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# A Novel Visible Range FRET Probe for Monitoring Acid Sphingomyelinase Activity in Living Cells

Christian Kappe,[a] Zainelabdeen H. Mohamed,[a, b] Eyad Naser,[c] Alexander Carpinteiro,[c] and Christoph Arenz*[a]

**Abstract:** Activity of acid sphingomyelinase has been implicated in a number of diseases like acute lung injury, sepsis or metastasis of melanoma cells. Here, we present a sphingomyelinase FRET probe based on FAM/BODIPY dyes for real-time monitoring of acid sphingomyelinase. The probe gives rise to a tremendous increase in fluorescence of the fluorescein FRET donor upon cleavage and we show that this is, to a significant part, due to cleavage-associated phase transition, suggesting a more systematic consideration of such effects for future probe development. The probe allows for the first time to monitor relative sphingomyelinase activities of intact living cells by flow cytometry.

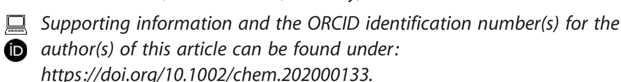
Sphingolipids form a class of membrane lipids that are ubiquitous in the plasma membranes of eukaryotes.[1] Less complex intermediates of membrane sphingolipid biosynthesis and degradation such as ceramide, sphingosine and sphingosine-1-phosphate are important regulators of cell fate and are therefore the focus of various pharmaceutical strategies.[2–4] Sphingomyelin, one of the most abundant lipids in eukaryotic plasma membranes, is cleaved by sphingomyelinases in a single step to yield ceramide and phosphoryl choline, thus serving as a reservoir for the above lipid mediators. Among different isoenzymes, the acid sphingomyelinase (ASM) has drawn most attention with regard to human disease.[5] ASM is a soluble lipid hydrolase and ubiquitous in the acidic compartments of eukaryotic cells. Different conditions can also trigger localization of ASM into lipid rafts of the outer leaflet of the plasma membrane.[6] Numerous reports suggest that inhibition of ASM could be a promising strategy for treating a variety of diseases like acute lung injury, major depression or metastasis of melanoma.[5,7] However, despite its potential as a drug target, no potent drug-like inhibitors are known so far.[8]

Given the importance of ASM as a potential drug target and the need for tools for convenient and effective testing of ASM activities, we decided to develop sphingomyelin FRET substrates. FRET probes, in contrast to quenched probes, turn-on or turn-off probes, offer the possibility of ratio measurements. The quotient of both, FRET donor and FRET acceptor fluorescence intensities reflects the degree of cleavage of the probe, independent of its concentration.[8,9] In a previous work we have already synthesized a FRET substrate for the downstream enzyme acid ceramidase. Although a nearly 100% FRET efficiency could be determined for the substrate, the ratio change was moderate with about 3.5-fold.[10] Likewise, we recently synthesized a FRET substrate for ASM with NBD as a FRET donor and Nile red as a FRET acceptor, where the ratio change was even lower, with a factor of about 3-fold. Closer investigation showed that the cause for the small signal change was quenching of the NBD dye upon cleavage and concomitant lipid-water phase transition.[11] This caused us to utilize the phase transition-triggered NBD quenching to develop a second ASM probe with coumarine as FRET donor and NBD as FRET acceptor. This simple trick yielded an about 80-fold ratio change upon cleavage.[11] The latter probe was highly suitable for monitoring enzymatic activity in vitro and due to its selective cleavage by ASM also suited for life cell imaging of enzyme activity. However, the coumarine FRET donor with an excitation optimum around 350 nm made use of a two-photon excitation microscope necessary.[11] To address this shortcoming, we now decided to make a new attempt towards a FRET substrate excitable in the visible range. In order to keep the polarity and membrane anchoring as close as possible to nature and to fall back on already known sphingomyelin derivatives, we decided to introduce a BODIPY dye into the fatty acid part, and a relatively polar fluorescein (FAM) residue (Exc. 485 nm/ Em. 518 nm) into the choline analog head group (Scheme 1). We were well aware of the pH dependence of fluorescence of FAM, but due to the low costs and the manifold possibilities of chemical modification, we decided for this dye combination. For the synthesis of the envisioned probe, we used the orthogonally protected sphingosyl-phosphorylethanolamine intermediate **1**, which was developed and described

[a] C. Kappe, Z. H. Mohamed, Prof. Dr. C. Arenz
Institut for Chemistry, Humboldt Universität zu Berlin
Brook-Taylor-Str. 2, 12489 Berlin (Germany)
E-mail: arenzchr@hu-berlin.de

[b] Z. H. Mohamed
Medicinal Chemistry Department, Faculty of Pharmacy
Assiut University, Assiut 71526 (Egypt)

[c] E. Naser, Dr. A. Carpinteiro
Department of Molecular Biology, University of Duisburg-Essen
Hufelandstr. 55, 45147 Essen (Germany)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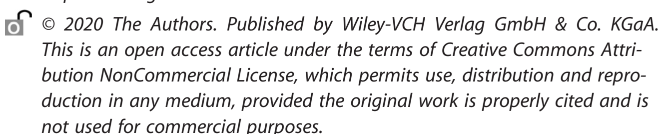
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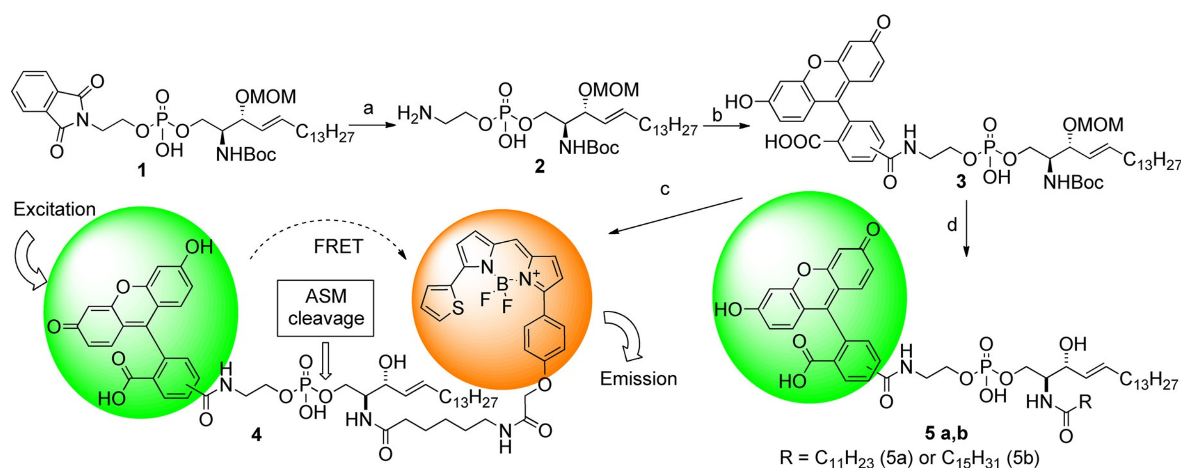


**Scheme 1.** Synthesis of probes **4** and **5**. Reagents and conditions: a) $H_4N_2 \cdot H_2O$, MeOH, 5.5 h, r.t., 84%; b) 5(6)-carboxyfluorescein, *N*-hydroxysuccinimide, DCC, $CH_2Cl_2$, 4 h, r.t., then $NEt_3$, DMF, 20 h, r.t., 66%; c) 4 M HCl dioxane/iso-PrOH, 4 h, 70 °C, then 1.3 equiv. BODIPY TR-X SE, $NEt_3$, pyridine/DMF, 10 h, r.t., 59% over 2 steps. d) 4 M HCl dioxane/iso-PrOH, 3.5 h, 70 °C, then 1.4 equiv. $C_{11}H_{23}COOSu$, $NEt_3$, iso-PrOH/THF, 30 h, r.t., 78% over 2 steps or 1.2 equiv. $C_{15}H_{32}COCl$, DIPEA, pyridine/$CH_2Cl_2$, 5.5 h, 0 °C, 12% over 2 steps.

by us previously.[11] After removal of the phthalimide protection group the resulting amine **2** was coupled to 5/6 carboxyfluorescein in the presence of *N*-hydroxysuccinimide (Scheme 1). The resulting intermediate **3** was treated with hydrochloric acid in dioxane to remove the remaining protecting groups. Finally, the crude deprotected intermediate was reacted with an active ester of BODIPY hexanoic acid to afford the desired FRET substrate **4**.

Upon incubation of a micellar solution of the novel substrate **4** with acid sphingomyelinase, the BODIPY acceptor fluorescence appeared to be almost unchanged, similar to results with the Nile red containing probes.[10–11] Surprisingly, the fluorescence of FAM increased significantly by more than 100-fold (Figure 1 A). Expectedly, the new substrate **4**, enabled us to differentiate between different concentrations/activities of ASM (Figure 1 B). The reaching of a plateau value for the fluorescence (Figure 1 B–D) indicated complete conversion of the probe, as shown by TLC separation after the experiments (Figure S2).

Surprised by the enormous sensitivity of the probe, we considered whether the unexpectedly high increase in FAM fluorescence may not have been fueled by the destruction of the FRET system alone, but also by the phase transition of the dye from the micelle environment into the aqueous solution. This would be the exact opposite of our very first ASM FRET probe, where the released dye was quenched by transition from the lipid micellar phase to the aqueous phase.

To investigate this further, we decided to synthesize a corresponding substrate having only a FAM but not a BODIPY residue. Towards this end, intermediate **3** was coupled with an unlabeled fatty acid. Since palmitoylation resulted in a completely insoluble and therefore hardly fluorescent product **5 b**, a lauric acid residue was introduced using the corresponding succinimide active ester (Scheme 1). As suspected beforehand, even the mono-labeled probe **5 a** without a FRET acceptor showed a significant increase in fluorescence upon cleavage by ASM (Figure 1 C). Indeed, this increase was still about 20-fold. We therefore concluded that the FAM dye alone was subject to some mode of quenching, while being bound to lipid micelles. Interestingly, both probes yielded virtually the same end-point fluorescence at 518 nm after cleavage, but the initial fluorescence of the doubly labeled probe **4** was significantly lower (≈7.5-fold), suggesting that this part of FAM quenching was due to FRET. The direct comparison also showed that the mono-labeled probe was cleaved much faster than the double-labeled FRET probe (Figure 1 C). A detailed kinetic characterization confirmed this observation (**4**, $K_M$ = 8.9 μM; $V_{max}$ = 3.1 μmol/(mg h); **5 a**, $K_M$ = 4.2 μM, Vmax = 7.1 μmol/(mg h); see Figures S3–S10). As in the case of our previously published probe, no cleavage of the two probes by cell lysates of cells overexpressing neutral sphingomyelinase 2 at neutral pH could be detected (data not shown).

Since we knew about the pH dependence of FAM fluorescence, we wanted to investigate this effect for our probe in more detail. Therefore, we set up the same enzymatic reaction at four different pH values, 4.5, 5.0, 5.5 and 7.0 using sodium acetate buffer or TRIS/HCl buffer, respectively (Figure 1 D). In the literature, the pH optimum of acid sphingomyelinase is usually given as "about 5". An early study reports a value of 4.4, which was determined with radioactively labelled substrate.[12] Another study gives a pH optimum of 5.0 to 5.5, but the values were obtained with hexadecanoyl-*p*-nitrophenyl phosphorylcholine as substrate.[13] From the recorded graphs using the probe **5 a** it was clearly visible, that the absolute fluorescence increase was the largest at pH 5.5. This behavior is easily explained by the fact that the fluorescence of fluorescein is increasing with higher pH.[14] However, direct comparison showed that initial slopes decreased with higher pH and that the curve at pH 4.5 entered a plateau phase, while the curves at higher pH values were still increasing, clearly demonstrating a pH optimum below 5.0 under these conditions (Figure 1 D). At pH 7, the curve showed a significantly elevated initial fluorescence and only a marginal increase in fluorescence, which seems to match with reports that acid sphingomyeli-


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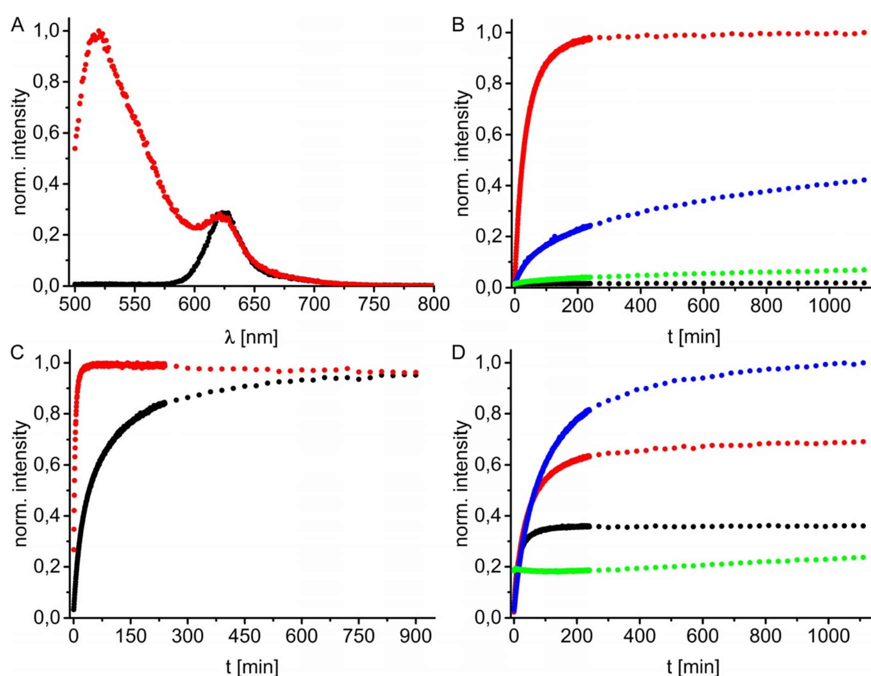


**Figure 1.** A) Probe **4** (1 μM, exc. 485 nm) before (black) and after (red) incubation with ASM. B) Probe **4** (1 μM, exc. 485 nm, em. 518 nm) in presence of 0 (black)/0.02 (green)/0.2 (blue)/2.0 (red) $\mu g\,mL^{-1}$ ASM, over time. C) Comparison of probe **4** (black) with probe **5a** (red), each 1 μM, with 2.0 $\mu g\,mL^{-1}$ ASM. D) Probe **4** (1 μM) with 2.0 $\mu g\,mL^{-1}$ ASM at pH 7 (green), pH 4.5 (black), pH 5.0 (red) and pH 5.5 (blue). Normalization was performed to the maximum observed fluorescence, respectively.

nase shows only about 5% of its maximum activity at neutral pH.[15] We were uncertain whether the pH dependence of the FAM dye and the localization of ASM in the acidic compartments of the cell would be compatible with a quantification of ASM activities in living cells. However, an even bigger problem with the use of confocal microscopy could be that, upon cleavage of the probe, FAM would transfer from a concentrated, membrane-bound state into the aqueous phase and would therefore be subject to a dilution effect that could counteract the strong increase in fluorescence. Therefore, we decided to use an application of the probe for flow cytometry (FC), because this method, in contrast to confocal methods, considers the total fluorescence of the cell. In fact, FC-based analysis of cellular ASM activity would provide a very fast and convenient way to examine clinical samples or the in-cellulo activity of potential ASM inhibitors. Until now, an in-cellulo characterization of ASM inhibitors can only be performed by a tedious procedure in which the cells are incubated with a radioactive or fluorescent probe together with the inhibitor. Finally, after pulse/chase, lysis of the cell and extraction of the lipids and subsequent concentration and separation by TLC or HPLC analysis, the in cellulo activity can be calculated, as reported very recently.[16] To test, whether this procedure could be overcome by using FC, we incubated L929 murine fibroblasts with the FRET probe 4 (1 μM) and the fluorescence after different incubation times was analyzed in the green (FAM) and red (BODIPY) channel, respectively (not shown). The peak fluorescence in the FAM channel (about 5-fold compared to background) was measured after 30 minutes, whereupon this incubation time was used as standard for all further measurements. This increase in fluorescence is small compared to the increase in vitro, which is likely attributed to the relatively high background fluorescence of the cells together with probably uncomplete cleavage of the probe. Further optimization in the future may better exploit the inherent features of the probe. To prove that this increase in fluorescence was really due to the activity of the acid sphingomyelinase, cells were pre-treated with 10 μM amitriptyline for 24 h. This compound accumulates in the endolysosomal compartment of the cell and leads to a proteolytic degradation of ASM and some other lysosomal lipid hydrolases.[6] Incubation with this drug led to significant decrease of the mean fluorescence intensity (MFI) of the cells in the FAM channel compared to vehicle-treated cells (Figure 2A). The MFI of the BODIPY channel, however, was indistinguishable from the one for those cells that were not treated with the inhibitor (Figure S1). A more detailed dose-dependent study showed a very high agreement of the remaining ASM activities for both the conventional and the FC-based method (Figure 2B). Therefore, we concluded that we have successfully established a FC-based assay for acid sphingomyelinase in living cells.

FRET probes are superior tools for monitoring enzyme activities in real time at high sensitivity and with high spatial resolution.[8] Above all, the possibility of ratiometric measurements recommends FRET probes compared to other concepts such as quenched probes. Here, we present a new FRET substrate for the acid sphingomyelinase. Probe **4** shows a 100-fold ratio change and we are not aware of a higher value from the literature. As in an earlier example in which a FRET acceptor led to a dramatic amplification of the probe by a lipid-water phase

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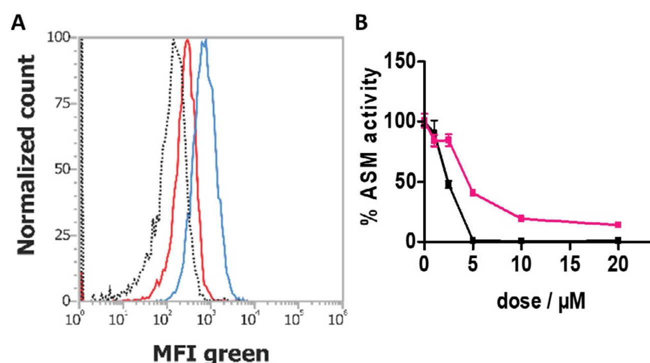


**Figure 2.** A) Flow cytometry (FC) analysis of L929 cells: Mean fluorescence intensities (MFI) in the green channel. Black curve = cell w/o probe; blue curve = cell with probe **4**, but w/o inhibitor; red curve = cells with probe **4** plus 10 µM amitriptyline (24 h pre-treatment). B) ASM activity assay after treatment with Amitriptyline for 24 h at the indicated concentrations. Black curve: conventional ASM assay. Magenta curve: FC-based assay using probe **4**.

transition, this principle was now shown in reverse for a FRET donor. Indeed, also the mono-labeled probe **5a** can be used for indicating ASM activities in vitro, but without the possibility of ratio measurement. Phase transitions upon FRET probe cleavage can be "friend or foe" and there is much to be suggested that such effects should be investigated more thoroughly and included in the planning of new FRET probes in the future. Moreover, we show that our new probe is suitable for a fast, easy and reproducible assessment of cellular ASM activities. The FC-based method could be superior for testing potential ASM inhibitors because the conventional assay introduces lots of artifacts with the real inhibition being often underestimated or lost. Finally the FC-method could also be used in clinical environments, for example, for an easy diagnosis of Niemann-Pick disease type A and B, an inborn ASM deficiency.[17]

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## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** enzyme assays · flow cytometry · Förster resonance energy transfer · live cell assays · sphingolipids

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