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A Novel Biotinylated Suicide Inhibitor for Directed Molecular Evolution of Lipolytic Enzymes

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Abstract—A bifunctional activity label (**8**) for directed molecular evolution of lipolytic enzymes has been designed and synthesized. The structure is composed of a 4-nitrophenyl activated phosphonate, that is, a suicide substrate of lipases/esterases, connected to a biotin moiety through a spacer containing a disulfide bridge. The phosphonate (**3**) was prepared by Michaelis–Arbuzov reaction of trimethylsilyl-protected 11-bromoundecanol (**2**) with triethyl phosphite. The deprotected ω-hydroxyalkylphosphonate (**4**) was transformed into an active *N*-hydroxysuccinimide carbonate (**5**) followed by 4-nitrophenyl activation of the phosphonate using standard procedures. The biotinylated phosphonate inhibitor (**8**) was then synthesised by coupling the phosphonate inhibitor (**6**) to the ϵ-amino-caproic acid and cystamine containing biotinyl spacer (**7**). The function of all relevant groups of the final activity label (**8**) (biotin-label, cleavable disulfide bridge, phosphonate-inhibitor) have been successfully tested with the commercial lipase Lipolase® (Novo Nordisk). Hence, a tool for directed molecular evolution of lipolytic enzymes has been developed.

Introduction

One of the goals of protein engineering is to produce enzymes with altered properties (e.g. changes in catalytic properties, stability, specificity, or pH profile). Based on careful examination of primary sequence and three-dimensional structure such changes can be achieved by site-directed mutagenesis of selected amino acids. The prediction, however, of changes needed to alter the properties of an enzyme in any desired direction still remains difficult particularly when dealing with catalysis due to our limited understanding of such characteristics.

An alternative to rational design is directed molecular evolution. In an attempt to mimic nature, the first step of the evolution in the laboratory is the generation of a large library of variants by random mutagenesis. The identification of individual mutants with desired properties within the library then relies on the design of efficient selection or screening protocols. Either the fittest variant is used as the starting point for a new round of mutagenesis or a number of the isolated and improved variants are recombined and the screening/selection is repeated using the so called shuffling approach.[1] The screening/selection is carried out under increasingly more stringent conditions. The evolutionary scheme is repeated for the necessary number of cycles until the desired properties have been achieved.

Often it has been difficult to correlate one particular property of an enzyme to a selectable phenotype of a living cell and selection schemes cannot be utilized. Screening protocols are typically laborious and only variant libraries of relatively limited size can be thoroughly screened. Consequently it would be desirable to develop new methods that would allow for enrichment for particular properties at the molecule level amongst huge populations of variants. One of the requirements for such experiments is the direct coupling of phenotype and genotype such that the enzyme is displayed and available for interactions with an inhibitor or a ligand, which can be achieved by various methods such as ribosomal display,[2] or display of oligo-peptides or polypeptides on phages,[3] virus[4] or cells.[5]

Amongst these, phage display, in which functional polypeptides are displayed on the surface of filamentous bacteriophages, has become the most developed and commonly used method. The gene encoding the enzyme is packaged within the bacteriophage and the enzyme

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itself is displayed on the phage surface as a fusion protein, thereby providing the direct link between the desired property of a selected variant and the DNA encoding it.

Another important prerequisite for the overall efficiency of this technique is the design and the application of suitable and effective enrichment protocols.[6,7]

The use of phage display to select for catalytic activity has been reported: mixtures of displayed active or inactive enzymes are incubated with a bifunctional activity label that features a mechanism-based inactivator (or suicide inhibitor) of the target enzyme. The suicide inhibitor is connected to an affinity label through a linker including a disulfide bond which can be reductively cleaved. On incubation of a mixture of phage enzymes, those phages showing catalytic activity on the inhibitor under the conditions of the experiment are labeled with the affinity ligand. These phages can be recovered on an affinity support. Cleavage of the disulfide bond releases these phages displaying the formerly active enzymes from the support.[8–10]

Mechanism-based inhibitors are, ideally, relatively unreactive organic compounds that become activated by the target enzyme itself using its specific mechanism. So far bifunctional activity labels for selection of filamentous bacteriophages displaying β-lactamase and penicillin binding proteins based on the above design have been prepared and proven successful. A mixture of phages displaying β-lactamase and an inactive mutant was incubated first with the biotyinlated suicide inhibitor, then with streptavidin coated beads, the immobilised phages were recovered and analyzed; the mixture was not only shown to be enriched in active enzymes but the most active phage-enzymes were selected from the mixture.[7,11]

Lipases (EC 3.1.1.3.) are serine hydrolases and catalyze the hydrolysis of organic esters as well as esterfication. The natural substrates of lipases are triglycerides of long-chain fatty acids (fats and oils), but they also accept other hydrophobic esters of primary and secondary alcohols. The capability of degrading fatty stain to a mix of more soluble free fatty acids, diglycerides, monoglycerides, and glycerol has led to the common application of lipases, for example, Lipolase®, in detergents[12] and in fat and oil processing.[13] The finding that lipases can act in organic solvents has expanded their applicability in a wide variety of chemical reactions. At present they constitute the largest groups of synthetically useful enzymes.[14,15]

This publication describes the design and synthesis of a biotinylated suicide-inhibitor to be used for directed molecular evolution of lipolytic enzymes. We present experimental proof of the functionality of the various parts of the inhibitor, viz. the interaction with the *Humicola lanuginosa* lipase commercially available as Lipolase® (Novo Nordisk), the interaction of the affinity label with the affinity support, and the cleavage of the linker by reduction.

Results and Discussion

Affinity label design

The structure features a 4-nitrophenyl (PNP) activated phosphonate, that is, a suicide substrate of lipolytic enzymes connected to a biotin moiety (affinity handle) through a spacer containing a disulfide bridge (reductive cleavage site). Affinity handle and cleavage site are identical to the β-lactamase label described by Fastrez and co-workers.[7]

The inhibitor is of the phosphonate irreversible type which mimics esters and triglycerides: the enzyme–inhibitor complex simulates the tetrahedral intermediate formed during lipolytic enzyme-catalyzed hydrolysis of natural substances. A direct covalent adduct between the nucleophilic hydroxyl group of the active site serine residue and the phosphorus atom of the inhibitor is formed releasing a nucleofug from the phosphonate.[16–19]

The linkage between the phosphonate and the reductive cleavage site was chosen to be a C11-alkyl chain in order to ensure a certain length to be able to fit into the hydrophobic cleft of lipase variants leaving the cleavage site outside.[20]

4-Nitrophenol was chosen as a leaving group to ensure efficient reactivity towards the active site and at the same time stability towards hydrolysis with water. In principle the sensitivity of the suicide substrate could be adjusted by the choice of leaving groups as the inactivation rate of phosphonates depends on the chemical identity of the leaving group (Fig. 1).[21,22]

Chemistry

Michaelis–Arbuzov reaction[23] of trimethylsilyl-protected 11-bromoundecanol (**2**)[24] with triethyl phosphite yielded the phosphonate **3**. Protection of **1** was necessary in order to avoid side reactions of the terminal hydroxyl group: intramolecular or intermolecular transesterfications[25] have otherwise led to significant losses and made product isolation difficult.

Deprotection of **3** using sulphuric acid/acetone gave the ω-hydroxyalkylphosphonate (**4**). Compound **4** was treated with phosgene, producing a chloroformate endgroup, which was then reacted with *N*-hydroxysuccinimide. This process resulted in a complete conversion of the end group to the ω-succinimidyl carbonate activated alkylphosphonate (**5**), which is capable of reacting with primary amines to give a stable carbamate bond.[26] This phosphonate (**5**) was transformed into the corresponding ethylphosphonochloridate by oxalyl chloride which was esterfied with 4-nitrophenol to the phosphonate inhibitor (**6**).[22] Finally, **6** was coupled with the primary amine **7** (biotinyl-*N*-ϵ-aminocaproyl-*N*-cystamine-*N*′-cystaminammonium trifluoroacetate), which was prepared in a six-step synthesis according to ref 7, to give the final biotinylated suicide inhibitor (**8**) (Scheme 1).

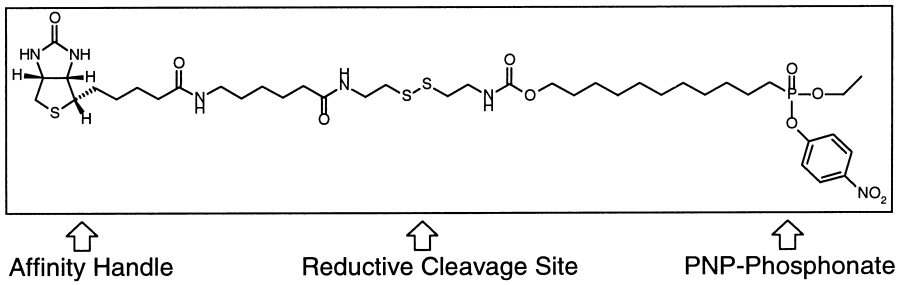
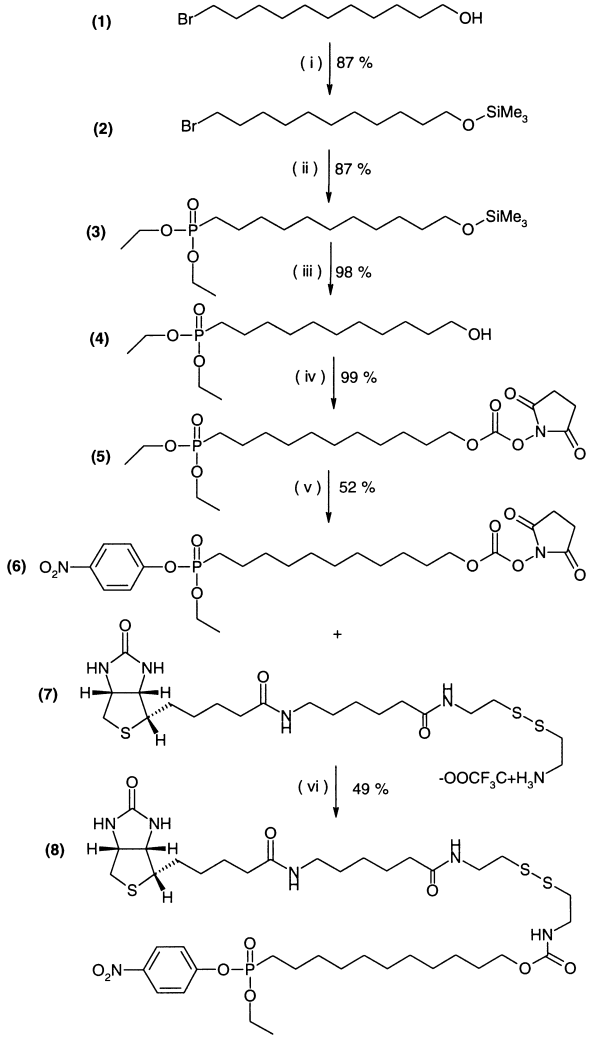


Figure 1. Structure of the affinity label: a 4-nitrophenyl activated phosphonate, that is, a suicide substrate of lipolytic enzymes is connected to a biotin moiety (affinity handle) through a spacer containing a disulfide bridge (reductive cleavage site).



Scheme 1. Reagents and conditions: (i) Me_3SiCl, Et_3N, CH_2Cl_2/ether; (ii) $(EtO)_3P$/reflux; (iii) H_2SO_4/acetone; (iv) 1. $COCl_2$, 2. *N*-hydroxysuccinimide/Et_3N; (v) 1. $(COCl)_2$, 2. 4-nitrophenol/Et_3N; (vi) DMF/Et_3N.

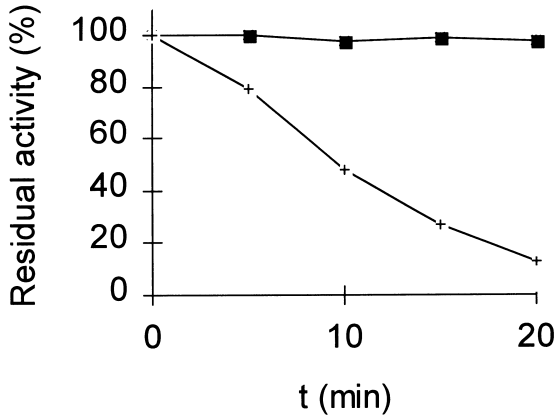


Figure 2. Inibition of Lipolase® by the suicide inhibitor (**8**) in a time dependent manner: Lipolase® was mixed with the suicide inhibitor (**8**) and the residual activity (crosses) was determined after 0, 5, 10, and 20 min by measuring the release of 4-nitrophenol in a PNP-butyrate assay at 405 nm. The residual activity of uninhibited Lipolase® is shown as a control (filled squares).

Functional analysis of the suicide inhibitor (8)

The inhibitor **8** inactivates native Lipolase® in a pseudo-first-order kinetics with a *t*-half of inactivation at about 12 min (Fig. 2). Thus the rate constant of inactivation has the same order of magnitude as that found for other sterically less hindered 4-nitrophenyl activated phosphonates and digestive lipases.[22] The inactivation of Lipolase® was found to be irreversible; no recovery of lipase activity was observed after overnight dialysis of inhibited Lipolase® against 500 volumes 100 mM Tris–HCl, pH 7.5 (data not shown).

Inactivated Lipolase® should be affinity tagged by biotin due to the covalent bond formed between the enzyme and the inhibitor. We, therefore, determined the affinity of inhibited Lipolase® for streptavidin by ELISA using horseradish peroxidase (HRP) conjugated anti-Lipolase® antibodies. As can be seen in Figure 3, inhibited Lipolase® has affinity for streptavidin: a strong positive signal is obtained for inhibited Lipolase®, whereas no signal is obtained using uninhibited Lipolase® as control.

In order to further assess the functionality of the inhibitor, Lipolase® was inhibited, and then bound onto streptavidin coated magnetic beads. The beads were washed carefully and then resuspended in a DTT (dithiothreitol) containing buffer to allow reduction of the disulfide bridge within the inhibitor. After 1 h incubation in the reducing buffer the beads were removed and the eluate was subjected to SDS–PAGE gelelectrophoresis. Following electrophoresis, proteins were blotted onto a nitrocellulose membrane. The membrane

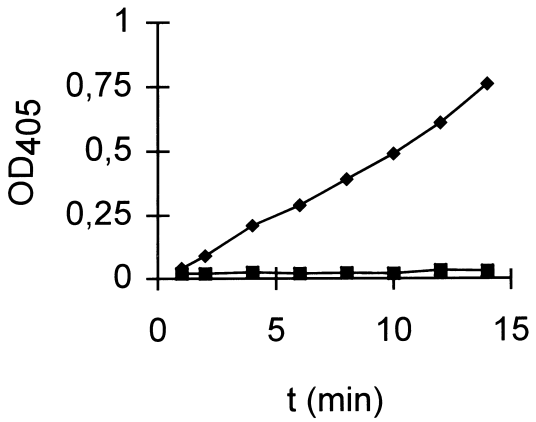


Figure 3. Lipolase®, inactivated by the inhibitor (**8**), and the affinity of inactivated Lipolase® for streptavidin coated surfaces, has been quantified by ELISA using horseradish peroxidase (HRP) conjugated anti-Lipolase® antibodies. Protocol: 1. streptavidin, 2. Lipolase®, 3. α-Lipolase®, 4. HRP α(α-Lipolase®), 5. detection reagent (1,2-phenylenediamine dihydrochloride). The figure shows the colour development at 405 nm by HRP catalyzed oxidation of 1,2-phenylenediamine dihydrochloride (inhibited Lipolase®: filled diamonds; active Lipolase®: filled squares). Thus inhibited Lipolase® binds to streptavidin coated plates whereas active Lipolase® does not.

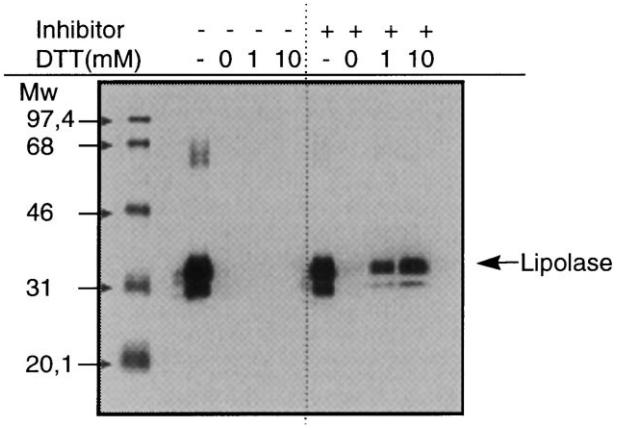


Figure 4. Elution of inhibited or active Lipolase® from streptavidin coated magnetic beads after washing and incubation with buffer containing DTT (dithiothreitol) at indicated concentrations. M: marker; Lane 1: 25 ng active Lipolase®; Lanes 2–4: elution of non-inhibited Lipolase® after wash and incubation with buffer containing 0, 1, or 10 mM DTT. Lane 5: 25 ng inhibited Lipolase®; Lanes 6–8: elution of inhibited Lipolase® after wash and incubation with buffer containing 0, 1, or 10 mM DTT. Lipolase® capture and detection was performed as described in experimental procedures.

was developed by treatment first with rabbit anti-Lipolase® antibodies and then with HRP conjugated porcine anti rabbit antibodies. As shown in Figure 4, inhibited Lipolase® is bound by the magnetic beads, but can be released again when the disulfide bridge is reduced.

Conclusion

A bifunctional activity label (**8**) for selection of displayed lipases/esterases has been designed and a practical synthesis developed. All three functions of **8**, such as affinity handle, reductive cleavage site, and inhibitor were shown to fulfill the properties necessary to be used for the selection of lipolytic enzymes in directed molecular evolution experiments. The new suicide inhibitor (**8**) will presumably be an important tool in the development of new, promising enzymes within this industrially very important enzyme class.

Experimental

Syntheses

^{1}H and ^{13}C NMR spectra were taken on a Bruker DRX 400 spectrometer. ^{31}P NMR spectra were recorded on a Varian Mercury 400 spectrometer using H_3PO_4 as internal standard. FAB mass spectra were recorded on a AutoSpec Ultima instrument double focussing mass spectrometer (EBE geometry) interfaced to a data system. Caesium fast ions generated with 33 kV and 2 μA emission current were used for bombardment (glycerol matrix, 8-kV accelerating potential, ion source temperature about 50 °C). The electrospray ionisation (ESI) mass spectrometry was performed on a Finnigan LCQ ion trap mass spectrometer equipped with a syringe pump inlet (source voltage: 4.5 kV, capillary temperature: 235 °C). Mass spectrometry, where the sample was introduced using a direct inlet probe (DIP), was performed on a Finnigan MAT 70B TSQ triple quadrupole mass spectrometer with a data processing system. The sample was analysed in the full scan mode with electron impact ionisation (EI) at 70 eV (temperature programming: 60 °C (0.1 min) −300 °C, 50 °C/min thereafter 25 °C/min to 350 °C, hold for 10 min, source temperature: 150 °C). Elemental analyses were determined by PD Analytical Laboratories (Novo Nordisk A/S, Måløv). Melting points were determined on a Büchi 510 and are uncorrected. All chemicals used were from commercial sources (Aldrich, Fluka, Avocado). Merck silica gel 60, 70–230 mesh ASTM was employed for column chromatography. Thin-layer chromatography was performed using Alugram SIL G/UV_{254} TLC-plares from Macherey-Nagel. Visualization was effected with UV-light, a spray of $KMnO_4$, and a spray of *p*-dimethylaminocinnalaldehyde[27] (DMACA) which is specific for biotin derivatives.

[(11-Bromoundecyl)oxy](trimethyl)silane (2). Freshly distilled trimethyl chlorosilane (23.8 g, 219.1 mmol) dissolved in diethylether (75 mL) was added dropwise to a solution of (**1**) (50.0 g, 199.0 mmol) and dry triethylamine (22.2 g, 219.4 mmol) in CH_2Cl_2 (100 mL) and diethylether (100 mL) with stirring so that the temperature remained below 5 °C (inert atmosphere). After stirring overnight, precipitated triethylamine hydrochloride was filtered off and the solvents removed in vacuo. The residual oil was distilled under high vacuum (bp 130–135 °C/1 mbar; Lit.[24] 123–127 °C 2 mmHg) to give **2** as a colorless liquid (56.0 g, 87%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.05 (s, 9H), 1.77 (m, 2H), 1.36 (m, 4H), 1.2–1.3 (m, ca. 12H), 3.35 (t, 2H), 3.51 (t, 2H).

Diethyl 11-[(1,1,1-trimethylsilyl)oxy]undecylphosphonate (3). A mixture of [(11-bromoundecyl)oxy](trimethyl)-silane (**2**) (50.0 g, 154.6 mmol) and triethyl phosphite (51.4 g, 309.3 mmol) was slowly heated to 156 °C and this temperature was maintained overnight. The ethyl bromide evolved was trapped with a condenser and a receiver cooled in an ice bath. Excess triethyl phosphite was removed under reduced pressure (1 mbar) and the residual oil was fractionally distilled under high vacuum (bp >157 °C/0.02 mbar) to give **3** as a colorless liquid (51.2 g, 87%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.05 (s, 9H), 1.20–1.35 (m, ca. 14H), 1.21 (t, 6H), 1.42 (m, 4H), 1.65 (m, 2H), 3.50 (t, 2H), 3.95 (dq, 4H). MS (ESI): m/z 403 (M + Na); ^{31}P NMR (162 MHz, $CDCl_3$) δ 33.27.

Diethyl (11-hydroxyundecyl)phosphonate (4). Compound **3** (15.0 g, 39.4 mmol) was dissolved in acetone (100 mL). Dilute H_2SO_4 (5%, 100 mL) was added and the mixture stirred at room temperature for 30 min. The mixture was diluted with water (1 L) and diethylether (200 mL) was added to improve phase separation. The organic phase was separated and the water phase was extracted 2× with diethylether. The combined organic phases were dried over $MgSO_4$ and the solvent was removed in vacuo after filtration to give **4** as a colorless liquid (11.91 g, 98%), which was used in the next step without purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25–1.40 (m, ca. 14H), 1.32 (t, 6H), 1.57 (m, 4H), 1.72 (m, 2H), 3.63 (t, 2H), 4.08 (m, 4H). MS (ESI): m/z 309 (M + H); ^{31}P NMR (162 MHz, $CDCl_3$) δ 33.34.

Diethyl [11-([(2,5-dioxotetrahydro-1*H*-1-pyrrolyl)oxy]-carbonyloxy)undecyl]phosphonate (5). Compound **4** (11.91 g, 38.6 mmol) was dissolved in CH_2Cl_2 (200 mL). Phosgene (1.93 M solution in toluene) (100 mL, 193.0 mmol) was added to the cooled mixture (ice bath) with stirring. The mixture was stirred overnight slowly allowing it to reach room temperature. The solvents and excess phosgene were removed in vacuo. The intermediate [11-(chlorocarbonyloxy)undecyl](diethoxy) was redissolved in dry CH_2Cl_2 (200 mL) followed by addition of *N*-hydroxy succinimide (4.54 g, 39.4 mmol). Dry triethylamine (6.76 g, 66.8 mmol) was added dropwise to the solution with stirring at room temperature. The solvents were removed in vacuo after stirring overnight. The remainder was redissolved in a small volume of diethylether, insoluble triethylamine hydrochloride was filtered off using a glass frit (5). Evaporation of the solvents in vacuo yielded an oil, which crystallized on standing (17.17 g, 99%): mp 58–59 °C (ether); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25–1.43 (m, ca. 14H), 1.32 (t, 6H), 1.60 (m, 2H), 1.73 (m, 4H), 2.84 (s, 4H), 4.08 (m, 4H), 4.31 (t, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.45 ($J_{c,p}$ = 5 Hz), 22.35 ($J_{c,p}$ = 5 Hz), 25.35, 25.41, 25.62 ($J_{c,p}$ = 140 Hz), 28.29, 28.99, 29.17, 29.25, 29.27, 29.34, 30.53 ($J_{c,p}$ = 17 Hz), 61.23 ($J_{c,p}$ = 6 Hz), 71.50, 151.24, 168.30; m/z 450 (M + H); ^{31}P NMR (162 MHz, $CDCl_3$) δ 33.38. Anal. calcd for $C_{20}H_{36}NO_8P$: C, 53.44; H, 8.07; N, 3.12. Found: C, 53.22; H, 8.08; N, 3.36.

Ethyl (4-nitrophenyl)[11-([(2,5-dioxotetrahydro-1*H*-1-pyrrolyl)oxy]carbonyloxy)undecyl]phosphonate (6). Compound **5** (4.50 g, 10.0 mmol) was dissolved in a mixture of dry CH_2Cl_2 (50 mL). Oxalyl chloride (3.17 g, 25.0 mmol) was added at 0 °C with stirring under an inert atmosphere. The mixture was slowly allowed to reach room temperature and stirred for 18 h at this temperature. The solvents and excess oxalyl chloride were removed in vacuo. The intermediate monochlorophosphonate (^{31}P NMR (162 MHz, $CDCl_3$) δ 46.03) and 4-nitrophenol (1.39 g, 10.0 mmol) were dissolved in dry CH_2Cl_2 (60 mL). Triethylamine (2.02 g, 20.0 mmol) was added dropwise at room temperature and the mixture was stirred for 3 h. The triethylamine hydrochloride formed was filtered off using a glass frit (G5). A yellow oil was obtained after evaporation of the solvent in vacuo, which was purified by flash chromatography (silical gel, CH_2Cl_2:AcOEt (7:1, v:v)) to give pure **6** as an oil (2.8 g, 52%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.2–1.4 (m, ca. 14H), 1.24 (t, 3H), 1.56 (m, 2H), 1.67 (m, 2H), 2.01 (m, 2H), 2.81 (s, 4H), 4.14 (m, 2H), 4.33 (t, 2H), 7.47 (d, 2H), 8.29 (d, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.39 ($J_{c,p}$ = 6 Hz), 22.23 ($J_{c,p}$ = 5 Hz), 25.83, 25.50, 26.09 ($J_{c,p}$ = 137 Hz), 28.38, 28.99, 29.04, 29.26, 29.31, 29.36, 30.42 ($J_{c,p}$ = 17 Hz), 62.91 ($J_{c,p}$ = 7 Hz), 71.64, 120.98 ($J_{c,p}$ = 5 Hz), 125.67, 144.57, 151.65, 155.85 ($J_{c,p}$ = 8 Hz), 168.67. MS (EI) m/z 543 (M + H); ^{31}P NMR (162 MHz, $CDCl_3$) δ 31.17. Anal. calcd for $C_{24}H_{35}N_2O_{10}P$: C, 53.13; H, 6.50; N, 5.16. Found: C, 52.89; H, 6.61; N, 5.24.

Ethyl (4-nitrophenyl)(11-[(2-[(2-[6-(5-[(3a*R*,4*R*,6a*S*)-2-oxoperhydrothieno[3,4-*d*]imidazol-4-yl]pentanoylamino)-hexanoyl]aminoethyl)disulfanyl]ethylamino)carbonyl]oxy-undecyl) phosphonate (8). Compound **6** (264 mg, 0.49 mmol) and biotinyl-*N*-e-aminocaproyl-*N*-cystamine-*N*′-cystaminammonium trifluoroacetate[7] (297 mg, 0.49 mmol) were dissolved in dry DMF (30 mL) under an inert atmosphere. Dry triethylamine (149 mg, 1.47 mmol) was added and the mixture was stirred for 4 h. The mixture was poured into water (500 mL) and the water phase was extracted with AcOEt (3×150 mL). The combined organic phases were dried over $MgSO_4$ and the solvent was removed in vacuo after filtration to give an oil, which was purified by flash chmromatography (silical gel, CH_2Cl_2:MeOH (8:1, v:v)) to give pure **8** as a white solid (220 mg, 49%). ^{1}H and ^{13}C NMR see Table 1, mp 127–132 °C. MS (FAB+) m/z 919 (M + H), ^{31}P NMR (162 MHz, $CDCl_3$) δ 31.71. Anal. calcd for $C_{40}H_{67}N_6O_{10}PS_3$: C, 52.27; H, 7.35; N, 9.14. Found: C, 52.10; H, 7.31; N, 8.93.

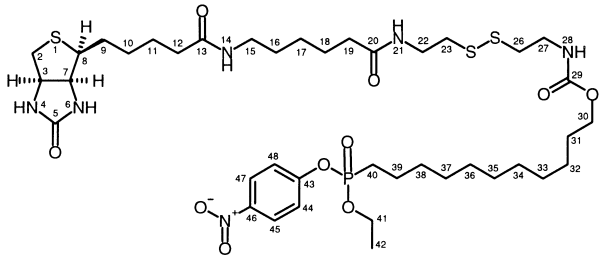
Enzyme activity and inhibition

Purified recombinant Lipolase® (100 μg/mL in 100 mM Tris–HCl, 150 mM NaCl, 0.3 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 7.5) was mixed with suicide inhibitor **8** (50 mM in DMSO) to a final inhibitor concentration of 25 μM). At t = 0, 5, 10 and 20 min 10 μL samples were withdrawn and added to 990 μL lipase assay buffer (80 μM PNP-butyrate in 50 mM Tris–HCl, 0.1% Triton X-100, 10 mM $CaCl_2$, pH 7.5). Release of 4-nitrophenol was recorded continuously at OD_{405} on a Milton Roy 1201 spectraphotometer.

Table 1.

Position[a]	^{1}H	^{13}C[b]
2	2.82 (dd, 5 and 13 Hz)	39.82
	2.58 (d, 13 Hz)	
3	4.30 (m)	59.13
4	6.34 (s(br))	
5		162.59
6	6.41 (s(br))	
7	4.13 (dd, 7 and 8 Hz)	60.99
8	3.09 (m)	55.37
9	1.62 (m)	27.93
	1.47 (m)	
10	1.30 (m)	28–29
11	1.53 (m)	25.2
12	2.04 (t, 7 Hz)	35.20
13		171.67
14	7.71 (t(br), 6 Hz)	
15	2.99 (q(br), 6 Hz)	38.20
16	1.37 (m)	29.88
17	1.22 (m)	26.02
18	1.49 (m)	24.87
19	2.05 (t, 6 Hz)	35.12
20		172.09
21	7.94 (t(br), 6 Hz)	
22	3.31 (q(br), 6 Hz)	37.87
23	2.76 (t, 7 Hz)	37.4
26	2.76 (t, 7 Hz)	37.4
27	3.25 (q(br), 6 Hz)	39.66
28	7.23 (t(br), 6 Hz)	
29		156.23
30	3.92 (t, 7 Hz)	63.75
31	1.52 (m)	28.6
32	~1.3 (m)	25.2
33–37	~1.3 (m)	28–29
38	~1.3 (m)	29.47 (16 Hz)
39	1.56 (m)	21.73 (5 Hz)
40	2.00 (m)	24.69 (137 Hz)
41	4.14 (m)	62.31 (7 Hz)
42	1.25 (m)	16.05 (6 Hz)
43		155.44 (8 Hz)
44/48	7.47 (dd, 9 and 1 Hz[c])	121.22 (4 Hz)
45/47	8.28 (d, 9 Hz)	125.71
46		143.90

[a]Numbering according to formula below.
[b]Carbon-phosphorous coupling constants (J_{cp}) in parentheses.
[c]$^4J_{HP}$.



Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Maxisorp; Nunc; Roskilde; Denmark) were coated overnight with 10 ng/well NeutrAvidin biotin-binding protein (Pierce, Illinois, USA) in 50 mM $NaHCO_3$, pH 9.6; blocked with 3% (wt/vol) milkpowder in Tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20 (TBST) for 2 h and washed five times with 0.5 M NaCl, 0.05% Tween 20. Active or inhibited Lipolase® (100 μg/mL) was diluted 1/250 in 1% (w/v) milkpowder in TBST. 100 μL (40 ng) active or inhibited Lipolase® was placed in coated wells and allowed to bind for 1 h after which wells were washed five times with 0.5 M NaCl, 0.05% Tween 20. Primary antibody: 100 μL of anti-Lipolase® antibodies (rabbit) diluted 1:5000 in 1% (w/v) milkpowder in TBST were placed in the wells for 1 h. Secondary antibody was a peroxidase conjugated porcine-anti rabbit IgG (Dako) diluted 1:5000 in 1% (w/v) milkpowder in TBST. After 1 h of incubation, the reactions were revealed by using 1,2-phenylenediamine dihydrochloride (OPD, DAKO) and H_2O_2 in citrate buffer (0.1 M citric acid; 67 mM Na_2HPO_4; pH 5.0). Colour development was monitored continuously at OD_{405} in an UVmax kinetic microplate reader from Molecular Devices.

Western blotting and immunodetection

2.5 μL inhibited Lipolase® (ca. 250 ng) was mixed with 50 μL Dynabeads M-280 streptavidin coated magnetic beads (Dynal, Norway) in TBS (100 mM Tris–HCl, 150 mM NaCl, pH 7.5), and incubated at 15 °C for 4 h with occasional agitation. Beads were then washed five times in 500 μL TBST (TBS, 0.1% Tween-20), and dissolved in 50 μL TBS containing either 0, 1, or 10 mM DTT. After 1 h incubation at 25 °C 10 μL of the supernatant was withdrawn and mixed with 10 μL SDS–PAGE sample buffer, the samples were heated to 98 °C for 4 min and then subjected to 12.5% Laemmli SDS–PAGE gelelectrophoresis on an Xcell II Mini-Cell™ chamber (Novex). Following electrophoresis, proteins were transferrred from the gel onto a nitrocellulose membrane (Schleicher and Schuell) using Novex Xcell II Blot module. The nitrocellulose membrane was blocked, washed, and developed using ECL Western blotting protocols (Amersham International, England) as recommended by supplier. The primary antibody (rabbit anti-Lipolase®) was diluted 1:8000 in TBST, and the secondary antibody, which was peroxidase conjugated porcine-anti rabbit IgG (Dako), was diluted 1:8000 in 1% (wt:vol) milkpowder in TBST. Protein molecular weight markers were from Amersham International and were applied to the gel and developed as recommended by supplier.

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