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Design, synthesis and evaluation of two diastereoisomeric phosphonate inhibitors of lamb pregastric lipase

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Abstract

A mixture of the two diastereoisomers of (2*R*)-1,2-di-*O*-butyl-*sn*-glycerol-3-*O*-*p*-nitrophenyl-*n*-propylphosphonate, varying only in their configuration at phosphorus, was synthesised from (*R*)-3-*O*-benzyl-*sn*-glycerol. The diastereoisomers were separated by normal phase HPLC and fully characterised. Both diastereoisomers were irreversible inhibitors of the activity of lamb pregastric lipase (LPGL), although the isomer which eluted first in the HPLC separation was significantly more effective.

Keywords: Chirality; Inhibition; Phosphonate; 4-Nitrophenylacetate; Pregastric lipase; Triacylglycerol

1. Introduction

Pregastric lipases (PGL) are important to the dairy industry for the catalysed hydrolysis of milk fat, flavour enhancement of cheeses, and related applications. These enzymes hydrolyse triacylglycerols early in the digestive tract. Most PGL preferentially hydrolyse esters at the *sn*-3(1) positions [1], although this preference is complicated by the fact that short-chain fatty acids occur in milk fat more frequently at these positions than they do at the internal *sn*-2 position [2].

Lamb pregastric lipase (LPGL) is isolated from the tongue and epiglottal region of lamb. It has been shown to hydrolyse triacylglycerols containing short-chain fatty acids [3–5]. O'Connor and coworkers have shown that the preferred substrate of LPGL is tributyrin (**1**) [6]. It has been demonstrated that LPGL is *sn*-3 selective, i.e. prefers the *R*-configuration at C2 of the glycerol backbone (see Scheme 1) [5,7].

Inhibitors have already provided important structural information on the molecular basis for lipase activity [8], and some lipase inhibitors have medicinal applications, e.g. the use of tetrahydrolipstatin (Xenical^{TM}) in the treatment of clinical obesity [9,10]. We proposed to use a pair of inhibitors to investigate further how a prochiral substrate is positioned at the active site of the enzyme [11,12].

Phosphonates are an important class of lipase inhibitor [13–16], which have been shown, by X-ray crystallography, to bind irreversibly at the active site of the enzyme [17–20]. In terms of geometry and charge distribution, the phosphonate functional group mimics the first transition state during the hydrolysis of natural substrates (Scheme 2). The design of our inhibitors was based on tributyrin (**1**), the preferred substrate of

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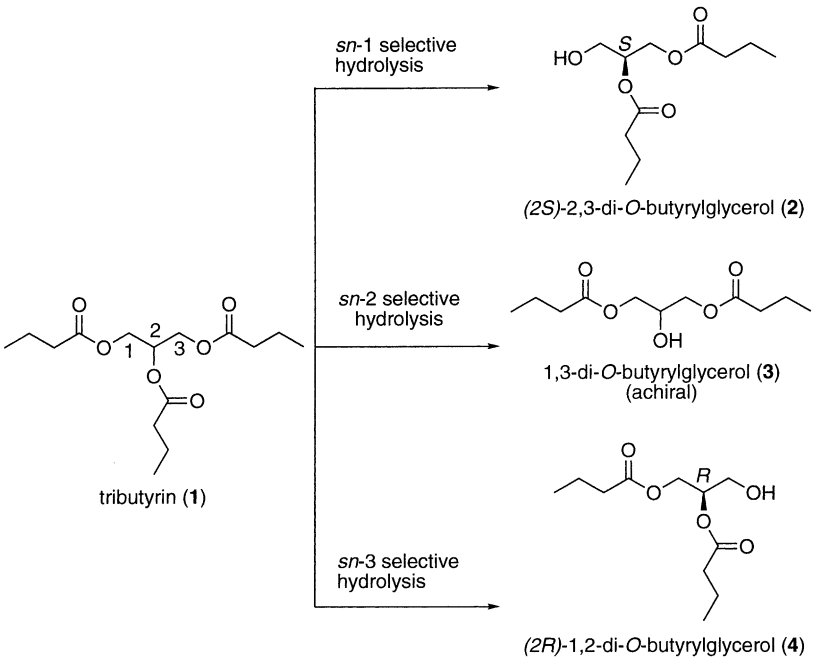


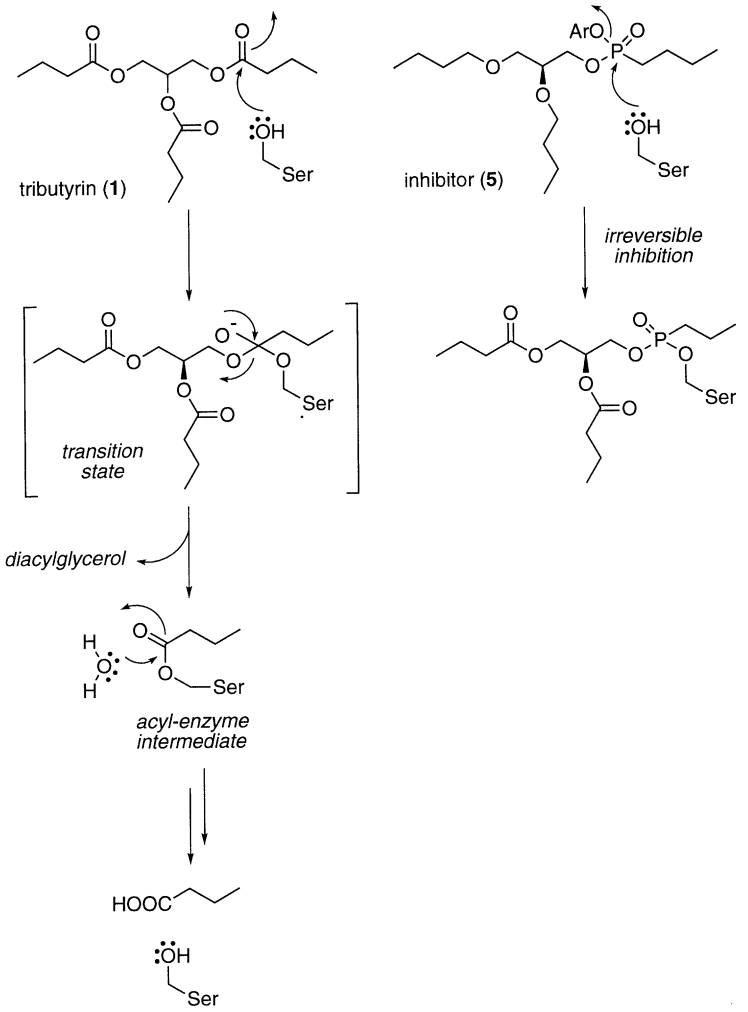
Scheme 1. Stereoselective hydrolysis of tributyrin (**1**).

LPGL, and the established *sn*-3-selectivity. To mimic tributyrin, we chose four carbon chains (or three carbon plus one phosphorus) for the glycerol. In their recent paper [21], Marguet et al. "intentionally retained the two carbonyl ester linkages in order to mimic as closely as possible the structure of acylglycerols". We, however, elected to follow the approach of Stadler et al. [22], invoking ether linkages. Ethers should not be susceptible to hydrolysis by the enzyme during assays, hopefully resulting in unambiguous interactions at the *sn*-3 position. We chose the *R*-enantiomer of 1-*O*-benzyl-*sn*-glycerol (**6**) as our starting material, in order to emulate hydrolysis at the *sn*-3 position. Another important design feature was the incorporation of a good leaving group at phosphorus, in the form of a *p*-nitrophenyl ester. The proposed mode of inhibition is illustrated in Scheme 2.

2. Materials and methods

(*R*)-3-*O*-benzyl-*sn*-glycerol (**6**) was purchased from Fluka, sodium hydride, *n*-bromobutane and propylphosphonic dichloride (**9**) were from Aldrich, and tetrabutylammonium iodide, 10% Pd/C and tributyrin (**1**) were from Acros. Dichloromethane was freshly distilled from calcium hydride. Sodium dihydrogen phosphate monohydrate was purchased from Merck. DMF was dried and distilled from BaO and stored over 4 Å molecular sieves. Triethylamine was dried and distilled from calcium hydride and stored over potassium hydroxide pellets. Bis–Tris propane [1,3-bis(tris-hydroxymethyl)methylaminopropane] and 4-nitrophenylacetate (PNPA) were purchased from Sigma. Triton X-100 was purchased from BDH.

NMR spectra were recorded on either a Bruker AM-200 or Bruker DRX-400 spectrometer. Proton NMR spectra are referenced to tetramethylsilane ($\delta = 0.00$) as an internal standard, ^{13}C NMR are referenced to $CDCl_3$ ($\delta = 77.0$) as an internal standard, ^{31}P NMR are referenced to 85% aqueous H_3PO_4 ($\delta = 0.0$) as an external standard. Mass spectra were recorded on a VG 7070 mass spectrometer, operating at a nominal accelerating voltage of 70 eV. Electron impact ionisation (EI) and chemical ionisation (CI) techniques used ammonia as the carrier gas. Fast atom bombardment (FAB) was performed using *m*-nitrobenzyl alcohol as



Scheme 2. Proposed mechanism for hydrolysis of **1** and inhibition by **5**.

matrix and Xe as carrier gas. Optical rotations were measured on a Perkin-Elmer 341 polarimeter.

2.1. Synthesis of inhibitors

(2*R*)-3-*O*-benzyl-1,2-di-*O*-butyl-*sn*-glycerol (**7**). Sodium hydride (308 mg, 70%, 8.97 mmol, 3.0 equiv.) was added to a solution of (*R*)-3-*O*-benzyl-*sn*-glycerol (**6**) (545 mg, 2.99 mmol, 1.0 equiv.), 1-bromobutane (1.28 ml, 11.96 mmol, 4.0 equiv.) and tetrabutylammonium iodide (353 mg, 0.96 mmol, 0.32 equiv.) in DMF (1 ml) at 0°C under a nitrogen atmosphere. Additional DMF (1.3 ml) was added to ensure continued stirring of the viscous mixture. The mixture was warmed to 55°C and stirred for 1.5 h. The mixture was cooled to room temperature, additional sodium hydride (84 mg, 70%, 3.5 mmol, 1.2 equiv.) was added and the mixture stirred for 17 h. The peach-coloured mixture was diluted with dichloromethane (25 ml) and washed with water (20 ml), saturated aq. Na_2CO_3 (20 ml) and brine (2 ml × 20 ml). The organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash column chromatography, eluting with 10:1 hexanes–EtOAc to

give (2*R*)-3-*O*-benzyl-1,2-di-*O*-butyl-*sn*-glycerol (**7**) as a colourless oil (602 mg; 69%). R_f 0.74 (4:1 hexanes–EtOAc); $[\alpha]_{20}^{D} = -0.46^{\circ}$ (*c* 2.9, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.91 (t, $J = 7.3$ Hz, 6H), 1.36 (m, $J = 7.5$ Hz, 4H), 1.51–1.59 (m, 4H), 3.42–3.62 (m, 9H), 4.55 (s, 2H), 7.25–7.33 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.7, 13.8, 19.2, 31.7, 32.1, 70.2, 70.2, 70.6, 71.2, 73.3, 77.9, 127.4, 127.5, 128.2, 138.4; HRMS (EI^+) calcd. for $C_{18}H_{30}O_3$ (M^+): 294.21950; observed: 294.21945.

(2*R*)-1,2-di-*O*-butyl-*sn*-glycerol (**8**). Palladium (10%) on carbon (19 mg) was added to a solution of (2*R*)-1,3-di-*O*-benzyl-1,2-di-*O*-butyl-*sn*-glycerol (**7**) (103 mg, 0.350 mmol) in 95% ethanol (2 ml). The suspension was stirred under an atmosphere of hydrogen for 18 h at room temperature. The suspension was filtered, and the carbon residue washed well with EtOAc. The filtrate was concentrated to give (2*R*)-1,3-di-*O*-butyl-*sn*-glycerol (**8**) as a colourless oil (67 mg; 94%) which was not purified further. R_f 0.33 (4:1 hexanes–EtOAc); $[\alpha]_{20}^{D} = -21.83^{\circ}$ (*c* 1.7, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.91 (t, $J = 6.9$ Hz, 6H), 1.27–1.46 (m, 4H), 1.48–1.64 (m, 4H), 2.26 (t, $J = 5.9$ Hz, 1H), 3.42–3.71 (m, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 13.9, 19.3, 31.7, 32.1, 63.1, 70.0, 70.9, 71.5, 78.2; HRMS (CI^+) calcd. for $C_{11}H_{25}O_3$ $(M + H)^+$: 205.18037; observed: 205.18024.

(2*R*)-1,3-di-*O*-butyl-*sn*-glycerol-*3*-*O*-*p*-nitrophenyl-*n*-propylphosphonate (**5**). A solution of (2*R*)-1,3-di-*O*-butyl-*sn*-glycerol (**8**) (195 mg, 0.956 mmol, 1.0 equiv.) and triethylamine (240 μl, 1.729 mmol, 1.8 equiv.) in CH_2Cl_2 (1 ml; 2 ml rinse) was added dropwise to a solution of propylphosphonic dichloride (**9**) (140 μl, 1.22 mmol, 1.2 equiv.) in CH_2Cl_2 (2 ml). The orange mixture was stirred at room temperature for 3 h under a nitrogen atmosphere. A solution of *p*-nitrophenol (**11**) (199 mg, 1.431 mmol, 1.5 equiv.) and triethylamine (240 μl, 1.729 mmol, 1.8 equiv.) in CH_2Cl_2 (1 ml; 2 ml rinse) was then added dropwise and the mixture stirred for an additional 1 h at room temperature after the addition was complete. The mixture was diluted with CH_2Cl_2 (15 ml) and washed with 5% K_2CO_3 (4 ml × 15 ml) and brine (2 ml × 15 ml). The organic layer was dried over $MgSO_4$, filtered and concentrated to give the product mixture as a light yellow oil (289 mg).

2.2. Isolation and separation of the diastereoisomers *5A* and *5B*

HPLC was performed using a Waters 600 Controller and pump equipped with a Rheodyne injector, and a Waters 996 Photodiode Array Detector, all interfaced with a PC using Millennium-32 software.

The residue from Section 2.1 was purified by semi-preparative RP-HPLC on an Econosil, C18, 10 μ, 21 mm × 25 mm column. A linear gradient was employed: 5–100% acetone in acetonitrile, over 20 min, with a flow rate of 11.5 ml min^{-1}. The composition of the eluant was monitored at 350 nm. Bis-*O*-(*p*-nitrophenyl)-*n*-propylphosphonate (**12**) eluted at R_T 5 min, followed by the mixture of diastereoisomers (**5**) which gave rise to a broad peak at R_T 8.5 min. After concentration of the appropriate eluant, the mixture of diastereoisomers was obtained as a colourless oil (50 mg; 13%).

The two diastereoisomers were separated by semi-preparative normal phase HPLC on an Econosil silica 10 μ, 10 mm × 25 mm column. An isocratic solvent system was employed: 99:1 (v/v) heptane–isopropanol, with a flow rate of 3 ml min^{-1}. The composition of the eluant was monitored at 234 nm. Baseline resolution of the two peaks was achieved and the two diastereoisomers were isolated on concentration of the appropriate eluant.

5A: R_T 30.2 min; R_f 0.35 (98:2 $CHCl_3$–MeOH); $[\alpha]_{20}^{D} = -5.9^{\circ}$ (*c* 0.23, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.86 (td, $J = 7.3$, 2.4 Hz, 6H), 1.07 (td, $J = 7.4$, 1.5 Hz, 3H), 1.30–1.38 (m, 4H), 1.48–1.55 (m, 4H), 1.73–1.78 (m, 2H), 1.93–1.99 (m, 2H), 3.40–3.58 (m, 7H), 4.06–4.09 (m, 1H), 4.31–4.34 (m, 1H), 7.39 (d, $J = 8.9$ Hz, 2H), 8.22 (d, $J = 8.9$ Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.8, 14.1, 15.2 ($^3J_{CP} = 17.9$ Hz), 16.0 ($^2J_{CP} = 5.7$ Hz), 19.2, 27.9 ($^1J_{CP} = 139.6$ Hz), 31.7, 32.0, 65.9 ($^2J_{CP} = 7.1$ Hz), 69.3, 70.3, 71.5, 77.2 ($^3J_{CP} = 6.1$ Hz), 121.0 ($^3J_{CP} = 4.6$ Hz), 125.6, 144.5, 155.7 ($^2J_{CP} = 8.5$ Hz); ^{31}P NMR (162 MHz, $CDCl_3$) δ: 28.7; HRMS (CI^+) calcd. for $C_{20}H_{35}NO_7P$ (M^+): 432.21398; observed: 432.21512.

5B: R_T 34.8 min; R_f 0.35 (98:2 $CHCl_3$–MeOH); $[\alpha]_{20}^{D} = -17.0^{\circ}$ (*c* 0.21, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.90 (td, $J = 7.4$, 3.7 Hz, 6H), 1.07 (td, $J = 7.3$, 1.7 Hz, 3H), 1.31–1.37 (m, 4H), 1.49–1.54 (m, 4H), 1.70–1.80 (m, 2H), 1.92–1.96 (m, 2H),

3.41–3.53 (m, 7H), 4.18–4.22 (m, 2H), 7.40 (d, J = 8.2 Hz, 2H), 8.22 (d, J = 9.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.8, 13.9, 15.2 ($^3J_{CP}$ = 18.0 Hz), 16.0 ($^2J_{CP}$ = 5.6 Hz), 19.2, 19.3, 27.9 ($^1J_{CP}$ = 139.5 Hz), 31.7, 32.0, 66.1 ($^2J_{CP}$ = 6.9 Hz), 69.2, 70.3, 71.5, 77.2 ($^3J_{CP}$ = 6.6 Hz), 121.0 ($^3J_{CP}$ = 4.7 Hz), 125.6, 144.5, 155.7 ($^2J_{CP}$ = 8.6 Hz); ^{31}P NMR (162 MHz, $CDCl_3$) δ: 28.5; HRMS (CI^+) calcd. for $C_{20}H_{35}NO_7P$ (M^+): 432.21398; observed: 432.21512.

2.3. Enzyme

Crude LPGL was supplied by the New Zealand Rennet Co., Ltd. The partial purification of LPGL was performed as described previously [23,24]. The enzyme was shown to have lipase activity of 1.271 μmol min^{-1} mg^{-1} of enzyme against tributyrin (**1**) (6.8 mM), at 35°C and pH 6.5. The lipase was freshly dissolved in Bis–Tris propane buffer (50 mM, pH 7). To ensure stability, the enzyme preparations, in the presence or absence of added inhibitor, were stored on ice for the duration of the experiment. Active and concentrated enzyme preparations were necessary to allow the rate of hydrolysis to be resolved from the system noise, in order to confirm that LPGL was indeed inactivated irreversibly by the inhibitor.

2.4. Enzyme assays

2.4.1. PNPA as substrate

A stock solution (60 mM) of PNPA was prepared in dry acetonitrile. The initial rate of LPGL catalysed hydrolysis of PNPA was monitored spectrophotometrically by measuring the increase in absorbance at 400 nm, pH 7.24 (50 mM Bis–Tris propane), 37°C and in the presence of various concentrations (0.26–1.05 mM) of a diastereoisomeric mixture of **5A** and **5B**. Each quartz cell contained 3 ml buffer, 20 μl lipase solution (12–16 g l^{-1} concentration adjusted to ensure that the initial rate constant of hydrolysis was >3 × 10^{-3} A/s), 50 μl PNPA solution and 10 μl of inhibitor mixture.

All rate constants were constants were corrected for background hydrolysis and the stability of the enzyme in the absence of inhibitor, determined over 3 min periods, was monitored for at least 60 min. In the inhibition studies, varying concentrations of the inhibitor were

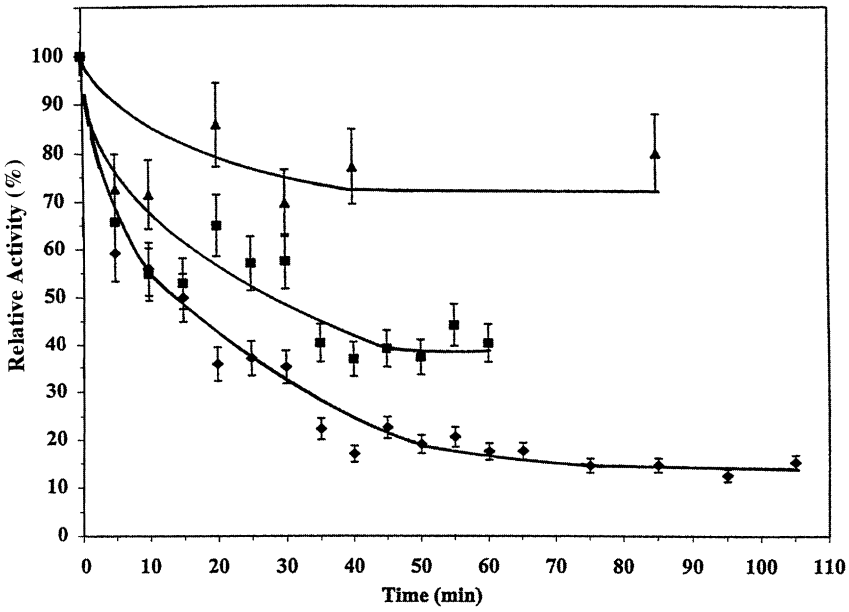


Fig. 1. Enzyme activity as a function of time for LPGL catalysed hydrolysis of PNPA at 37°C, pH 7.24, in the presence of a diastereoisomeric mixture of **5A** and **5B** at concentrations: (▲) (0.26 mM); (■) (0.66 mM); (◆) (1.05 mM).

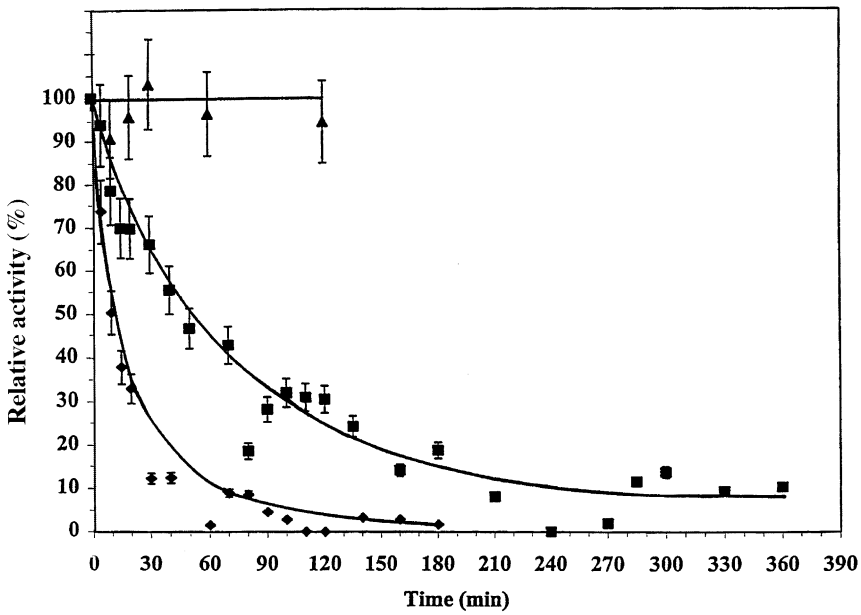


Fig. 2. Enzyme activity as a function of time for LPGL catalysed hydrolysis of tributyrin (**1**) at 35°C, pH 6.5. (▲) Control (lipase only); (◆) in the presence of diastereoisomer **5A**; (■) in the presence of diastereoisomer **5B**.

added to the lipase solution at zero time, and aliquots were removed from this mixture every 5 min in order to determine the activity against PNPA. Typical data for relevant percent activity are shown in Fig. 1.

2.4.2. Tributyrin as substrate

A stock solution of tributyrin (**1**) (6.8 mM) was prepared in a 1% (v/v) solution of Triton X-100 in Milli-Q water. Homogeneity of the mixture was ensured by sonicating the mixture for 15 min, followed by continuous stirring over a hot plate throughout the experiment. Phosphate buffer ($NaH_2PO_4 \cdot H_2O$)(5.1 g, 37 mM) was added to the stirred emulsion. The initial rate of hydrolysis of **1** in the presence of LPGL, and the lipase inhibitor complexes, was assayed by a Metrohm 736-6P autotitrator in pH-stat mode (pH 6.5) at 35°C.

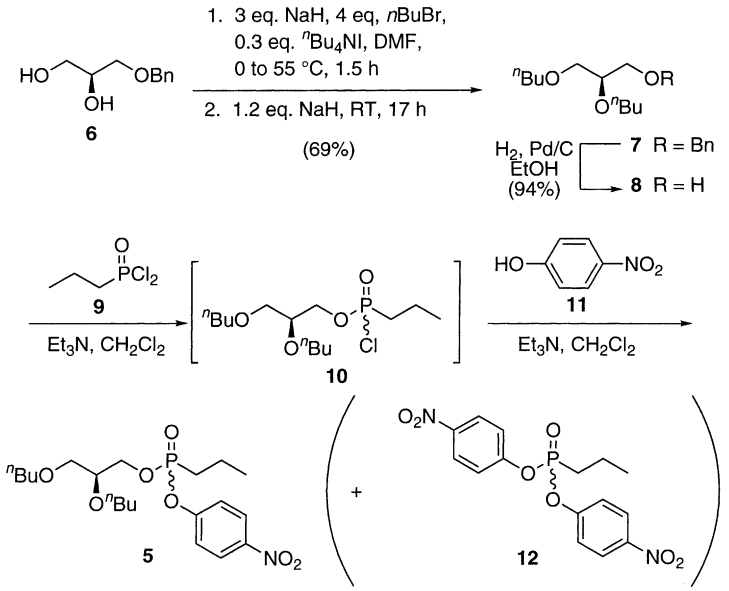
In the control study, a 100 μl aliquot of lipase solution ($14.8\,g\,l^{-1}$) was added to the emulsified substrate and the reaction was monitored by titration of released butyric acid with standardised NaOH. The stability of the lipase in the absence of inhibitor was monitored for 3 min titration periods for at least 60 min.

In the inhibition studies, an aliquot (12 μl) of inhibitor solution (50–250 mM) was added to 2.5 ml lipase solution ($14.8\,g\,l^{-1}$) at zero time. Aliquots (100 μl) were then withdrawn at 5 min intervals and added to 50 ml emulsified substrate and the initial rate of hydrolysis of tributyrin (**1**) was monitored by titration of released butyric acid with 0.01 M NaOH over a 3 min period. The percent activity, relevant to that of the enzyme in the absence of inhibitor, was then determined. Typical plots in the presence of 1.25 mM **5A** and **5B** are shown in Fig. 2. The background rate of hydrolysis, measured in the absence of lipase but in the presence of 1.25 mM inhibitor was shown to be negligible.

3. Results and discussion

3.1. Synthesis and separation of the inhibitors

Our synthesis began with commercially available (*R*)-1-*O*-benzyl-*sn*-glycerol (**6**) as illustrated in Scheme 3. We first needed to convert the two free alcohols to their *n*-butyl ethers. Similar transformations had been reported using an alkyl methanesulfonate, in the presence of a base (typically KOH), in an



Scheme 3. Synthesis of the inhibitors.

aromatic hydrocarbon solvent at reflux [25]. In the present case, this method gave extremely low yields (<5%) of alkylated product **7**. More satisfactory results were obtained using an excess of sodium hydride and bromobutane, in the presence of catalytic tetrabutylammonium iodide. The benzyl ether was cleaved hydrogenolytically to liberate the primary alcohol **8**.

n-Propylphosphonic dichloride (**9**) was then allowed to react with alcohol **8** in the presence of triethylamine. ^{31}P NMR analysis of the reaction mixture indicated that **9** ($\delta = 48.7$) was being converted to a mixture of the two diastereoisomeric esters **10** ($\delta = 43.2$ and 43.4). When no further change was taking place, a solution of *p*-nitrophenol (**11**) and triethylamine was added. This led to the formation of the two diastereoisomeric esters **5** ($\delta = 28.4$ and 28.6). It was evident from the ^{31}P NMR spectra, run throughout the reaction, that many side reactions were also taking place. A significant by-product was bis-*O*-(*p*-nitrophenyl)-*n*-propylphosphonate (**12**).

The mixture of diastereoisomers was isolated from the crude product mixture, in low yield, using RP-HPLC. The two isomers were then separated, using normal phase HPLC conditions described previously by Marguet [21]. The two inhibitors were designated **5A** and **5B**, in order of their elution by normal phase HPLC. Both compounds were characterised and their ^{1}H, ^{13}C and ^{31}P NMR spectra fully assigned. Unfortunately, no evidence was obtained for the configuration at phosphorus.

3.2. Inhibition of Lipase

Preliminary tests (Fig. 1) were conducted to test the ability of **5A** and **5B** to act as inhibitors of the activity of LPGL catalysed hydrolysis of PNPA. The time course for activity of LPGL against ester substrate in the presence of a diastereoisomeric mixture of **5A** and **5B** showed that, in each case, the enzymatic activity decreased steadily to reach a plateau value which remained constant; ca. 30, 50 and 90% loss in activity after ca. 20, 40 and 50 min incubation time, respectively, in the presence of 0.26, 0.66 and 1.05 mM mixture of diastereoisomeric inhibitors, respectively.

Further tests were then conducted to test the effect of the individual diastereomers, **5A** and **5B**, on their activity against the LPGL catalysed hydrolysis of tributyrin (**1**). Both were found to be inhibitory, as shown in Fig. 2, with distinctive rates of inactivation. No reactivation was observed after a further 6 h incubation, indicating irreversible inhibition. The half-life for lipase-catalysed hydrolysis of tributyrin

(**1**) was 12 ± 2.5 min in the presence **5A**; this inhibitor completely shut down lipolytic activity after 2 h. Compound **5B** was less effective, resulting in a half-life for lipase-catalysed hydrolysis of tributyrin (**1**) of 44 ± 9 min, with enzyme activity reaching a plateau of 10–15% after 5 h. Similar results were shown with another partially purified LPGL preparation of similar activity (data not shown). This demonstrates that LPGL is stereoselective vis-à-vis the configuration at phosphorus. Without knowing the configuration at phosphorus in the inhibitors or the enzyme-inhibitor adducts, it is not possible to draw any further conclusions about the facial selectivity of the lipase-catalysed hydrolysis of tributyrin (**1**).

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