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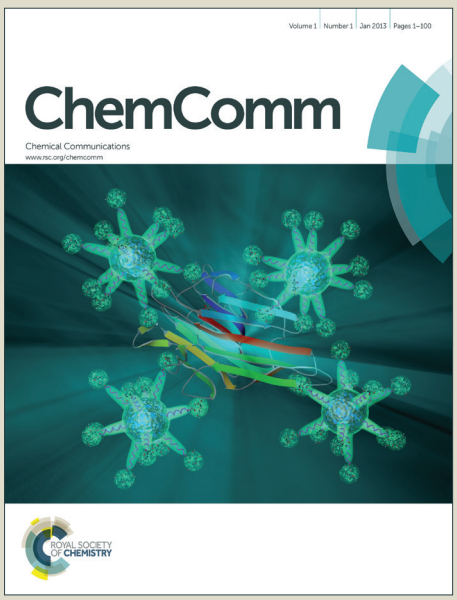

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Syntheses of Lactosyl Ceramide Analogues Carrying Novel Bifunctional BODIPY Dyes Directed towards the Differential Analysis of Multiplexed Glycosphingolipids by MS/MS using iTRAQ

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2013,
Accepted 00th January 2013

DOI: 10.1039/x0xx00000x

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Lactosyl ceramide analogues carrying novel bifunctional BODIPY-based fluorescent tags were designed and synthesised for live cell imaging. Addition of azide functionality on the fluorophore facilitated isobaric tagging for quantitative multiplexed analysis of biomolecules based on tandem mass spectrometry.

Glycosphingolipids (GSLs) are known to play important roles in a wide variety of cell functions, such as cell-cell interaction, modulation of cell growth and differentiation, and intracellular signalling. [1] However, very little is known about the global regulation of glycan synthesis and degradation, although considerable efforts have been made to study the functional roles of individual glycans of GSLs in the cell. Several difficulties arise when attempting to study the global regulatory mechanism of glycan structures in cells. Glycan synthesis and degradation is a complex process that depends on various factors, such as involvement of multiple enzymes and vesicular transport and/or the maturation of Golgi cisternae. In order to improve our understanding of the transformation process during glycan synthesis and degradation, quantitative analysis of individual molecules is required for systematic investigation. This analysis should provide spatiotemporal resolution [2,3] and molecular structure. [4–6]

To gain access to global information on the changes in glycan structure occurring within the cell, we had recently described a new

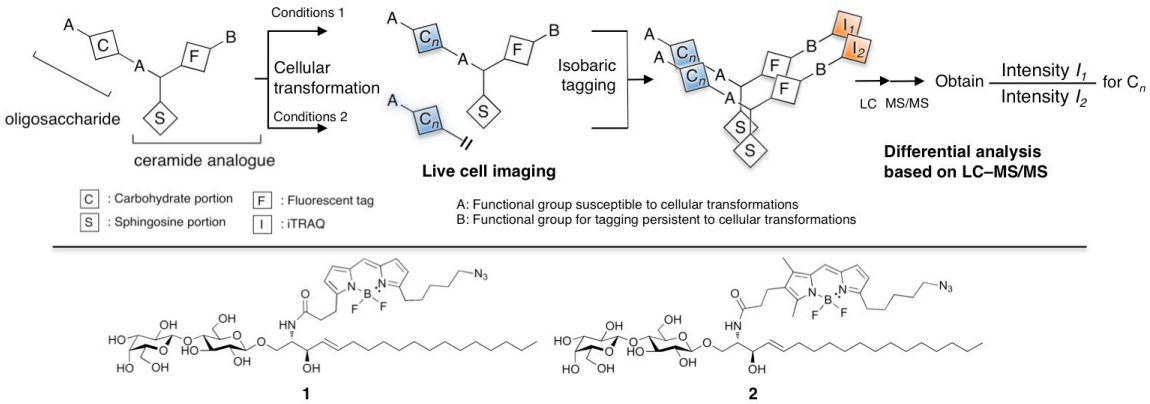

Fig. 1 Overall strategy of investigating the regulatory mechanism in glycan synthesis taking place inside the cell and the structures of target compounds

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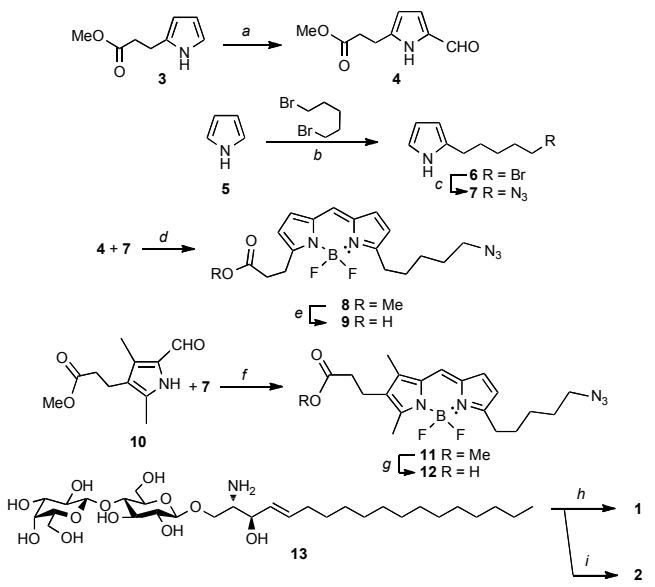
method using a series of analysis platforms, such as confocal laser scanning microscopy, nanoliquid chromatography (nanoLC), and nano electrospray (ESI)-tandem mass spectrometry (MS/MS). [7,8] Confocal microscopy can be used to visualise the intracellular distribution and trafficking of molecules in living cells. The nanoLC-MS/MS technique provides quantitative information on each resolved GSL and structural information for individual molecule. However, it is technically difficult to obtain accurate relative quantities of GSLs between multiple samples obtained from cultured cells grown under different conditions, due to the experimental error associated with run-to-run variations in nanoLC performance. Thus, we attempted to develop a simple and efficient quantitation strategy for the differential analysis of multiple GSL samples by MS/MS using isobaric tags for relative and absolute quantitation (iTRAQ). [9,10] Compounds carrying iTRAQ are not distinguishable by mass number, but the series of fragments (product ions) of a specific ion generated from a compound by MS/MS, provides information about the structure and the origin of the ion species as it can be decoded by specific reporter ion. We planned to introduce the isobaric tag into the fluorescent moiety



Scheme 1 a) $POCl_3$, DMF, $(CH_2Cl)_2$, reflux, 15 min then AcONa, 83%; b) BuMgCl, Et_2O, 0 °C, 3 h, 11%; c) NaN_3, DMSO, 50 °C, quant; d) 1) HBr (48% in H_2O), EtOH, 0 °C, 1 h, 2) Et_3N, BF_3•Et_2O, 0 °C, 3 h, 18% over 2 steps; e) Lipase PA Amano, DMF–KPB, 91%; f) 1) HBr (48% in H_2O), EtOH, 0 °C, 1 h, 2) Et_3N, BF_3•Et_2O, 0 °C, 3 h, 63% over 2 steps; g) Lipase PA Amano, DMF–KPB, 90%; h) **9**, DMT-MM, THF–H_2O, 82%; i) **12**, DMT-MM, THF–H_2O, 84%

(dipyrromethene boron difluoride; BODIPY) of the GSL samples. The localisation of the fluorescent probe molecules can be visualised, and this procedure has been performed for closely related compounds. Lactosyl sphingosine (LacSph) carrying BODIPY fluorophores is known to mimic the behaviour of natural GSLs in the cell. [8,11,12] We hypothesized that BODIPY could easily be modified for the incorporation of iTRAQ without affecting the behaviour of GSLs (Fig. 1). On the basis of these considerations, we designed two novel bifunctional azido-BODIPY acid molecules carrying a carboxylic acid and an azide functionality (Scheme 1). The carboxylic acid functionality on the bifunctional BODIPY can be incorporated into LacSph under standard amidation conditions. The azide functional group can be labelled with an alkyne group that carries iTRAQ. The isobaric tag is introduced into the GSL samples after imaging of cellular events, through Click reactions. [13,14]

Here, we describe the preliminary results of our overall approach for the accurate relative quantitation of GSL samples. Fluorescently tagged lactosyl ceramide (LacCer) analogues **1** and **2** (Scheme 1a), based on the two novel bifunctional azido-BODIPY acid derivatives **9** and **12**, were successfully synthesized. We further examined the uptake and intracellular distribution of the novel fluorescent LacCer analogues **1** and **2** in PC 12D cells. We labelled the LacSphBODIPY derivative **2** with iTRAQ113 in order to ascertain whether MS/MS could provide structural information for glycan as well as the iTRAQ reporter ion, for future investigation.

The first step was the preparation of the necessary pyrrole derivatives toward the synthesis of bifunctional azido-BODIPY acids **9** and **12**. Since the LacSph carrying BODIPY C-5 has been shown to be an excellent probe molecule for the investigation of glycan transformation, we decided to modify the structure to include azido functionality without major changes to its chemical properties. Our concerns in the designing the fluorophore are the overall structure of the fluorescent tag and the partition coefficient between water and octanol. The calculated LogP values for native LacCer with stearic acid and LacSphBODIPY C-5 are 8.7 and 6.7, respectively. Based on these considerations, we designed two LacCer analogues, **1** (ClogP = 8.4) and **2** (ClogP = 9.2), containing new BODIPY dyes **9** and **12**. The pyrroles **3** and **10** were prepared according to previously described procedures. [15,16] As a common precursor for the synthesis of **9** and **12**, the pyrrole **7** with azide functionality was prepared *via* Friedel-Crafts alkylation of pyrroles with 1,5-dibromopentane followed by nucleophilic substitution of the bromo moieties with an azide. During the Friedel-Crafts reaction, two regioisomers were formed in which the ratio of the 2- and 3-substituted pyrroles were 3:1. The resulting regioisomeric mixture was used unpurified for the next step. Pyrrole **3** was subjected to Vilsmeier-Haack formylation. A high yield of pyrrole **4** was obtained by the addition of the Vilsmeier reagent, generated from

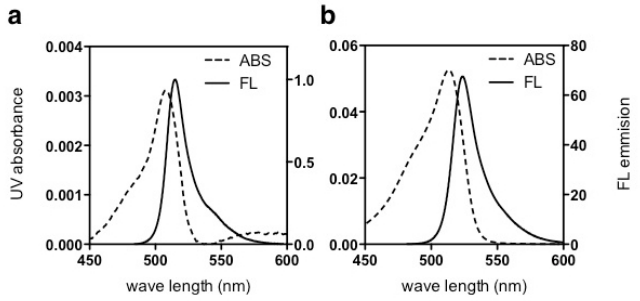


Fig. 2 Absorbance and fluorescent spectra of compounds **9** and **12**

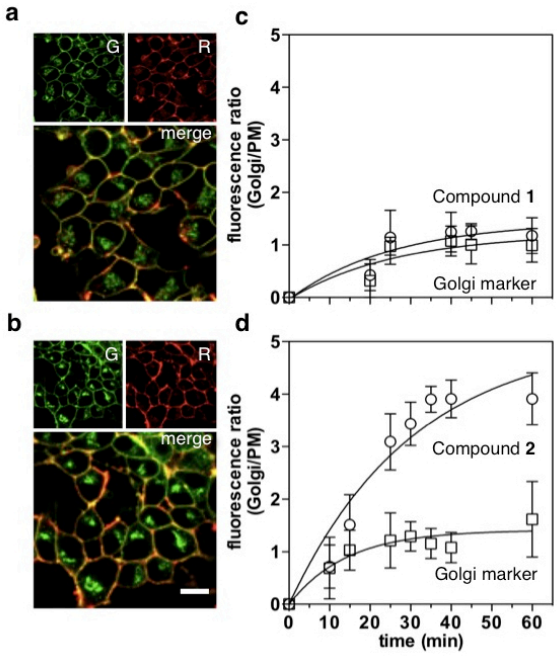


Fig. 3 Intracellular distribution of **1** and **2** in PC 12D cells and time course study of internalization. (a) Co-staining of **1** (G) and Golgi marker (LacSphBODIPY-TR-X; R). **1** co-localized with Golgi marker (merge). (b) Co-staining of **2** (G) and Golgi marker (LacSphBODIPY-TR-X; R). **2** co-localized with Golgi marker (merge). Scale bar, 10 μm. (c) Time course study for **1** reaches to Golgi apparatus from PM as indicated by the ratio of mean for fluorescence intensity at the Golgi to PM (mean ± sem. n = 5). (d) Time course study for **2** reaches to Golgi apparatus from PM. For a description of the quantification procedure, see supplementary.

phosphoryl chloride and dimethylformamide, from pyrrole **3**. Respective condensations of carboxyaldehyde pyrroles **4** and **10** with **7**, under the influence of hydrobromic acid, and subsequent complexation with boron trifluoride, yielded the desired azido-BODIPY methyl esters **8** (18%) and **11** (63%). The above-mentioned undesired regioisomer generated in Friedel-Crafts reaction could be easily separated by chromatography at this stage. Next, various lipases were screened for the hydrolysis of methyl esters of the BODIPY FL C3 acid because of the well-known sensitivity of BODIPY-based dyes to alkaline conditions. The lipase PS from *Burkholderia cepacia* was found effective for this purpose. Thus, methyl esters in the BODIPYs **8** and **11** were hydrolysed using the lipase to generate azido-BODIPY acids **9** and **12** in high yields. The absorbance and fluorescence spectra for **9** (maximum absorbance: 507; emission maximum: 514) and **12** (maximum absorbance: 514; emission maximum: 525) are shown in Figure 2. After the synthesis of azido-BODIPY acid derivatives **9** and **12**, we synthesised LacCers carrying the azido-functionalised BODIPYs **1** and **2**. In our previous studies, it was shown that the use of the condensation agent DMT-MM [17] was highly suitable for incorporation of BODIPY acids into *N*-hexyl-4-aminobutyl lactosides. [18] The approach was successfully adopted for the synthesis of **1** and **2**.

To investigate whether the fluorescent moiety in the fatty acid region affects the cellular uptake and intracellular distribution and trafficking of the new LacCer analogues **1** and **2**, we incubated PC-12D cells in the presence of **1** or **2**, each complexed with BSA and Golgi marker, and observed their distribution within the cells by confocal microscopy. It is apparent from Figure 3a and 3b that both compounds were taken up by the cell and accumulated in the Golgi apparatus. The Golgi marker lactosyl sphingosin carrying BODIPY TR-X has been shown to undergo glycosylation. Therefore, compounds **1** and **2** are believed to be excellent probes for investigating the process of glycan synthesis. However, our preliminary results indicated that the two compounds were internalised at different speeds. Thus, we conducted a time-course study of the accumulation of **1** and **2** in the Golgi apparatus. The ratio of mean fluorescence intensity in the Golgi region to that in the plasma membrane (PM) region was calculated. It was shown that the LacCer analogue **2** accumulated considerably faster in the Golgi apparatus than compound **1** or the known Golgi marker (Fig. 3c and 3d). Because the carbohydrate portion is constant, the observed difference in transportation speed is considered to reflect the partition coefficient of the structurally different synthesised BODIPYs. Regardless of the difference in the speed of internalisation, it was confirmed that both newly synthesised LacCer analogues could be used to study the transformation process.

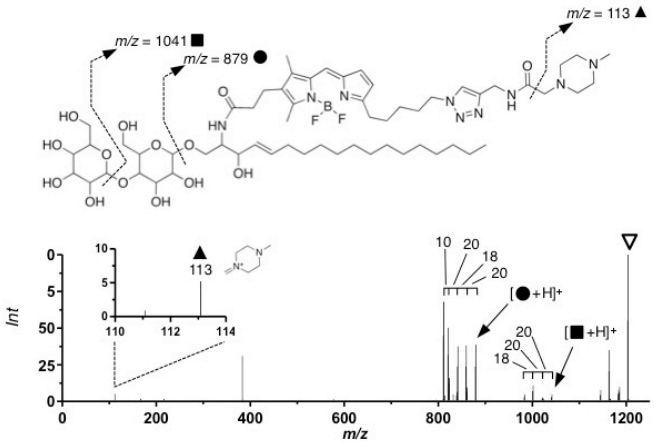


Fig. 4 MS/MS spectrum of fluorescent LacCer analogue **2** labeled with the 113 iTRAQ.

A mass spectrometer capable of performing MS/MS for the analysis of glycan structure and resolution of the iTRAQ reporter ions is required for quantitative GSL profiling. To ensure successful analysis of glycan transformation and the isobaric reporter ion, a hybrid quadrupole time-of-flight (Qq-TOF) mass spectrometer, which had been reported to be suitable for quantitative comparison of multiplexed samples, [9] was used for the MS/MS analysis of LacCer analogue **2** labelled with iTRAQ113. The Click reaction between the azido group and the tag carrying the alkyne group was carried out in the presence of $CuSO_4$, producing the conjugate in 91% yield. The MS/MS spectrum showed a series of product ions corresponding with the rupture of interglycosidic linkages, together with the reporter ion (*m/z* 113) (Fig. 4). The result demonstrated that a Qq-TOF mass spectrometer can be used for iTRAQ-based quantitative differential analysis of GSL samples.

In summary, LacCer analogues **1** and **2** carrying the novel bifunctional azido-BODIPY acid derivatives **9** and **12** were synthesised for quantitative comparison of multiplexed GSL samples by MS/MS using iTRAQ. It was shown that these fluorescent LacCer analogues were taken up by the cells and accumulated in the Golgi apparatus; analogue **2** was transported more rapidly than analogue **1**. Furthermore, a Qq-TOF mass spectrometer was shown to be suitable for the analysis of iTRAQ-tagged LacCer analogues, using LacCer analogue **2**. We anticipate that our results can be applied for quantitative multiplexed analysis of GSL samples from different cellular states.

Notes and references

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Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c000000x/

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