL for three times). The combined organic phases were concentrated under vacuum and purified by column chromatography using  $v(CH_2Cl_2)/v(CH_3OH) = 100:1$  as the eluent. The dye fractions are concentrated under vacuum, and the residue is dissolved in 5 mL ethanol. After addition of 10 mL perchloric acid (note: the operation should be executed very carefully due to the instability of perchlorate salts), the solution was precipitated by dropwise addition of water (150 mL). Compound **CR3** was obtained by filtration as dark green solid (982 mg, 33%). mp 290-292 °C; <sup>1</sup>H NMR (400 MHz, DMSO) δ (ppm): 8.04-8.03 (m, 1H), 7.74-7.62 (m, 3H), 7.28-7.26 (m, 1H), 6.92-6.64 (m, 3H), 3.49-3.36 (m, 8H), 2.81-2.78 (m, 2H), 2.65-2.64 (m, 2H), 1.94-1.83 (m, 4H), 1.16-1.13 (t, 6H, J = 6.9 Hz).<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD/CD<sub>2</sub>Cl<sub>2</sub>) δ (ppm): 167.1, 161.8, 157.4, 157.3, 156.5, 152.3, 152.0, 136.3, 132.5, 131.8, 130.9, 129.4, 129.3, 127.6, 122.3, 122.1, 115.2, 115.1, 106.3, 103.5, 100.0, 96.9, 50.8, 50.3, 46.2, 27.2, 20.6, 19.6, 19.5, 11.9. ESI-HRMS m/z calcd for  $[C_{33}H_{31}N_2O_5]^+$  535.22275, found 535.22213. Elem. anal. calcd for C<sub>33</sub>H<sub>31</sub>ClN<sub>2</sub>O<sub>9</sub>: C, 62.41; H, 4.92; N, 4.41, found: C, 63.28; H, 5.46; N, 4.14.

Synthesis of compound CR1E. Concentrated H<sub>2</sub>SO<sub>4</sub> (0.5 mL) was added to an ethanol solution of compound CR1 (120 mg, 0.2 mmol), and the solution was heated to reflux for 2 h.<sup>25</sup> After cooling, ethanol was removed under vacuum and the residue was neutralized with 20 mL 10% NaOH aqueous solution to collect the resulting precipitate. After drying, it was purified by silica gel chromatography using v(CH<sub>2</sub>Cl<sub>2</sub>)/v(CH<sub>3</sub>OH) = 100:1 as the eluent to give CR1E as dark green solid (39 mg, 31%). mp 272-274 °C; <sup>1</sup>H NMR (400 MHz, DMSO)  $\delta$  (ppm): 8.20-8.17 (m, 2H), 7.83-7.71 (m, 2H), 7.40-7.31 (m, 2H), 7.20-7.07 (m, 2H), 6.90-6.75 (m, 2H), 4.10-4.08 (q, 2H, *J* = 7.1 Hz), 3.71-3.60 (m, 8H), 1.23-1.18 (m, 12H), 1.07-1.04 (t, 3H, *J* = 7.1 Hz). <sup>13</sup>C NMR (100 MHz, DMSO)  $\delta$  (ppm): 164.8, 161.8, 161.2, 157.2, ACS Paragon Plus Environment

156.9, 156.6, 156.4, 155.1, 135.6, 133.0, 132.9, 131.4, 130.1, 129.5, 128.3, 126.9, 116.7, 115.4, 111.8, 103.6, 99.9, 97.2, 96.9, 61.1, 54.9, 45.8, 45.0, 13.6, 12.4. ESI-HRMS m/z calcd for  $[C_{33}H_{35}N_2O_5]^+$ 539.25405, found 539.25309. Elem. anal. calcd for  $C_{33}H_{35}CIN_2O_9$ ·1/2H<sub>2</sub>O: C, 61.16; H, 5.60; N, 4.32, found: C, 61.29; H, 5.61; N, 4.23.

Synthesis of compound CR2E. Concentrated H<sub>2</sub>SO<sub>4</sub> (1 mL) was added to an ethanol solution of compound CR2 (505 mg, 0.8 mmol), and the solution was heated to reflux for 2 h.<sup>25</sup> After cooling, ethanol was removed under vacuum and the residue was neutralized with 40 mL 10% NaOH aqueous solution to collect the resulting precipitate. After drying, it was purified by silica gel chromatography using v(CH<sub>2</sub>Cl<sub>2</sub>)/v(CH<sub>3</sub>OH) = 100:1 as the eluent to give CR2E as dark green solid (350 mg, 66%). mp 279-280 °C; <sup>1</sup>H NMR (400 MHz, DMSO)  $\delta$  (ppm): 8.20-8.18 (m, 1H), 7.90-7.71 (m, 3H), 7.33-7.31 (m, 2H), 7.16-7.13 (m, 1H), 6.88-6.84 (m, 1H), 6.64 (s, 1H), 4.12-4.06 (q, 2H, *J* = 7.0 Hz), 3.69-3.68 (m, 4H), 3.58-3.55 (m, 2H), 3.15 (s, 3H), 2.87-2.84 (t, 2H, *J* = 5.7 Hz), 1.96-1.94 (m, 2H), 1.23 (s, 6H), 1.07-1.03 (t, 3H, *J* = 7.1 Hz). <sup>13</sup>C NMR (100 MHz, DMSO)  $\delta$  (ppm): 164.9, 161.3, 161.1, 156.8, 156.5, 156.4, 156.2, 154.3, 135.6, 132.9, 131.3, 130.1, 129.5, 128.4, 128.3, 123.1, 122.9, 116.3, 115.1, 103.5, 99.6, 97.0, 96.0, 61.1, 54.9, 51.0, 45.8, 26.4, 20.5, 13.7, 12.5. ESI-HRMS m/z calcd for [C<sub>33</sub>H<sub>33</sub>N<sub>2</sub>O<sub>5</sub>]<sup>+</sup> 537.23840, found 537.23773. Elem. anal. calcd for C<sub>33</sub>H<sub>33</sub>ClN<sub>2</sub>O<sub>9</sub>·1/2H<sub>2</sub>O: C, 61.35; H, 5.30; N, 4.34, found: C, 61.46; H, 5.35; N, 4.30.

Synthesis of compound CR3E. Concentrated H<sub>2</sub>SO<sub>4</sub> (1 mL) was added to an ethanol solution of compound CR3 (430 mg, 0.7 mmol), and the solution was heated to reflux for 2 h.<sup>25</sup> After cooling, ethanol was removed under vacuum and the residue was neutralized with 40 mL 10% NaOH aqueous solution to collect the resulting precipitate. After drying, it was purified by silica gel chromatography using  $v(CH_2Cl_2)/v(CH_3OH) = 100:1$  as the eluent to give CR3E as dark green solid (3908mg, 68%). mp 273-274 °C; <sup>1</sup>H NMR (400 MHz, DMSO)  $\delta$  (ppm): 8.20-8.18 (m, 1H), 7.83-7.70 (m, 3H), 7.32-7.30 (m, ACS Paragon Plus Environment

2H), 7.12-7.10 (m, 1H), 6.84-6.82 (m, 1H), 4.09-4.07 (m, 2H), 3.68-3.66 (m, 4H), 3.51-3.48 (m, 4H), 2.86 (s, 2H), 2.69 (s, 2H), 1.95-1.90 (m, 4H), 1.23 (s, 6H), 1.06-1.03 (t, 3H, J = 7.0 Hz). <sup>13</sup>C NMR (100 MHz, DMSO)  $\delta$  (ppm): 164.8, 161.0, 160.9, 156.8, 156.5, 156.0, 151.6, 151.3, 135.7, 132.8, 131.2, 130.1, 129.4, 128.2, 122.0, 121.6, 115.9, 114.8, 105.7, 103.3, 99.3, 97.0, 61.0, 50.3, 49.7, 45.6, 26.7, 20.3, 19.4, 19.1, 13.6, 12.5. ESI-HRMS m/z calcd for [C<sub>35</sub>H<sub>35</sub>N<sub>2</sub>O<sub>5</sub>]<sup>+</sup> 563.25405, found 563.25315. Elem. anal. calcd for C<sub>35</sub>H<sub>35</sub>ClN<sub>2</sub>O<sub>9</sub>·1/2H<sub>2</sub>O: C, 62.54; H, 5.40; N, 4.17, found: C, 62.59; H, 5.37; N, 4.15.

Synthesis of compound CR1S. Compounds CR1 (150 mg, 0.25 mmol) and phosphorus oxychloride (1 mL) was added into 1,2-dichloromethane (5 mL) and heated to reflux for 4 h.<sup>15</sup> After cooling, the reaction mixture was concentrated under vacuum to obtain crude acyl chloride. 5 mL of saturated Na<sub>2</sub>S aqueous solution (1.2g) was then added to the crude acyl chloride and the mixture was stirred for 3 h at room temperature. Following extraction with ethyl acetate (total 100 mL for three times), the organic phase was purified by column chromatography using CH<sub>2</sub>Cl<sub>2</sub> as the eluent to afford the pale yellow solid CR1S in 56% yield (72 mg). mp 301-302 °C; 1H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  (ppm): 7.88-7.85 (m, 2H), 7.51-7.41 (m, 2H), 7.21-7.19 (m, 1H), 6.72-6.64 (m, 2H), 6.44-6.38 (m, 2H), 3.45-3.33 (m, 8H), 1.23-1.17 (m, 12H). 13C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  (ppm): 197.4, 159.7, 158.0, 156.8, 155.6, 151.4, 149.8, 148.5, 136.5, 133.8, 130.4, 128.0, 125.4, 124.4, 123.0, 110.2, 108.9, 108.8, 102.0, 97.4, 97.1, 95.8, 58.5, 44.9, 44.5, 12.7, 12.5. ESI-HRMS m/z calcd for [C<sub>31</sub>H<sub>30</sub>N<sub>2</sub>O<sub>4</sub>S+H]<sup>+</sup> 527.19990, found 527.20044. Elem. anal. calcd for C<sub>31</sub>H<sub>30</sub>N<sub>2</sub>O<sub>4</sub>S·1/4H<sub>2</sub>O: C, 70.10; H, 5.79; N, 5.27, found: C, 70.19; H, 5.91; N, 5.19.

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**Supporting Information Available:** X-ray structure details of compounds CR2, CR3, CR2E, CR3E and CR1S (CIF); photophysical data; imaging data; sensor data and <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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