Available online at www.sciencedirect.com
ScienceDirect
Chemistry and Physics of Lipids 144 (2006) 60–68
CPL
CHEMISTRY AND PHYSICS OF LIPIDS
www.elsevier.com/locate/chemphyslip

A versatile library of activity-based probes for fluorescence detection and/or affinity isolation of lipolytic enzymes

Heidrun Susani-Etzerodt, Hannes Schmidinger, Gernot Riesenhuber, Ruth Birner-Gruenberger, Albin Hermetter*

Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria

Received 2 February 2006; received in revised form 21 June 2006; accepted 28 June 2006
Available online 21 July 2006

Abstract

This work describes the synthesis of a library of fluorescent and/or biotinylated alkylphosphonate inhibitors being reactive towards serine hydrolases, especially lipases and esterases. Fluorescent inhibitors can be used for sensitive and rapid detection of active proteins by gel electrophoresis. Biotinylated inhibitors are applicable for the enrichment and isolation of active enzymes. Functionality as well as the different detection methods of the synthesized inhibitors were successfully tested with an enzyme preparation, namely cholesterol esterase from porcine pancreas (ppCE). Moreover, a biotinylated inhibitor was employed to enrich ppCE on avidin beads. Hence, our set of phosphonate inhibitors can be used for the detection and/or isolation of active serine hydrolases.

Keywords: Activity-based enzyme probes; Alkylphosphonic acid ester; Biotin-labeled inhibitors; Cholesterol esterase; Fluorescent lipids; Serine hydrolases

1. Introduction

Specific and even less-specific inhibitors are useful tools in enzyme biochemistry and biophysics provided the mechanisms underlying enzyme function are known (Campbell and Szardenings, 2003). In this context, *p*-nitrophenyl or fluoroalkylphosphonic acid esters turned out to be useful suicide inhibitors of serine hydrolases, a large and diverse family which comprises numerous lipases, esterases and proteases. These inhibitors have been applied to investigate enzyme–lipid interactions with respect to substrate- and stereoselectivity (Berman et al., 1985; Brzozowski et al., 1991; Egloff et al., 1995; Hamilton et al., 1998; Mannesse et al., 1995; Taylor et al., 2001) and have been established as powerful probes for enzyme analytics.

The reaction of alkylphosphonic acid esters with serine hydrolases depends on the activities of these enzymes. The mechanism comprises the nucleophilic attack of the active serine (which is part of the catalytic triad Ser-His-Asp/Glu) on the phosphorus of the inhibitor and the simultaneous release of the *p*-nitrophenyl residue which is a very efficient leaving group. The serine remains covalently and irreversibly bound to the inhibitor, mimicking the first (tetrahedral) transition state of carboxylic acid ester hydrolysis (Bjorkling et al., 1994; Cygler et al., 1994; Moreau et al., 1991; Ransac et al., 1997).

One bioanalytical technique using these activity-sensing probes is based on the measurement of *p*-nitrophenol release from *p*-nitrophenyl esters due to

* Corresponding author. Tel.: +43 3168736457; fax: +43 3168736952.
E-mail address: albin.hermetter@tugraz.at (A. Hermetter).

0009-3084/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.chemphyslip.2006.06.018

nucleophilic substitution by the active serine of the enzyme (Rotticci et al., 2000; Scheib et al., 1998; Stadler et al., 1996). However, large amounts of enzyme are required to generate measurable quantities of the analyte.

To overcome this limitation, much higher sensitivity can be gained by fluorescence labeling of the inhibitor. Fluorescent phosphonate inhibitors form covalent and stoichiometric (1/1, mol/mol) complexes even with low abundant enzymes in complex samples, that can easily be visualized after separation by one- or two-dimensional gel electrophoresis. Therefore, these probes are important tools for functional proteomic research.

Biotin tagging of inhibitors is the method of choice for detection of several proteins (i.e. very large and small proteins, highly basic and acidic proteins and membrane proteins, which are unsuitable for 2-D electrophoresis) (Adam et al., 2001). Biotinylated probes allow enrichment and purification of such proteins using avidin chromatography procedures.

Here, we report on a set of fluorescently labeled *p*-nitrophenyl phosphonate probes for the rapid and sensitive in-gel visualization (Birner-Gruenberger et al., 2005; Oskolkova et al., 2003; Schmidinger et al., 2005; Scholze et al., 1999) as well as a set of biotinylated inhibitors applicable for affinity isolation (Adam et al., 2001; Deussen et al., 2000; Kidd et al., 2003; Liu et al., 1999) of enzyme–inhibitor complexes. In addition to their tags (fluorophore or biotin) the phosphonates also differ in their alcohol moieties (see insert to Fig. 2) which mimic the structures of natural lipase and esterase substrates and are therefore responsible for inhibitor specificity towards various types of enzymes. This work gives an overview of the respective lipase and esterase inhibitors including known compounds and the novel probes (**3b** and **c**, **4a–f**, **5d–f**) which are described in detail.

2. Materials and methods

2.1. Inhibitor syntheses

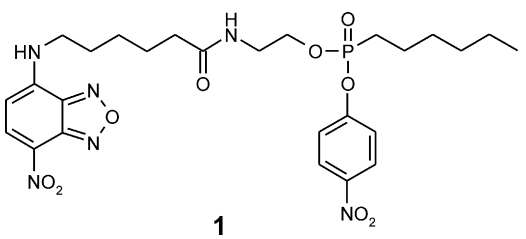
Chemicals for lipid synthesis were purchased from commercial suppliers and used without further purification unless otherwise stated. Triethylamine was freshly distilled over calcium hydride. TLC was carried out on silica gel 60 F_{254} aluminium sheets (0.2 mm, MERCK, Whitehouse Station, NJ, USA). The compounds were visualized under UV light (360 nm), with *p*-dimethylaminocinnamaldehyde (DMACA) or by charring at 120 °C after spraying with 50% sulfuric acid. Phosphorus containing compounds were detected after spraying with phosphomolybdic acid. Flash chromatography was performed on silica gel 60 (230–400 mesh, MERCK). NMR spectra were recorded using a VARIAN INOVA-500 spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts (δ) are given in parts per million (ppm) using tetramethylsilane as standard. Multiplicities are abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; bs, broad signal. Mass spectra were obtained using a MALDI micro MX^{TM} (Waters, Milford, MA, USA) with a nitrogen UV laser (337 nm wavelength) and a time-lag focusing unit. Reflectron mode was positive, source voltage 15 kV and pulse voltage were 1950 V. Matrix suppression was 600 Da and sampling rate was 0.5 ns. Calibration was performed with a suitable polyethylene glycol mixture, as well as with Bradykinin, Angiotensin I, Substance-P or Glu-fibrino peptide as internal standards. Samples were prepared by mixing solutions of matrix (10 mg/mL α-cyano-4-hydroxy cinnamic acid in EtOH/acetonitrile/aqueous 0.1% TFA 495/495/10, v/v/v) and analyte (0.01–1 mg/mL in $CHCl_3$/MeOH 2/1, v/v) at the ratio 10:1 (v/v). The mixture (0.5 μL) was spotted on a target plate (stainless steel) and allowed to air-drying prior to analysis.

2.2. Synthesis of NBD-HE-HP (*1*)

NBD-HE-HP (**1**) was synthesized as published (Oskolkova et al., 2003; Schmidinger et al., 2005) (Fig. 1).

2.3. Synthesis of inhibitor groups *3*, *4* and *5*

Fig. 2 depicts the general procedure for the preparation of inhibitor groups **3**, **4** and **5** following published procedures (Deussen et al., 2000; Reetz et al., 2002; Schmidinger et al., 2005). To a solution of 62 mg (0.1 mmol) of compound **2a** (Reetz et al., 2002) in 1 mL absolute dichloromethane 40 μL (0.3 mmol) bromotrimethylsilane were added. Stirred at room temperature for 40 h. After removal of all volatile components under reduced pressure the orange residue



1

Fig. 1. Structure of NBD-HE-HP (**1**).

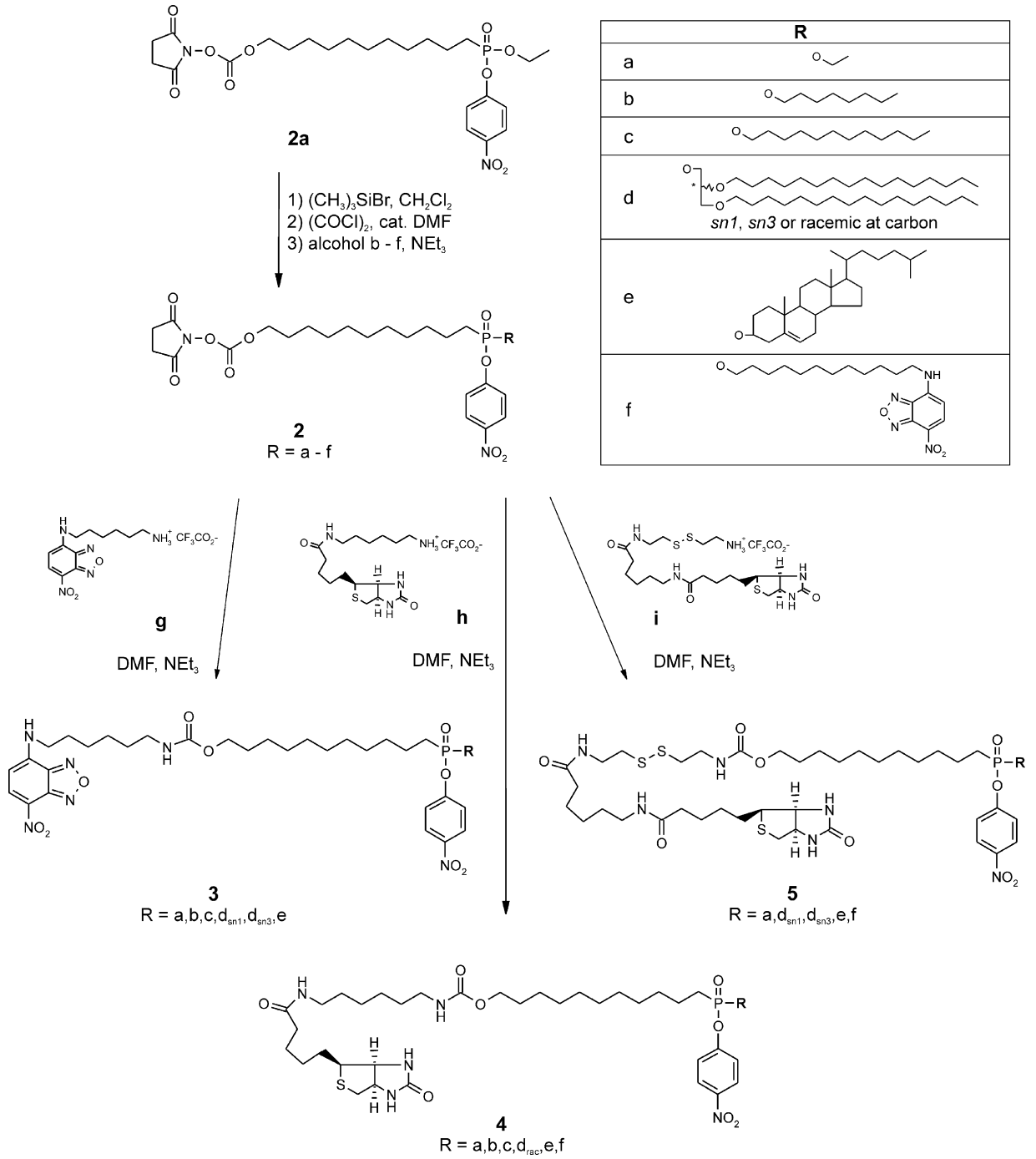


Fig. 2. Synthetic routes for the preparation of inhibitor groups **3**–**5**. For substituents **R** (a–f) see insert.

was dissolved in a mixture of 1 mL dichloromethane and 26 μL (0.3 mmol) oxalyl dichloride followed by addition of 1.5 μL (0.02 mmol) DMF. This mixture was heated under reflux for 18 h. The volatile components were removed, and the residue was dissolved in 1 mL CH_2Cl_2. To this solution 0.1 mmol of the alcohol components **b**–**f** (see insert to Fig. 2) and 18 μL (0.3 mmol) of triethylamine were added and the mixture was stirred at room temperature for 8 h. After evaporation of the solvent, the intermediate compounds **2b**–**f** were purified by flash chromatography on 25 mL swollen silica gel.

2b: yellowish oil; yield 69%; $R_f = 0.3$ in CH_2Cl_2/EtOAc 8/1 (v/v); **2c**: yellowish oil, yield 68%, $R_f = 0.4$ in CH_2Cl_2/EtOAc 8/1 (v/v); $\mathbf{2d}_{sn1}$: yellowish oil, yield 50%, $R_f = 0.3$ in CH_2Cl_2/EtOAc 94/6 (v/v); $\mathbf{2d}_{sn3}$: equivalent to $\mathbf{2d}_{sn1}$; **2e**: yellowish oil, yield 52%, $R_f = 0.4$ in cyclohexane/$CHCl_3$ 3/1 (v/v); **2f**: orange oil, yield 21%; $R_f = 0.4$ in $CHCl_3$/EtOAc 88/12 (v/v).

The purified intermediates **2a**–**f** (0.03 mmol) were dissolved in 1.5 mL dry DMF, respectively, and 0.03 mmol **g**, **h** or **i** (Schmidinger et al., 2005; Vanwetswinkel et al., 1995) and 13 μL (0.09 mmol) triethylamine were added. The mixture was stirred at room temperature for 2 h. After removal of the solvent in vacuo the products (inhibitor groups **3**, **4** and **5**) were purified by flash chromatography on 20 mL swollen silica gel. Yields and solvents for flash chromatography as well as MS and NMR data are only indicated for compounds which have not been characterized yet.

3b: orange oil; yield 63%; $R_f = 0.5$ in $CHCl_3$/EtOAc 3/1 (v/v); MS: $m/z = 813.873$, $C_{38}H_{59}N_6O_{10}P + Na^+$ requires 813.873; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.43$ (d, $J = 8.6$ Hz, 1H, Ar), 8.16 (d, $J = 9.2$ Hz, 2H, Ar), 7.32 (d, $J = 9.2$ Hz, 2H, Ar), 6.46 (bs, 1H, NH), 6.11 (d, $J = 8.7$ Hz, 1H, Ar), 4.62 (bs, 1H, NH), 4.13 − 4.06 (m, 1H), 4.04 − 3.95 (m, 3H), 3.43 (q, $J = 5.8$ and 5.7 Hz, 2H), 3.13 (q, $J = 6.6$ and 6.6 Hz, 2H), 1.91 − 1.83 (m, 2H), 1.78 − 1.14 (m, 38H), 0.81 (t, $J = 6.8$ Hz, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 157.2$, 156.1, 144.7, 144.5, 144.2, 136.7, 125.9, 121.1, 98.7, 67.1, 65.3, 43.9, 40.7, 32.0, 30.6, 30.5, 30.2, 29.7, 29.6, 29.5, 29.4, 29.2, 28.5, 26.7, 26.4, 26.2, 26.0, 25.7, 25.6, 22.9, 22.4, 14.3.

3c: orange oil; yield 75%; $R_f = 0.5$ in $CHCl_3$/EtOAc 3/1 (v/v); MS: $m/z = 869.979$, $C_{42}H_{67}N_6O_{10}P + Na^+$ requires 869.979.

4a: yellowish oil; yield 41%; $R_f = 0.6$ in $CHCl_3$/MeOH 7/1 (v/v); MS: $m/z = 792.920$, $C_{36}H_{60}N_5O_9PS + Na^+$ requires 792.919; ^{1}H NMR (500 MHz, DMSO): $\delta = 8.27$ (d, $J = 9.1$ Hz, 2H, Ar), 7.73 (bs, 1H, NH), 7.45 (d, $J = 9.0$ Hz, 2H, Ar), 7.01 (bs, 1H, NH), 6.41 (bs, 1H, NH), 6.35 (bs, 1H, NH), 4.29 (t, $J = 5.1$ Hz, 1H, CH biotin), 4.18 − 4.09 (m, 3H), 3.88 (t, $J = 6.6$ Hz, 2H), 3.08 (m, 1H), 2.98 (q, $J = 6.7$ and 6.1 Hz, 2H), 2.92 (q, $J = 6.6$ and 6.4 Hz, 2H), 2.80 (q, $J = 6.7$ and 6.1 Hz, 1 H), 2.56 (d, $J = 12.5$ Hz, 1H), 2.02 − 1.95 (m, 4H), 1.62 − 1.15 (m, 35H); ^{13}C NMR (125 MHz, DMSO): $\delta = 172.5$, 163.4, 157.5, 156.2, 144.8, 126.6, 122.1, 64.2, 63.2, 61.7, 59.9, 56.2, 40.0, 39.0, 36.0, 30.3, 30.2, 30.1, 29.8, 29.6, 29.4, 29.1, 28.9, 28.7, 26.8, 26.7, 26.1, 24.9, 22.5, 16.9.

4b: yellowish oil; yield 32%; $R_f = 0.5$ in $CHCl_3$/MeOH 8/1 (v/v); MS: $m/z = 877.079$, $C_{42}H_{72}N_5O_9PS + Na^+$ requires 877.079; ^{1}H NMR (500 MHz, CD_3OD): $\delta = 8.31$ (d, $J = 9.2$ Hz, 2H, Ar), 7.45 (d, $J = 8.4$ Hz, 2H, Ar), 4.62 (bs, 1H, NH), 4.49 (q, $J = 4.9$ and 3.0 Hz, 1H, CH–CH_2 biotin), 4.30 (q, $J = 4.5$ and 3.4 Hz, 1H, CH biotin), 4.22 − 4.10 (m, 2H), 4.05 (bs, 1H, NH), 4.00 (t, $J = 6.6$ Hz, 2H, decyl-CH_2–O), 3.23 − 3.13 (m, 3H), 3.08 (t, $J = 7.0$ Hz, 2H), 2.93 (q, $J = 5.0$ and 12.7 Hz, 1H), 2.71 (d, $J = 12.7$ Hz, 1H), 2.20 (t, $J = 7.3$ Hz, 2H), 2.06 (m, 2H), 1.78 − 1.22 (m, 46H), 0.89 (t, $J = 6.8$ Hz, 3H, CH_3); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 174.8$, 164.9, 158.2, 155.6, 145.1, 125.7, 121.4, 67.3, 64.6, 62.2, 60.5, 55.9, 40.4, 39.9, 39.1, 35.7, 31.8, 30.3, 30.2, 30.1, 29.7, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 28.6, 28.3, 26.5, 26.3, 25.8, 25.6, 25.4, 24.5, 22.5, 22.2, 22.1, 13.3.

4c: yellowish oil; yield 49%; $R_f = 0.5$ in $CHCl_3$/MeOH 9/1 (v/v); MS: $m/z = 933.180$, $C_{46}H_{80}N_5O_9PS + Na^+$ requires 933.185; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.24$ (d, $J = 9.2$ Hz, 2H, Ar), 7.39 (d, $J = 7.3$ Hz, 2H, Ar), 5.91 (bs, 1H, NH), 5.76 (bs, 1H, NH), 5.04 (bs, 1H, NH), 4.80 (bs, 1H, NH), 4.52 (t, $J = 5.5$ Hz, 1H, CH–CH_2 biotin), 4.33 (t, $J = 5.9$ Hz, 1H, CH biotin), 4.20 − 4.12 (m, 1H), 4.11 − 4.00 (m, 3H), 3.23 (q, $J = 6.6$ and 6.2 Hz, 2H), 3.19 − 3.11 (s, 3H), 2.93 (q, $J = 5.0$ and 7.7 Hz, 1H), 2.74 (d, $J = 12.9$ Hz, 1 H), 2.25 − 2.17 (m, 2H), 1.98 − 1.89 (m, 2H), 1.80 − 1.21 (m, 52H), 0.89 (t, $J = 6.8$ Hz, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 173.2$, 163.5, 156.2, 125.9, 121.2, 120.5, 67.2, 65.2, 62.1, 60.4, 40.9, 39.4, 32.2, 30.6, 30.1, 29.9, 29.8, 29.7, 29.6, 29.5, 29.3, 28.3, 26.8, 26.1, 25.7, 22.9, 14.4.

$\mathbf{4d}_{rac}$: white solid; yield 36%; $R_f = 0.5$ in $CHCl_3$/MeOH 10/1 (v/v); MS: $m/z = 1287.769$, $C_{69}H_{126}N_5O_{11}PS + Na^+$ requires 1287.795; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.23$ (d, $J = 9.1$ Hz, 2H, Ar), 7.40 (q, $J = 4.6$ and 4.2 Hz, 2H, Ar), 5.96 (bs, 1H, NH), 5.86 (bs, 1H, NH), 5.10 (bs, 1H, NH), 4.81 (bs, 1H, NH), 4.52 (t, $J = 7.2$ Hz, 1H, CH–CH_2 biotin), 4.33 (t, $J = 5.6$ Hz, 1H, CH biotin), 4.22 − 4.15 (m, 1H), 4.10 − 4.00 (m, 3H), 3.61 − 3.38 (m, 8H), 3.22 (m, 2H), 3.16 (m, 3H), 2.92 (q, $J = 4.9$ and 7.9 Hz, 1H), 2.74 (d, $J = 12.9$ Hz, 1H), 2.23 − 2.18 (m, 2H), 2.01 − 1.92 (m, 2H), 1.77 − 1.18 (m, 87H), 0.88 (t, $J = 6.8$ Hz, 6H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 173.3$, 163.7, 156.0, 144.7, 125.9, 121.3, 72.1, 71.0, 69.6, 66.4, 65.2, 62.0, 60.3, 55.7, 40.8, 39.5, 36.2, 32.2, 30.8, 30.7, 30.2, 30.1, 30.0, 29.9, 29.8, 29.7, 29.6, 29.3, 28.3, 26.7, 26.5, 26.3, 26.1, 25.8, 25.6, 25.0, 22.9, 22.5, 22.4, 14.4.

4e: white solid; yield 37%; $R_f = 0.5$ in $CHCl_3$/MeOH 10/1 (v/v); MS: $m/z = 1133.504$, $C_{61}H_{100}N_5O_9PS + Na^+$ requires 1133.505; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.23$ (m, 2H, Ar), 7.39 (m, 2H, Ar), 6.23 (bs, 1H,

NH), 6.13 (bs, 1H, NH), 5.37 (bs, 1H, C=CH–CH_2 chol), 5.29 (m, 1H), 4.86 (bs, 1H, NH), 4.51 (t, J = 5.1 Hz, 1H, CH–CH_2 biotin), 4.39 – 4.30 (m, 2H), 4.02 (t, J = 6.7 Hz, 2H, decyl-CH_2–O), 3.22 (d, J = 6.5 Hz, 2H), 3.15 (q, J = 7.2 and 4.8 Hz, 3H), 2.91 (q, J = 4.9 and 8.0 Hz, 1H), 2.74 (d, J = 12.8 Hz, 1H), 2.46 – 2.35 (m, 1H), 2.21 (t, J = 7.5 Hz, 2H), 2.02 – 1.03 (m, 61H), 1.00 (s, 3H), 0.90 (d, J = 6.5 Hz, 3H), 0.86 (q, J = 2.3 and 4.3 Hz, 6H), 0.66 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.4, 164.0, 157.2, 156.2, 144.6, 139.3, 125.9, 123.6, 121.3, 77.9, 65.1, 62.0, 60.4, 56.8, 56.4, 55.8, 50.2, 42.5, 40.9, 40.8, 40.5, 39.9, 39.7, 39.4, 37.1, 36.6, 36.4, 36.2, 36.0, 32.1, 32.0, 30.7, 30.6, 30.2, 30.1, 29.9, 29.7, 29.6, 29.5, 29.3, 29.2, 28.4, 28.3, 28.2, 27.4, 23.1, 22.8, 22.6, 22.5, 21.2, 19.5, 18.9, 12.1.

4f: orange oil; yield 37%; R_f = 0.4 in $CHCl_3$/MeOH 10/1 (v/v); MS: m/z = 1111.311, $C_{52}H_{82}N_9O_{12}PS + Na^+$ requires 1111.290; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.53 (d, J = 8.9 Hz, 1H, Ar), 8.29 (d, J = 9.2 Hz, 2H, Ar), 7.44 (d, J = 9.1 Hz, 2H, Ar), 6.36 (d, J = 8.9 Hz, 1H, Ar), 4.62 (bs, 1H, NH), 4.49 (q, J = 4.9 and 2.9 Hz, 1H, CH–CH_2 biotin), 4.30 (q, J = 4.5 and 3.4 Hz, 1H, CH biotin), 4.21 – 4.09 (m, 2H), 4.04 (bs, 1H, NH), 3.99 (t, J = 6.6 Hz, 2H decyl-CH_2–O), 3.53 (bs, 2H), 3.22 – 3.14 (m, 3H), 3.07 (t, J = 7.0 Hz, 2H), 2.92 (q, J = 5.0 and 7.8 Hz, 1H), 2.70 (d, J = 12.7 Hz, 1H), 2.19 (t, J = 7.4 Hz, 2H), 2.06 (m, 2H), 1.78 – 1.20 (m, 61H); ^{13}C NMR (125 MHz, CD_3OD): δ = 178.1, 161.3, 155.1, 147.7, 145.9, 142.7, 128.8, 125.6, 121.4, 119.8, 110.0, 78.3, 64.6, 62.2, 60.5, 55.9, 39.9, 29.5, 29.1, 28.9, 28.7, 28.4, 25.8, 25.4.

5d$_{sn1}$: white solid; yield 42%; R_f = 0.3 in CH_2Cl_2/MeOH 8/1 (v/v); MS: m/z = 1437.032, $C_{73}H_{133}N_6O_{12}PS_3 + Na^+$ requires 1437.032; ^{1}H NMR (500 MHz, DMSO): δ = 8.22 (d, J = 9.1 Hz, 2H, Ar), 7.93 (t, J = 5.4 Hz, 1H, NH), 7.71 (t, J = 5.4 Hz, 1H, NH), 7.42 (m, 2H, Ar), 7.18 (bs, 1H, NH), 6.42 (s, 1H, NH), 6.35 (s, 1H, NH), 4.29 (t, J = 7.4 Hz, 1H, CH biotin), 4.12 (t, J = 5.4 Hz, 1H), 3.88 (t, J = 6.1 Hz, 2H), 3.23 (q, J = 5.6 and 6.3 Hz, 4H), 3.08 (m, 1H), 2.98 (q, J = 6.5 and 6.2 Hz, 3H), 2.80 (q, J = 5.1 and 7.4 Hz, 1H), 2.73 (t, J = 6.6 Hz, 8H), 2.63 (s, 1H), 2.58 (d, J = 3.2 Hz, 1H), 2.55 (s, 1H), 2.53 (bs, 2H), 2.51 (t, J = 1.8 Hz, 8H), 2.46 (s, 1H), 2.36 (s, 1H), 2.05 – 2.00 (m, 6H), 1.62 – 1.12 (m, 76H), 0.80 (t, J = 6.9 Hz, 6H).

5d$_{sn3}$: white solid; yield 56%; R_f = 0.3 in CH_2Cl_2/MeOH 8/1 (v/v); MS and NMR equivalent to **5d**$_{sn1}$.

5e: white solid; yield 46%; R_f = 0.6 in CH_2Cl_2/MeOH 89/11 (v/v); MS: m/z = 1282.766, $C_{65}H_{107}N_6O_{10}PS_3 + Na^+$ requires 1282.741; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.24 (q, J = 8.9 Hz, 2H, Ar), 7.39 (q, J = 9.3 Hz, 2H, Ar), 5.35 (d, J = 4.9 Hz, 1H, C = CH–CH_2 chol), 4.49 (bs, 1H, CH–CH_2 biotin, NH), 4.36 (bs, 1H, CH–O chol), 4.04 (t, J = 5.4 Hz, 2H, decyl-CH_2–O), 3.60 (bs, 1H, CH–CH, biotin), 3.27 (bs, 4H), 2.34 (t, J = 2.7 Hz, 4H), 2.00 – 0.65 (m, 89H).

5f: orange oil; yield 21%; R_f = 0.3 in CH_2Cl_2/MeOH 8/1 (v/v); MS: m/z = 1260.525, $C_{56}H_{89}N_{10}O_{13}PS_3 + Na^+$ requires 1260.527.

2.4. Activity tagging and detection procedures

2.4.1. Fluorescent inhibitors

Triton X-100 (20 nmol, stock solution 1 mM in $CHCl_3$) and fluorescent inhibitor **1**, **3a–e**, **4f** or **5f** (see Figs. 1 and 2) (0.4 nmol, stock solution 0.1 mM in $CHCl_3$/MeOH 2/1, v/v) were mixed in Eppendorf tubes and the organic solvent was evaporated under a stream of nitrogen. Then, 0.5 μg of ppCE (Cholesterol esterase from porcine pancreas, Fluka, Buchs, Switzerland) dissolved in 20 μL of 50 mM Tris–HCl (pH 7.4) were added to the solvent-free lipid–detergent mixture and incubated under protection from light at 37 °C and 550 rpm for 2 h. The final concentrations of fluorescent inhibitor and Triton X-100 in the aqueous solution were 20 μM and 1 mM, respectively. Reactions were stopped by adding 5 μL of reducing 1D sample buffer (20 mM KH_2PO_4, 6 mM EDTA, 60 mg/mL SDS, 100 mg/mL glycerol, 0.5 mg/mL bromophenol blue and 20 μL/mL mercaptoethanol, pH 6.8) followed by heating the samples at 95 °C for 3 min. Inhibitor-labeled proteins were separated by SDS-PAGE which was performed in a Tris–glycine buffer system (5% stacking gel, 10% resolving gel) at 20 mA constant current (Mini PROTEAN 3 or PROTEAN II xi multi-cell, BIORAD, Hercules, CA, USA). Gels were subsequently fixed in 7.5% acetic acid and 10% ethanol and visualized by scanning at 530 nm with a Molecular Imager^{TM} FX Pro Plus (BIORAD). Excitation wavelength was 488 nm, scanning resolution was 100 μm.

2.4.2. Biotinylated inhibitors without a disulfide bridge

For labeling 0.5 μg of ppCE, 0.08 nmol of biotinylated inhibitor without a disulfide bridge (inhibitor group **4**, see Fig. 2) were used (final concentration 4 μM). For control experiments protein samples were heated at 95 °C for 5 min prior to treatment with inhibitors to distinguish between specific (heat-sensitive) and non-specific (heat-insensitive) protein reactivity. All other preparation steps were performed as described above for the fluorescent inhibitors. After SDS-PAGE, enzyme–inhibitor complexes were transferred onto

nitrocellulose membranes (Protran BA85, Schleicher & Schuell, Dassel, Germany) by electroblotting (Mini Trans-Blot® Electrophoretic Transfer Cell, BIORAD). The membranes were blocked in 5% (w/v) non-fat dry milk in TBS-T (150 mM NaCl, 100 mM Tris–HCl pH 7.4, 1% Tween®20) at room temperature for 1 h and subsequently treated with an avidin-Alexa Fluor® 555 conjugate (synthesized as recommended by the manufacturer (Molecular Probes, Eugene, OR, USA)) (dilution 1:2000 in TBS-T) at room temperature for 30 min followed by three washings in TBS-T (10 min each). For detection of fluorescent bands, membranes were laser-scanned at an excitation wavelength of 532 nm with fluorescence detection at 555 nm (Molecular Imager™ FX Pro Plus, BIORAD).

2.4.3. Biotinylated inhibitors with a disulfide bridge

Biotinylated inhibitors containing a disulfide bridge (inhibitor group **5**, see Fig. 2) (0.08 nmol, final concentration 4 μM) were used for labelling of 0.5 μg of ppCE. Labeling and control reactions were performed in the same manner as described before. Reactions were stopped by adding 5 μL of non-reducing 1D sample buffer (20 mM KH_2PO_4, 6 mM EDTA, 60 mg/mL SDS, 100 mg/mL glycerol, 0.5 mg/mL bromophenol blue, pH 6.8) followed by heating of samples at 95 °C for 3 min. Enzyme–inhibitor complexes were resolved by non-reducing SDS-PAGE since the biotin tags would otherwise be cleaved off. After SDS-PAGE and electroblotting with subsequent blocking (see above) membranes were treated with an avidin–horseradish peroxidase (HRP) conjugate from BIORAD (dilution 1:2000 in TBS-T) at room temperature for 30 min. After three washing steps using TBS-T (10 min each), blots were incubated with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and then exposed to an X-ray film (Kodak, Rochester, NY, USA) for 2–45 min.

2.5. Avidin-based affinity purification

For affinity isolation, 5 μg of ppCE were incubated with 0.1 nmol of inhibitor **4a** (final concentration 4 μM) and 25 nmol Triton X-100 (final concentration 1 mM) in 25 μL 50 mM Tris–HCl (pH 7.4) at 37 °C and 550 rpm for 2 h. After addition of 300 μL Tris-buffered saline (TBS, 150 mM NaCl, 100 mM Tris–HCl pH 7.4) containing 0.5% SDS (w/v), the solution was heated at 95 °C for 10 min to ensure accessibility of biotin moieties (Kidd et al., 2003). The sample was further diluted 2.5-fold with TBS (to a final concentration of 0.2% SDS, w/v) followed by incubation with 20 μL avidin–agarose beads (suspension in 50% glycerol, 0.15 M sodium phosphate, pH 6.8, containing 15 ppm Kathon® CG/ICP II, Sigma, St. Louis, MO, USA) on a rotator at room temperature for 2 h. After removal of the supernatant, the beads were washed twice with TBS containing 0.2% SDS (w/v) and twice with TBS alone. Bound protein was subsequently eluted with 20 μL of reducing 1D sample buffer (see above) at 95 °C for 10 min. Proteins in the supernatant were precipitated in 10% trichloroacetic acid on ice for 60 min and collected by centrifugation at 4 °C at 10,000 × *g* for 15 min. The pellet was washed once with ice-cold acetone, resuspended in 20 μL reducing 1D sample buffer (see above), heated at 95 °C for 3 min and subjected to SDS-PAGE and Western blotting together with the eluate. Detection of enzyme–inhibitor complexes was performed as indicated for biotinylated inhibitors without a disulfide bridge.

3. Results and discussion

This work provides a comprehensive library of alkylphosphonic acid esters with a fluorescent or a biotin tag to be used as mechanism-based inhibitors that covalently react with their target enzymes (i.e. serine hydrolases including lipases and esterases). The respective lipids include known compounds and novel probes (**3b–c**, **4a–f**, **5d–f**) which are described in detail.

Fluorescent inhibitors containing NBD as a fluorophore (see Figs. 1 and 2) are very sensitive enzyme probes. The respective enzyme–inhibitor complexes can rapidly be detected by in-gel fluorescence scanning after protein separation by one- as well as two-dimensional gel electrophoresis as the small and uncharged NBD is compatible with isoelectric focusing (Birner-Gruenberger et al., 2005; Schmidinger et al., 2005). Moreover, NBD as a reporter fluorophore has the great advantage that absorption and emission wavelengths are long enough compared with the unspecific fluorescence background of many biological samples.

Biotinylated phosphonate inhibitors (see Fig. 2) can be utilized for avidin-based enrichment and isolation of low abundant and membrane-bound enzymes which are difficult to analyze by 2D-gel electrophoresis. We used avidin agarose for affinity isolation of enzymes from complex proteomes (inhibitor group **4**, Fig. 2). Biotin–avidin interactions were cleaved by heating the enzyme–inhibitor complexes under denaturing conditions at 95 °C (Adam et al., 2001; Kidd et al., 2003). If the biotinylated inhibitors contain disulfide bridges (inhibitor group **5**, Fig. 2), these can be cleaved under mild reductive conditions according to Deussen

et al. (2000), releasing the biotin moiety from the enzyme–inhibitor complexes.

Trifunctional inhibitors containing a fluorophore as well as a biotin tag without (**4f**) and with (**5f**) a disulfide bridge (see Fig. 2) were also synthesized. These inhibitors may facilitate the detection of enzyme–inhibitor complexes after affinity isolation with avidin–agarose beads avoiding time-consuming analytical procedures (Adam et al., 2002).

3.1. Synthesis of p-nitrophenylphosphonate inhibitors

NBD-HE-HP (**1**) (Fig. 1) was prepared by coupling an NBD-labeled alcohol to an unlabeled phosphonic acid dichloride (Oskolkova et al., 2003). In addition, a set of alkylphosphonic acid esters containing the label (fluorophore or biotin) in the phosphorus-alkyl chain was synthesized (Fig. 2). Starting from the central bifunctional synthon **2a** (Reetz et al., 2002) the ethoxy group was replaced by TMS-Br. The resulting intermediate was subsequently reacted with oxalyl chloride to form an activated *p*-nitrophenylphosphonochloridate which was converted with the alcohol components **b**–**f** (see insert in Fig. 2). Intermediates **2a**–**f** have a second reactive group, namely ω-succinimidyl carbonate which was reacted with the primary amines **g**, **h** and **i** to give stable carbamate bonds resulting in three main groups of alkylphosphonates. The first group (inhibitor group **3**) contains 7-nitrobenz-2-oxa-1,3-diazole (NBD) as a fluorescent tag, the second group (inhibitor group **4**) contains a biotin tag but no disulfide bridge, and the third group (inhibitor group **5**) has an affinity tag (biotin) and a disulfide bridge. Each of the three groups shown in Fig. 2 comprises phosphonic acid esters with alcohol components of different polarity. These components mimic the structures of natural lipase and esterase substrates and show therefore considerable specificity towards various types of lipolytic enzymes. Inhibitors $\mathbf{3d}_{sn1}$, $\mathbf{3d}_{sn3}$, $\mathbf{4d}_{rac}$, $\mathbf{5d}_{sn1}$ and $\mathbf{5d}_{sn3}$ correspond to glycerolipids but contain ether instead of carboxylester bonds in the *sn*-2 and -3(1) positions of glycerol that are not cleavable by lipase-catalyzed hydrolysis. Therefore, the *p*-nitrophenyl ester bonds in the *sn*-1(3) positions are the sole reaction sites available for the nucleophilic serine of lipolytic enzymes.

All synthesized inhibitors are racemic at phosphorus. It is known that the S_P isomers preferably react with serine hydrolases. However, the presence of the R_P isomers does not interfere with this process (Birner-Grunberger et al., 2004; Scholze et al., 1999; Zandonella et al., 1999). Therefore, the cheaper racemates (at P) were used for enzyme analytics.

3.2. Application of suicide inhibitors to functional enzyme analysis

In order to assess functionality of the synthesized fluorescent and biotinylated inhibitors they were incubated with an enzyme preparation of known lipolytic activity and selectivity, namely cholesterol esterase from porcine pancreas (ppCE). The labeled protein preparations were subjected to 1D-gel electrophoresis and the labeled protein bands were detected as described under Section 2. Fig. 3 shows that each of the synthesized inhibitors reacted more or less efficiently with this enzyme. Neither unlabeled nor thermally denatured proteins showed signals. Thus, labeling is specific for active enzymes. All fluorescent phosphonates displayed in Panel A efficiently inhibited ppCE irrespective of polarity of the alcohol residue. Both groups of biotinylated inhibitors (with and without disulfide bridge) reacted similarly (Panels B and C). The polar phosphonic acid esters **4a–c**, **4f**, **5a** and **5f** show good reactivities towards ppCE whereas inhibitors $\mathbf{4d}_{rac}$, $\mathbf{5d}_{sn1}$, $\mathbf{5d}_{sn3}$ and **5e**, which contain hydrophobic alcohol moieties, display a rather weak signal. These bulky alcohol residues could sterically hinder interactions between the biotin tags of the enzyme–inhibitor complexes and the avidin conjugates that are necessary for signal detection. Proteins labeled with biotinylated inhibitors containing a disulfide bridge (Panel C) had to be separated by non-reducing SDS-PAGE (without mercaptoethanol) in order to detect the biotin tags by avidin blotting, which would otherwise be cleaved off. Because detection of biotinylated phosphonates is very time-consuming, the major application of these inhibitors is protein purification using solid supports (Panel D). ppCE was affinity tagged with **4a**, bound onto avidin–agarose beads and carefully washed. Proteins were eluted from the beads by heating with 1D sample buffer. Compared to the supernatant (s), which was obtained after centrifugation of the beads, labeled protein was highly enriched in the eluate (el). Control experiments with unlabeled or preheated protein did not show any unspecific reactivity with the avidin–agarose beads or the inhibitors. Cholesterol esterase labeled by the trifunctional probes **4f** and **5f** can be detected either by NBD-fluorescence or by avidin blotting (see Panels A–C). These results show that the trifunctional probes are reactive towards lipolytic enzymes. Since the labeled enzyme binds to avidin, these inhibitors are applicable for avidin-based affinity isolation. Finally, they allow rapid and sensitive in-gel detection of enzyme–inhibitor complexes by NBD-fluorescence. They are promising tools for the isolation and subsequent detection of active serine hydrolases though it has to be emphasized that

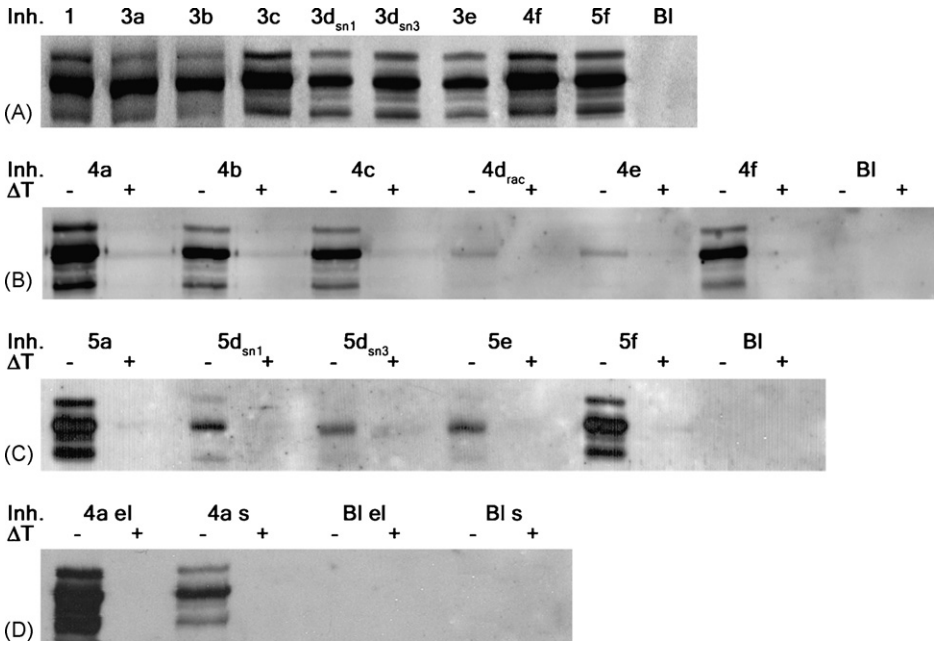


Fig. 3. Functional analysis of suicide inhibitors. Cholesterol esterase from porcine pancreas (ppCE) was incubated with 20 μM fluorescent inhibitor (A), or with 4 μM biotinylated inhibitor without disulfide bridge (B), or with 4 μM biotinylated inhibitor with disulfide bridge (C). ppCE was also affinity isolated by avidin–agarose beads after reaction with **4a** (D). Numbering of inhibitors corresponds to Figs. 1 and 2. Labeling and detection was performed as described in Section 2. Unlabeled (Bl) and preheated (Δ*T*+) samples were used for control reactions. el: eluate; s: supernatant.

their structures are more complex than the molecules of the natural enzyme substrates.

In conclusion, we have developed a library of fluorescent and/or biotinylated alkylphosphonate inhibitors containing alcohol moieties of different polarity for detection and identification of serine hydrolases as well as for discrimination of lipases and esterases. Functional enzyme analysis performed with the synthesized inhibitors has shown that all of them reacted with cholesterol esterase from porcine pancreas (ppCE). Some of the fluorescent inhibitors (**1**, **3a**, **3d**$_{sn1}$, **3d**$_{sn3}$ and **3e**) have already been used for screening and discrimination of lipolytic enzymes in enzyme preparations (Schmidinger et al., 2005) as well as for mapping the lipolytic proteome of mouse adipose tissue (Birner-Gruenberger et al., 2005). Biotinylated inhibitors turned out to be useful for microarray screening of lipase and esterase activity (Schmidinger et al., 2006), for molecular evolution screening of lipases by phage display (Danielsen et al., 2001) and for enzyme enrichment (see above and Deussen et al., 2000). They will hopefully promote the discovery of lipolytic enzymes which may have escaped detection by fluorescent inhibitors using 2D-gel electrophoresis. The whole set of activity-based probes is currently used for mapping the lipolytic proteomes of organs and tissues that play essential roles in murine and human lipid metabolism.

Acknowledgements

This work has been financed by the projects Genomics of Lipid-associated Disorders (GOLD II) (http://gold.uni-graz.at/) and Click it: In vivo functional proteomic screening and cell imaging of lipases in the framework of GEN-AU (GENome research in AUstria) (http://www.gen-au.at/) which is funded by the Austrian Federal Ministry for Education, Science and Culture (bm:bwk).

References

Adam, G.C., Sorensen, E.J., Cravatt, B.F., 2002. Trifunctional chemical probes for the consolidated detection and identification of enzyme activities from complex proteomes. Mol. Cell Proteomics 1, 828–835.

Adam, G.C., Cravatt, B.F., Sorensen, E.J., 2001. Profiling the specific reactivity of the proteome with non-directed activity-based probes. Chem. Biol. 8, 81–95.

Berman, H.A., Olshefski, D.F., Gilbert, M., Decker, M.M., 1985. Fluorescent phosphonate labels for serine hydrolases. Kinetic and spectroscopic properties of (7-nitrobenz-2-oxa-1,3-diazole)aminoalkyl

methylphosphonofluoridates and their conjugates with acetylcholinesterase molecular forms. J. Biol. Chem. 260, 3462–3468.

Birner-Gruenberger, R., Susani-Etzerodt, H., Waldhuber, M., Riesenhuber, G., Schmidinger, H., Rechberger, G., Kollroser, M., Strauss, J.G., Lass, A., Zimmermann, R., Haemmerle, G., Zechner, R., Hermetter, A., 2005. The lipolytic proteome of mouse adipose tissue. Mol. Cell Proteomics 4, 1710–1717.

Birner-Grunberger, R., Scholze, H., Faber, K., Hermetter, A., 2004. Identification of various lipolytic enzymes in crude porcine pancreatic lipase preparations using covalent fluorescent inhibitors. Biotechnol. Bioeng. 85, 147–154.

Bjorkling, F., Dahl, A., Patkar, S., Zundel, M., 1994. Inhibition of lipases by phosphonates. Bioorg. Med. Chem. 2, 697–705.

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A., Thim, L., 1991. A model for interfacial activation in lipases from the structure of a fungal lipase–inhibitor complex. Nature 351, 491–494.

Campbell, D.A., Szardenings, A.K., 2003. Functional profiling of the proteome with affinity labels. Curr. Opin. Chem. Biol. 7, 296–303.

Cygler, M., Grochulski, P., Kazlauskas, R.J., Schrag, J.D., Bouthillier, F., Rubin, B., Serreqi, A.N., Gupta, A.K., 1994. A structural basis for the chiral preferences of lipases. J. Am. Chem. Soc. 116, 3180–3186.

Danielsen, S., Eklund, M., Deussen, H.J., Graslund, T., Nygren, P.A., Borchert, T.V., 2001. In vitro selection of enzymatically active lipase variants from phage libraries using a mechanism-based inhibitor. Gene 272, 267–274.

Deussen, H.J., Danielsen, S., Breinholt, J., Borchert, T.V., 2000. A novel biotinylated suicide inhibitor for directed molecular evolution of lipolytic enzymes. Bioorg. Med. Chem. 8, 507–513.

Egloff, M.P., Marguet, F., Buono, G., Verger, R., Cambillau, C., van Tilbeurgh, H., 1995. The 2.46 Å resolution structure of the pancreatic lipase–colipase complex inhibited by a C11 alkyl phosphonate. Biochemistry 34, 2751–2762.

Hamilton, R., Walker, B., Walker, B.J., 1998. Synthesis and proteinase inhibitory properties of diphenyl phosphonate analogues of aspartic and glutamic acids. Bioorg. Med. Chem. Lett. 8, 1655–1660.

Kidd, D., Liu, Y., Cravatt, B.F., 2003. Profiling serine hydrolase activities in complex proteomes. Biochemistry 40, 4005–4015.

Liu, Y., Patricelli, M.P., Cravatt, B.F., 1999. Activity-based protein profiling: the serine hydrolases. Proc. Natl. Acad. Sci. U.S.A. 96, 14694–14699.

Mannesse, M.L.M., Boots, J.-W.P., Dijkman, R., Slotboom, A.T., van der Hijden, H.T.W.M., Egmond, M.R., Verheij, H.M., de Haas, G.H., 1995. Phosphonate analogues of triacylglycerols are potent inhibitors of lipase. Biochim. Biophys. Acta 1259, 56–64.

Moreau, H., Moulin, A., Gargouri, Y., Noel, J.P., Verger, R., 1991. Inactivation of gastric and pancreatic lipases by diethyl paranitrophenyl phosphate. Biochemistry 30, 1037–1041.

Oskolkova, O.V., Saf, R., Zenzmaier, E., Hermetter, A., 2003. Fluorescent organophosphonates as inhibitors of microbial lipases. Chem. Phys. Lipids 125, 103–114.

Ransac, S., Gargouri, Y., Marguet, F., Buono, G., Beglinger, C., Hildebrand, P., Lengsfeld, H., Hadvary, P., Verger, R., 1997. Covalent inactivation of lipases. Lipases Meth. Enzymol. 286, 190–231.

Reetz, M.T., Ruggeberg, C.J., Droge, M.J., Quax, W.J., 2002. Immobilization of chiral enzyme inhibitors on solid supports by amide-forming coupling and olefin metathesis. Tetrahedron 58, 8465–8473.

Rotticci, D., Norin, T., Hult, K., Martinelle, M., 2000. An active-site titration method for lipases. Biochim. Biophys. Acta 1483, 132–140.

Scheib, H., Pleiss, J., Stadler, P., Kovac, A., Potthoff, A.P., Haalck, L., Spener, F., Paltauf, F., Schmid, R.D., 1998. Rational design of *Rhizopus oryzae* lipase with modified stereoselectivity toward triradylglycerols. Protein Eng. 11, 675–682.

Schmidinger, H., Birner-Gruenberger, R., Riesenhuber, G., Saf, R., Susani-Etzerodt, H., Hermetter, A., 2005. Novel fluorescent phosphonic acid esters for discrimination of lipases and esterases. Chembiochem 6, 1776–1781.

Schmidinger, H., Susani-Etzerodt, H., Birner-Gruenberger, R., Hermetter, A., 2006. Inhibitor- and protein microarrays for activity-based recognition of lipolytic enzymes. Chembiochem 7, 527–534.

Scholze, H., Stutz, H., Paltauf, F., Hermetter, A., 1999. Fluorescent inhibitors for the qualitative and quantitative analysis of lipolytic enzymes. Anal. Biochem. 276, 72–80.

Stadler, P., Zandonella, G., Haalck, L., Spener, F., Hermetter, A., Paltauf, F., 1996. Inhibition of microbial lipases with stereoisomeric triradylglycerol analog phosphonates. Biochim. Biophys. Acta 1304, 229–244.

Taylor, C.M., O'Connor, C.J., Chamberlain, J.L., Sun, C.Q., 2001. Design, synthesis and evaluation of two diastereoisomeric phosphonate inhibitors of lamb pregastric lipase. J. Mol. Catal. B: Enzym. 15, 15–22.

Vanwetswinkel, S., Touillaux, R., Fastrez, J., Marchand-Brynaert, J., 1995. Bifunctional activity labels for selection of filamentous bacteriophages displaying enzymes. Bioorg. Med. Chem. 3, 907–915.

Zandonella, G., Stadler, P., Haalck, L., Spener, F., Paltauf, F., Hermetter, A., 1999. Interactions of fluorescent triacylglycerol analogs covalently bound to the active site of a lipase from *Rhizopus oryzae*. Eur. J. Biochem. 262, 63–69.