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Synthesis of a Fluorogenic Analogue of Sphingosine-1-Phosphate and Its Use to Determine Sphingosine-1-Phosphate Lyase Activity

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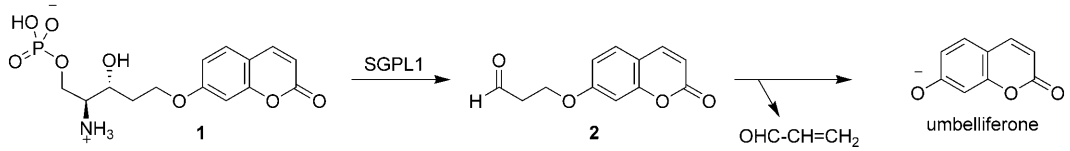
Sphingosine-1-phosphate lyase (SGPL1), encoded by *Sgpl1*, is an endoplasmic reticulum membrane protein that catalyses the pyridoxal 5′-phosphate-dependent cleavage of sphingosine-1-phosphate to ethanolamine phosphate and hexadecenal.[1–3] SGPL1 serves central roles in development,[4–6] chemotaxis[7] and in preventing defects in reproductive structures and function.[8] On the other hand, SGPL1 acts as a tumor suppressor,[9,10] has a role in chemoresistance[11–14] and it is also implicated in immunity.[15] Collectively, SGPL1 represents a novel target for cancer therapy and immunosuppression.[16] The research of SGPL1-mediated biology and the identification of novel small chemical entities that modify SGPL1 activity in drug discovery programmes would benefit from the availability of an easy SGPL1 assay amenable to high-throughput screening (HTS) formats. SGPL1 determination has been carried out using a rather tedious radiometric procedure. The enzyme activity is measured by following the formation of labelled hexadecanal from [4,5-^{3}H]dihydrosphingosine-1-phosphate.[17] After enzyme incubation and lipid extraction, the aldehyde is separated by thin-layer chromatography and quantified by using liquid-scintillation counting. A new assay using a fluorescent substrate has been reported, but it still requires lipid extraction and chromatographic separation.[18]

The use of a fluorogenic substrate as SGPL1 sensor was envisaged after the pioneering works by Reymond and co-workers.[19] In this context, the coumarinic sphinganine 1-phosphate analogue **1** was designed as potential fluorogenic SGPL1 substrate. Since both saturated and unsaturated as well as truncated base phosphates are transformed by SGPL1 and the reaction is highly stereoselective for the D-erythro isomer,[1] we reasoned that compound **1** contains the required structural features to behave as a SGPL1 substrate. Upon SGPL1 cleavage, the aldehyde **2** is produced, which should undergo subsequent β-elimination at neutral-alkaline pH to release the fluorescent product umbelliferone and acrolein[20] (Scheme 1).

The synthesis of **1** (Scheme 2) was accomplished from alcohol **3**, which was obtained in five steps from Garner's aldehyde as described.[21] Deprotection of **3** under *p*-toluenesulfonic acid catalysis in methanol gave diol **4**, which was selectively phosphorylated at the primary alcohol function using dimethylchlorophosphate to afford **5**. Finally, concurrent hydrolysis of the two methyl phosphate esters and the *tert*-butoxycarbonyl group was carried out using trimethylsilylbromide, giving **1** in 45% yield.[22] Furthermore, aldehyde **2** was also synthesized following reported procedures[23] to investigate its conversion into umbelliferone in the enzyme reaction conditions.

To confirm that SGPL1 was specifically involved in the production of fluorescence from **1**, the assay was performed with lysates of murine embryonic fibroblasts (MEF) prepared from both homozygous (−/−) and heterozygous (+/−) knockout



Scheme 1. SGPL1-catalyzed fluorescence release from **1**.

and wild-type (wt) embryos, obtained by crossing Sgpl1+/− mice.[24] Fluorescence released upon incubation of (−/−) cell lysates with increasing concentrations of **1** was barely above background (Figure 1 A), while a concentration dependent production of fluorescence was observed with (+/−) and wt cells, with higher enzyme activities at all concentrations in the latter case. Moreover, no fluorescence was released from 250 μM substrate and lysates of wt cells in the absence of pyridoxal phosphate, which further confirmed the specificity of this substrate for SGPL1. In wt cells, production of fluorescence from **1** was dependent on the amount of protein, reaching a plateau at around 1 mg/well (10 mg mL^{-1}).

The effect of the incubation mixture components on both maximum fluorescence and signal/background ratio were

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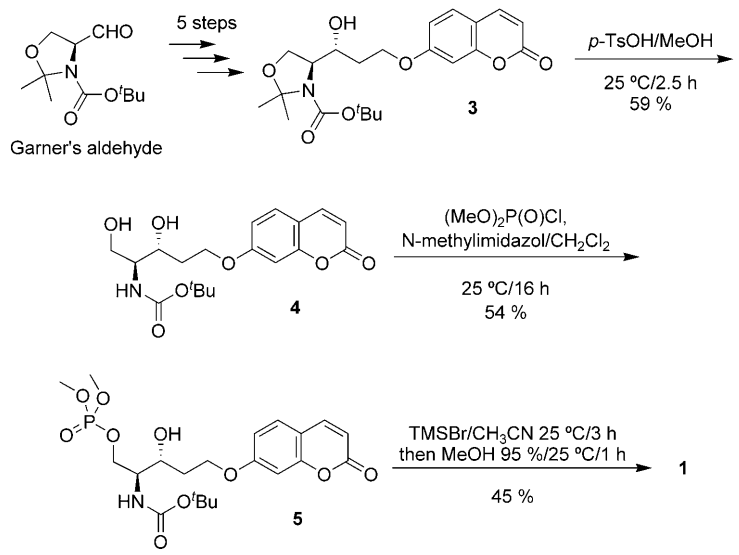

Scheme 2. Synthesis of **1**.

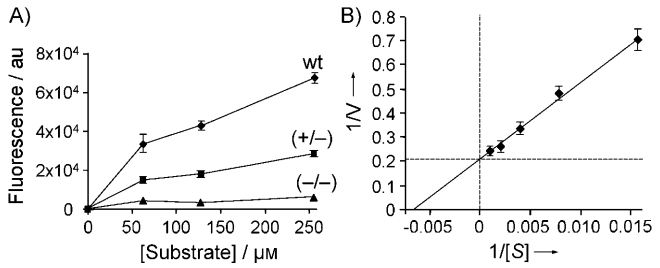

Figure 1. Enzymatic cleavage of **1**. A) Cleavage of **1** by lysates of either homozygous (−/−), heterozygous (+/−) or wt mouse embryonic fibroblasts. Cell lysates (9 mg protein per mL) were incubated with **1** in 0.5 M phosphate buffer pH 7.4 with 0.25 mM pyridoxal phosphate at 37 °C for 6 h. Data are means ± SD of one experiment with triplicates; B) Lineweaver–Burk plots for cleavage of **1**. Experiments were carried out with wt cell lysates (9 mg mL^{-1}) with the same buffer as in (A) plus 25 μM Na_3VO_4. Data are means ± SD of one representative experiment with triplicates.

examined with **1** using wt cell homogenates and compared to the fluorescence released from pure aldehyde **2** and that of umbelliferone submitted to the same incubation conditions. Although BSA is commonly used as catalyst in this type of proton transfer reaction,[25] its reactivity with pyridoxal phosphate[26,27] precluded its use here. Nevertheless, a time dependent release of umbelliferone from **2** was observed in the absence of BSA, and it reached completeness after 60 min (Figure 2B). Therefore, BSA was not necessary to fulfil the β-elimination reaction. SGPL1 assays use Triton-X100 for substrate solubilization. In our hands, production of fluorescence from **2** was dramatically decreased in the presence of Triton-X100 (Figure 2B), possibly due to the engulfing of umbelliferone in the detergent micelles. Thus, fluorescence of umbelliferone was reduced about sixfold in the presence of Triton-X100 (Figure 2A), which was therefore avoided in the reaction mixture. Other additives commonly used to measure SGPL1 activity, such as NaF and Na_3VO_4, had no effect on the fluorescence released either from **2** or from umbelliferone (Figure 2A, B). However, although NaF did not affect the production of fluorescence from **1**, significantly more umbelliferone was released in the presence of Na_3VO_4 (Figure 2C). This result is in agreement with sphingosine-1-phosphate phosphatase belonging to the PAP2-like superfamily of phosphatases,[28,29] which includes vanadate-sensitive lipid phosphate phosphohydrolases.[30] Importantly, although dithiothreitol (DTT) had no effect on the fluorescence produced from **2** or that of umbelliferone (Figures 2A, 2B), it brought about a significant reduction in the fluorescence generated from **1**. These results suggested that the deleterious effect of DTT occurred at the SGPL1 reaction level, although in the presence of Triton X-100 and when using sphinganine-1-phosphate as substrate, DTT is not inhibitory.[31] Site-directed mutagenesis of the human cDNA disclosed the importance of the cysteine residues C218 and C317 for the cleavage reaction,[31] in agreement with the *N*-ethylmaleimide sensitivity.[31] The reason for this discrepancy is currently not known.

From these overall results, incubation of **1** (125 μM) with 100 μL of a cell lysate (about 10 mg mL^{-1}) in 0.5 M potassium phosphate pH 7.5 containing Na_3VO_4 (25 μM) and pyridoxal phosphate (0.25 mM) for 6 h was established as optimal. The SGPL1 kinetic constants for **1** under these conditions were $K_M = 152$ μM and $V_{max} = 4.8$ pmol min^{-1} mg (Figure 1B), and the coumarinic aldehyde was detected at concentrations as low as

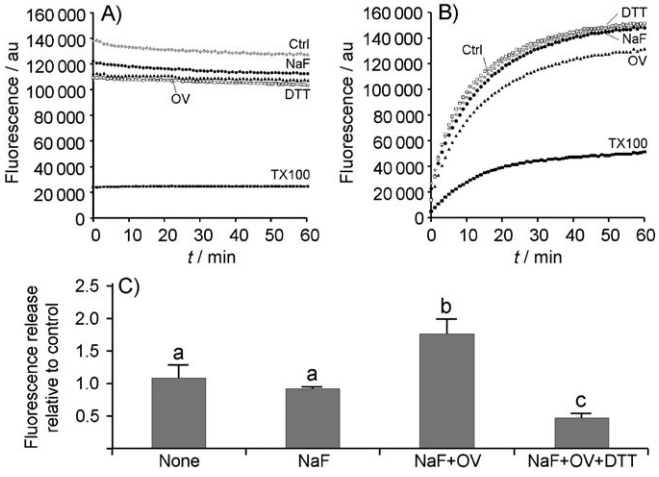

Figure 2. Effect of incubation mixture components. A) Effect on umbelliferone fluorescence; B) effect on β-elimination of aldehyde **2** and C) effect on SGPL1 cleavage of **1**. In (A) and (B), the control reaction mixture contained: 0.5 M phosphate buffer pH 7.4 (85 μL) containing 0.25 mM pyridoxal phosphate and 125 μM of either aldehyde **2** (A) or umbelliferone (B) solution. Both were added in methanol (15 μL), except in the experiment with Triton-X100, in which they were added as 0.25% Triton-X100 solution in phosphate buffer. Other compounds and concentrations were: DTT, 5 mM; Na_3VO_4, 25 μM; NaF, 25 mM. Incubations were carried out at 37 °C. In (C), substrate **1** (125 μM) and wt cell lysates (10 mg protein mL^{-1}) were incubated (37 °C/6 h) in 0.5 M phosphate buffer (pH 7.4) containing 0.25 mM pyridoxal phosphate (control) and, when indicated, 25 mM NaF, 25 μM Na_3VO_4 and 5 mM DTT. Letters atop each bar denote statistical significance (unpaired 2 tail *t* test, $p \leq 0.05$). Abbreviations are: Ctrl, control; OV, Na_3VO_4; TX100, Triton-X100.

50 nM (50 pmol mL^{-1}). Compared to both the physiological[1] and the fluorescent substrates[18] previously reported, the K_M is about ten-fold higher; this is likely due to the bulky ω-group in **1**. Therefore, SPL affinity for substrate **1** is about ten-fold lower than that for either the radioactive or the fluorescent substrates reported. V_{max} are not comparable because of the different enzyme sources used, which may contain different SPL concentrations.

In summary, an easy procedure to measure SGPL1 activity using a fluorogenic substrate sensor has been reported. The measurements can be carried out directly in microtiter plates without the need of separation of the reaction products, which represents a significant improvement of the previously reported methods. This procedure should become a suitable tool for the discovery of new enzyme modulators within combinatorial libraries, as well as in research to decipher the role of SGPL1 in disease outcome and progression.

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