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Cell-Permeable Iminocoumarine-Based Fluorescent Dyes for Mitochondria

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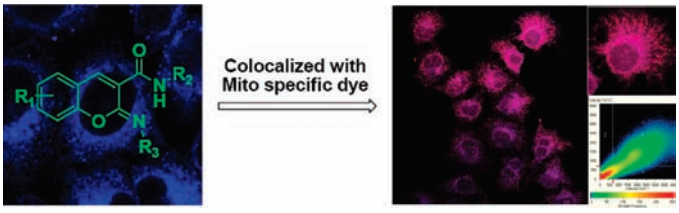
ABSTRACT



A class of small molecule fluorophores, 2-iminocoumarin-3-carboxamide derivatives, has been developed by a rapid microwave-assisted process. These fluorescent probes are cell membrane permeable with low cytotoxicity and able to selectively stain organelles in living cells.

Fluorescent probes with high sensitivity and ease of handling have been extensively employed in biological science, clinical diagnosis, and drug discovery.[1] The development of fluorescent probes, particularly synthetic small molecular fluorescent probes with various core skeletons, such as fluorescein, BODIPY, and rhodamine, has received special attention.[1] The use of small molecule fluorescent dyes in designing imaging probes is generally advantageous as they offer a greater number of options for optimizing delivery, and a single dye can be used on multiple targeting carrier molecules.[1e] Although significant progress has been made in this field, there is still a great demand for novel fluorescent core skeletons with flexible synthetic strategies and good photophysical properties for bioimaging.[2]

Coumarin-based fluorescent dyes have been used extensively in biological studies,[3] and iminocoumarins have recently been investigated as a sensitive zinc sensor[4] and a selective detector of dual-specific protein tyrosine

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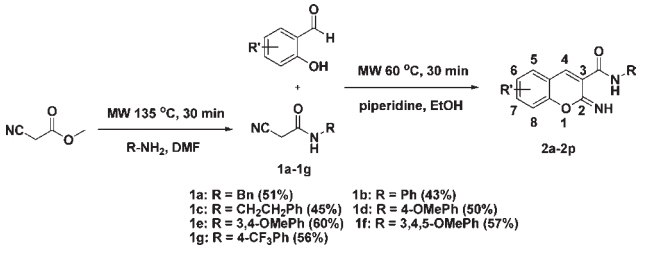
phosphatases in vitro.[5] Iminocoumarins can be excited at longer wavelengths than coumarin analogues, hence increasing their suitability for biological applications.[5] However, there have been very few investigations of their 3-carboxamide analogues as a scaffold for fluorescence probes. In our ongoing efforts to develop novel approaches to the synthesis of biologically active heterocyclic compounds,[6] we have designed and synthesized a 2-iminocoumarin-3-carboxamide[7] fluorescent core skeleton with good photophysical properties, which can be easily derivatized and potentially used for fluorescent intracellular imaging.[8]

Table 1. Synthesis of Compounds **2a**–**2p**



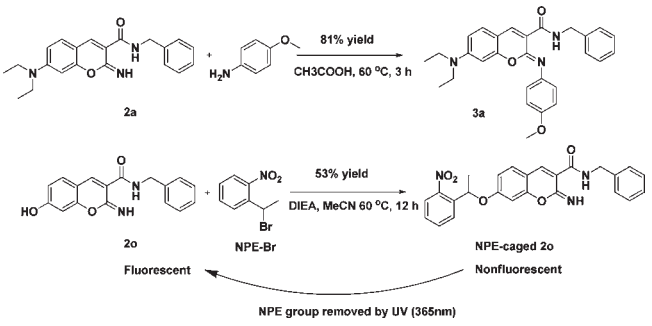
entry	1	R	R′	2	yield[a] (%)
1	**1a**	Bn	7-Diethylamino	**2a**	76
2	**1b**	Ph	7-Diethylamino	**2b**	95
3	**1c**	CH_2CH_2Ph	7-Diethylamino	**2c**	67
4	**1d**	4-OMeBn	7-Diethylamino	**2d**	78
5	**1e**	3,4-OMeBn	7-Diethylamino	**2e**	55
6	**1f**	3,4,5-OMeBn	7-Diethylamino	**2f**	73
7	**1g**	4-CF_3Bn	7-Diethylamino	**2g**	80
8	**1a**	Bn	–	**2h**	64
9	**1a**	Bn	5-Methoxy	**2i**	57
10	**1a**	Bn	6-Methoxy	**2j**	97
11	**1a**	Bn	7-Methoxy	**2k**	85
12	**1a**	Bn	8-Methoxy	**2l**	78
13	**1a**	Bn	6-Bromo	**2m**	95
14	**1a**	Bn	6-Hydroxy	**2n**	73
15	**1a**	Bn	7-Hydroxy	**2o**	70[b]
16	**1a**	Bn	8-Hydroxy	**2p**	72

[a] Isolated yields via filtration. [b] Purification by chromatography.

Target compounds containing the core skeleton were successfully synthesized under microwave irradiation via a two-step process (Table 1). First, efficient amidation[9] of methyl cyanoacetate with aryl amines under microwave irradiation at 135 °C gives rise to the key cyanoacetamide intermediates **1a**–**1g** in 30 min. Then, convenient condensation of the corresponding cyanoacetamides and commercially available substituted salicylaldehydes via a Knoevenagel reaction at 60 °C under microwave irradiation produces the desired 2-iminocoumarin-3-carboxamide **2a**–**2p** derivatives after an additional 30 min. It is important to emphasize that in most cases analytically pure products are obtained in moderate to good yields (ranging from 55% to 97%, Table 1) from the reaction mixture via simple filtration the exception being compound **2o**.

In an endeavor to further examine the fluorescent dyes with good photophysical properties and potential biological applications, we attempted to further modify the 2-iminocoumarin-3-carboxamide probes with direct derivation (Scheme 1). For example, by direct reaction of **2a** and *p*-anisidine in acetic acid at 60 °C for 3 h, product

Scheme 1. Synthesis of **3a** and **NPE-caged 2o**



3a possessing a 4-methoxyphenylimino substituent at position 2 was obtained in 81% yield. Now that the photocaged fluorescent probes[10] have wide applications in tracking the spatiotemporal dynamics of molecular movements in biological systems, **NPE-caged 2o** was prepared in moderate yield by incorporating a 1-(2-nitrophenyl)ethyl (NPE)[10a] group onto the 7-hydroxyl group of **2o**. **NPE-caged 2o** is nonfluorescent before

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activation; however, the photolabile protecting group can be removed by ultraviolet (UV) illumination (365 nm) to progressively generate the fluorescent probe **2o** (Figure S2 shows HPLC chromatograms of **NPE-caged 2o** and its photolysis products).

Table 2. Photophysical Data of **2a**–**2p** and **3a**[a]

compd	λ_{abs} (nm)[b]	λ_{em} (nm)[c]	Stokes shift [nm]	log ε_{max} (M^{-1} cm^{-1})[d]	Φ_f[e]
2a	397	475	78	4.61	0.59
2b	414	480	66	4.54	0.20
2c	402	470	68	4.28	0.39
2d	396	470	74	4.56	0.51
2e	401	473	72	4.41	0.42
2f	403	475	72	4.52	0.50
2g	402	473	71	4.50	0.61
2h	352	407	55	4.03	<0.05
2i	357	446	89	4.04	<0.05
2j	381	449	68	3.95	<0.05
2k	365	442	77	4.25	0.09
2l	382	449	67	3.19	<0.05
2m	363	445	82	3.91	<0.05
2n	382	466	84	3.93	<0.05
2o	370	483	113	4.31	0.16
2p	350	453	103	3.81	<0.05
3a	418	504	86	4.18	0.15

[a] Determined in chloroform. [b] Absorption maxima. [c] Emission maxima. [d] Maximum molar extinction coefficient. [e] Absolute quantum yield determined by a calibrated integrating sphere system.

With the fluorescent compound library in hand, we investigated the optical properties of the compounds, and the results are summarized in Table 2. In general, the iminocoumarin derivatives containing an electron-donating group R′ at position 7 and an electron-withdrawing group CONHR at position 3 afforded a strong fluorescence emission. The introduction of a benzyl group at position R with a fixed R′ substituent such as a diethylamino group leads to larger Stokes shifts and higher quantum yields than with a phenyl or phenethyl group (**2a** vs **2b**–**2c**). However, no significant change in the fluorescence properties is observed with alternation of the substituents on the benzyl group (**2d**–**2g**). Furthermore, the fluorescence emissions, in agreement with the electron-donating potentials of group R′, are increased in the following order: bromo (**2m**) < methoxy (**2i**–**2k**) < hydroxyl (**2n**–**2p**) < diethylamino (**2a**–**2g**). On the other hand, the substituted position of the electron-donating group R′ is crucial for the photonic property. For example, compounds with a substituent at position 7 show relatively higher quantum yields and extinction coefficients (**2i**–**2l** and **2n**–**2p**). It is interesting that introduction of a 4-methoxyl-phenyl group at position 2 of compound **2a** generates a large red shift in the excitation wavelength, owing to both the extension of the electronic conjugation and the electron-withdrawing effect of the added aromatic ring (**3a**). Next, the solvatochromic behavior of compound **2a** was examined; it can be observed that the extinction coefficients and fluorescence emission brightness going from organic to water media largely decreased (Figure S1 and Table S1).

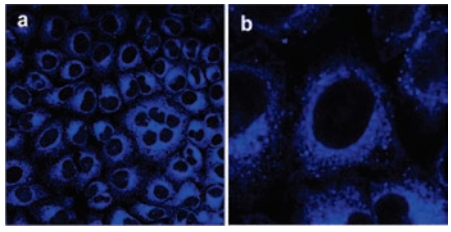

Figure 1. (a) Fluorescence images of HeLa cells initially incubated with 1 μM **2a** for 30 min in DMEM medium (with 10% FBS and 1% penicillin/streptomycin) and then fixed with 4% paraformaldehyde for 10 min (λ_{ex} = 390 nm); (b) 4× amplification image of (a).

In order to examine the potential use of these 2-iminocoumarin-3-carboxamide dyes for biological imaging, we initially incubated HeLa cells with a representative compound **2a** (1 μM) at 37 °C for 30 min. As shown in Figure 1, strong blue fluorescence was detected inside the cells (except for the nucleus) with excitation at 390 nm, which indicates that compound **2a** has good cell membrane permeability. HeLa cells were extensively stained at either 1 or 5 μM (Figure S4) incubation concentration (Figure 1). On the basis of cell morphology, compound **2a** appears to have low cell toxicity. We further evaluated the toxicity of the compounds **2a** and **2k** to HeLa and 7702 (Normal Human Liver cell line) cells; the IC_{50} values are over 100 μM (Figure S3). Moreover, **2a** can be used in either HBSS buffer (Figure S4a and S4b) or DMEM medium (Figure 1a and 1b), which indicates its stability when used for live cell imaging.

To further investigate the subcellular localization of compound **2a**, organelle-specific fluorescent dyes were employed for colocalization studies. As shown in Figure 2, we found that compound **2a** can colocalize strongly with MitoTracker Red CMXRos in mitochondria, with weighted colocalization coefficient values to 0.992 (Figure 2c and 2f, analyzed using LSM 510 software). The extensive overlapping with MitoTracker Red CMXRos indicated that mitochondria are probably the main site of compound **2a** accumulation. In cell biology, mitochondria are membrane-enclosed energy producing organelles found in most eukaryotic cells, they participate in a range of cellular processes, such as signaling, cellular differentiation, and cell death. Mitochondrial dysfunctions or defects involved in a variety of diseases ranging from aging and metabolic diseases to degenerative, hyper-proliferative diseases and cancers.[11] The imaging of

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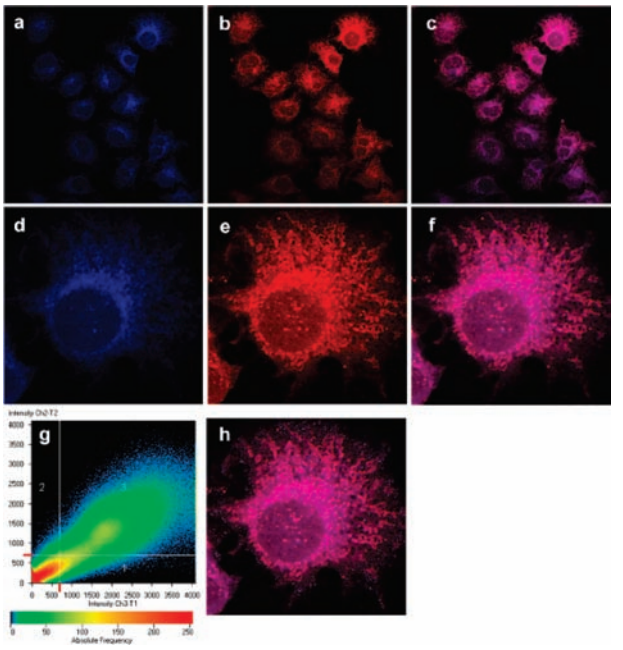

Figure 2. Confocal images of **2a** and mitochondrial specific dye in HeLa cells. (a) Fluorescence image of HeLa cells stained with compound **2a**; (b) Fluorescence image of HeLa cells stained with mitochondrial specific dye (MitoTracker Red CMXRos); (c) Merged image of (a) and (b); (d) 4× amplification image of single cell stained with compound **2a**; (e) 4× amplification image of single cell stained with MitoTracker Red CMXRos; (f) Merged image of (d) and (e); (g) and (h) colocalization analysis of compound **2a** and MitoTracker Red CMXRos in image (f).

mitochondria with novel synthetic mitochondrial specific probes will undoubtedly provide a more detailed understanding about the mitochondrial biology and signal propagation.[11e] Additionally, mitochondria possess a net negative charge on the matrix side of the membrane; hence, common markers for this organelle tend to be moderately lipophilic fluorophores with delocalized positive charges or with depolarizing effects on mitochondria.[8b,c] Bearing this in mind, the lipohilic property of compound **2a**, and its potential depolarizing effects, may contribute to its accumulation in mitochondria.

We further employed ER tracker Red dye (used to label the endoplasmatic reticulum) and Golgin-97 (an antibody used to label the Golgi complex) to examine whether **2a** can be localized within other subcellular domains. There is a relatively low colocalization of compound **2a** with ER tracker Red dye (Figure S5) and within the Golgi complex (Figure S6), with weighted colocalization coefficient values only to 0.569 (Figure S5g and S5h) and 0.392 (Figure S6g and S6h) respectively.

In summary, we have developed a small molecule fluorescent core skeleton, 2-iminocoumarin-3-carboxamide, with convenient synthesis and easy derivation. The library of fluorophores can undergo a dramatic change in fluorescence properties simply by changing the substituents at the two variation points of the fluorophore. Furthermore, the dyes can permeate the living cell membranes with low cytotoxicity and accumulate preferentially in the mitochondria, thus possessing potential utility in intracellular imaging.

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Supporting Information Available. Full experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.