CHEMBIOCHEM

DOI: 10.1002/cbic.200500013

Novel Fluorescent Phosphonic Acid Esters for Discrimination of Lipases and Esterases

Hannes Schmidinger,[a] Ruth Birner-Gruenberger,[a] Gernot Riesenhuber,[a] Robert Saf,[b] Heidrun Susani-Etzerodt,[a] and Albin Hermetter*[a]

Lipases and esterases are responsible for carboxylester hydrolysis inside and outside cells and are useful biocatalysts for (stereo)-selective modification of synthetic substrates. Here we describe novel fluorescent suicide inhibitors that differ in structure and polarity for screening and discrimination of lipolytic enzymes in enzyme preparations. The inhibitors covalently react with the enzymes to form fluorescent lipid–protein complexes that can be resolved by gel electrophoresis. The selectivities of the inhibitors were determined by using different (phospho)lipase, esterase and cholesterol esterase preparations. The results indicate that formation of an inhibitor–enzyme complex is highly dependent on the chemical structure of the inhibitor. We identified inhibitors with very low specificity, and other derivatives that were highly specific for certain subgroups of lipolytic enzymes such as lipases and cholesterol esterases. A combination of these substrate-analogous activity probes represents a useful toolbox for rapid identification and classification of serine hydrolase enzymes.

Introduction

Screening of proteomes and subproteomes for active enzymes is a major task in various fields of biosciences including biocatalysis, biotechnology and biomedicine. For this purpose, fast and selective methods are required for the detection of the proteins of interest in complex biological samples. Once identified, the novel enzymes can be overexpressed and characterized with respect to activity and selectivity on biological or synthetic substrates of interest.

Here we report a fluorescence technology for selectively screening for lipolytic enzymes. Lipase- and esterase-mediated hydrolysis of acyl esters is based on a mechanism involving a nucleophilic serine, which in most cases is part of a catalytic triad with histidine and aspartate. In the first reaction step, the active serine attacks the carbonyl group of the scissile fatty acid to give a tetrahedral transition state. This intermediate state is cleaved to yield the free fatty acid, the alcohol component and the nucleophilic serine. Although many lipases show strong structural and sequential similarities,[1–3] their substrate and stereospecificities can vary significantly.[4,5] It is known that *p*-nitrophenylesters of alkylphosphonic acids irreversibly and stoichiometrically react with the nucleophilic serine of lipases and esterases, freezing the reaction at the point of the tetrahedral transition state. In the past, they have been applied to the determination of serine hydrolase activity by using different approaches.[6,7] If fluorescent inhibitors are used, the tagged enzyme becomes "visible".[8] Thus, it can be detected and quantified on the basis of its fluorescent signal.[9] Fluorescently labelled alkyl phosphonates detect active enzymes in electrophoretically pure proteins and in complex proteome samples.[10–15] In this work, we describe a series of novel fluorescent inhibitors that differ with respect to structure and polarity. Fourteen enzyme probes have been characterized by using commercial enzyme preparations. These compounds show different reactivities towards lipases and esterases, and can therefore be used for discrimination of these enzyme subgroups.

Results and Discussion

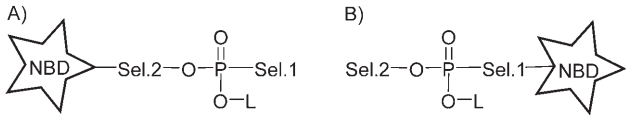
It was the aim of this work to prepare *p*-nitrophenol esters of suitably substituted phosphonic acid derivatives as high- and low-affinity probes for the discovery, identification and characterization of different lipases and esterases in a given sample.[14] Both enzyme classes act on a wide range of carboxylic esters of different structure, polarity and stereochemistry. Therefore, compounds with a reactive phosphonic acid *p*-nitrophenyl ester bond, a fluorescent reporter and two selector domains Sel.1 and Sel.2 (Scheme 1) differing in polarity and stereochemistry were synthesized. Variation of the Sel.1 and Sel.2 domains led to a variety of enzyme probes that can be considered as components of a toolbox for identifying lipases and esterases.

Two basic strategies were applied to prepare the inhibitors. One was based on nitrobenz-2-oxa-1,3-diazole (NBD) labelled

[a] *Dr. H. Schmidinger, Dr. R. Birner-Gruenberger, G. Riesenhuber, H. Susani-Etzerodt, Prof. Dr. A. Hermetter*
Institut für Biochemie, Technische Universität Graz
Petersgasse 12/II, 8010 Graz (Austria)
Fax: (+43) 316-873-6952
E-mail: albin.hermetter@tugraz.at

[b] *Prof. Dr. R. Saf*
Institut für Chemische Technologie organischer Stoffe ICTOS
Technische Universität Graz
Stremayrgasse 16 (Austria)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.



Scheme 1. A) Fluorescent reporter (NBD) attached to selector 2 (compounds **1**, **2** and **10**–**14**), selector 1 is unmodified; B) NBD attached to the ω-position of selector 1 (compounds **3**–**9**), selector 2 is unmodified. L: *p*-nitrophenol leaving group.

alcohols (Sel.2) that were coupled to unlabelled phosphonic acid dichlorides. This reaction led to the formation of phosphonates in which the fluorescent tag was bound to the alcohol component of the inhibitor (Scheme 1 A). Phosphonate derivatives with different polarity that contained either methyl (**10**–**13**) or hexyl (**1**, **2**, **14**) chains as selector domains (Sel.1) linked to the phosphorus atom were prepared.

In a second approach (Scheme 1 B), compound **27** (see Table 1) served as a central alkyl phosphonic acid synthon for preparation of inhibitors containing the reporter fluorophore in the phosphorus-alkyl chain (Sel.1). Thus, the fluorescent phosphonic acid esters **3**–**9** were obtained by ester exchange with unlabelled alcohol components (Sel.2). In these compounds, the fluorescently labelled alkylphosphonyl moiety mimics the scissile acyl chain in biological and synthetic carboxylic acid esters. The complete list of inhibitors is shown in Table 1.

The synthetic phosphonates were all racemic at phosphorus. It is known that the configuration at phosphorous determines the reactivity of the inhibitor towards the nucleophilic serines of lipases, the S_P isomer being the preferred reactant. However, the presence of the R_P compound in the reaction mixture does not interfere with the inhibition.[9,14]

Solubilization of inhibitors, especially of the very hydrophobic derivatives **3**, **4** and **5**, turned out to be critical. In order to keep solubilization conditions as mild as possible, a Triton X-100/Tris-HCl buffer system was used.[14] Preliminary experiments had shown that a minimum Triton X-100 concentration of 1 mM was necessary for proper enzyme labelling. An increase of detergent concentration above 2–4 mM, depending on the probed enzyme, resulted in a decrease of labelling efficiency. In this study, all experiments were performed with 1 mM Triton X-100 in order to maintain standard reaction conditions for inhibitor-based enzyme screening. Selectivity was determined as the ratio of bound inhibitor to total protein contained in an electrophoresis band (NBD fluorescence of inhibitor/RuBPS fluorescence of total protein).[16] For comparison, activities of a given enzyme towards the individual inhibitors were expressed as a percentage of the maximum activity observed for this enzyme.

Our inhibitor toolbox for lipases and esterases comprises three groups of NBD-labelled inhibitors, differing by the length of the alkylphosphonyl chain, the polarity and the stereochemistry of the alkoxy group. Two groups comprise inhibitors in which a fluorescent alcoxy residue and a methyl or hexyl substituent are bound to phosphorus. The inhibitors **10**–**14**, containing optically active amino alkoxy residues were selected to screen the enzyme preference of diastereomers (considering the racemic phosphorus). A third group of inhibitors contains a fluorescently labelled P-alkyl chain and optically active glycerolipids as biologically relevant alkoxy components (e.g., 1,2(2,3)-di-*O*-hexadecyl-*sn*-glyceryl). These compounds were used to probe the regio- and stereoselectivity of lipases and esterases.

Lipolytic enzymes including lipases, esterases, a cutinase from *Fusarium solari* and cholesterol esterases were studied. For this purpose, protein samples were incubated with inhibitors and then subjected to 1D SDS-PAGE. After protein separation, the fluorescent bands corresponding to the labelled (active) enzymes were detected by using a laser gel scanner. Typical examples are shown for bovine pancreatic cholesterol esterase and esterase B from *Burkholderia gladioli* in Figures 1 and 2, respectively.

The cholesterol esterase preparation contained several active enzymes. Besides cholesterol esterase (<), the highest activity originated from a 55 kDa protein (<<). Cholesterol esterase was active towards most inhibitors of the library. Low activity was found with inhibitor **5**, and the short, bulky, inhibitors **10**–**14**. Cholesterol esterase was only labelled by the S_c

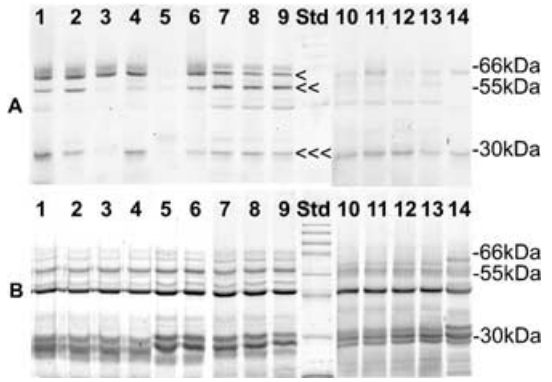


Figure 1. Probing active lipolytic enzymes in a cholesterol esterase preparation from bovine pancreas. A) fluorescently labelled enzymes; B) RuBPS image: whole protein stain. Lane numbering corresponds to the inhibitor numbers given in Table 1. <: cholesterol esterase, << and <<<: unknown enzymes (note that these proteins are not labelled with inhibitor **3**); Std: molecular-weight standard.

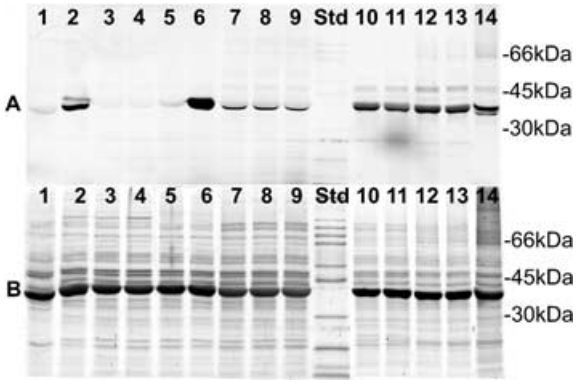
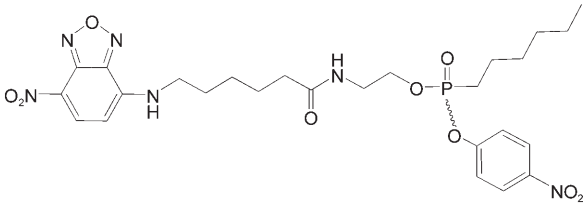
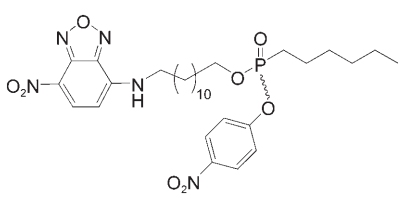


Figure 2. Probing active lipolytic enzymes in a recombinant esterase B preparation from *Burkholderia gladioli*. A) fluorescently labelled enzymes; B) RuBPS image; whole protein stain. Lane numbering corresponds to the inhibitor numbers given in Table 1; Std: molecular-weight standard.

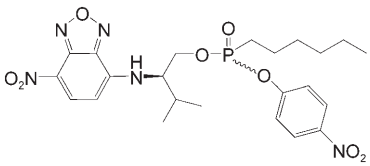
Table 1. Fluorescent activity recognition probes for lipases and esterases.



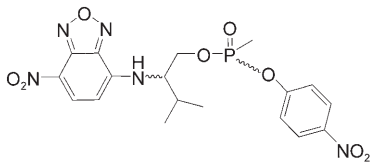
1



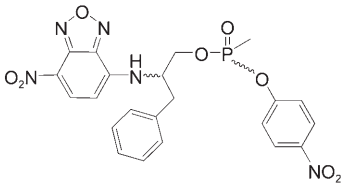
2



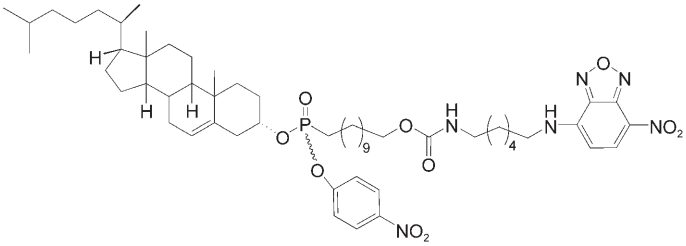
14



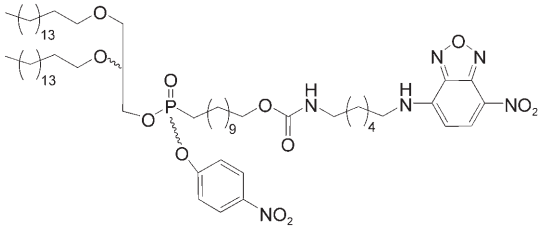
S = **10**
R = **11**



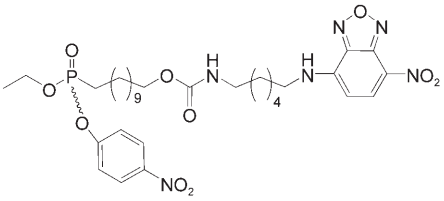
S = **12**
R = **13**



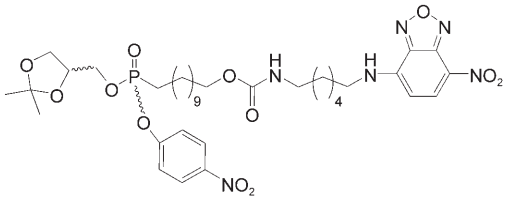
3



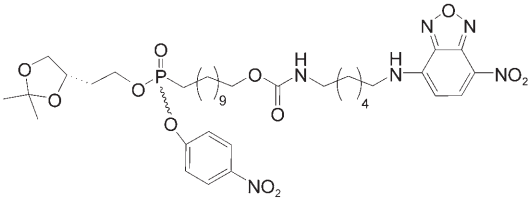
S = **4** (*sn*-1)
R = **5** (*sn*-3)



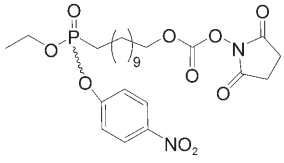
6



S = **7** (*sn*-1)
R = **8** (*sn*-3)



9



27

(*sn*-1) trialkylglycerol isomer **4**. In contrast, the 55 kDa protein that reacted with other inhibitors (<<) was not labelled by the cholesterol and triacylglycerol-analogous inhibitors. Inhibitor **3**, which is a cholesterol ester analogue, showed a unique behaviour as it selectively labelled cholesterol esterase. Thus, it may be considered a highly specific affinity tag for cholesterol ester-hydrolyzing enzymes. The most abundant protein, according to the whole protein stain, had a molecular weight close to 46 kDa and was weakly labelled by the inhibitors bearing a polar moiety in close vicinity to the reactive phosphorus (**1**, **7**–**13**). A highly abundant 28 kDa (<<<) protein was detected by all inhibitors, except the cholesterol ester analogue.

Esterase B from *Burkholderia gladioli* (EP6), was highly active towards inhibitors **2** (54% activity) and **6** (100% activity), and to a lower extent, towards the more hydrophilic inhibitors **10**–**14** (Figure 2).

The cholesterol and triacylglycerol derivatives **3**–**5** did not react with this esterase. It is important to note that the results are in accordance with the reported EP6 preference for bulky acetates and tertiary alcohols, and its low activity towards hydrophobic triglycerides.[17] EP6 strongly discriminated between inhibitors **2** and **1**. Whereas inhibitor **1** was active towards most of the lipases and esterases in our study, EP6 was labelled poorly by this lipid.

The time-dependent formation of enzyme–inhibitor complexes was determined for EP6 and bovine cholesterol esterase (bCE; Figure 3). EP6 reacted slowly with compounds **1** and **2**.

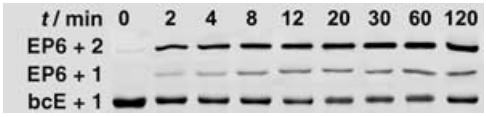


Figure 3. Time-dependent formation of enzyme complexes with inhibitors **1** and **2**. Enzymes were esterase B from *Burkholderia gladioli* (EP6) and bovine cholesterol esterase (bCE).

Compound **2** showed 25-fold inhibition of EP6 after 2 h as compared to **1**. It has to be noted at this point that detergents affect enzyme activity. As shown for EP6, 50 mM Triton X-100 can reduce activity to zero.[17] Inhibitors **2** and **6** were much more reactive towards EP6 than inhibitor **1** under the same buffer conditions (Figure 2). In contrast to EP6, inhibition of bCE by the same inhibitors was much faster, leading to immediate labelling of the enzyme after a few seconds.

The above results show that the application of several inhibitors makes detection of all serine hydrolase enzymes in a complex sample much more likely. To test the hypothesis that a toolbox of various mechanism-based enzyme probes increases the probability of the detection of all active proteins in a sample. The analysis of enzyme activity was extended to a total of 19 enzyme preparations of different substrate preference and from various sources.

We have shown that reactivity towards a single inhibitor strongly depends on the probed enzyme (Table 2). In general, inhibitor diastereomers **10**–**14**, containing the S_C-configured alcohol component were much more reactive than the R_C isomer. In most cases, replacement of the methyl residue at phosphorus by a hexyl chain (**10**→**14**) improved the reactivity towards lipases or esterases. The polar moieties close to the reactive phosphorous significantly affect inhibitor reactivity. This becomes evident when the activities of EP6, FSC, EX9 and PAF acetylhydrolase towards inhibitors **1** and **2** are compared. Whereas EP6 and FSC reacted with inhibitor **2**, EX9 and PAF-AH were preferably labelled by the amide-containing derivative **1**. The diastereomers of dialkylglycerolipids **4** and **5** showed very different reactivities. Inhibitor **4** with the S_C configuration at glycerol was active on PSL, psLPL, MME, CAL A, ppCE and bCE, whereas the R_C-configured analogue bound only weakly to the same proteins. Although the lipolytic enzymes showed

Table 2. Relative activities of lipolytic enzymes towards various inhibitors. The values indicated for a given enzyme and a given inhibitor are expressed as percent of the maximum activity observed with the "best inhibitor" for the same enzyme (mean values of two experiments). For better visualization 90–100% activities are depicted in bold and 60–89% in italics.

	Inhibitor Number													
	3	**4**	**5**	**7**	**8**	**9**	**6**	**1**	**2**	**14**	**10**	**11**	**12**	**13**
length of P-alkyl chain				C_{11}					C_6				C_1	
PCL	4	9	7	6	12	10	48	**92**	**100**	27	10	7	5	9
PCL-W	52	58	48	26	34	15	15	*75*	**100**	*68*	30	38	**99**	38
PSL	29	*88*	18	32	*70*	**100**	**92**	*84*	**100**	9	98	11	34	11
psLPL	6	36	6	25	22	24	*68*	*86*	**90**	**100**	54	19	29	18
MME	7	*69*	46	*86*	**100**	**100**	*70*	*69*	*72*	6	0	0	1	1
EP10	15	25	21	**100**	*80*	*76*	34	42	51	55	11	12	19	9
EP6	1	1	3	10	10	9	**100**	2	54	15	22	13	22	14
EX9	5	3	4	15	8	10	16	41	11	28	23	25	**100**	29
CAL-A	24	60	2	7	9	4	25	*78*	**100**	8	6	2	16	2
ANL	16	20	17	41	50	42	49	**100**	29	37	6	6	7	5
ROL	2	41	4	*68*	**100**	*84*	56	*69*	*70*	34	23	4	*77*	21
ROL-F	5	9	4	*66*	**100**	*71*	48	48	56	2	7	2	11	9
bLPL	1	1	1	*70*	*72*	*69*	37	42	29	42	**100**	16	*76*	23
FSC	7	32	14	*73*	*76*	*75*	*82*	42	**95**	34	**98**	**91**	**90**	**100**
ppCE	*73*	*78*	24	36	41	43	54	52	*69*	52	**100**	**92**	19	*69*
bCE	32	27	0	32	38	21	35	50	24	27	*83*	**100**	23	30
ppL	1	1	2	20	21	20	12	10	18	**100**	4	7	4	4
RML	2	18	6	33	42	30	26	28	33	**100**	23	3	35	0
PAF-AH	8	14	12	29	14	13	14	55	13	35	52	*72*	**100**	45

Enzymes: PCL *Pseudomonas cepacia* lipase; PCL-W: *Pseudomonas cepacia* lipase L287W, PSL: *Pseudomonas species* lipase, psLPL: *Pseudomonas species* lipoprotein lipase, MME: *Mucor miehei* esterase; EP10: *Burkholderia gladioli* esterase A; EP6: *Burkholderia gladioli* esterase B; EX9: *Xanthomonas vesicatoria* esterase E; CAL-A: *Candida antarctica* lipase A; ANL: *Aspergillus niger* lipase; ROL: *Rhizopus oryzae* lipase; ROL-F: *Rhizopus oryzae* lipase L258F L⁻254F; bLPL: lipoprotein lipase from bovine milk; FSC: *Fusarium solari* cutinase; ppCE: porcine pancreatic cholesterol esterase; bCE: bovine pancreatic cholesterol esterase; ppL: porcine pancreatic lipase; RML: *Rhizomucor miehei* lipase; PAF-AH: human recombinant platelet activating factor acetyl hydrolase.

a preference for the S_C isomer of the dialkylglycero inhibitors, such an effect was not observed with the isopropylideneglycerol-based derivatives **7**–**9**, which lack the hydrophobic chains. The R_C and S_C isomers of these compounds were equally active towards the same enzymes.

Our data suggest that enzyme screening of a complex protein sample with only one of the inhibitors would result in an incomplete list of activities, as none of the inhibitors labelled all 19 enzymes of this study within a reasonable time. Inhibitor reactivity was mainly determined by the length of the alkylphosphonyl chain (**10** to **13** as compared to **14**). Thus, the influence of the alkoxy component can only be studied if inhibitors with the same alkylphosphonyl chain lengths are compared. Inhibitors **10**–**14**, which contain a bulky alcohol and a short alkylphosphonyl chain preferably detected esterase and PAF-AH activities, whereas the triacylglycerol analogue **4** was specific for lipases. The cholesterol ester analogue preferably labelled cholesterol esterases. It is clearly the most specific inhibitor in the toolbox. We believe that these probes will be widely used for the screening and discovery of enzymes in various fields of bio(medical) sciences, for example, to find novel "drugable" enzymes, enzyme markers for medical diagnosis or biocatalysts for synthetic chemistry.

Experimental Section

Synthesis

N-*(2-hydroxyethyl)-6-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanamide* ***15***: Succinimidyl 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)hexanoate (12.5 mg, 32.0 μmol) was dissolved in dry THF (2 mL). Freshly distilled 2-aminoethanol (7 μL, 116.0 μmol) was added, and after 2 h all volatile components were evaporated under reduced pressure. The residue was solubilized in $CHCl_3$/MeOH/H_2O (65:25:4; 5 mL) and incubated with Dowex 50Wx8 (H^+ form, 200–400 mesh) for 30 min. The ion-exchange material was filtered off, and the filtrate was dried to yield pure **15** (99%). $R_f = 0.4$ ($CHCl_3$/MeOH/H_2O 65:25:4).

General procedure for compounds ***1–2, 10–14***: The NBD-labelled amino alcohols (see Supporting Information) were dissolved in dry dichloromethane. After addition of *N*-methylimidazol (5 equiv) and tetrazol (0.10 equiv), the dichlorophosphonate (3 equiv) was added, and the mixture was stirred for 3 h. A mixture of 4-nitrophenol (5 equiv) and *N*-methylimidazol (5 equiv) was added, and the resulting solution was stirred overnight at room temperature. All volatile components were evaporated under reduced pressure, and the residue was purified by flash chromatography.

General procedure for the synthesis of compounds ***3–9***: Compound **27** (60.2 mg, 120 μmol) was dissolved in CH_2Cl_2 (5 mL), and $(CH_3)_3SiBr$ (55.1 mg, 360 μmol) was added. After the mixture had been stirred for 40 h, all volatile components were removed under reduced pressure. The orange residue was dissolved in CH_2Cl_2 (10 mL). Then oxalylchloride (45.7 mg, 360 μmol) and DMF (3 μL) were added, and the resulting mixture was kept under reflux for 18 h. After removal of all volatile components under reduced pressure, the residue was dissolved in CH_2Cl_2 (2 mL), the alcohol component (1 equiv) was added together with triethylamine (3 equiv). After the mixture had been stirred for 8 h, the solvent was evaporated, and the intermediate compound was subjected to flash chromatography. The purified intermediate was dissolved in DMF (1.5 mL; in acetonitrile for compound **3**), containing triethylamine (3 equiv). Compound **28** (1 equiv) was added to this solution, and the resulting mixture was stirred for 2 h, followed by purification by flash chromatography.

N-*(7-nitro-2,1,3-benzoxadiazol-4-yl)hexane-1,6-diamine (as TFA salt)* ***28***: BOC–diaminohexane hydrochloride (1.26 g, 5 mmol) was dissolved in Na_2CO_3 (10%, 15 mL), and NBD-Cl (0.50 g, 2.5 mmol) was added. After the mixture had been stirred at room temperature overnight, water (50 mL) was added, and the product was extracted with diethyl ether (50 mL). The organic phase was washed with brine (4×10 mL), dried over Na_2SO_4 and purified by flash chromatography ($CHCl_3$/EtOAc, 88:12). The BOC protecting group of the purified intermediate was removed by using freshly distilled trifluoroacetic acid (1 mL) at room temperature. After the mixture had been stirred overnight, excess TFA was evaporated, and the oily product was treated with diethyl ether to yield the product as orange crystals (423 mg).

For detailed inhibitor characterization, as well as data acquisition and analysis, the reader is referred to the Supporting Information.

Preparation of inhibitor–enzyme complexes: Stock solutions of fluorescent inhibitors in $CHCl_3$ (100 μM) were prepared. Enzymes were incubated with inhibitor (1 nmol = at least twofold molar excess). For this purpose, inhibitor stock (10 μL) was transferred together with a Triton X-100 solution (2 μL, 10 mM in $CHCl_3$) to a sterile Eppendorf tube. The organic solvent was removed under an argon stream, and the residue was dried under vacuum for 30 min. The enzyme solution (20 μL, see Supporting Information) was added to the tube. The sample was shaken vigorously for 2 min, spun down briefly and incubated in an Eppendorf thermomixer at 37 °C and 550 rpm for 2 h. After incubation, sample loading buffer (5 μL) was added. The mixture was incubated at 95 °C for 5 min, then subjected to SDS-PAGE (10% resolving and 4.5% stacking gel) at 20 mA/gel constant current.

Time-dependent inhibition of EP6 and bCE: Inhibitors (11 nmol, 110 μL stock) were transferred together with a Triton X-100 solution (22 μL, 10 mM in $CHCl_3$) to a sterile 1.5 mL Eppendorf tube. The organic solvent was removed under an argon stream, and the residue was dried for 30 min under vacuum. The enzyme solution (220 μL) was preincubated at 37 °C for 5 min and added to the tube. The samples were shaken, and aliquots (20 μL) were withdrawn after 0, 2, 4, 8, 12, 20, 30, 60 and 120 min. The aliquots were immediately added to fivefold-concentrated SDS-PAGE sample buffer (5 μL) at 95 °C. After incubation at 95 °C for 5 min, the denatured samples were subjected to SDS-PAGE and processed as described above.

CAUTION: Compounds **10** and **11** are active on acetylcholine esterase (AChE) from *Electrophorus electricus* (electric eel) and therefore should be handled with extreme caution (see Supporting Information).[18]

Acknowledgements

We are indebted to Prof. Dr. Kurt Faber and Prof. Dr. Helmut Schwab for the generous supply of enzymes. Financial support from bm:bwk (GOLD, C4, a subproject of GEN-AU) is gratefully acknowledged.

Keywords: alkyl phosphonates · esterases · fluorescent probes · inhibitors · lipases

[1] J. Pleiss, M. Fischer, R. D. Schmid, *Chem. Phys. Lipids* **1998**, *93*, 67 – 80.
[2] R. J. Kazlauskas, *Trends Biotechnol.* **1994**, *12*, 464 – 472.
[3] J. L. Arpigny, K. E. Jaeger, *Biochem. J.* **1999**, *343*(1), 177 – 183.
[4] M. Martinelle, M. Holmquist, K. Hult, *Biochim. Biophys. Acta* **1995**, *1258*, 272 – 276.
[5] A. M. Brzozowski, U. Derewenda, Z. S. Derewenda, G. G. Dodson, D. M. Lawson, J. P. Turkenburg, F. Bjorkling, B. Huge-Jensen, S. A. Patkar, L. Thim, *Nature* **1991**, *351*, 491 – 494.
[6] O. V. Oskolkova, R. Saf, E. Zenzmaier, A. Hermetter, *Chem. Phys. Lipids* **2003**, *125*, 103 – 114.
[7] D. Rotticci, T. Norin, K. Hult, M. Martinelle, *Biochim. Biophys. Acta* **2000**, *1483*, 132 – 140.
[8] M. L. M. Mannesse, J.-W. P. Boots, R. Dijkman, A. T. Slotboom, H. T. W. M. van der Hijden, M. R. Egmond, H. M. Verheij, G. H. de Haas, *Biochim. Biophys. Acta* **1995**, *1259*, 56 – 64.
[9] H. Scholze, H. Stutz, F. Paltauf, A. Hermetter, *Anal. Biochem.* **1999**, *276*, 72 – 80.
[10] B. F. Cravatt, E. J. Sorensen, *Curr. Opin. Chem. Biol.* **2000**, *4*, 663 – 668.
[11] Y. Liu, M. P. Patricelli, B. F. Cravatt, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 14 694 – 14 699.
[12] A. E. Speers, B. F. Cravatt, *ChemBioChem* **2004**, *5*, 41 – 47.
[13] G. C. Adam, E. J. Sorensen, B. F. Cravatt, *Mol. Cell. Proteomics* **2002**, *1*, 781 – 790.
[14] R. Birner-Grunberger, H. Scholze, K. Faber, A. Hermetter, *Biotechnol. Bioeng.* **2004**, *85*, 147 – 154.
[15] D. C. Greenbaum, W. D. Arnold, F. Lu, L. Hayrapetian, A. Baruch, J. Krumrine, S. Toba, K. Chehade, D. Bromme, I. D. Kuntz, M. Bogyo, *Chem. Biol.* **2002**, *9*, 1085 – 1094.
[16] T. Rabilloud, J. M. Strub, S. Luche, A. van Dorsselaer, J. Lunardi, *Proteomics* **2001**, *1*, 699 – 704.
[17] E. I. Petersen, G. Valinger, B. Solkner, G. Stubenrauch, H. Schwab, *J. Biotechnol.* **2001**, *89*, 11 – 25.
[18] Y. Bourne, J. Grassi, P. E. Bougis, P. Marchot, *J. Biol. Chem.* **1999**, *274*, 30 370 – 30 376.

Received: January 12, 2005
Published online on August 11, 2005

