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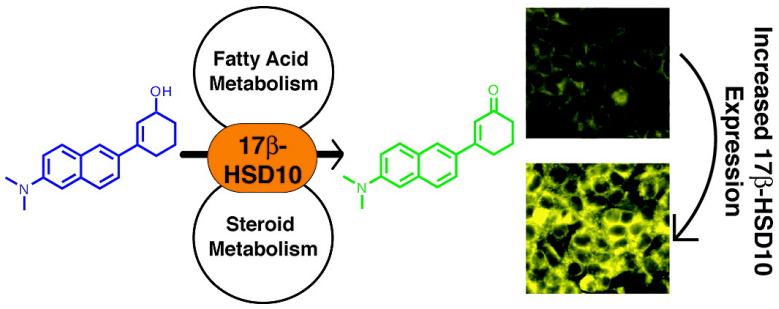

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Harnessing Functional Plasticity of Enzymes: A Fluorogenic Probe for Imaging 17β-HSD10 Dehydrogenase, an Enzyme Involved in Alzheimer's and Parkinson's Diseases

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Abstract: In this paper, we describe the development of a fluorogenic substrate for 17β-hydroxysteroid-dehydrogenase type 10 (17β-HSD10), which is a multifunctional metabolic enzyme fulfilling several metabolic roles (β-oxidation of fatty acids, catabolism of isoleucine, and metabolism of steroids). In recent years, it has emerged as an important stress and pathological marker in neurons and glial cells (expression down-regulation in Parkinson's disease, up-regulation and association with β-amyloid peptide in Alzheimer's disease). Through the iterative molecular design and chemical synthesis described herein, compound **1** was developed, which possesses all required properties for a selective optical reporter substrate: alcohol−ketone optical switching, the ability to function as a good enzyme substrate (expressed in kinetic parameters), cell permeability, and cell retention. Probe **1** provides a blue-to-green/yellow bright switch and enables non-invasive, real-time imaging of 17β-HSD10 in live human cells. The selectivity of reporter **1** was established by the quantitative correlation of metabolic activity to protein expression in human kidney cell line HEK-293T.

Introduction

Optical cell imaging has become an indispensable tool for the life sciences. However, few fluorescent probes are available for direct read-out of enzyme activity in cells.[1] As part of a broad research program, we are developing optical reporter substrates for important metabolic and signaling enzymes.[2] In this paper, we report a new fluorogenic probe that enables the direct activity measurement of 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10) in live human cells, through fluorescent microscopy. Other names for 17β-HSD10 have also been used in the literature including endoplasmic reticulum-associated amyloid β-binding protein (ERAB), amyloid β-peptide-binding alcohol dehydrogenase (ABAD), short chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD), hydroxyacyl-CoA dehydrogenase (HAD), and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD).

17β-HSD10 Is an Important Physiological and Pathological Marker. 17β-HSD10 is a multifunctional enzyme, fulfilling several metabolic roles. It is the third enzyme in the β-oxidation cycle, oxidizing 3-hydroxyacyl-CoAs with a preference for short-chain substrates.[3] 17β-HSD10 is also required for catabolism of isoleucine, as it accepts branched hydroxyacyl-CoA substrates.[4] Moreover, it has been proposed that 17β-HSD10 is involved in steroid metabolism (Figure 1).[5] Several cases of inherited 17β-HSD10 deficiency have been documented, and this condition results in a loss of mental and motor skills.[4] Reduced expression of this protein was found in brains of Parkinson's patients, while increased expression levels showed protective effects in mouse models of Parkinson's disease[6] and brain ischemia.[7] Further, 17β-HSD10 binds β-amyloid peptide, the pathological marker for Alzheimer's disease, and it was proposed that the resulting complex potentiates the oxidative stress and loss of neuronal function indicative of this disease.[8] Despite the importance of this emerging physiological and pathological marker, there are no agents for direct imaging of 17β-HSD10 in live cells and tissues. The functional plasticity

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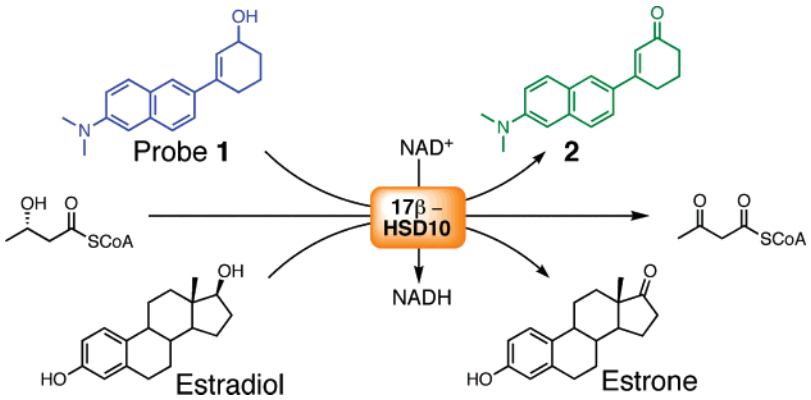

Figure 1. 17β-HSD10 dehydrognease metabolizes fatty acids, amino acids, and steroids. The enzyme's inherent substrate plasticity enabled the development of reporter probe **1** for direct imaging of 17β-HSD10 activity in intact human cells. 17β-HSD10 is defined as 17β-hydroxysteroid dehydrogenase type 10.

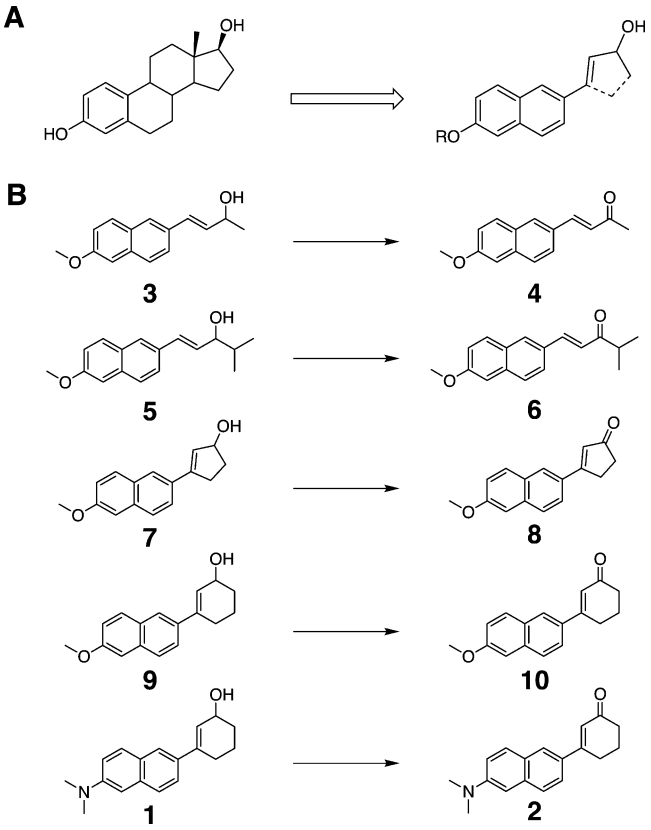

Figure 2. (A) Probe design: the 2,6-disubstituted naphthalene core mimics the size and shape of 17β-estradiol, one of the natural substrates of 17β-HSD10. (B) Reporter substrate/product pairs for 17β-HSD10 that were synthesized and examined.

of this enzyme, required for its multiple metabolic functions, suggested the possibility of designing a fluorogenic reporter substrate.

Results

***De Novo* Design of Optical Probes for Alcohol-to-Ketone Transformations.** Examining the enzyme's natural substrates, the preferred strategy was to design a fluorogenic steroid mimic. A 2,6-disubstituted naphthalene core was chosen to approximate the size and shape of 17β-estradiol (Figure 2A). As 17β-HSD10 is a dehydrogenase, operating in the oxidation direction, an alcohol probe needed to be designed that, upon oxidation to the corresponding ketone, would afford a fluorogenic (increase in emission intensity) or fluoromorphic (shift in emission maximum) response (i.e., the off/on or on/on alcohol → ketone switch). This represented the first key challenge to be addressed, as ketones usually quench fluorescence due to facile intersystem crossing to a non-radiative triplet excitation state.[9] Our design was founded on the idea that the quenching effect may be diminished by engaging the electron-withdrawing ketone in a strong electronic coupling with a well-placed electron-donating group, affording a "push−pull" system,[9,10] which is the defining element of many bright organic fluorophores. Specifically, an electron-donating methoxy group was placed in the 6-position to enable the electronic resonance with the ketone in the 2-position of naphthalene (Figure 2B). Initially, we synthesized methyl alcohol **3** and the corresponding ketone **4**. The latter showed a major red-shift in fluorescence; however, significant quenching still occurred, resulting in a decrease in emission intensity (Figure 3A). Despite the low quantum yield of ketone **4** (0.01, Table 1), its fluorescence is 2 orders of magnitude greater than that of alcohol **3** at 510 nm (fluoromorphic switch), which allows for easy fluorimetric monitoring of the enzyme-catalyzed oxidation. To our satisfaction, an *in vitro* kinetic assay with the purified enzyme demonstrated that probe **3** was a substrate for 17β-HSD10 (see the next section).

In order to improve the photophysical properties as well as the kinetic parameters, derivatives **5**−**10** were synthesized. The isopropyl alcohol−ketone pair **5**/**6** has photophysical properties similar to those of the parent couple **3**/**4**. In contrast, the fluorescence of cyclic ketone **8** is not quenched; in fact, ketone **8** is much more fluorescent than alcohol **7**, with a quantum yield of 0.71 in aqueous buffer, affording an excellent fluorogenic switch (Figure 3E). This fortunate outcome may be rationalized by increased electronic communication between the conformationally rigid cyclic ketone and the electron-rich naphthalene. Similarly, cyclohexenone **10** is not quenched in comparison to cyclohexenol **9**, constituting a fluoromorphic couple where the alcohol and ketone show comparable fluorescence intensities, with the maxima separated by more than 100 nm (Figure 3G).

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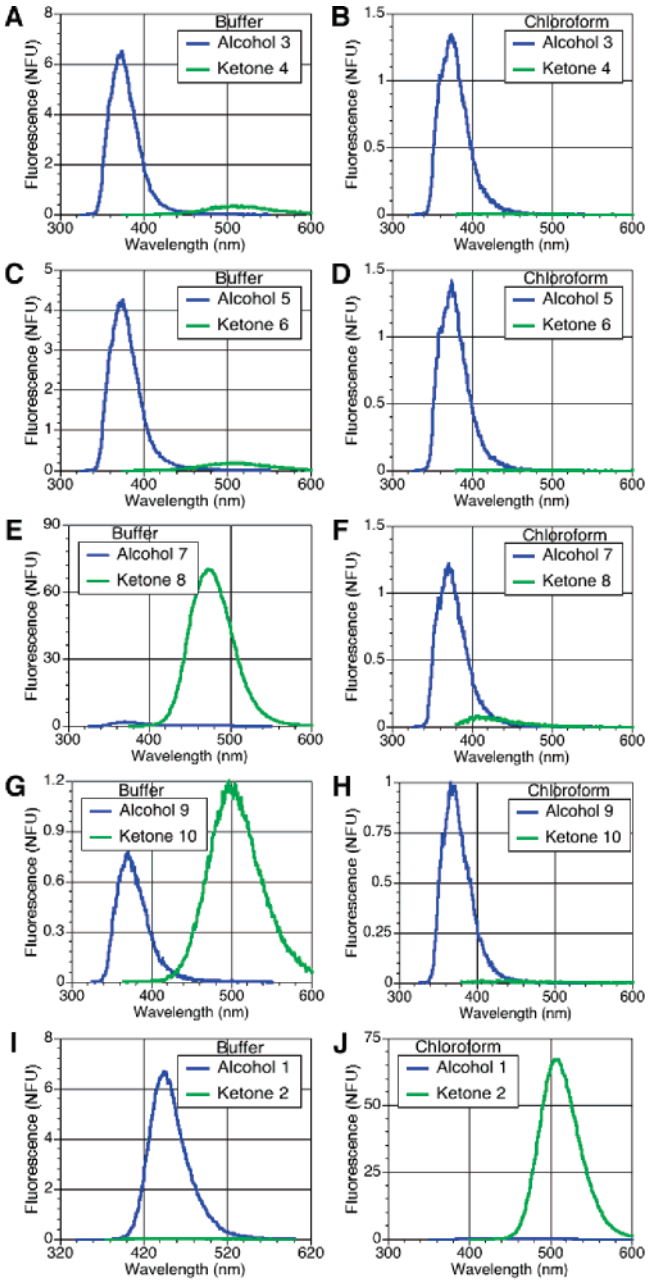

Figure 3. Fluorescent spectra for probes **1**−**10** in aqueous buffer and chloroform. Probes are at a concentration of 50 μM in potassium phosphate buffer (0.1 M, pH 7.0, 1% DMSO; A, C, E, G, I) or chloroform (B, D, F, H, J), excited at the excitation maximum for each compound (Table 1). All spectra are normalized to alcohol **9** in chloroform. NFU is defined as "normalized fluorescence units".

It is noteworthy that the emission of the cyclic ketones as well as the acyclic ketones is completely quenched in chloroform (Figure 3).

Last, we prepared the pair **1**/**2**, where the 6-position bears a strongly electron-donating dimethylamino group. While ketone **2** is not fluorescent in potassium phosphate buffer, it gives bright green-yellow emission in chloroform and in cells, affording an excellent fluorogenic switch (Figure 3I,J). This photophysical profile proved highly advantageous for cell microscopy imaging, as is discussed below. We propose that fluorescence in chloroform (or cell membranes) originates from the charge-transfer excited state.[11] Indeed, charge-transfer fluorophores display polarity-sensitive emission which is brightest in nonpolar solvents.[12]

Enzyme Kinetic Parameters for Reporter Probes Obtained with Purified 17β-HSD10. The first reporters we prepared, namely acyclic alcohols **3** and **5**, proved to be good substrates, albeit with relatively high K_m values (**3**, K_m = 100 μM; **5**, K_m = 70 μM; Table 2). We hypothesized that cyclic alcohols **7** and **9** would be better mimics of 17β-estradiol. This indeed is the case: the K_m value for substrates **7** and **9** is 8-fold lower than that for compound **3**. In terms of K_m, probes **7** and **9** match the physiological substrate 17β-estradiol (K_m = 15 μM); however, the reporter substrates out-perform 17β-estradiol in terms of turnover rates and catalytic efficiencies (Table 2). Similarly, probe **1**, containing the dimethylamino group in the 6-position, exhibits excellent kinetic properties. The systematic exercise synchronizing the desired photophysical properties and the substrate kinetic profiles generated three excellent reporter candidates for the cell imaging studies.

Direct Imaging of 17β-HSD10 Activity in Cells. The next key goal was to examine the metabolism of the probes in intact human cells. To this end, human kidney cells (HEK-293T) were transiently transfected with a 17β-HSD10 plasmid and incubated with probes **3**, **5**, **7**, and **9**. Probe conversion was examined by fluorimetry (fluorescence of the media) and fluorescent microscopy. Conversion of the probe to product was easily monitored by fluorimetry for probes **3**, **7**, and **9**. Importantly, the probes clearly showed faster oxidation with the transfected cells in comparison to the null-transfected control. However, microscopic imaging was precluded by the low cellular retention of the probes and the high background fluorescence.

To overcome this problem, the 6-methoxy group in substrate **9** was replaced with a more basic and more polar dimethylamino group, yielding probe **1**. While this alteration had only a minor effect on the *in vitro* kinetic parameters (see above), it led to a dramatic increase in cell retention, enabling the imaging of probe **1** metabolism via fluorescent microscopy. Furthermore, ketone **2** is not fluorescent in potassium phosphate buffer, whereas it is bright green-yellow in chloroform (a mimic of cell membranes) and in cells. This optical property proved advantageous for cell microscopy, eliminating the background fluorescence of the medium. Transfected HEK-293T cells indeed showed a faster rate of probe oxidation in comparison to null-transfected cells (Figure 4). Upon addition of probe **1** to the cellular media, the oxidation to ketone **2** could be monitored in the 585 nm filter cube, and the increase in fluorescence was linear for the first five hours of incubation. The increase in cell fluorescence corresponds to probe metabolism, which was rigorously confirmed by HPLC analysis of medium extracts (see Supporting Information). Furthermore, the quantitative comparison between the metabolic rates (formation of product **2**) and varying amounts of 17β-HSD10 DNA transfected into cells revealed a linear correlation, thus demonstrating that probe **1** reports on 17β-HSD10 activity in human cells (Figure 4).

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Table 1. Photophysical Properties of 17β-HSD10 Probes

	alcohol						ketone				
	λ_{abs} (nm)	ϵ (M^{-1} cm^{-1})	$\lambda_{max,ex}$ (nm)	$\lambda_{max,em}$ (nm)	ϕ^a		λ_{abs} (nm)	ϵ (M^{-1} cm^{-1})	$\lambda_{max,ex}$ (nm)	$\lambda_{max,em}$ (nm)	ϕ^b
					In Buffer[c]						
1	305	8 900	313	444	0.44	**2**	360	5 000	350	500	0.01
3	298	12 000	297	370	0.19	**4**	334	18 000	350	510	0.01
5	298	12 000	298	372	0.11	**6**	334	12 000	350	511	0.01
7	298	7 700	300	370	0.15	**8**	334	23 000	350	473	0.71
9	298	5 700	280	370	0.11	**10**	334	16 000	340	500	0.04
					In Chloroform						
1	320	18 000	330	370	0.01	**2**	390	15 000	385	510	0.70
3	300	16 000	303	374	0.06	**4**	334	22 000			0.0
5	300	15 000	303	374	0.05	**6**	334	17 000			0.0
7	300	16 000	304	370	0.05	**8**	334	26 000			0.0
9	300	13 000	300	370	0.04	**10**	334	20.000			0.0

[a] Relative to 2-aminopyridine in 0.1 N H_2SO_4 ($\phi = 0.6$) as a standard (excited at 300 nm). [b] Relative to quinine bisulfate in 0.1 N H_2SO_4 ($\phi = 0.46$) as a standard (excited at 350 nm). [c] Potassium phosphate buffer (0.1 M, pH 7.0). Extinction coefficients and quantum yields have an error of $\pm$10%.

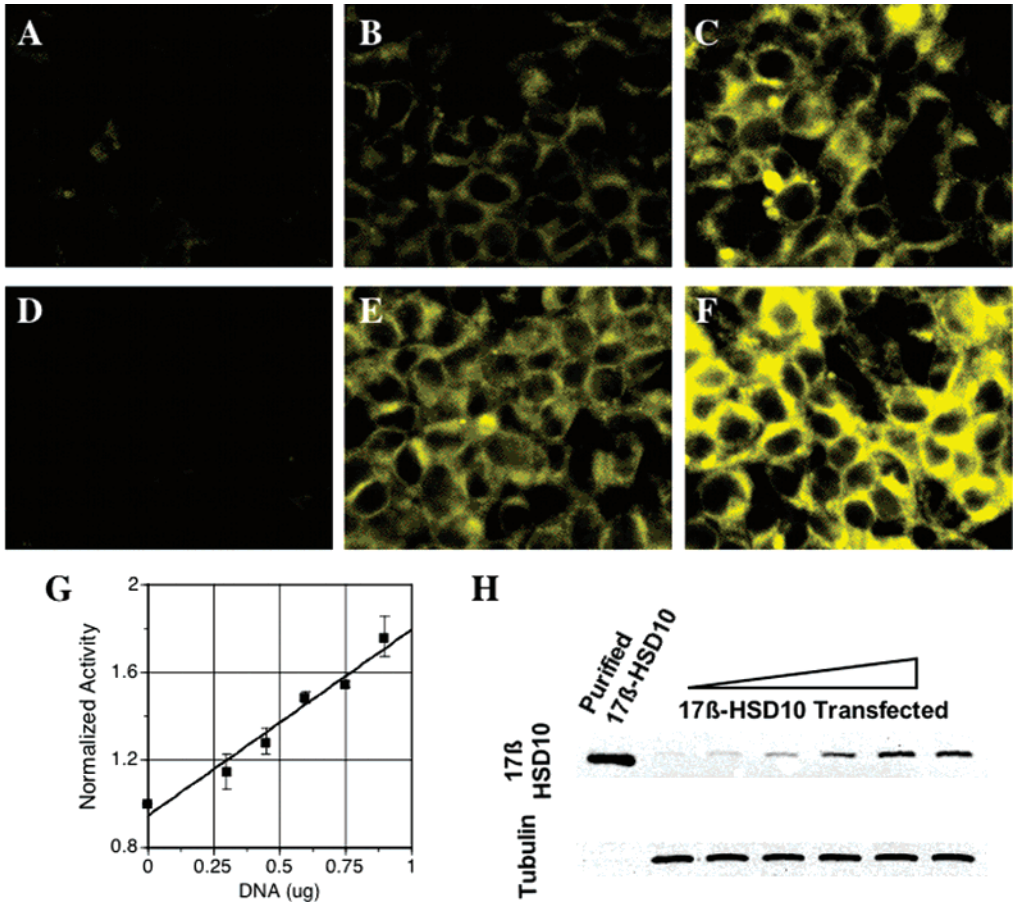

Figure 4. Metabolism of alcohol **1** in 17β-HSD10 transfected HEK-293T cells monitored by fluorescent microscopy (A−F). Images were taken after incubating cells with probe **1** for 0 (A,D), 2 (B,E), and 4.5 h (C,F). (A−C) Null-transfected and (D−F) 17β-HSD10 transfected. Dark circles are unstained nuclei. (G) Correlation of the metabolic rate of probe **1** to the amount of 17β-HSD10 DNA transiently transfected into HEK-293T cells. [Probe **1**] = 20 μM; rate of probe metabolism was measured as the mean fluorescence intensity of fluorescent images over time and normalized to null-transfected cells. Data shown are the average $\pm$ SD of three independent experiments, as described in the Supporting Information. (H) Western Blot of HEK-293T cells transfected with 0−0.9μg 17β-HSD10. Expression of 17β-HSD10 and α-tubulin was detected in cell lysates, as described in the Supporting Information.

Conclusions

Using rational design principles, we developed probes **1**, **3**, **5**, **7**, and **9** as fluorescent reporter substrates for 17β-HSD10. Cyclic derivatives **7** and **9** offered key advantages over the acyclic compounds **3** and **5** in terms of improvements in both kinetic and fluorescent properties. Although probes **7** and **9** are not suited for fluorescent microscopy studies, they can be used for fluorimetric cellular assays. Substitution of the 6-methoxy group for a dimethylamino group afforded alcohol−ketone pair

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Table 2. Kinetic Data for 17β-HSD10 Probes

substrate	K_m[a] (μM)	SA (μmol min^{-1} mg^{-1})	k_{cat} (min^{-1})
1	15 ± 3	0.65 ± 0.07	22 ± 3
3	100 ± 11	0.41 ± 0.02	13.7 ± 0.7
5	70 ± 10	0.41 ± 0.03	12 ± 1
7	13 ± 2[b]	0.15 ± 0.01	4.8 ± 0.4
9	12 ± 3	1.7 ± 0.1	55 ± 5
17β-estradiol	15 ± 7[c]		0.53 ± 0.07
	15 ± 3[d]	0.073 ± 0.08[e]	0.53 ± 0.07

[a] Assay was run in 0.1 M potassium phosphate buffer (pH 9), NAD^+ (500 μM), substrate, and 17β-HSD10 (0.1−0.8 ng); change in fluorescence or absorbance was monitored as described in the Supporting Information. [b] Substrate inhibition occurs. [c] Value taken from ref 13. [d] Determined by competitive two-substrate assay using probe **3** (see ref 2c). [e] Determined by following the formation of NADH at 340 nm.

1/2, which exhibits excellent properties for fluorescent microscopy studies. Ketone **2** is non-fluorescent in water but is fluorescent in chloroform and cell membranes, which eliminates background fluorescence for microscopy. As a result, fluorogenic probe **1** enables non-invasive and continuous measurement of 17β-HSD10 activity *in cells*, through fluorescent microscopy. This new imaging agent will be used to elucidate the biological functions of this important physiological marker.

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Supporting Information Available: Synthetic procedures for compounds **1**−**10**, enzymology assay, cell imaging protocols, and complete ref 8. This material is available free of charge via the Internet at http://pubs.acs.org.

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