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Fluorescent organophosphonates as inhibitors of microbial lipases

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Abstract

Short- and long-chain 1-*O*-alkyl-2-acylaminodeoxyglycero- and alkoxy-alkylphosphonic acid *p*-nitrophenyl esters were synthesized as inhibitors for analytical and mechanistic studies on lipolytic enzymes. The respective compounds contain perylene or nitrobenzoxadiazole as reporter fluorophores covalently bound to the ω-ends of the respective 2-acylamino- and alkoxy- residues. Their inhibitory effects on the activities of three selected lipases showing different substrate preferences were determined, including the lipases from *Rhizopus oryzae*, *Pseudomonas species*, and *Pseudomonas cepacia*. *R. oryzae* lipase reacted much better with the single-chain inhibitors than the two-chain deoxyglycerolipids. In contrast, *P. cepacia* lipase was inactivated by perylene-containing two-chain phosphonate (**XXII**) to a larger extent as compared to the other inhibitors whereas *Pseudomonas species* lipase interacted efficiently and without any preferences with all inhibitors used in this study. In summary, the different lipases show a very characteristic reactivity pattern not only with respect to triacylglycerol substrates but also to their structurally related inhibitors. Thus, the novel phosphonates might be useful tools not only for analysis and discrimination of known lipolytic enzymes but also for discovery of yet unknown lipases/esterases in biological samples.

Keywords: Microbial lipases; Synthesis of inhibitors; Fluorescent triacylglycerol analog; Covalent inhibition

1. Introduction

Lipolytic enzymes are widely used biocatalysts in research and industry to achieve chemical reactions with high regio- and stereoselectivity yielding enantiomeric alcohols or amines (Jaerger and Reetz, 1998; Schmid and Verger, 1998; Schmid et al., 2001; Koeller and Wong, 2001; Klibanov, 2001). Lipases (EC 3.1.1.3) catalyze hydrolysis and synthesis of triacylglycerols. All lipases accept esters of medium (C4) and long-chain (C16) saturated fatty acids as substrates (mainly at *sn*-1 or *sn*-3 positions) (Rangheard et al., 1992; Kirk et al., 1992; Ransac et al., 1990; Rogalska et al., 1993). The mechanism of action of these enzymes involves the nucleophilic cleavage of an ester bond by an activated serine which belongs to the catalytic triad Ser-His-Asp/Glu (Cygler and Schrag, 1997; Jaerger and Reetz, 1998; Schmid and Verger, 1998). Lipophilic *p*-nitrophenyl phosphonate esters are convenient tools in lipase research, e.g. for studies of substrate–enzyme interactions on the molecular level (Ransac et al., 1997) and functional analysis, e.g. to determine the active enzyme fraction of crude or pure protein preparations (Scholze

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et al., 1999; Rotticci et al., 2000; Hermetter et al., 2001). These inhibitors react with the nucleophilic serine of lipases, thus leading to the formation of covalent and equimolar lipid–protein complexes that are stable in aqueous and organic solutions (Rotticci et al., 2000; Ransac et al., 1997; Bjoerkling et al., 1994; Zandonella et al., 1999). Such complexes represent "open" lipase conformations and mimic the substrate–enzyme interactions in the first (tetrahedral) transition state. Fluorescent labels in the hydrophobic tail of the organophosphonates are not only useful for quantitative analysis of lipases but also for studying lipid–protein interactions in the first transition state under different environment (solvent) conditions (Oskolkova and Hermetter, 2002; Zandonella et al., 1999).

The phosphonates described in the present work were tested as inhibitors of lipases from *Rhizopus oryzae*, *Pseudomonas species*, and *Pseudomonas cepacia*. Comparative experiments showed that the novel compounds have a very different inhibitory profile towards the individual enzymes. Only one of the compounds under investigation, namely perylenebutanoylamino phosphonate (**XXII**), preferably inactivates PCL in comparison to other inhibitors. PSL was almost quantitatively inhibited by all organophosphonates used in this study.

2. Experimental procedures

Standard chemicals were obtained from Merck. Mercaptoethanol and *p*-nitrophenol were from Sigma; methyl phosphonic acid dichloride, *n*-hexylphosphonic acid dichloride and methyl suberyl chloride were from Aldrich. 1-Methylimidazole, perylene, 3-carbomethoxypropionyl chloride, and *N*-hydroxysuccinimide were obtained from Fluka. Succinimidyl 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoate was purchased from Molecular Probes, 12-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoic acid from Lambda Fluoreszenztechnologie GmbH (Graz, Austria). Activated succinimid esters of fluorescently labeled acids were synthesized from appropriate fatty acids and *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide by a procedure essentially described elsewhere (Lapidot et al., 1967). The synthesis of 2-amino-2-deoxy-1(3)-*O*-trityl-3(1)-*O*-alkyl-*sn*-glycerols was performed in our laboratory according to (Bucher, Ribitsch et al., unpublished data).

Crude *Pseudomonas species* lipase was purchased from Nagase Biochemicals Ltd. (Japan), the pure lipase from *P. cepacia* (PCL) was obtained from R.D. Schmid, University of Stuttgart, Germany. *R. oryzae* lipase was kindly provided by F. Spener and L. Haalck, University of Münster, Germany.

Dichloromethane (Riedel-de Haën) was dried by refluxing with phosphorus pentoxide (Merck) and subsequently distilled. Other solvents were of analytical grade. TLC was carried out on silica gel 60 F_{254} aluminum sheets (0.2 mm, Merck) using following mixtures: petroleum ether/ether, 2:1 (v/v) (system 1), chloroform/methanol/acetone, 10:0.2:0.2 (v/v/v) (system 2), chloroform/methanol/acetone, 10:0.5:0.5 (v/v/v) (system 3), chloroform/methanol, 10:3 (v/v) (system 4), or ether/tetrahydrofurane, 2:1 (v/v) (system 5) as developing solvents. For preparative TLC purification silica gel 60 aluminum sheets (0.2 mm, Merck) without fluorescent indicator were used. Compounds were visualized under UV light (360 nm) and by charring at 120 °C after spraying with 50% sulfuric acid. Phosphorus-containing compounds were visualized on TLC plates by staining with phosphomolybdic acid (Dittmer and Lester, 1964), or quantitatively determined in solutions by the method of Broekhuyse (1968). Short column chromatography was performed on Kieselgel 60 (230–400 mesh, Merck).

^{1}H NMR spectra were recorded in deuterated solvents at 199.97 MHz, using a Varian Gemini 200 spectrometer. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as a standard.

Positive ion ESI mass-spectra were recorded on a standard Kratos electrospray ion source that was fitted to a Kratos Profile HV-4 double-focusing magnetic sector instrument (acceleration voltage 2 kV, *m*/*z*-range 25–2400 Da, scan speed 10 s/Dec, resolution 1700 (10% valley)). The potentials applied to the ESI source (temperature 50 °C, countercurrent flow of nitrogen 150 ml/min) were +5.98 kV at the spraying capillary, +3.12 kV at the cylinder, and +2.57 kV at the endplate. A Harvard Apparatus 22 syringe pump was used to deliver a constant flow (6 μl/min) of dry methanol containing CsI (250 mg/l). Solutions of the samples (50 μM) in the same solvent were injected via a 100-μl sample loop. The *m*/*z* values are given for the most intense peak of any isotope distribution.

2.1. 3-(3-Perylenoyl)propanoic acid methyl ester

To a solution of 3-carbomethoxypropionyl chloride (51.8 μl, 0.415 mmol) in 10 ml dichloromethane, 64.7 mg (0.476 mmol) aluminum chloride were added in portions under stirring at 0 °C. The reaction was kept at this temperature for 1 h and then 100.0 mg (0.396 mmol) perylene were added portionwise. The mixture was kept at 0 °C for 1 h and then overnight at room temperature, poured on about 10 g of ice, acidified with one to three drops of concentrated hydrochloric acid. The product was extracted with dichloromethane (6 × 50 ml), washed with water (2 × 10 ml). The organic layers were dried over sodium sulfate. After evaporation, the residue was purified by column chromatography on silica gel eluting with chloroform to give the pure product perylenoylpropanoic acid methyl ester (121.2 mg, 83.5%). R_f 0.16 (system 1). ^{1}H NMR ($CDCl_3$) (δ, ppm): 2.87 (t, 2H, $J_{a,b} = J_{2,3}$ 6.5 Hz, 2–CH_2–COO–), 3.40 (t, 2H, $J_{a,b} = J_{2,3}$ 6.5 Hz, 3–CH_2–CO–Ar), 3.77 (s, 3H, CH_3), 7.47–7.65 (m, 3H, Ar), 7.74 (t, 2H, J 8.6 Hz, Ar), 7.95 (d, 1H, *J* 8.0 Hz, Ar), 8.14–8.31 (m, 4H, Ar), 8.56 (d, 1H, *J* 8.6 Hz, Ar).

2.2. 4-(3-Perylenyl)butanoic acid

To a suspension of 3-perylenoyl propanoic acid methyl ester (360.2 mg, 0.983 mmol) in 2.0 ml diethylene glycol, 98% hydrazine hydrate (150.6 μl, 2.949 mmol) and powdered potassium hydroxide (275.3 mg, 4.916 mmol) were added. The reaction mixture was heated to 140 °C for 2 h, then to 190 °C and kept over night, cooled to room temperature, diluted with 20 ml water, acidified with 0.5 ml concentrated hydrochloric acid, the precipitate formed was filtered, washed with water to pH 7.0, dried on air or washed with acetone, then with a mixture of chloroform/methanol (8:2, v/v). The filtrate was concentrated in vacuo. The crude product was applied into a silica gel column and eluted with chloroform to 20% methanol in chloroform to give perylenebutanoic acid (228.9 mg, 68.8%). R_f 0.47 (system 3). ^{1}H NMR ($CDCl_3$/DMSO-d_6, 20:1) (δ, ppm): 1.92 (quin., 2H, 3-CH_2), 2.38 (t, 2H, *J* 7.0 Hz, 2-CH_2), 3.03 (t, 2H, *J* 7.0 Hz, 4-CH_2), 7.41 (d, 1H, *J* 7.8 Hz, Ar), 7.43–7.64 (m, 3H, Ar), 7.76 (d, 1H, *J* 3.4 Hz, Ar), 7.80 (d, 1H, *J* 3.4 Hz, Ar), 8.00 (d, 1H, *J* 8.5 Hz, Ar), 8.21–8.60 (m, 4H, Ar).

2.3. 7-(3-Perylenoyl)heptanoic acid methyl ester

The synthesis and purification of perylenoylheptanoic acid methyl ester was achieved as described for perylenoyl propanoic acid methyl ester starting from suberic acid monomethyl ester chloride and perylene. Yield 73.7%. R_f 0.23 (system 1). ^{1}H NMR ($CDCl_3$) (δ, ppm): 1.43 (m, 4H, 4-CH_2, 5-CH_2), 1.67 (m, 2H, 3-CH_2), 1.82 (m, 2H, 6-CH_2), 2.32 (t, 2H, $J_{a,b} = J_{2,3}$ 7.4 Hz, 2-CH_2), 3.05 (t, 2H, $J_{a,b} = J_{6,7}$ 7.4 Hz, 7-CH_2), 3.69 (s, 3H, CH_3), 7.43–7.64 (m, 3H, Ar), 7.74 (t, 2H, *J* 8.3 Hz, Ar), 7.84 (d, 1H, *J* 7.9 Hz, Ar), 8.13–8.30 (m, 4H, Ar), 8.48 (d, 1H, *J* 7.5 Hz, Ar).

2.4. 8-(3-Perylenyl)octanoic acid

The synthesis and isolation was accomplished under identical conditions as described for perylenebutanoic acid staring from 7-(3-perylenoyl)heptanoic acid methyl ester yielding 60.0% perylenyloctanoic acid. R_f 0.54 (system 3). ^{1}H NMR ($CDCl_3$/CD_3OD, 9:1) (δ, ppm): 1.25–1.50 (m, 6H, 3 CH_2), 1.59 (m, 2H, 3-CH_2), 1.72 (m, 2H, 7-CH_2), 2.26 (dt, 2H, $J_{a,b}$ 1.8 Hz, $J_{2,3}$ 7.5 Hz, 2-CH_2), 2.97 (t, 2H, $J_{7,8}$ 7.7 Hz, 8-CH_2), 7.28 (d, 1H, *J* 8.0 Hz, Ar), 7.43 (m, 3H, Ar), 7.59 (d, 1H, *J* 3.4 Hz, Ar), 7.63 (d, 1H, *J* 3.4 Hz, Ar), 7.83 (d, 1H, *J* 8.5 Hz, Ar), 8.10–8.22 (m, 4H, Ar).

2.5. 2-(4-(3-Perylenyl)butanoyl)amino-2-deoxy-3-O-octyl-sn-glycerol (**XII**)

(A) To a solution of 12.0 mg (0.027 mmol) perylenebutyric acid succinimide ester (**I**) in 3 ml THF, 2-amino-2-deoxy-1-*O*-trityl-3-*O*-octyl-*sn*-glycerol (**V**) (12.2 mg, 0.027 mmol) was added. The reaction mixture was kept at room temperature overnight, evaporated under a stream of argon. The residue was applied into a silica gel column and eluted with chloroform. Fractions containing the product were collected and the solvent was removed under reduced pressure. The compound (**XI**) (20.8 mg) was obtained with 98.6% yield. R_f 0.81 (system 2). Removal of the trityl blocking group from 2-perylenebutanoylamino-1-*O*-trityl-3-*O*-octyl-*sn*-glycerol (**XI**) was carried out with boron trifluoride–

methanol in absolute dichloromethane under procedure elaborated by Hermetter et al. (1989). R_f 0.13 (system 2). Yield 70.0%. (B) The compound (**XII**) was achieved by an analogous procedure described above starting from perylenebutanoic acid succinimide ester (**I**) and 2-amino-2-deoxy-3-*O*-octyl-*sn*-glycerol (**VI**) with 91.0% yield. The products obtained in both cases were identical.

*2.6. 2-(4-(3-Perylenyl)butanoyl)amino-2-deoxy-1-O-octyl-sn-glycerol (**XIV**)*

The compound (**XIV**) was obtained from an equimolar mixture of perylenebutanoic acid succinimide ester (**I**) and 2-amino-2-deoxy-1-*O*-octyl-3-*O*-trityl-*sn*-glycerol (**VII**) under identical conditions described in method (A) for (**XII**). Overall yield after two stages was 88.3%.

*2.7. 2-(8-(3-Perylenyl)octanoyl)amino-2-deoxy-1-O-hexadecyl-sn-glycerol (**XVI**)*

This product was synthesized using the same procedure as for (**XII**) (procedure A) starting from peryleneoctanoic acid succinimide ester (**II**) and 2-amino-2-deoxy-1-*O*-hexadecyl-3-*O*-trityl-*sn*-glycerol (**VIII**). Yield (after two stages) is 90.0%.

*2.8. 2-(8-(3-Perylenyl)octanoyl)amino-2-deoxy-3-O-hexadecyl-sn-glycerol (**XVIII**)*

The compound (**XVIII**) was prepared in 67.9% yield by the same procedure as described for (**XII**) (A), starting from amino glycerol (**IX**) and peryleneoctanoic acid succinimidyl ester (**II**).

*2.9. 2-(6-NBD-hexanoyl)amino-2-deoxy-3-O-octyl-sn-glycerol (**XX**)*

The amino glycerol (**XX**) was obtained from NBD-hexanoic acid succinimide ester (**III**) and 2-amino-2-deoxy-1-*O*-trityl-3-*O*-octyl-*sn*-glycerol (**V**) as described for (**XII**) (A). Yield 86.0%. Alternatively, the same compound (**XX**) was prepared under identical conditions as described for (**XII**) (method (B)) but using succinimide ester (**III**) and 2-amino-2-deoxy-3-*O*-octyl-*sn*-glycerol (**VI**) with a 100% yield. R_f 0.16 (system 2).

*2.10. 2-(6-NBD-dodecanoyl)amino-2-deoxy-3-O-hexadecyl-sn-glycerol (**XXI**)*

The synthesis of the long-chain alkyl acylamino glycerol (**XXI**) was achieved under identical conditions as described for the compound (**XII**) (method B) starting from NBD-dodecanoic acid succinimid ester (**IV**) and 2-amino-2-deoxy-3-*O*-hexadecyl-*sn*-glycerol (**X**). The yield comprised 98.0%.

*2.11. 2-(4-(3-Perylenyl)butanoyl)amino-2-deoxy-3-O-octyl-sn-glycero-1-O-(n-hexyl)phosphonic acid p-nitrophenyl ester (**XXII**)*

To a solution of the glycerol derivative (**XII**) (20.0 mg, 0.038 mmol), 40 μl triethylamine, and 3.5% tetrazole solution in acetonitrile (7.4 μl, 0.004 mmol) in 2 ml dichloromethane, hexyl phosphonic acid dichloride (12.9 μl, 0.076 mmol) was added at 5 °C. After 1 h when the reaction was complete, *p*-nitrophenol (10.6 mg, 0.076 mmol) was added and the reaction mixture was kept overnight at room temperature. The solvent was removed under a stream of nitrogen. The product was isolated by preparative TLC in the enveloping system chloroform/methanol/acetone, 10:0.2:0.2 (v/v/v). Yield was 13.1 mg (43.3%). ^{1}H NMR ($CDCl_3$) (δ, ppm): 0.87 (t, 6H, $2CH_3$), 1.05–2.00 (m, 22H, CH_2-alkyl), 2.13 (m, 2H, 2-CH_2-acyl), 2.30 (m, 2H, 3-CH_2-acyl), 3.05 (m, 2H, CH_2-*O*-alkyl), 3.40 (m, 4H, CH_2-*O*-glycerol, 4-CH_2-acyl), 4.25 (m, 3H, CH_2-*O*-P, CH-N), 6.18 (dd, 1H, NH), 7.29-7.40 (m, 3H, Ar), 7.50 (m, 3H, Ar), 7.66 (d, 1H, *J* 2.4 Hz, Ar), 7.70 (d, 1H, *J* 2.2 Hz, Ar), 7.89 (dd, 1H, *J* 3.4 Hz, *J* 8.1 Hz, Ar), 8.17 (m, 6H, Ar).

*2.12. 2-(4-(3-Perylenyl)butanoyl)amino-2-deoxy-1-O-octyl-sn-glycero-3-O-(n-hexyl)phosphonic acid p-nitrophenyl ester (**XXVI**)*

To a solution of 2-perylenebutanoylamino-1-*O*-octyl-*sn*-glycerol (**XIV**) (8.1 mg, 0.016 mmol) and *N*-methylimidazole (6.0 μl, 0.070 mmol) in 2 ml dichloromethane, *n*-hexylphosphonic dichloride (10.8 μl, 0.063 mmol) was added. The reaction mixture was kept at room temperature for 3 h. After the reaction was complete, a mixture of *p*-nitrophenol (10.6 mg, 0.076 mmol) and *N*-methylimidazole (6.0 μl, 0.070 mmol) were added. After 18 h, the solvent was

evaporated under a stream of argon. The phosphonate (**XXVI**) was purified by TLC in system 2. Yield 0.7 mg (5.7%).

*2.13. 2-(8-(3-Perylenyl)octanoyl)amino-2-deoxy-3-O-hexadecyl-sn-glycero-1-O-(n-hexyl)phosphonic acid p-nitrophenyl ester (**XXIII**)*

The phosphonate (**XXIII**) was prepared under identical conditions as described for (**XXII**) with 6.3% yield and under conditions as for (**XXIV**) as well (yield 31.6%) starting from (**XVIII**).

*2.14. 2-(8-(3-Perylenyl)octanoyl)amino-2-deoxy-1-O-hexadecyl-sn-glycero-3-O-(n-hexyl)phosphonic acid p-nitrophenyl ester (**XXVII**)*

The synthesis and isolation was accomplished according to the procedure described for (**XXII**) starting from the amino glycerol analog (**XVI**). Yield 26.1%.

*2.15. 2-(NBD-hexanoyl)amino-2-deoxy-3-O-octyl-sn-glycero-1-O-(n-hexyl)phosphonic acid p-nitrophenyl ester (**XXIV**)*

The product (**XXIV**) was obtained from fluorescent labeled glycerol (**XX**) under identical conditions as described for (**XXII**) with 27.8% yield.

*2.16. 2-(NBD-dodecanoyl)amino-2-deoxy-3-O-hexadecyl-sn-glycero-1-O-(n-hexyl)phosphonic acid p-nitrophenyl ester (**XXV**)*

The compound (**XXI**) was phosphorylated as described for the preparation of phosphonate (**XXII**) using acylamino *O*-alkyl glycerol (**XXI**) as a starting material. The yield comprised 4.4%.

*2.17. 3-Perylenebutanol (**XXVIII**)*

To a solution of perylenebutanoic acid (50.0 mg, 0.148 mmol) in 3.5 ml toluene, Red-Al (0.67 ml, 2.232 mmol) was added carefully. After 1 h, when the reaction was complete, the mixture was cooled in an ice bath, and 25% sulfuric acid was added to pH 5 to decompose the unreacted Red-Al. Then water (30 ml) was added and the product was extracted with chloroform (5 × 50 ml). The organic phase was washed with water (2 × 20 ml), dried over sodium sulfate, evaporated under reduced pressure. The product was purified on a silica gel column eluting with chloroform. R_f 0.57 (system 3). Yield 31.7 mg (66.2%).

*2.18. O-(4-(3-Perylene)butyl)-O-methylphosphonic acid p-nitrophenyl ester (**XXIX**)*

One-chain phosphonate inhibitor (**XXIX**) was obtained from perylenebutanol (**XXVIII**) analogous to the procedure described for phosphonate (**XXVI**), but using methyl phosphonic acid dichloride as phosphorylating reagent. Yield 57.6%.

*2.19. ((6-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl)aminoethanol (**XXX**)*

A mixture of NBD-hexanoic acid succinimidyl ester (**III**) (12.5 mg, 0.0319 mmol) and 2-aminoethanol (2 μl, 0.0324 mmol) in 2 ml THF was kept for 30 min. After TLC showed the reaction complete, the solvent was removed under a stream of argon. The residue was dissolved in a chloroform/methanol/water mixture (65:25:4, v/v/v) (5 ml), and 1 g of a resin Dowex 50Wx8 (H^+-form) was added. After stirring for 30 min, the product (**XXX**) dissolved was filtered and the resin was washed with the same solvent system (3 × 5 ml). The filtrate was evaporated under reduced pressure. TLC analysis showed single fluorescent spot with R_f 0.69 (system 4) and no aminoethanol after spraying with ninhydrine. Yield 10.6 mg (98.3%).

*2.20. O-((6-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino)hexanoyl)aminoethyl-O-(n-hexyl)phosphonic acid p-nitrophenyl ester (**XXXI**)*

Using the method described for phosphonate (**XXII**), NBD-labeled hexylphosphonate was obtained starting from the respective alcohol (**XXX**). The yield comprised 11.1%.

2.21. Determination of lipase activity

Inhibition of lipase activity by the water-insoluble phosphonates was performed in Triton X-100 micelles (2 mM final concentration) and measured by a continuous fluorescence method according to Duque et al. (1996) using 3-*O*-hexadecyl-2-pyrenedecanoyl-1-tri-

nitrophenylaminododecanoyl-*sn*-glycerol as a substrate. Lipase activity was determined at 30 °C from the dequenching of pyrene fluorescence at 378 nm (excitation at 342 nm, slit widths 5.0 nm each) (the initial linear increase in fluorescence intensity after addition of the lipase was used to measure enzyme activity). The measurements were performed in ethanol/water (1:3, v/v) as a solvent.

2.22. Inhibition experiments

Triton X-100 specially purified for membrane research (Hofmann La Roche) (10 μl of a 100 mM stock solution in chloroform) and the appropriate volume of a phosphonate solution in chloroform were mixed and the solvent was removed under a stream of nitrogen. The residue was dispersed in an 100 μM Tris–HCl buffer (pH 7.40 at 37 °C) by vigorous vortexing followed by addition of an aliquot of a lipase solution, giving a total volume of 500 μl. Incubations were performed at 4 °C. The percentage of inhibition after 16 h in the reaction mixture was determined by measuring the residual lipase activity of appropriate aliquots (3 μl) as indicated above. Controls were run in parallel under identical conditions but in the absence of inhibitors.

2.23. Spectroscopic analyses

A molar absorbance coefficient of 22,000 M^{-1} cm^{-1} at 466 nm in methanol or 29,000 M^{-1} cm^{-1} at 442 nm in ethanol for phosphonate inhibitors labeled with NBD or perylene, respectively was used to determine the concentration of the fluorescent phosphonates (measurements were performed on a Hitachi U-3210 spectrophotometer) (Haugland, 1996). Fluorescence spectra of both perylene and NBD-labelled compounds in ethanol were recorded using a Shimadzu RF-5301PC spectrofluorophotometer in ethanol.

3. Results and discussion

3.1. Chemical synthesis

Eight novel phosphonic acid esters fluorescently labeled with perylenebutanoic, perylene octanoic, NBD-hexanoic or NBD-dodecanoic acid were synthesized. Perylene and NBD-labels were chosen because of the longer wavelength absorption and emission as compared to pyrene-containing inhibitors, which have been synthesized and characterized previously (Zandonella et al., 1999).

For the preparation of perylene-labeled lipase inhibitors, the corresponding fluorescently labeled perylene fatty acids were needed. Friedel–Crafts acylation of perylene with appropriate dicarbonic acid monomethyl ester chlorides gave perylenoyl propanoic acid methyl ester and perylenoyl heptanoic acid methyl ester. It is known that perylene is only acylated at C-3 position under these reaction conditions (Zinke et al., 1940). The ^{1}H NMR spectra of the synthesized perylenoyl propanoic and perylenoyl heptanoic acid esters (protons of the alkyl and aromatic moieties) confirmed the indicated structures of the acylation products. Kizhner–Wolf reduction of the keto-groups and the concomitant deblocking of the methyl protective groups gave perylenebutanoic and peryleneoctanoic acids, respectively. Proton NMR spectra were in agreement with the ascribed structures.

The synthetic routes leading to the fluorescently labeled triacylglycerol analogues are outlined in Fig. 1. The respective compounds are alkylacyl(amino)deoxyglycerophosphonic acids and alkyloxyphosphonic acid derivatives. Three major crucial steps are involved in their synthesis that are: (1) attachment of a fluorescent acyl chain to 1(3)-*O*-alkyl-2-aminodeoxy-3(1)-*O*-trityl-*sn*-glycerol, (2) detritylation, and (3) phosphorylation of the resultant alkylacyl(amino)deoxyglycerols. In principle, the acylation of an amino group can be performed using acid anhydride (Singh and Schmidt, 1989), acyl-halides (Zimmerman et al., 1988; Dijkman et al., 1990), mixed carbodiimide (Hammarstroem, 1971), or mixed anhydrides formed by ethylchloroformate and a fatty acid (Acquotti et al., 1986). These procedures have several disadvantages especially because they require a large molar excess of acylating reagents and long reaction times. The use of acyl chlorides was not convenient in our case because of the possible modification of fatty acid derivatives during acylation. Activated fatty acid esters (for example, *p*-nitrophenyl esters) have also successfully been applied, e.g. for sphingolipid synthesis (Tkaczuk and Thornton, 1981; Groenberg et al., 1991; Kann et al., 1991; Shibuya et al., 1992). However, acylation with

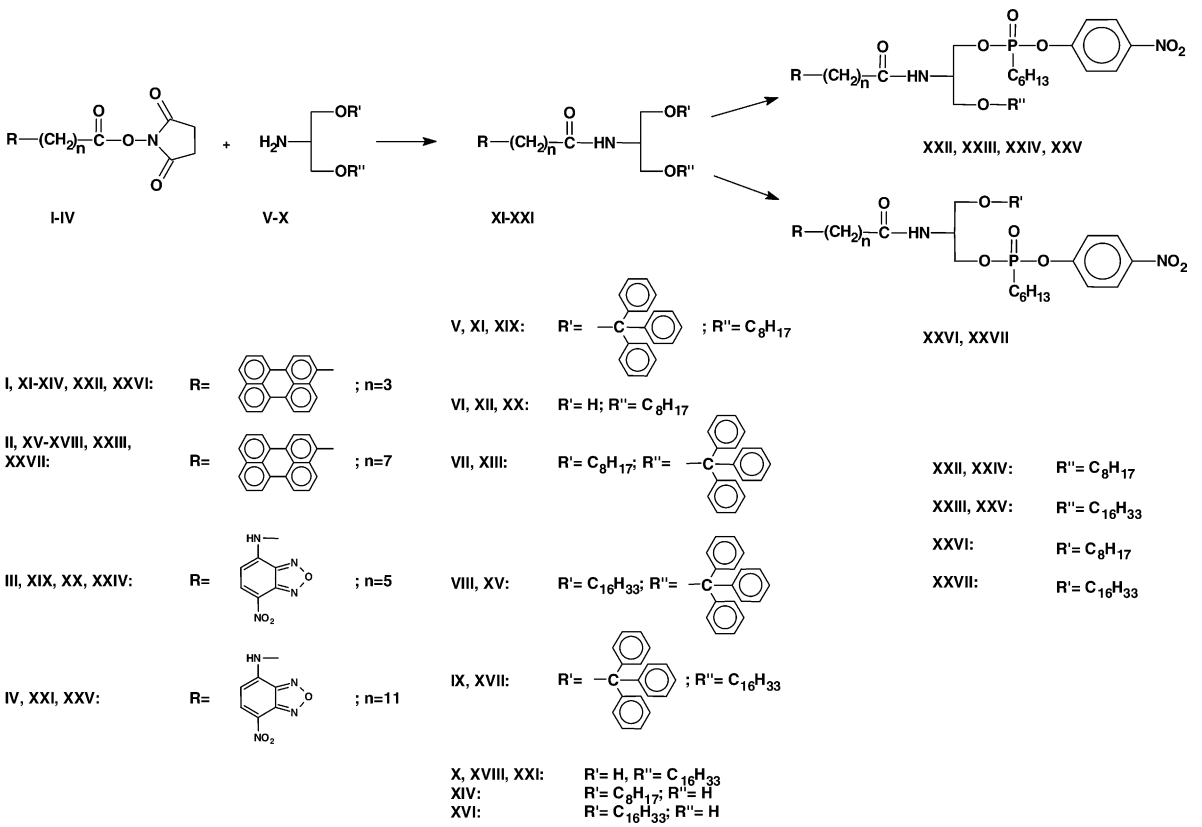


Fig. 1. Synthetic routes of fluorescently labeled organophosphonates.

p-nitrophenyl esters of perylenebutanoic and perylene octanoic acids may give only low yields of acylation products probably because of the close vicinity of a bulky fluorophore and the ester group (Oskolkova et al., 1999). Thus, we used *N*-hydroxysuccinimide esters of fluorescently labeled fatty acids to acylate selectively amino groups in the presence of free hydroxy groups with high yields (Julina et al., 1986). In the present study, this simple method gave high yields of acylation products (e.g. 91.0% yield of (**XII**) when 2-amino-2-deoxy-3-*O*-octyl-*sn*-glycerol (**VI**) was acylated with perylenebutanoic acid succinimide ester).

The protective trityl group of the labeled alkylacylaminodeoxytritylglycerols was removed under standard conditions as described for the synthesis of diradylglycerols (Hermetter et al., 1989) using boron trifluoride–methanol. The obtained fluorescent alkylacylaminodeoxyglycerols (**XI**–**XVIII**) showed fluorescence spectra typical for perylene-labeled compounds (Johanson et al., 1987).

Two further problems are encountered in the synthesis of fluorescent phosphonate inhibitors. Reaction yields of phosphorylation of glycerolipids tend to be low. Secondly the fluorescently labeled fatty acids and, as a consequence, the fluorescently labeled glycerolipid intermediates are expensive and that is one special reason, why yields should be as high as possible. The diradylglycerols were reacted with alkylphosphonic acid dichloride in the presence of *N*-methylmorpholine as a base followed by substitution of the second chloro atom at phosphorus by *p*-nitrophenol. Although these two steps were carried out consecutively in an one-pot reaction, product yields were rather low (5.7% for 2-deoxy-2-(4-perylenylbutanoyl)amino-1-*O*-octyl-3-*O*-hexylphosphonate (**XXVI**) or 6.3% for 2-deoxy-2-(8-perylenyloctanoyl)amino-3-*O*-hexadecyl-1-*O*-hexylphosphonate

(**XXIII**)) most likely because of the close vicinity of the amido group to the phosphoric acid moiety, which as a consequence, can lead to an intramolecular cyclic side-product (oxazaphospholane) in considerable amounts. Such compounds can in fact be prepared from 2-deoxy-2-amino-glycerophosphocholine in the presence of phosphoroxytrichloride (Deigner and Fyrnys, 1992). To avoid formation of such compounds we used a modification of the phosphorylation procedure as described by Zhao and Landry (1993) for the synthesis of phosphonate esters. This approach used tetrazole as a catalyst and has already been shown to be successful for the synthesis of phosphonates from sterically hindered alcohols such as menthol and testosterone (Zhao and Landry, 1993) or for the synthesis of organophosphonate esters (Rotticci et al., 2000). In our case, the yields of products were significantly improved when tetrazole was used as a catalyst (e.g. yields of 2-deoxy-2-(8-peryleneoctanoyl)amino-3-*O*-hexadecyl-1-*O*-hexylphosphonate (**XXIII**) were 6.3% in the absence of tetrazole and 31.6% in its presence, Table 1). For the other glycerolipid phosphonate analogues, yields were above 25% if synthesized by the tetrazole-catalyzed reaction (except of phosphonate **XXV**).

As a second class of lipase inhibitors, phosphonates containing single-chain alkoxy groups instead of glycerolipids were prepared as follows (Fig. 2). Perylenebutanoic acid was first reduced to perylenebutanol (**XXVIII**) by reaction with Red-Al. Phosphorylation of the alcohol (**XXVIII**) in the absence of tetrazole gave perylenebutyl methylphosphonate (**XXIX**) with high yield (57.6%). NBD-hexanoic acid succinimide ester reacted with excess aminoethanol yielding derivative (**XXX**). Phosphorylation of the latter compound gave phosphonate (**XXXI**) with 11.1% yield.

The proton signals in the ^{1}H NMR-spectrum of phosphonate (**XXII**) were consistent with the indicated molecular structure. Since the phosphorylation procedures for all glycerolipids in this study were identical, we present here only one proton NMR spectrum for the latter compound. UV and fluorescence spectra of all perylene-labeled organophosphorus compounds showed identical λ_{max} which are consistent with their assumed chemical structures. The perylene- (**XXII**, **XXIII**, **XXVI**, **XXVII**, **XXIX**) and NBD-phosphonates (**XXIV**, **XXV**, **XXXI**) had fluorescence maxima at 448 and 533 nm in ethanol typical for perylene (Haugland, 1996) and NBD containing derivatives (Nicols and Pagano, 1982), respectively. The ratio of fluorescent label and phosphorus content of all phosphonates was about 1–1.15, additionally confirming the structures of the compounds obtained. Moreover, in ESI mass-spectra of the compounds (**XXII**, **XXVI**, **XXIII**, **XXVII**, **XXXI**, **XXIV**, **XXV**) only signals corresponding to their molecular

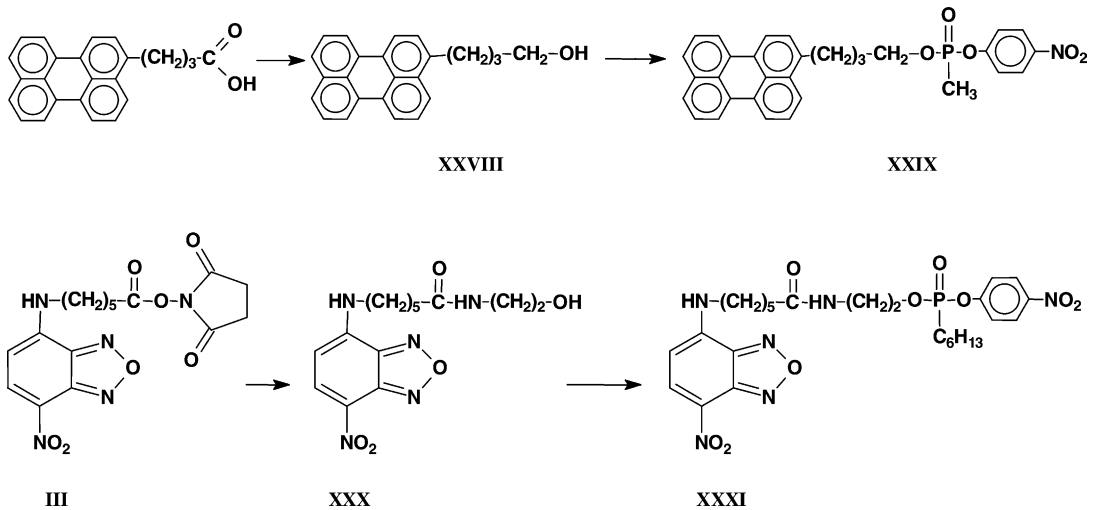
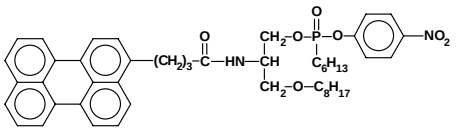
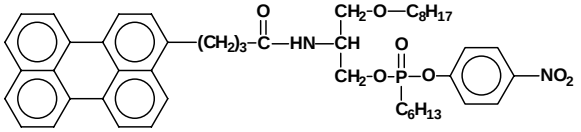
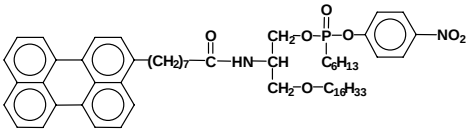
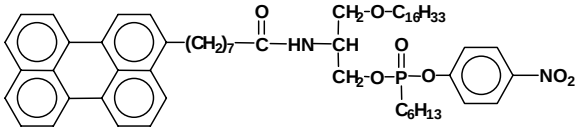
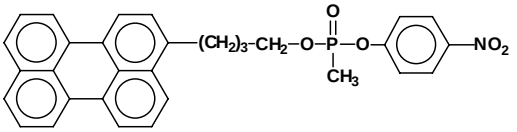
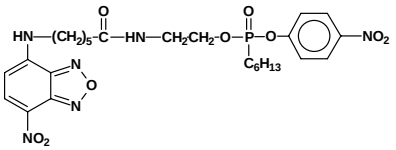
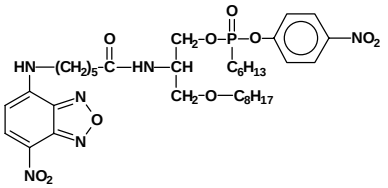
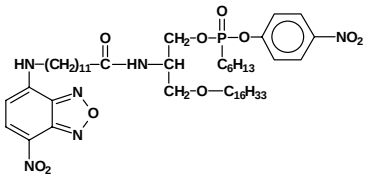


Fig. 2. Synthesis of one-chain perylene- and NBD-inhibitors.

Table 1
Comparison of chromatographic characteristics of synthetic fluorescent phosphonates and their yields after phosphorylation

Compounds	Number	Chromatographic behavior (R_f)	Yield of phosphorylation (%)	
			Without catalyst	With tetrazole as catalyst
	**XXII**	0.33[a]	–	43.3
	**XXVI**	0.33[a]	5.7	–
	**XXIII**	0.46[a]	6.3	31.6
	**XXVII**	0.46[a]	–	26.1
	**XXIX**	0.59[b]	57.6	–
	**XXXI**	0.22[c]	–	11.1
	**XXIV**	0.33[c]	–	27.8
	**XXV**	0.55[c]	–	4.4

Solvents for TLC: (a) chloroform/methanol/acetone, 10:0.2:0.2 (v/v/v); (b) ether/tetrahydrofurane, 2:1 (v/v) or (c) chloroform/methanol/acetone, 10:0.5:0.5 (v/v/v).

structures were observed (Table 2). Only in the case of phosphonate (**XXIX**) it was impossible to obtain a useful mass-spectrum.

3.2. Interaction of inhibitors with lipolytic enzymes

Di-*O*-alkylglycero-phosphonates have already been reported as useful inhibitors of lipase activity (Stadler et al., 1996; Zandonella et al., 1999). However, it was still an open question, whether microbial lipases would react with fluorescent phosphonate inhibitors containing amide bonds.

Thus, the novel compounds were tested with respect to their ability to inhibit three selected microbial lipases with different substrate preferences. The lipase from *R. oryzae* was efficiently inactivated by the perylene- and NBD-inhibitors (Fig. 3) at 4 °C for 16 h in 100 μM Tris–HCl buffer at inhibitor and lipase concentrations of 1.0 and 0.1 mM, respectively.

The two-chain phosphonate (**XXII**) was a much more potent inhibitor of the lipase from *P. cepacia* than the other inhibitors used (Fig. 3). In contrast, the *Pseudomonas species* lipase was quantitatively inactivated by all synthesized organophosphonates (Fig. 3).

Lipases may show very different steric constraints around and within their active site, and as a consequence, very different substrate and inhibitor preferences (Pleiss et al., 1998). The lipases ROL, PCL and PSL, which had been chosen for the inhibition experiments with organophosphonates, are typical examples for this structural diversity among highly homologous enzymes. Accordingly, they show different patterns of reactivity not only towards lipid substrates, but also to structurally related inhibitors as demonstrated in this study. Thus, the novel phosphonates might be useful tools not only for analysis and discrimination of known lipolytic enzymes but also for discovery of yet unknown lipases/esterases in biological samples.

As an alternative to water-soluble lipase monomers used in industry, cross-linked enzyme crystals (CLECs) (Khalaf et al., 1996; Lalonde et al., 1995; St. Clair and Navia, 1992) have been introduced to increase enzyme activity (Zelinski and Waldmann, 1997) and stability in organic solvents (Persichetti et al., 1995). This makes them useful catalysts which are easy to separate from the reaction mixture and can be repeatedly used after subsequent filtration and washing. Preliminary experiments have shown that

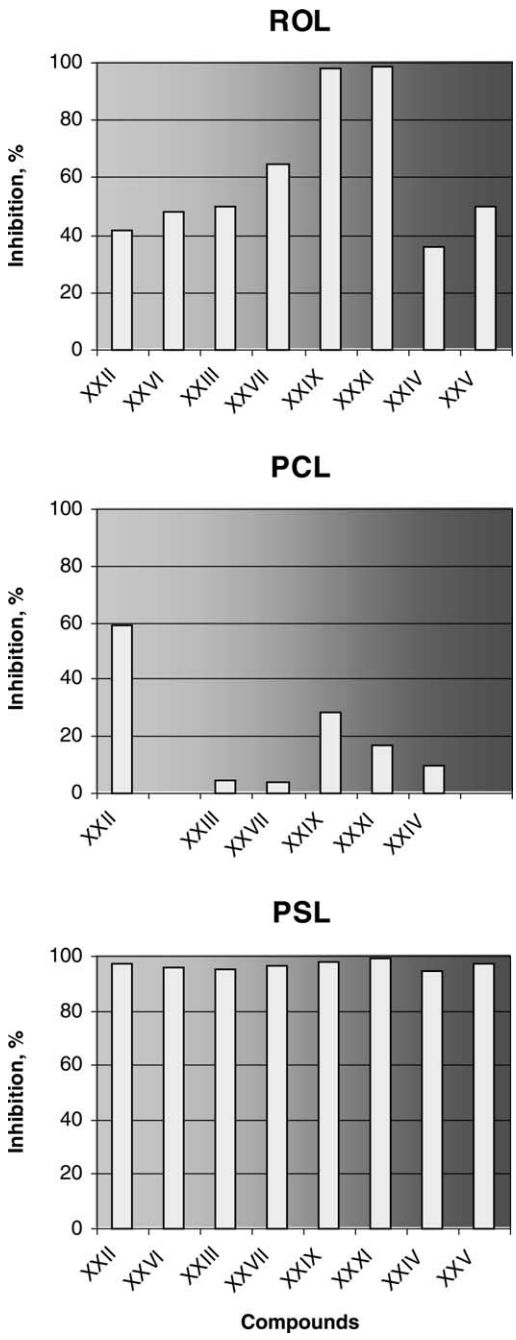


Fig. 3. Inhibition of microbial lipases from *Rhizopus oryzae* (ROL), *Pseudomonas cepacia* (PCL), and *Pseudomonas species* (PSL). Experimental conditions are described in Section 2. Values are percentages of inhibition of the indicated lipase in comparison to enzyme activity without inhibitor. Inactivation of PCL by organophosphonates **XXVI** and **XXV** was not determined.

Table 2
Results of mass-spectrometry analysis of organophosphonates. Mass-spectra were recorded as described in Section 2

Compound number	Formula	Molecular weight	Ions observed	m/z_{theor}	$m/z_{observed}$
XXII	$C_{47}H_{57}N_2O_7P$	792.39	$[C_{47}H_{57}N_2O_7P{\cdot}Cs]^+$	925.30	925.4
XXVI	$C_{47}H_{57}N_2O_7P$	792.39	$[C_{47}H_{57}N_2O_7P{\cdot}Cs]^+$	925.30	925.3
XXIII	$C_{59}H_{81}N_2O_7P$	960.58	$[C_{59}H_{81}N_2O_7P{\cdot}Cs]^+$	1093.48	1093.4
XXVII	$C_{59}H_{81}N_2O_7P$	960.58	$[C_{59}H_{81}N_2O_7P{\cdot}Cs]^+$	1093.48	1093.4
XXIX	$C_{31}H_{26}NO_5P$	523.15	n.d.		
XXXI	$C_{26}H_{35}N_6O_9P$	606.22	$[C_{26}H_{35}N_6O_9P{\cdot}Cs]^+$	739.13	739.3
XXIV	$C_{35}H_{53}N_6O_{10}P$	748.36	$[C_{35}H_{53}N_6O_{10}P{\cdot}Cs]^+$	881.26	881.3
XXV	$C_{49}H_{81}N_6O_{10}P$	944.58	$[C_{49}H_{81}N_6O_{10}P{\cdot}Cs]^+$	1077.48	1077.3

n.d.: not detectable.

the novel inhibitors described in this work might also be useful to characterize the functional quality of these systems which is otherwise difficult to determine. Work is in progress to characterize activity properties of CLECs in three dimensions using the fluorescent inhibitors.

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