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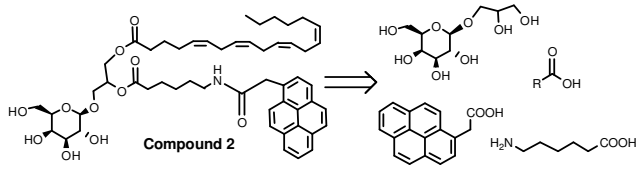

Design and Synthesis of Fluorescent Galactolipid Probes

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Abstract

Fluorescent galactolipid analogues (**1**, **1a** and **2**) have been synthesized with a pyrene group attached to the amino-terminal of a hexanoyl chain bound to an otherwise normal galactolipid structure. The synthetic lipids are obtained from peracetyl galactose by a general and versatile procedure based on the trichloroacetimidate methodology. The intense violet fluorescence (376 and 395 nm) and good photostability of pyrene make these compounds highly suitable as probes to study galactolipid metabolism. As proof of concept, we report that compound **2** is a valid tool to detect galactolipase activity in enzymatic preparations of potato tubers.

Keywords

Glycoglycerolipids, Fluorescence, Chemical Probes, Lipid Metabolism

Galactose-containing glycerolipids, commonly named galactolipids, are main components of chloroplast membranes but can be also present in non-photosynthetic tissues, such as potato tuber or apple. In shortage of phosphate, galactolipids can also substitute phospholipids in a process that has acquired growing consideration as the major mechanism of cell-membrane lipid homeostasis in plants.[1] The two most common galactolipids are mono- and di-galactosyldiacylglycerol (MGDG and DGDG) that basically differ for the presence of one or two sugars linked to glycerol. Both classes are characterized by uncommon levels of polyunsaturated fatty acids that in certain algae can account for more than 80% of total fatty acids. Recent studies have demonstrated that galactolipids play an important role in not only the organization of photosynthetic membranes, but also in their photosynthetic activities.[2] Furthermore, these lipids are crucial precursors of eco-physiological mediators, including jasmonate and volatile organic deterrents, in plants[3] and algae.[4] Galactolipids are a primary source of galactose and polyunsaturated fatty acids (PUFAs) in the human diet[5] and also exhibit COX mediated anti-inflammatory activity[6] and inhibitory effects on tumor promoter-induced Epstein-Barr virus (EBV) activation.[7]

Despite the growing body of evidence about the biological role, there is a general deficiency of cellular and molecular tools devised to investigate metabolism and functional role of galactolipids. Recently we have reported a novel synthetic approach for the preparation of galacto- and sulfoglyco-lipids.[8] The method is of general application and can be used to prepare natural or syntetic variants containing a regiospecific distribution of polyunsaturated fatty acids. In the present work we aim at describing the synthesis and the properties of pyrene- containing β-galactosyl-diacylglycerols that were specifically designed as fluorescent substitutes of natural galactolipids in functional and structural studies. As demonstration of the potential application of these molecules in studies on lipid

metabolism and signaling, we also report the use of the fluorescent compound **2** to monitor galactolipase activity in raw preparations of potato tubers.[9]

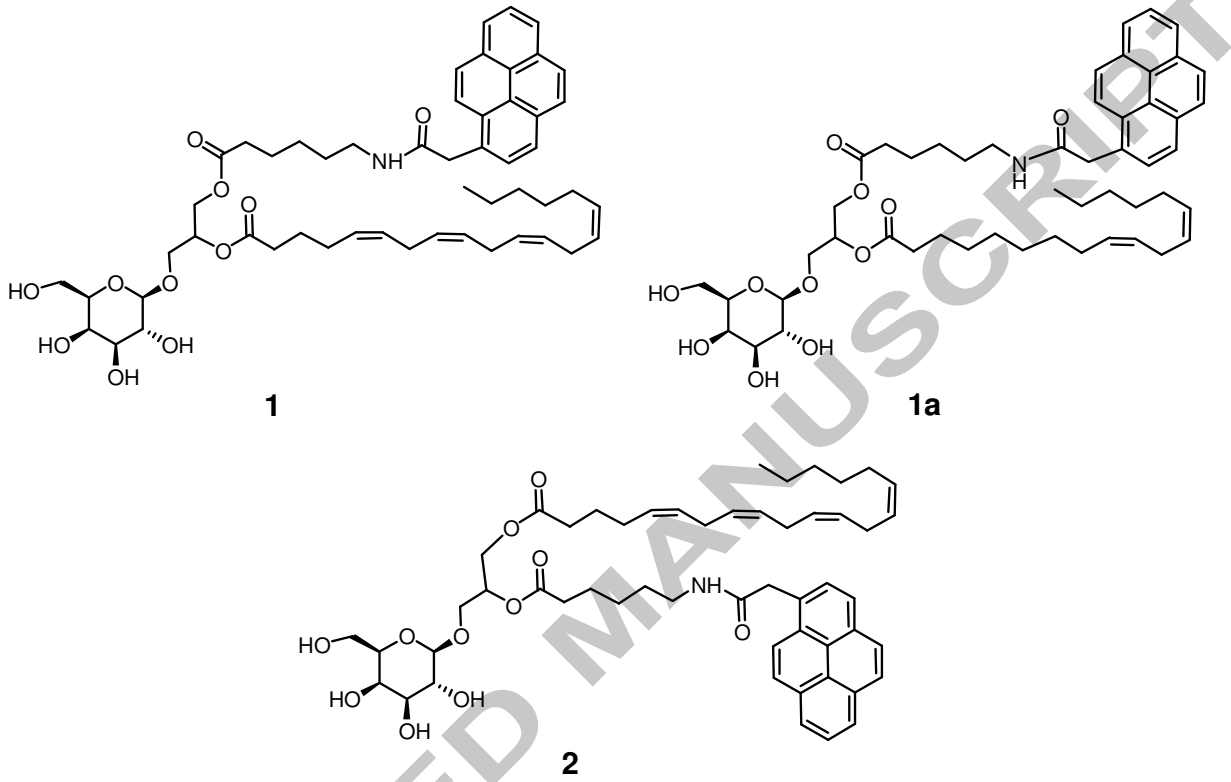
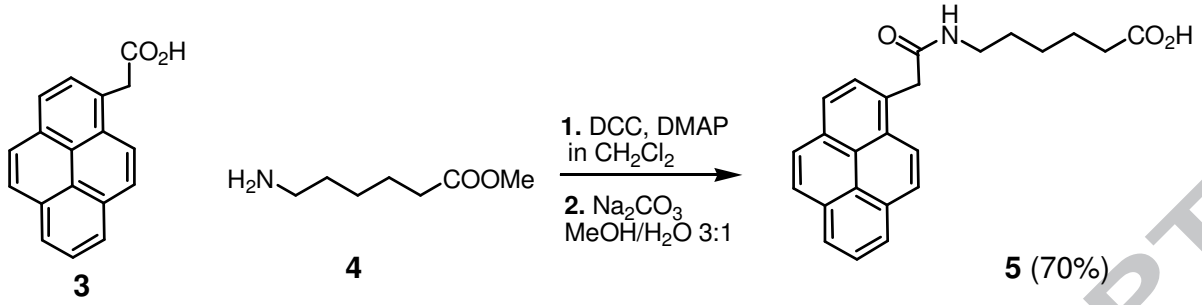


Figure 1. Structures of compounds **1, 1a** and **2**.

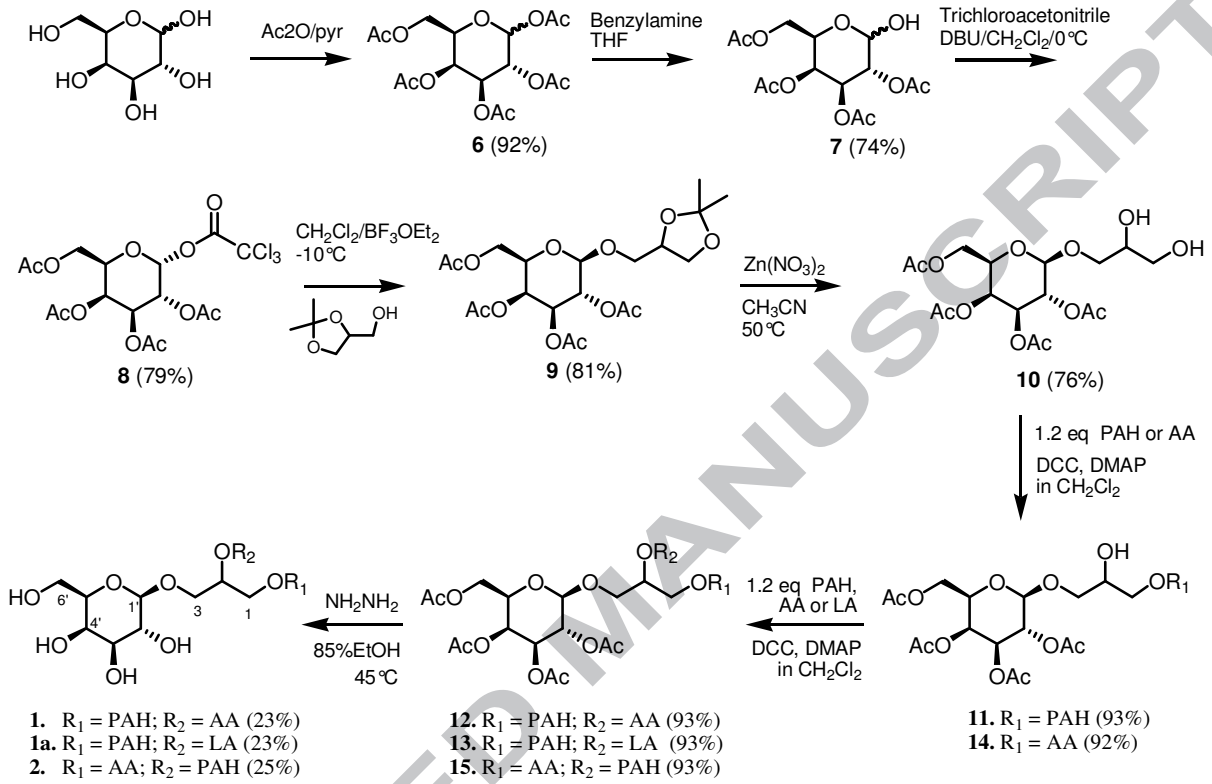
Pyrene (342 nm excitation wavelength and 376 nm emission wavelength) is one of the most common fluorophores and has been widely used as a fluorescent label in research on lipid metabolism.[10] Our choice to use pyreneacetic acid (**3**) as the fluorescent group to tag β-galactosyl-acylglycerols was due to chemical and biochemical considerations including high extinction coefficient with stable fluorescence, chemical and biological inertness, compatibility with biological systems and stability during chemical synthesis. In order to link the fluorescent tag to glycerol, 1-pyreneacetic acid (**3**) was functionalized by introducing a six carbon atom spacer (Scheme 1).



Scheme 1. Synthesis of the fluorescent-armed tag 6-pyreneacetamido-hexanoic acid (PAH) (**5**).

In a convergent synthetic strategy (Scheme 2), 6-pyreneacetamido-hexanoic acid (PAH) (**5**) was then condensed to 2',3',4',6'-tetracetylated-monogalactosyl glycerol (**10**) by DCC. This latter compound was prepared by trichloroacetimidate-mediated condensation of peracetyl D-galactose with glycerol acetonide,[8,11] followed by selective hydrolysis of the isopropylidene by zinc nitrate hexahydrate in acetonitrile.[8-12] Notably, equimolar amounts of reagents gave only introduction of the armed-pyrenyl substituent at the primary alcohol of glycerol, as indicated by long-range NMR-experiments that showed for **11** a diagnostic correlation between the carboxylic ester at δ 173.1 with the down-shifted methylene protons (δ 4.13) of glycerol. In the ^{1}H-NMR spectrum of compound **11**, anomeric signals of two diastereoisomers at δ 4.50 and 4.48 were distinguishable in a ratio of 1:1 due to use of racemic glycerol acetonide in the coupling reaction. Successive reaction of 3-O-[2',3',4',6'-tetracetyl-β-galactose]-1-[6''-pyreneacetamido-hexanoyl] glycerol (**11**) with equimolar concentration of arachidonic acid (AA) or linoleic acid (LA) in the presence of DCC gave derivatives **12** and **13** that were converted to the target product 1-[6''-pyreneacetamido-hexanoyl]-2-arachidonoyl-3-O-β-galactosyl glycerol (**1**) and 1-[6''-pyreneacetamido-hexanoyl]-2-linoleoyl-3-O-β-galactosyl glycerol (**1a**) after selective removal of acetyl groups by hydrazine hydrate (Scheme 2). Similarly, the regioisomeric product **2**, 1-

arachidonoyl-2-[6''-pyreneacetamido-hexanoyl]-3-O-β-galactosyl glycerol, was obtained from **10** by sequential condensation of arachidonic acid and 6-pyreneacetamido-hexanoic acid (**5**) as reported in Scheme 2.



Scheme 2. Synthesis of fluorescent β-galactolipids **1**, **1a** and **2**. PAH= 6-pyreneacetamido-hexanoic acid; AA= arachidonic acid; LA= linoleic acid.

In organic solvents and buffer solutions (pH =7.2), probes **1**, **1a** and **2** exhibited fluorescent spectra similar to that of pyrene with a major excitation peak at 342 nm and two emission bands at 376 and 395 nm (Figure 2). The fluorescent emission could be easily monitored on TLC plates with a detection limit that was 1 nmol by visualization over a 365 nm UV lamp. Emission intensity remained stable during preparation and development of TLC plate, as well as the fluorescent spot was still detectable after 30 min from the analysis.

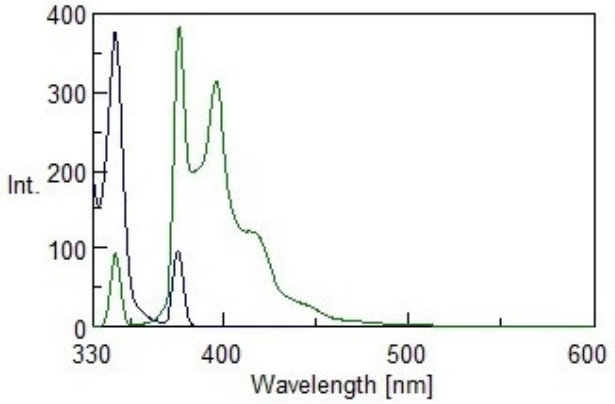


Figure 2. Excitation (black) and emission (green) spectra of 7.4 mM 3-*O*-β-galactosyl-1-[6"-pyreneacetamido-hexanoyl]-2-arachidonoyl-glycerol (**1**) in MeOH. Spectra of probes **1a** and **2** are identical.

Potato tubers contain galactolipid-hydrolyzing enzymes that are commonly referred to as patatins.[13] These proteins that belong to the class of lipolytic acyl hydrolases (LAHs) account for up to 40% of total proteins in the tubers and their activity is sufficient to rapidly hydrolyze native and model molecules of phospholipids and galactolipids.[9] For these reasons, we decided to test the hydrolysis of compounds **1** and **2** by potato homogenates in order to verify if the pyrenyl fluorophore affected LAH recognition of β-galactosyl diacylglycerols. Crude preparations of potato tuber proteins were carried out by precipitation with 40-70% ammonium sulfate followed by dialysis.[14]

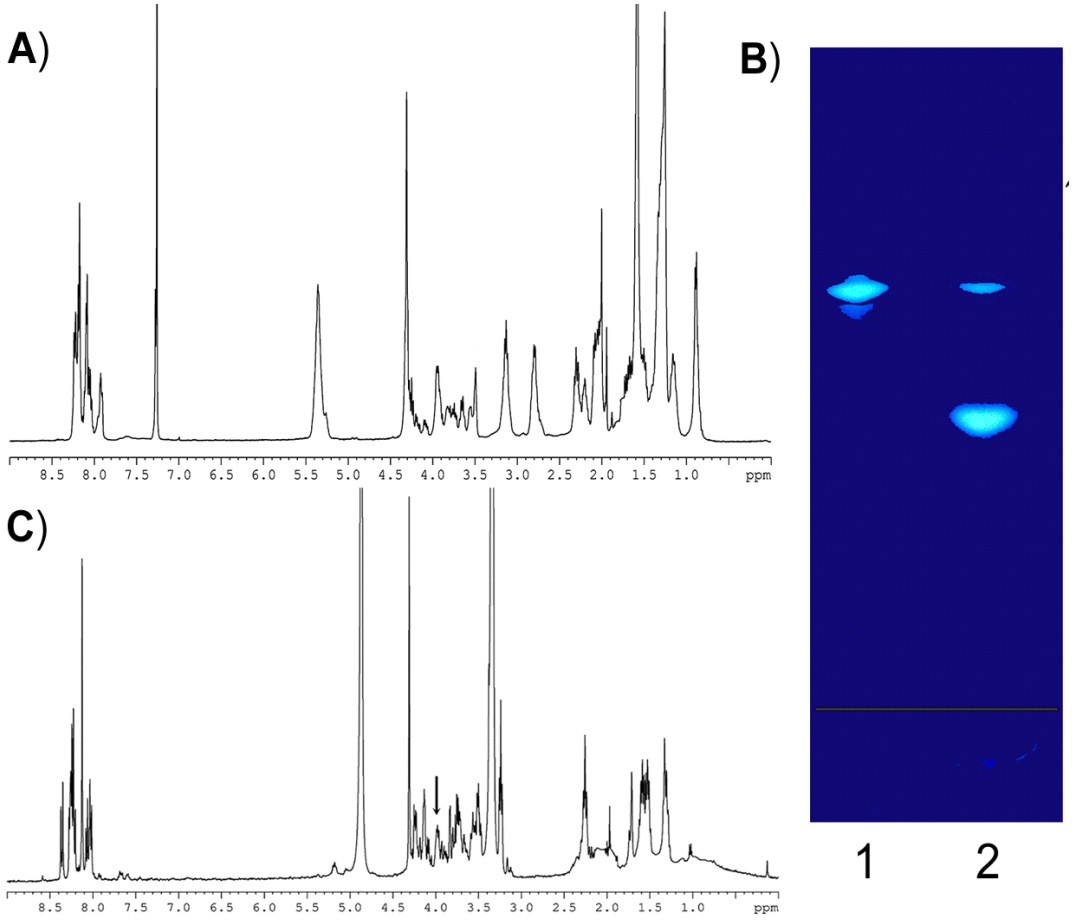


Figure 3. Hydrolysis of fluorescent probe **2** by potato tuber homogenates. A) ^{1}H-NMR ($CDCl_3$) of compound **2**; B) SiO_2-TLC ($CHCl_3$/MeOH/water 65:25:4) analysis of the reaction after 1h. Compound **2** R_f = 0.75; Compound **17** R_f = 0.60. C) ^{1}H-NMR (CD_3OD) of product (**17**) recovered after enzymatic reaction.

LAH activity of this preparation was first tested on natural MGDG isolated from the diatom *Skeletonema marinoi* according to ref. 4a, verifying the hydrolysis of complex lipids by GCMS on the corresponding methyl esters (data not shown).

Then the potato tuber homogenates were incubated with 200 µg of the fluorescent probes **1** and **2**. After 1 hour of incubation, TLC analysis showed the almost complete disappearance of probe **2** with formation of a fluorescent spot at lower R_f (Figure 3), whereas probe **1** remained unaltered (not shown). To verify these results, the incubation

buffers were extracted with diethyl ether and the resulting organic fractions were methylated by CH_2N_2. After purification on silica gel, compound **1** was recovered almost completely (82% recovery). On the other hand, the incubation of probe **2** resulted in a 15:85 mixture of compounds **16** and **17**, as proved by signal of H-2 of the acylated glycerol of **16** together with the signals of **17** in the spectra of the reaction mixture (Supporting Information). Both products derived by selective hydrolysis of arachidonic acid from the primary position of glycerol in compound **2**. It is worth noting that the 1-acyl derivative **17** is the product of the thermodinamically-favored intramolecular migration of the PAH from the secondary to the primary position of glycerol. These data were fully corroborated by GC-MS analysis of the extracts that revealed significant release of arachidonic acid only from compound **2** (Supporting Material). Even if compound **1** and **2** were in diasteroisomeric form, the LAH activity did not show diasterospecificity during the incubation. Nevertheless, we cannot rule out that the two diasteroisomers may be recognized in a different way in other assays.

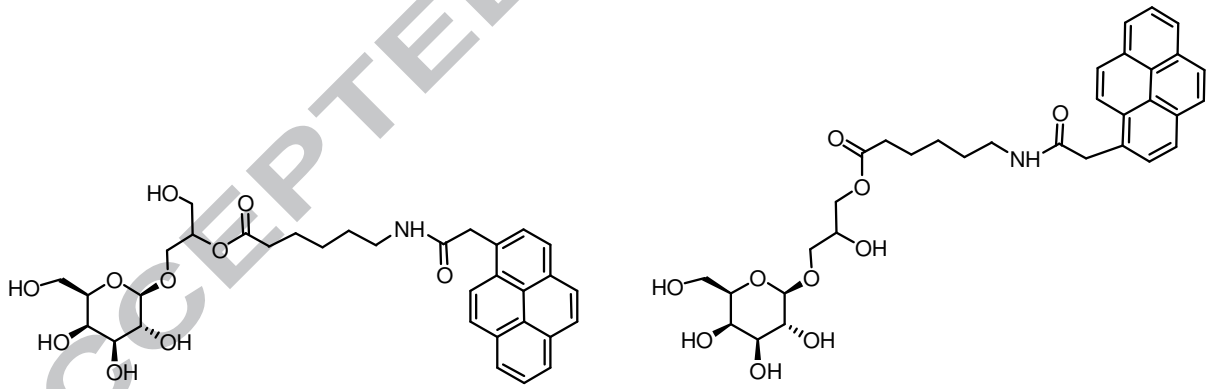


Figure 4. Structures of compounds **16** and **17.**

In this study we have reported the preparation and the characterization of three fluorescent galactolipid analogues (**1**, **1a** and **2**) that are designed to study galactolipid metabolism. In particular, compound **2** proved to be very effective to assess the hydrolytic activity of

patatins, members of a wide class of enzymes that play a fundamental role in lipid remodeling and plant signalling.[15] The TLC assays were very quick to perform and visualization of the fluorescent probes over a UV lamp was straightforward and readily usable for the study of biosynthesis, tailoring and relocation of MGDG and DGDG. On the other hand, the fluorescent galactolipid **1** was only marginally hydrolyzed by the potato homogenates thus suggesting that the large pyrene group at position *sn*-1 of glycerol both hampers a productive interaction with the hydrolytic enzyme(s) and lends to these molecules a biochemical stability that can be potentially used in fluorescence microscopy or DGDG biosynthesis. Galactolipid analogues like **1** and **2** are particularly attractive from a synthetic point of view since the PAH residue (a) can be easily introduced by DCC condensation, (b) is compatible with the use of polyunsaturated and monounsaturated fatty acids and (c) is stable under the very mild hydrolytic removal of the acetyl groups that are necessary for the trichloroacetimidate methodology of coupling. In this view, the synthetic strategy here reported can be readily used to prepare a wide variety of reliable and sensitive lipid probes.

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Supporting data

Supplementary data associated with this article can be found, in the online version, at

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