Fluorescent Probes Hot Paper

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A FRET Probe for Cell-Based Imaging of Ganglioside-Processing Enzyme Activity and High-Throughput Screening**

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Abstract: Gangliosides are important signaling molecules in the cell membrane and are processed by several enzymes. Deficiencies in these enzymes can cause human lysosomal storage diseases. Building an understanding of the pathways of glycosphingolipid catabolism requires methods for the analysis of these enzymatic activities A GM3-derived FRET probe was synthesized chemoenzymatically for the detection and quantitation of a range of ganglioside-degrading enzymes, both in cell lysates and in living cells. This is the first substrate that enables the ratiometric fluorogenic assay of sphingolipid ceramide N-deacylase and endoglycoceramidase and can detect and localize neuraminidase activity in living cells. It is therefore a valuable tool for building a better understanding of membrane-confined enzymology. It also enables the robust and reliable assay of ganglioside-degrading enzymes in a microtiter plate, thus opening the door to screening for novel or engineered biocatalysts or for new inhibitors.

Gangliosides are a group of glycosphingolipids bearing one or more sialic acid residues on their termini. They are often found associated with other sphingolipids, cholesterol, and some membrane proteins to form so-called "lipid raft" structures in the cell membrane. In this way, gangliosides can influence cell structure and interactions with the extracellular environment.[1] As such, they play extremely important roles in biochemical signaling, pathogen entry, membrane transport, and intracellular protein sorting.[2] In vertebrates, the ganglioside degradation system involves numerous enzymes, including neuraminidase on the plasma membrane and acid ceramidase in the lumen of endosomes and lysosomes.[2] Unsurprisingly, given their importance, disruption of these enzymes can result in disease states in humans.[3] Radiolabeled gangliosides have proved to be useful as tools for elucidating glycosphingolipid metabolism and as substrates for the assay of ganglioside-degrading enzymes. However, such assays, which are usually based on TLC separations of radioisotopically labeled compounds, are laborious, poorly reproducible, and cannot be used for real-time measurements.[4] New probes that overcome these limitations would therefore be of great value for understanding the cellular roles of this key class of molecules.

Over the past two decades, fluorescent probes have become valuable tools in cell biology for the imaging of enzymatic reactions and the localization of biomolecules.[5] For example, 4-methylumbelliferyl glycosides are used to measure the activity of a range of glycosidases, including that of ganglioside-degrading beta-hexosaminidase A for the analysis of GM2 gangliosidoses.[6] Of particular value have been fluorescence resonance energy transfer (FRET) probes that allow ratiometric measurement, thereby providing greater precision than measurement at a single wavelength.[7] The design of probes to interrogate membrane-associated proteins presents specific challenges,[8] with only a handful of successful examples having been used for the assay of phospholipase A2,[9] ceramidase,[10] GM2-activator protein, and glucocerebrosidase.[11] Herein, we present the design, synthesis, and biological application of a small-molecule FRET probe that can be used in the detection and quantitation of a range of ganglioside-degrading enzymes, both in cell lysates and in living cells. The utility of this reagent in the high-throughput assay of two ganglioside-degrading enzymes in crude cell lysates is demonstrated, as well as in the ratiometric imaging of neuraminidase activity on the plasma membrane of living cells.

Probe **1** is based on the structure of ganglioside GM3 and carries two fluorophores, 7-hydroxycoumarin and BODIPY, which are attached on either end of the molecule to serve as FRET donor and acceptor, respectively (Figure 1). Placement of the fluorophores at the extreme ends of the molecule yields a reagent that can be recognized and cleaved by several different ganglioside-degrading enzymes, namely sphingolipid ceramide N-deacylase (Enz1), endoglycoceramidase (Enz2), and neuraminidase (Enz3). Cleavage of the probe by any of these enzymes will yield a large decrease in FRET efficiency, thereby giving a detectable ratiometric fluorescent signal.

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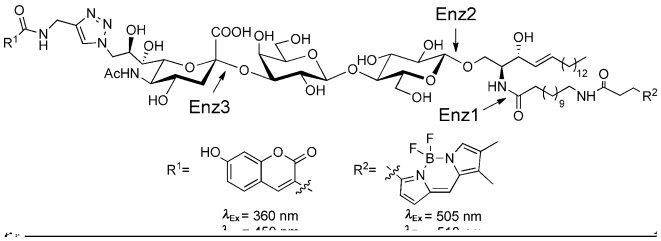
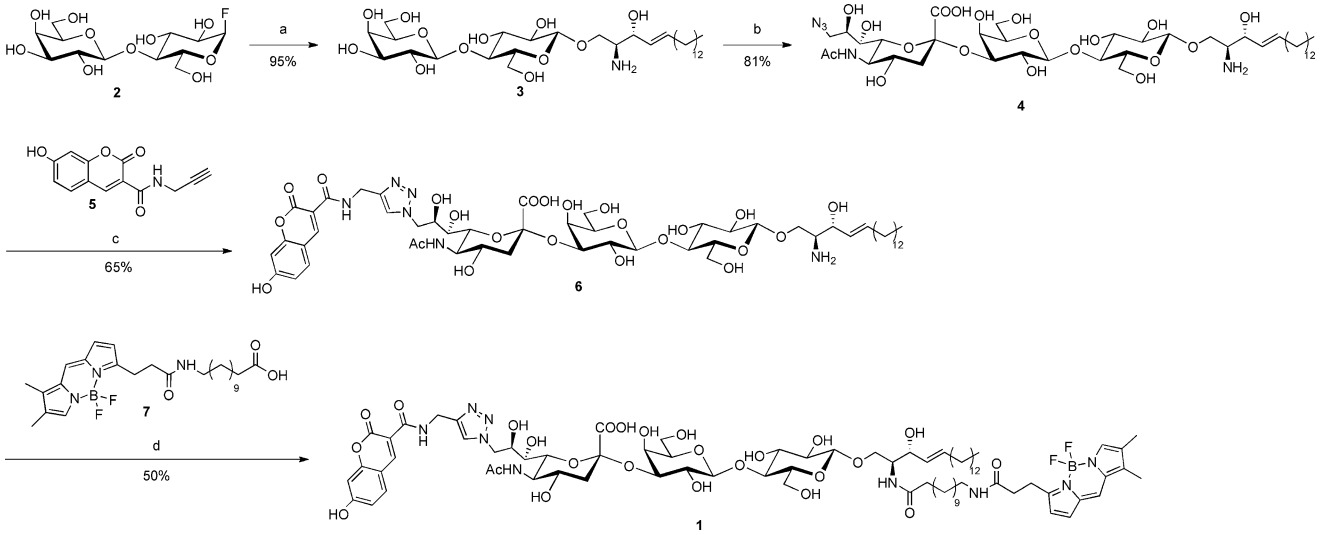


Figure 1. FRET probe 1 and its enzymatic cleavage points. The 7-hydroxycoumarin (R1) FRET donor is attached to the C9 position of sialic acid while the BODIPY FRET acceptor is attached to the terminus of the fatty acid alkyl chain. Enzymatic hydrolysis of the substrate by either sphingolipid ceramide N-deacylase (Enz1), endoglycoceramidase (Enz2), or neuraminidase (Enz3) will disrupt FRET. The arrows indicate where each enzyme cleaves the substrate.

The chemical synthesis of gangliosides and their derivatives can be a challenging task, especially when sensitive fluorophores are involved, owing to the many activation, coupling, protection, and deprotection steps required.[12] The use of enzymatic steps, where possible, can both simplify the synthesis and allow the use of milder reaction conditions, which in turn facilitates the final purification procedure. A key enzyme component in our strategy is an engineered endoglycoceramidase II (EGCase) from Rhodococcus sp. strain M-777.[13] While the natural role of this GH5 glycoside hydrolase is hydrolysis of the glycosyl ceramide linkage, it was converted into an efficient glycosynthase through two rounds of protein engineering and directed evolution.[13] By using this engineered glycosynthase, the synthesis of glycosyl sphingosines can be achieved from the corresponding glycosyl fluorides in near quantitative yields. Further elaboration to more complex glycosyl sphingosines can be achieved by using appropriate glycosyltransferases. Subsequent chemical N-acylation of these glycosyl sphingosines then yields the intact ganglioside structures. The utility of this highly flexible chemoenzymatic strategy has been demonstrated in the synthesis of several gangliosides, including the complex echinodermatous ganglioside LLG-3.[13,14] We further improved this strategy by introducing another enzyme, sphingolipid ceramide N-deacylase (SCDase), to carry out the final N-acylation step,[15] thereby enabling the fully enzymatic assembly of the fluorescent ganglioside GM3 derivative **1** (Scheme 1).

Lactosyl sphingosine (**3**) is first synthesized by using EGCase glycosynthase to couple lactosyl fluoride and d-erythro-sphingosine. The coupling of 9-azido sialic acid to **3** to yield **4** was then carried out using the sialyltransferase Cst-I from C. jejuni and CMP-9-azido sialic acid, itself synthesized from 9-azido sialic acid by using CMP-Neu5Ac synthetase.[16] Attachment of the coumarin derivative **5** to **4** was carried out by copper-catalyzed click chemistry to yield the fluorescent lyso-GM3 derivative **6**. Finally, SCDase from Shewanella alga G8 was used to catalyze the condensation between **6** and the BODIPY-coupled lauric acid **7**[15] to give the final product **1** in 50% yield, with unreacted **6** and **7** being easily recoverable (see the Supporting Information). The syntheses of the linker-modified fluorophores **5** and **7** are provided in the Supporting Information.

Compound **1**, as an analogue of ganglioside GM3, forms micelles in aqueous solution, which strongly affects its fluorescent properties owing to self-quenching of the probe. Triton X-100 can be used as a spacer surfactant to reduce the self-quenching, with the extent of the reduction being dependent on its molar ratio relative to **1** (Figure S1 in the Supporting Information). Under optimized conditions, **1** is incorporated into Triton X-100 micelles (containing approximately 140 molecules) in such a way that, on average, one in every five micelles contains one molecule of the substrate. Under these conditions, the emission of 7-hydroxycoumarin at around 450 nm is barely observed when excited at 360 nm, while a strong emission from BODIPY is seen around 518 nm.



Scheme 1. Assembly of FRET probe 1. a) EGCase glycosynthase, d-*erythro*-sphingosine, NaOAc buffer pH 5.3, 10% dimethoxyethane, 37 °C; b) CMP-9-azido-9-deoxy-sialic acid, α2,3-sialyltransferase, alkaline phosphatase, 50 mm Tris buffer (pH 7.5) containing 100 mm $MgCl_2$, RT; c) alkyne coumarin 5, 50 mm BTTES buffer, 200 mm sodium ascorbate, 50 mm $CuSO_4$, 50% DMF, 35 °C; d) BODIPY carboxylic acid 7, SCDase glycosphingolipid N-deacylase, 50 mm HEPES buffer (pH 7.5), 10% dimethoxyethane, 35 °C.

The FRET efficiency was calculated to be more than 95%, thus demonstrating that energy transfer proceeds efficiently in intact probe **1**. Upon individual addition of ganglioside-degrading enzymes: sphingolipid ceramide N-deacylase (Enz1) from Shewanella alga G8,[15] endoglycoceramidase (Enz2) from Rhodococcus strain M-777,[17] or neuraminidase (Enz3) from Micromonospora viridifaciens,[18] substantial increases in the fluorescent emission at 450 nm were observed, accompanied by decreases in the emission at 518 nm (Figure 2a). This is very evident in the change in color of the reaction solution from green to bright blue when observed under UV light (Figure 2b). TLC analysis of the end

Figure 2. Hydrolysis of compound 1 by sphingolipid ceramide N-deacylase (Enz1), endoglycoceramidase (Enz2), or neuraminidase (Enz3). Addition of the enzymes to a 0.1 mm solution of probe 1 in 20 mm Tris-HCl buffer (pH 7.5) with 1% Triton-X100 led to an increase in the coumarin fluorescence (blue) and a decrease in the BODIPY fluorescence (green). a) Fluorescence spectra before and after 30 min incubation with the different enzymes. b) Visual detection of the reactions under a UV lamp. c) Different hydrolysis patterns of probe 1 after hydrolysis by Enz1, Enz2, and Enz3. Neg: unreacted probe 1 as a negative control.

products formed in each case indicated completely different hydrolytic patterns as a consequence of cleavage by Enz1, Enz2, and Enz3 as would be expected, thus showing that the substrate can be used in a multi-enzyme system for the simultaneous detection of different enzymatic activities (Figure 2c). Changes in the ratio Em_{450}/Em_{518} of up to 70-fold were observed for Enz2 and Enz3, while with Enz1 the change was much smaller (7.3-fold) since an equilibrium is established between hydrolysis and synthesis.[15] These large changes in ratio render probe **1** highly suitable for ratiometric measurement within living cells. Measurements made ratiometrically greatly reduce the influence of environmental changes (such as pH, polarity, and temperature).

To explore the utility of probe **1** in live cells, we examined its ability to insert into the membrane of human lymphoma (Jurkat) cells. These cells have previously been found to express NEU3,[19] the membrane-associated form of human neuraminidase that processes ganglioside substrates. Indeed flow cytometry studies clearly showed fluorescent labelling of these cells (see Figure S2 in the Supporting Information). However microscopy studies were rendered more challenging by the rapid hydrolysis of **1** observed upon simple addition to the cell media as a result of neuraminidase activity. To minimize these problems, we used a microinjector to add a small amount of the substrate in the vicinity of cells as they were being imaged (Figure 3). Staining of the cells occurred primarily at the plasma membrane, as most clearly seen from the BODIPY emission, with clear labeling of the cell membrane over background regions. (see Supporting Information). Control experiments in cell-free systems and in regions away from the cell allowed us to assign the initial spike and drop of fluorescence as being due to the local introduction and diffusion of the fluorophores (Figures S3

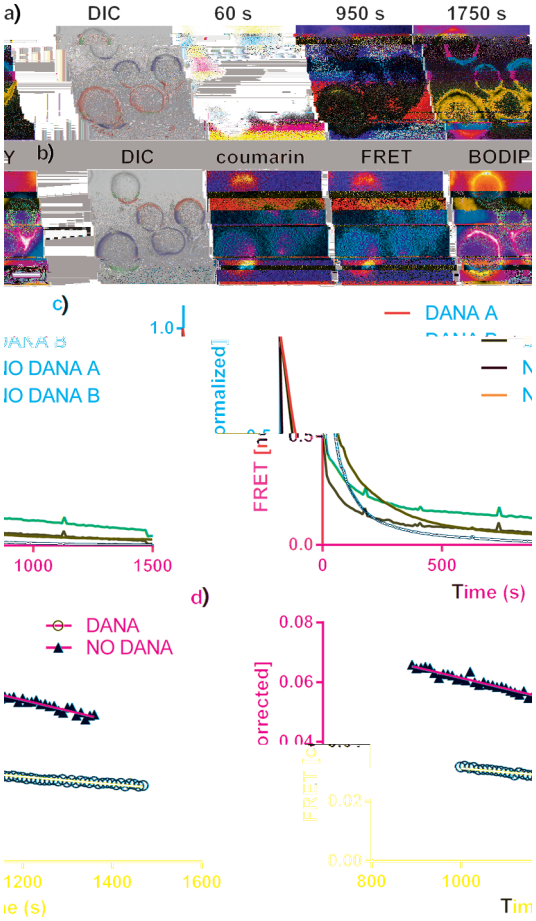


Figure 3. Observation of 1 in the cell membrane of Jurkat cells. a) Representative images from a video showing cell staining by compound 1 over time. The substrate was injected at 30 s. The differential interference contrast (DIC) image at time 0 is shown on the left. Images from the BODIPY channel are shown at the indicated times, with an initial high signal followed by a decrease in intensity. b) Jurkat cells stained with 1 are shown in three channels (140 s) to show coumarin (Ex_{365}/Em_{445}), FRET (Ex_{365}/Em_{515}), and BODIPY (Ex_{488}/Em_{515}) fluorescence. Fluorescence images are false-colored to enhance the contrast. c) A time course of FRET intensity from microscopy images of regions on the cells (A) or in the background (B). Experiments were run in the presence or absence of DANA. d) A linear segment of the FRET signal from cells stained with 1 in the presence or absence of DANA is shown after background subtraction as described in the Supporting Information. The decay rates for the region shown are ▫1.3▫0.1□10▫^{5} (DANA) and ▫3.6▫0.1□10▫^{5} (NO DANA), thus indicating approximately 3-fold faster decay in the absence of the neuraminidase inhibitor.

and S4), thus allowing us to correct the signals for this phenomenon. The subsequent time-dependent decrease of the fluorescence signal in the membrane is seen as a linear phase (ca. 1000 s; Figure S5). Linear regression of these data (Figure S6 and Table S1 in the Supporting Information) revealed that the later parts of the decay were highly dependent upon neuraminidase activity. The addition of 2,3-didehydro-N-acetyl-neuraminic acid (DANA), a general neuraminidase inhibitor that blocks the human enzymes,[20] strongly suppressed the decrease in fluorescence signal in all three channels, which suggests that the decrease monitored is primarily due to neuraminidase action.

By measuring the background-corrected FRET signal from cells labelled with **1** (Figure 3c, d), we could see large differences in the rate of decay in the FRET channel in the presence versus the absence of DANA. All of these observations support the notion that probe **1** inserts into the plasma membrane where it is acted on as a substrate of human neuraminidases, although we cannot rule out that other enzymes or physical processes may also contribute to the signal. The most likely candidate neuraminidase is NEU3, although our experiments cannot distinguish between specific isoenzymes such as NEU4 that may also cleave glycolipids.[21] In either case, probe **1** is the first reported compound that can be used to detect the activity of membrane-associated neuraminidase enzymes in real time.

Ganglioside-degrading enzymes are useful tools for remodeling gangliosides on cell surfaces and can therefore be used to probe the roles of these glycolipids. Access to methods for high-throughput screening of these enzymes would be useful for directed evolution or inhibitor discovery, but this is challenging since the hydrolysis of the natural substrates does not yield a useful, directly measureable signal. While chromogenic substrate surrogates are available for the screening of neuraminidases (Enz3), significant structural differences from the natural substrate limit their use in some applications. However, as far as we are aware, no colorimetric or fluorogenic substrates exist for monitoring sphingolipid ceramide N-deacylase (Enz1) or endoglycoceramidase (Enz2). Indeed detection of the activity of these enzymes relies on TLC analysis with radiolabeled ganglioside substrates,[4] an approach that is not well-suited to high-throughput screening. Probe **1** is ideal for the high-throughput assay of all of these enzymes, with the reaction being observable at high sensitivity and in real time. No background hydrolysis of **1** is seen in lysates of control E. coli BL21 cells, even after 12 h (data not shown). However, large dose-dependent changes in fluorescence are seen upon the addition of

Figure 4. Detection of the enzymatic activities of endoglycoceramidase (Enz2) from *Rhodococcus strain M-777* and neuraminidase (Enz3) from *Micromonospora viridifaciens* in cell lysates. a) Dependence of rate upon crude lysate concentration. Changes in fluorescence upon the addition to 1 of 0 μL, 5 μL, 10 μL, 20 μL, 30 μL, 40 μL or 50 μL of 100□diluted crude lysate from *E. coli* cells expressing Enz3. The relative amounts of enzyme and relative reaction rates are shown. b) Reproducibility of the assay when using probe 1. Crude lysate from *E. coli* cells expressing Enz2 or Enz3 was added to 1 in buffer in a 96-well plate. Lysate from *E. coli* BL21(DE3), which carries an empty pET28a plasmid, was used as a blank.

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