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Synthesis of substrates for periodate-coupled assay of phospholipases C and sphingomyelinases

Kira Løw Larsen, Rokhsana J. Andersen, Jesper Brask*

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

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ABSTRACT

A series of 4-nitrophenyl (pNP) and 4-methylumbelliferyl (4MU) substrate analogues of phosphatidyl choline (PC) and phosphatidic acid (PA) were synthesized from 4-bromo-1-butene by ether formation, olefin epoxidation and ring opening with the phosphate head group. The pNP PC analogue, 4-(4-nitrophenoxy)-2-hydroxy-butyl-1-phosphoryl choline (**1**) was evaluated in assays of fungal sphingomyelinases, also displaying phospholipase C activity. Reactions were terminated with a periodate-containing stop solution, leading to liberation of pNP, quantified spectrophotometrically in an end-point measurement. A kinetic evaluation of sphingomyelinases from *Kionochaeta* sp. and *Penicillium emersonii* showed relatively high K_M and low k_{cat} values for this substrate, limiting its practical applicability in assays with low sphingomyelinase concentrations.

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1. Introduction

Phospholipases C (PLCs) are phosphodiesterases that hydrolyze the bond between glycerol and phosphate in glycerophospholipids, yielding diacylglycerol and a phosphoryl headgroup, e.g. phosphorylcholine or phosphorylinositol. Sphingomyelinases catalyze the parallel reaction in sphingophospholipids, yielding ceramide and phosphorylcholine from sphingomyelin (Fig. 1) [1]. Both enzyme classes have diverse biological functions in membrane maintenance, regulation of cellular mechanisms, digestion, etc. Further, the isolated enzymes attract increasing interest for a number of technical applications [2]. For the study of their biological roles as well as for screening activities aimed towards a technical application, it is essential with specific and sensitive enzymatic assays to probe specificity and evaluate activity.

A synthetic substrate commonly used is 4-nitrophenyl phosphorylcholine (pNP-PC) (Fig. 1) [2,3]. Easily implemented in a high-throughput screening setup, this low-cost substrate will release the 4-nitrophenol (pNP) chromophore upon PLC/sphingomyelinase catalyzed hydrolysis. However, pNP-PC is typically only a substrate for enzymes with activity towards phosphorylcholine phospholipids. It has then been suggested to assay phosphatidyl inositol (PI) specific PLCs with the corresponding pNP-PI substrate [4]. Another general issue with these simple pNP-ester substrates is that they are only remotely structurally and electronically related to their natural lipid counterparts. This means, amongst other things, that pNP-PC may not be very specific for PLCs or sphingomyelinases. Indeed, it has been reported that several other hydrolytic enzymes, e.g. alkaline phosphatase, catalyze hydrolysis of pNP-PC [3,5].

Periodate-coupled assays are spectrophotometric assays where the chromophore or fluorophore is not released directly upon hydrolysis. The hydrolysis reaction is coupled with periodate oxidation of the primary hydrolysis product (a vicinal diol) and subsequent β-elimination to yield the chromophore/fluorophore. Several substrates for periodate-coupled assays of hydrolytic enzymes have been reported in the literature. The synthesis of pNP-based substrates for periodate-coupled chromogenic assays of esterases, sulfatases, and phosphatases was described by Rammler and coworkers back in 1973 [6,7]. More recently, Reymond and coworkers reported several structures for periodate-coupled chromogenic and fluorogenic assays, including substrates for acylases, phosphatases, epoxide hydrolases, lipases, esterases and proteases [8–11]. Most recently, a synthetic substrate was suggested for periodate-coupled assay of arabinofuranosidases [12]. A related concept is the fluorogenic cyanohydrin ester substrates also reported by Reymond and coworkers, in which the primary hydrolysis product (a cyanohydrin) spontaneously decompose with cyanide elimination and subsequent β-elimination, releasing the fluorophore (without a periodate oxidation step) [13].

On this background, we designed a class of synthetic substrates for assaying sphingomyelinases and/or PLC enzymes. Consisting of a C4 chain with an ether linkage to the chosen chromophore or flu-

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

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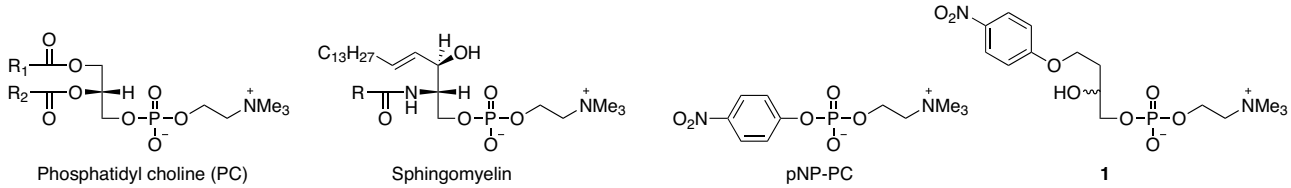


Fig. 1. Structure of the synthetic substrate (**1**) compared with the natural substrates phosphatidyl choline (PC) and sphingomyelin, and the synthetic substrate pNP-PC.

orophore on C-4, a free hydroxyl group on C-2 and a phosphate ester linkage on C-1, these substrates were envisioned as being more phospholipid-like than the simple pNP-PC (Fig. 1). Treating a substrate like (**1**) with an enzyme possessing PLC and/or sphingomyelinase activity, would yield the vicinal diol (**2**), which upon periodate oxidation and spontaneous β-elimination would liberate pNP, detected spectrophotometrically at 405 nm (Scheme 1). The assay could either be kinetic based, if the enzyme can tolerate a low concentration of periodate, or end-point based, in which case a periodate-containing stop solution is added to the enzymatic reaction after a certain incubation time.

2. Material and methods

^{1}H NMR, ^{13}C NMR and ^{31}P NMR were recorded at 400 MHz, 100 MHz and 162 MHz, respectively, on a Bruker Avance III HD spectrometer with a BBO SmartProbe. Spectra were recorded at 25 °C. LC–MS was performed on a Thermo Finnigan LCQ Deca XP Max system, using a C4 RP-column and a CH_3CN/H_2O solvent system with 0.15% HCOOH. Reactions were monitored using TLC, carried out on aluminum plates coated with 0.25 mm of Silica gel 60 F_{254}. The spots were visualized by UV-light. Column chromatography was done using Biotage or Telos silica flash cartridges and an Interchim Minibox II™ solvent pump. Absorbance was measured at 405 nm for pNP based substrates on a SpectraMax 190 Absorbance Microplate Reader from Molecular Devices.

2.1. 4-(4-Nitrophenoxy)-1-butene (*5*)

4-Nitrophenol (2 g, 14 mmol) was dissolved in anhydrous DMF (20 mL), K_2CO_3 (4.9 g) and KI (270 mg) were added followed by 4-bromo-1-butene (1.14 mL, 11 mmol, 0.8 eq). The reaction mixture was stirred overnight at 60 °C under N_2 atmosphere. The solvent was evaporated in vacuo and the crude product partitioned between aqueous NaOH (16 mL, 1 M) and diethyl ether (40 mL). The layers were separated and the aqueous phase extracted with ether (3 × 40 mL). The combined organic phases were washed with brine (30 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo to afford (**5**) as a yellow oil (819 mg, 38%). Rf (EtOAc/hexane 1:5) = 0.7. ^{1}H NMR ($CDCl_3$) δ = 8.19 (d, J = 9.2 Hz, 2H), 6.96 (d, J = 9.2 Hz, 2H), 5.90 (ddt, J = 17.3, 10.3, 6.6 Hz, 1H), 5.20 (dd, J = 1.4, 17.3 Hz, 1H), 5.15 (dd, J = 1.4, 10.3 Hz, 1H), 4.12 (t, J = 6.7, 2H), 2.59 (dt, J = 6.6, 6.7 Hz, 2H) ppm. ^{13}C NMR ($CDCl_3$) δ = 163.9, 141.4, 133.6, 125.9, 117.7, 114.5, 67.9, 33.3 ppm.

2.2. 4-(4-Nitrophenoxy)-1-butene-epoxide (*6*)

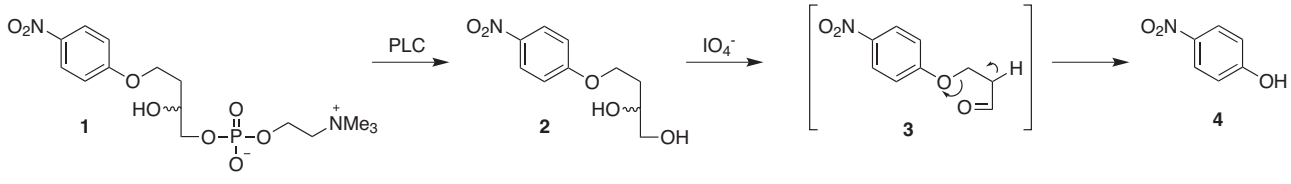
4-(4-Nitrophenoxy)-1-butene (**5**, 0.51 g, 2.6 mmol) was dissolved in DCM (15 mL). m-CPBA (1.14 g, 6.6 mmol, 2.5 eq) was dissolved in DCM (10 mL), and then added to the reaction mixture. The solution was stirred overnight at RT under N_2 atmosphere. Then more DCM (20 mL) was added and the reaction mixture washed with saturated $NaHCO_3$ (20 mL), H_2O (20 mL), and brine (20 mL). The organic phase was dried over Na_2SO_4, filtered, and evaporated in vacuo. The residue was purified by column chromatography (EtOAc/heptane 1:4 -> 1:3) to afford (**6**) as a light orange solid (203 mg, 37%). Rf (EtOAc/heptane 1:2) = 0.26. ^{1}H NMR ($CDCl_3$) δ = 8.21 (d, J = 9.2 Hz, 2H), 6.98 (d, J = 9.2 Hz, 2H), 4.28–4.18 (m, 2H), 3.17 (tdd, J = 6.8, 4.1, 2.6 Hz, 1H), 2.87 (dd, J = 4.9, 4.1 Hz, 1H), 2.61 (dd, J = 4.9, 2.6 Hz, 1H), 2.26–2.18 (m, 1H), 1.99–1.90 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$) δ = 163.8, 141.6, 125.9, 114.4, 65.6, 49.4, 47.2, 32.2 ppm.

2.3. 4-(4-Nitrophenoxy)-2-hydroxy-butyl-1-phosphoryl choline (*1*)

Phosphocholine calcium chloride salt (158 mg, 0.478 mmol) was dissolved in distilled water (0.5 mL) followed by addition of K_2HPO_4 (83 mg, 0.478 mmol) dissolved in distilled water (0.2 mL). The reaction mixture stirred for 2 h at RT. Formation of a white precipitate was observed. The epoxide (**6**) (100 mg, 0.478 mmol) was added and the reaction mixture stirred for 7 days at 80 °C. The reaction mixture was concentrated in vacuo and the crude product purified by column chromatography ($CHCl_3$/MeOH/H_2O 70:30:4 -> MeOH/H_2O 8:2 -> MeOH/H_2O 1:1) to afford the desired substrate (**1**) as a white solid (65 mg, 35%). Rf ($CHCl_3$/MeOH/H_2O 70:30:4) = 0.16. ^{1}H NMR (D_2O) δ = 8.05 (d, J = 9.3 Hz, 2H), 6.94 (d, J = 9.3 Hz, 2H), 4.26–4.21 (m, 2H), 4.16 (dd, J = 7.0, 5.4 Hz, 2H), 4.03–3.98 (m, 1H), 3.87 (ddd, J = 10.8, 5.5, 3.4 Hz, 1H), 3.76 (dt, J = 10.8, 6.2 Hz, 1H), 3.58–3.57 (m, 2H), 3.13 (s, 9H), 1.98 (dtd, J = 14.3, 7.0, 4.3 Hz,1H), 1.91–1.82 (ddt, J = 14.3, 8.8, 5.4 Hz, 1H) ppm. ^{13}C NMR (D_2O) δ = 163.9, 140.8, 126.1, 114.7, 69.4, 67.3, 66.0, 65.3, 59.4, 53.9, 31.5 ppm ^{31}P NMR (D_2O) δ = −0.15 ppm. LC–MS, calculated for the zwitterionic structure $C_{15}H_{25}N_2O_8P$, = 392.13 Da, found m/z: 393.07 $[M+H]^+$, 415.27 $[M+Na]^+$, 785.27 $[2M+H]^+$, 807.13 $[2M + Na]^+$.

2.4. 4-(4-Nitrophenoxy)-2-hydroxy-butyl-1-phosphate (*8*)

K_2HPO_4 (871 mg, 5 mmol) was dissolved in distilled water (10 mL) and epoxide (**6**) (131 mg, 0.627 mmol) was added. The reac-



Scheme 1. Mechanism of the assay.

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