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Chemoenzymatic synthesis of fluorogenic phospholipids and evaluation in assays of phospholipases A, C and D

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ABSTRACT

Phospholipases are ubiquitous in nature and the target of significant research aiming at both their physiological roles and technical applications in e.g. the food industry. In the search for sensitive and selective phospholipase assays, we have focused on synthetic FRET (Förster resonance energy transfer) substrates. This has led to the development of a facile, easily scalable and low cost synthesis of fluorogenic phospholipids featuring the dansyl/dabcyl fluorophore/quencher-pair on the fatty acid ω-position and on the phosphatidylethanolamine head group, respectively. Hence, the two substrates lyso-(dansyl-FA)-GPE-dabcyl (**6**) and (dansyl-FA)$_2$-GPE-dabcyl (**7**) were synthesized by a chemoenzymatic strategy, in which preparation of (**6**) further included a novel selective enzymatic esterification step. As proof of concept, activity of a handful of phospholipases, one from each of the PLA1, PLA2, PLC and PLD classes, were assayed using substrates (**6**) and (**7**), and the kinetic parameter k_{cat}/K_M was determined. The PLA1 (Lecitase Ultra™) was found to be highly active on both substrates, whereas the PLD (from white cabbage) had no activity, presumably due to steric effects associated with the dabcyl-functionalization of the head group. It was further substantiated that the substrates are specific towards phospholipase activity as the tested lipase (Lipolase™) showed close to zero activity.

1. Introduction

Phospholipases are abundant enzymes in nature and can be extracted from numerous sources such as bacteria, fungi, plants or mammals (De Maria et al., 2007). They act on phospholipids, one of the main constituents of biological membranes, and are involved in different biological mechanisms like cell signaling. Phospholipids are characterized by their head group structure; for instance phosphatidylcholine (PC) is found in the outer leaflet of eukaryotic plasma membranes, and phosphatidylserine (PS) and phosphatidylethanolamine (PE) are confined to the membrane's inner leaflet (Zwaal and Schroit, 1997). Phospholipases are divided into different classes; phospholipases A1 (PLA1) cleave the *sn*-1 ester bond, yielding a free fatty acid (FFA) and a 1-lysophospholipid; phospholipases A2 (PLA2) target the *sn*-2 ester; phospholipases C (PLC) hydrolyze the *sn*-3 phosphate ester bond on the glycerol side, yielding a diglyceride and the free head group; while phospholipases D (PLD) target the phosphate ester bond on the 'head group side'. Phospholipases B (PLB) are unspecific PLAs. Isolated phospholipases are commonly used in industrial processes such as cheese and bread production, and in vegetable oil degumming, a step of oil refinement where the phospholipids are removed from the oil (Sampaio et al., 2015; Lilbaek et al., 2006).

In this study, we describe an easy chemoenzymatic synthesis of two synthetic phospholipids and their use as substrates in phospholipase assays. Having efficient and sensitive assays is crucial in searching for phospholipases (e.g. high-throughput screening of protein-engineered variants) with improved properties for industrial applications. The synthetic phospholipids are fluorogenic, i.e. they become fluorescent upon enzymatic action (hydrolysis). This is obtained by derivatizing the phospholipid with a fluorophore and a quencher. As long as those two groups are close to each other the fluorescence is quenched. However, when the phospholipase cleaves the substrate, the fluorophore and the quencher diffuse apart and fluorescence appears.

This phenomenon is based on FRET (Förster resonance energy transfer), first described in 1948 (Forster, 1948). It generally relies on a donor/acceptor couple. The donor is excited into an electronic higher energy state due to an incident light beam. Then, instead of emitting light to go back to its ground state, it will transfer its energy to an acceptor nearby without emitting radiation. This transfer is thought to be based on dipole–dipole interactions

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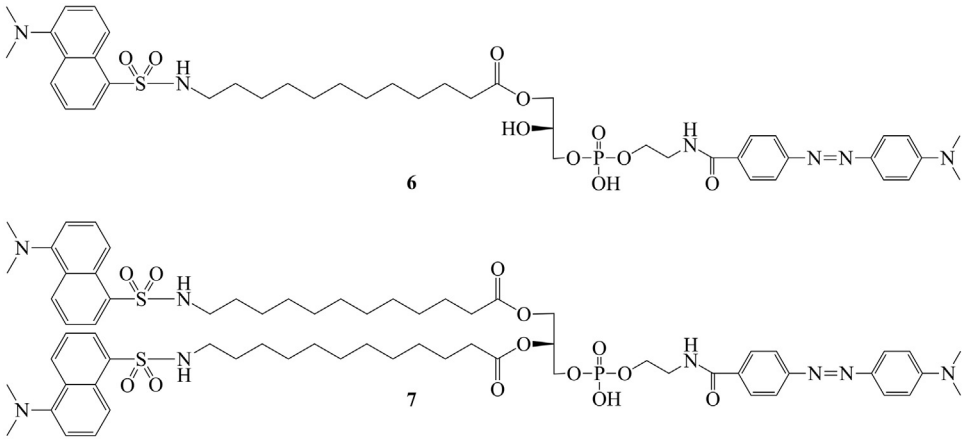


Fig. 1. Lyso-(dansyl-FA)-GPE-dabcyl (**6**) and (dansyl-FA)$_2$-GPE-dabcyl (**7**).

(Sahoo, 2011). Through this mechanism, the donor will go back to its ground state whereas the acceptor will be excited. Next, the acceptor will then go back to its ground state either by emitting light (if the acceptor is a fluorophore) or by heat transfer (when the acceptor is a non-fluorescent molecule). In the latter case, quenching of the donor's fluorescence is observed. There are four requirements to observe FRET: 1) the donor emission spectra and the acceptor absorption spectra should be overlapping; 2) the distance between the donor and the acceptor should be small, typically between 1 and 10 nm; 3) there must be a specific dipole orientation between the couple; and 4) the fluorescence of the donor should last long enough for the energy transfer between the donor and the acceptor to occur.

A number of phospholipase FRET substrates have been described in the literature, mostly targeted towards the study of biologically interesting PLA2 enzymes (Manna and Cho, 2007; Rose and Prestwich, 2006a; Wichmann and Schultz, 2001; Wichmann et al., 2007). This includes substrates with the FRET donor and acceptor both positioned at the ω-position of the two fatty acids at *sn*-1 and *sn*-2, such as "BBPC" and "DBPC" reported by Prestwich and coworkers. Here, "BBPC" is a PC analogue with two bodipy fluorophores, whereas "DBPC" is a PC with a dabcyl quencher and a bodipy fluorophore (Feng et al., 2002). With two identical fluorophores (having overlapping excitation and emission spectra), self-quenching is observed (homo-FRET). Examples of this include the PC analogues with fatty acid functionalized with coumarins introduced by Hajdu and coworkers, who claim that the smaller coumarins interfere less with the physical-chemical properties of the lipids (Wang et al., 2013). Other substrates use the phospholipid head group as a handle for attachment of a reporter group. This includes (lyso)PLC/PLD substrates from the Prestwich group, resembling either a PE structure with a reporter group attached to the amino-functionality by an amide bond, or a PC structure with a reporter group attached via a quaternary ammonium ion (Rose and Prestwich, 2006b; Ferguson et al., 2006). Obviously, PLC and PLD activity can only be assayed by FRET substrates with a reporter group located on the head group. Phospholipase FRET substrates are also commercially available, e.g. the EnzChek brand from ThermoFischer/Invitrogen. The commercial substrates are however expensive and may not cover the specific need (e.g. enzyme specificity or fluorescent properties), thereby justifying an in-house synthesis approach.

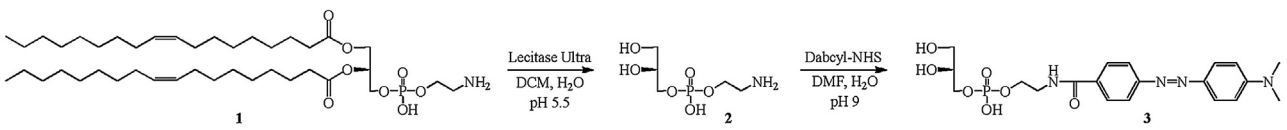
2. Results and discussion

For the substrates presented in this study, we prioritized a short synthesis route using relatively inexpensive starting materials. This would allow for scale up to quantities required to high-throughput screening. Hence, a commercially available PE was chosen as the starting point. The fatty acids were removed with a PLA, the PE head group was derivatized with a quencher, and a fluorescently labelled fatty acid was coupled back on to the glycerol backbone, resulting in a lyso-PE and a PE analogue (Fig. 1).

The choice was made to use dansyl as a fluorescent donor and dabcyl as the non-fluorescent acceptor/quencher. Indeed, the dabcyl group, having absorption maximum between 430 and 500 nm has been widely applied in fluorogenic assays, while the dansyl group qualifies as a low-cost fluorophore with fluorescence emission between 400 and 650 nm. The substrate design was further inspired by our previous work with fluorogenic triglycerides (Andersen and Brask, 2016). Holding a quencher in the headgroup and a fluorophore in both fatty acids, substrate (**7**) would have the potential of being a generic substrate for all phospholipase classes. The lyso-substrate (**6**) was synthesized to assess both phospholipase and lysophospholipase activities.

2.1. Synthesis

sn-Glycero-3-(*N*-dabcyl-phosphoethanolamine) (GPE-dabcyl, **3**) was synthesized from commercially available 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (**1**) by PLA catalyzed hydrolysis, followed by acylation of the amino headgroup with 4-[4-(dimethylamino)phenylazo]benzoic acid *N*-succinimidyl ester (dabcyl-NHS) (Scheme 1). Despite being a PLA1, a large concentration of commercial Lecitase Ultra was found to hydrolyze both fatty acids in an overnight reaction. It was not investigated if this could be explained by acyl migration or the enzyme being somewhat unspecific under these conditions (Pluckthun and Dennis, 1982). Following extraction and freeze-drying of the aqueous phase, crude *sn*-glycero-3-phosphoethanolamine (GPE, **2**)



Scheme 1. Synthesis of GPE-dabcyl (**3**).