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# Synthesis and evaluation of fluorogenic triglycerides as lipase assay substrates

Rokhsana J. Andersen, Jesper Brask*

*Novozymes A/S, Krogshøjvej 36, 2880 Bagsværd, Denmark*

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ABSTRACT

Three racemic fluorogenic triglycerides are synthesized and evaluated as lipase assay substrates. The presented synthesis route goes through a key triglyceride intermediate which can be chemoselectively functionalized with a wide range of different probes. Hence the substrate can be tailor-made for a specific assay, or focus can be on low cost in larger scale for applications in high-throughput screening (HTS) assays. In the specific examples, TG-ED, TG-FD and TG-$F_2$ are assembled with the Edans-Dabcyl or the fluorescein-Dabcyl FRET pair, or relying on fluorescein self-quenching, respectively. Proof-of-concept assays allowed determination of 1st order kinetic parameters ($k_{cat}/K_M$) of 460 $s^{-1}$ $M^{-1}$, 59 $s^{-1}$ $M^{-1}$ and 346 $s^{-1}$ $M^{-1}$, respectively, for the three substrates. Commercially available EnzChek lipase substrate provided 204 $s^{-1}$ $M^{-1}$. Substrate concentration was identified as a critical parameter, with measured reaction rates decreasing at higher concentrations when intermolecular quenching becomes significant.


## 1. Introduction

Lipases are interfacially activated enzymes that catalyze the hydrolysis of lipids on the water/oil interphase (Singh and Mukhopadhyay, 2012; Svendsen, 2000). Microbial lipases are produced in large scale by heterologous expression and applied in the detergent industry, but also for baking, dairy, biodiesel, biocatalysis, etc (Hasan et al., 2006). Development of efficient assays for characterization and high-throughput screening of lipases and their protein engineered variants is therefore of significant interest. Many methods have been reported to follow lipase reactions (Stoytcheva et al., 2012; Hasan et al., 2009), typically based on the liberation of free fatty acids (titration of pH or indicator based). For a high-throughput screening (HTS) setup it is often required that lipase activity generates a color or – preferred – a fluorescent response (Schmidt and Bornscheuer, 2005). As there are limitations to the indicator-based assays, synthetic chromogenic or fluorogenic substrates, such as simple alkyl esters of 4-nitrophenyl (pNP) or 4-methylumbelliferone (4MU), have found wide use (Stoytcheva et al., 2012). The price for their convenience is however that these structures are only very remotely related to natural lipids.

In this paper we describe the synthesis of three fluorogenic triglycerides (TG-ED, TG-FD and TG-$F_2$, Fig. 1) and provide initial data for their use in lipase assays. The concept of having both a fluorophore and a quencher (or more general a donor and an acceptor) in the same substrate, positioned in such way that enzymatic action (hydrolysis) will remove the quencher and allow the product to become fluorescent, is well-known. Often referred to as FRET (Förster resonance energy transfer) substrates, these have been reported for most classes of hydrolytic enzymes, including proteases (Wang et al., 1990; Thompson et al., 2000), amylases (Oka et al., 2012), DNAses (Ghosh et al., 1994) and phospholipases (Wichmann et al., 2007).

FRET is the radiationless energy transfer between two adjacent molecules. The efficiency of energy transfer is related to the sixth power of the ratio of the distance R between donor and acceptor and the Förster radius $R_0$ (Mueller et al., 2013). The Förster radius $R_0$ is the distance between donor and acceptor at which the energy transfer is half-maximal and is usually in the range of 1.5–6 nm.

Also a number of FRET-type lipase substrates have been published in the literature. Duque and coworkers synthesized a triglyceride analogue with a pyrene fluorophore and a trinitrophenylamino quencher (Duque et al., 1996), whereas Mitnaul and coworkers have suggested to use self-quenching Bodipy-functionalized triglyceride and phosphatidylcholine substrates (Mitnaul et al., 2007). Further, Yang and coworkers synthesized and evaluated a number of non-glyceride FRET-based lipase substrates (Yang et al., 2006).

* Corresponding author.
*E-mail address:* jebk@novozymes.com (J. Brask).

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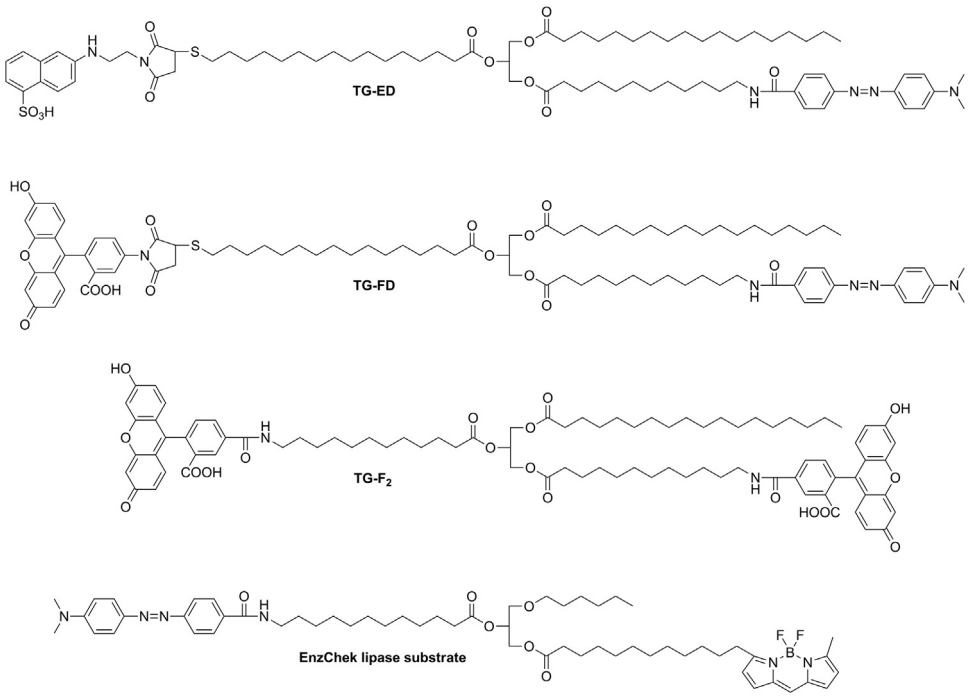


**Fig. 1.** Structures of the three lipase substrates, compared with commercially available EnzChek lipase substrate.

A number of FRET substrates are commercially available from Invitrogen/ThermoFisher under the brand EnzChek®. There is also a commercial EnzChek lipase substrate (cat. no. E33955), featuring a Bodipy-Dabcyl fluorophore-quencher pair, and an ether bond in the 3-position, presumably to avoid unproductive lipase hydrolysis (Fig. 1) (Basu et al., 2011). The advantages of in-house synthesis over a commercial substrate are related to both price and flexibility. The EnzChek lipase substrate is very costly (approx. 300 USD for 100 μg), severely constraining its use in a HTS setup. Synthesis of the triglyceride substrates presented here easily yields 10–1000 mg per batch, and can be upscaled further. In addition, the chemistry is highly flexible since the presented methodology can be readily modified to synthesize substrates with other fatty acids or fluorophores/quenchers. Hence, it can be imagined that the generic approach of using chemoselective reactions to derivatize a functionalized triglyceride scaffold can be further expanded to include not only other fluorophores and quenchers, but also other chromophores, nanoparticles, affinity ligands, etc.

Another interesting difference between the commercial EnzCheck lipase substrate and TG-ED and TG-FD is the position of the fluorophore. In the EnzChek substrate, a typical 1,3-specific lipase will hydrolyse the lauric acid carrying the BODIPY fluorophore from the 1-position of the glycerol backbone (Fig. 1). This is in contrast to TG-ED and TG-FD in which the fluorophore is on the 2-position and a 1,3-specific lipase will therefore produce a fluorescent mono/diglyceride structure. Whereas this may be irrelevant for solution-based assays, the difference could potentially be used for imaging purposes to follow the fate of the hydrolysis products in various biological and technical contexts.

## 2. Experimental

### 2.1. General procedures

All chemicals were purchased from Sigma-Aldrich, except for Edans C2 maleimide which was from AnaSpec (Fremont, CA, USA) (cat. no. AS-81432) and the EnzChek lipase substrate green (cat. no. E33955) from Thermo Fisher Scientific. Flash chromatography was carried out with pre-packed silica gel cartridges from Biotage. Analytical TLC was performed on Merck silica gel 60 $F_{254}$ plates. Spots were detected by a UV lamp at 254 nm or 366 nm or by staining with aqueous $H_2SO_4$ or $KMnO_4$ followed by heating with a hot air gun. $^1H$ and $^{13}C$ NMR data were acquired at 300 K on a Bruker Avance III HD 400 MHz instrument equipped with a SmartProbe™. MALDI-MS data were acquired on a Bruker Ultraflex Extreme, using a Super-DHB matrix with 0.1% TFA. Fluorescence measurements (for kinetic assay data) were performed at room temperature (25 °C) with a SpectraMax M2 from Molecular Devices.

### 2.2. Synthesis of FA-NHMmt **2** and FA-SMmt **4**

#### 2.2.1. FA-NHMmt **2**

12-Aminododecanoic acid (FA-$NH_2$, **1**) (1.08 g, 5 mmol) was suspended in $CHCl_3$-MeCN (5:1, 18 mL) and chloro trimethylsilane (TMS-Cl, 0.63 mL, 1 eq) was added. The mixture was heated to reflux (65 °C) under $N_2$ for 2 h. Remains a suspension. After cooling to RT, triethylamine (1.39 mL, 2 eq) was added. Then addition of 4-methoxytrityl chloride (Mmt-Cl), (1.54 g, 1 eq) dissolved in $CHCl_3$ (10 mL). The turbid orange solution was stirred at RT ON. MeOH (1.0 mL, 5 eq) was then added. The orange solution slowly turned yellow. TLC (heptane-EtOAc, 3:1) confirmed full conversion of Mmt-Cl (Rf 0.40) to product (Rf 0.15). Evaporated to an oil, of which 1.7 g is purified by flash chromatography, eluting with heptane-EtOAc (3:1, later 3:2). The product containing fractions were identified by TLC, pooled and evaporated to yield 1.4 g (57%) of yellow viscous oil.

$^1H$ NMR ($CDCl_3$, selected signals in ppm): 7.49 (d, 4H, Mmt), 7.40 (d, 2H, Mmt), 7.29 (t, 4H, Mmt), 7.20 (t, 2H, Mmt), 6.83 (d, 2H, Mmt CH next to –OMe), 3.81 (s, 3H, –OMe), 2.36 (t, 2H, –$CH_2$COOH), 2.16 (t, 2H, –NHC$H_2$–), 1.68–1.60 (m, 2H, –C$H_2$$CH_2$COOH), 1.53–1.46 (m, 2H, –NHC$H_2$C$H_2$–).

$^{13}C$ NMR ($CDCl_3$, selected signals in ppm): 113.0 (Mmt CH next to –OMe), 55.2 (–OMe), 43.7 (–$NHCH_2$–), 34.1 (–$CH_2$COOH), 30.6 (–$NHCH_2CH_2$–), 24.8 (–$CH_2CH_2$COOH).