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Design and Synthesis of Triglyceride Analogue Biotinylated Suicide Inhibitors for Directed Molecular Evolution of Lipolytic Enzymes

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Abstract—The design, synthesis, and inhibition properties of two new triglyceride analogue biotinylated suicide inhibitors **(2)** and **(3)** for directed molecular evolution of lipolytic enzymes by phage-display is described.

Introduction

Directed molecular evolution is an attempt to mimic nature, in producing enzymes with altered properties (e.g., changes in catalytic properties, stability, specificity, or pH profile). Large libraries comprising up to 10^{10} variants can today easily be created by different methods of random mutagenesis in the laboratory. The current bottle-neck is the selection and screening of the library in order to identify individual mutants with the desired properties. Therefore, the development of appropriate search systems is of key importance to the whole process of directed evolution.[1] Phage display[2] has become the most developed and commonly used method in direct coupling of phenotype and genotype of enzymes: The gene encoding the enzyme is packaged within a bacteriophage and the enzyme itself is displayed on the phage surface as a fusion protein. Those phages displaying the desired enzymes on the surface can be selected from a phage library by biopanning: Mixtures of displayed active or inactive enzymes are incubated with a bifunctional activity label that features a mechanism-based inactivator[3] (or suicide inhibitor) of the target enzyme. The suicide inhibitor is connected to an affinity label (e.g., biotin) 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 streptavidin coated affinity support. Cleavage of the disulfide bond releases these phages displaying the formerly active enzymes from the support.[2b,4] The captured phages with high affinity for the ligand can be enriched, amplified and further characterized. The evolutionary scheme is repeated for the necessary number of cycles until the desired properties have been achieved. So far bifunctional activity labels for selection of filamentous bacteriophages displaying β-lactamase and penicillin binding proteins,[4,5] β-galactosidase,[6] and Lipolase® (a commercially available *Humicola lanuginosa* lipase from Novo Nordisk),[7] based on the above design, have been used. Lipases (EC 3.1.1.3.) are serine hydrolases and catalyze the hydrolysis of organic esters as well as esterification. The natural substrates of lipases are triglycerides of long-chain fatty acids (fats and oils). The capability of degrading fatty stain to a mixture of more soluble free fatty acids, diglycerides, monoglycerides, and glycerol has led to the common application of lipases, e.g. Lipolase®, in detergents[8] and in fat and oil processing.[9] Lipases constitute furthermore the largest group of synthetically useful enzymes.[10]

Affinity Label Design

The structure of the suicide inhibitor of Lipolase® **(1)**, which was synthesized earlier according to the design discussed above,[7] is shown in Figure 1. The inhibitor is of the phosphonate irreversible type, which mimics the tetrahedral transition state intermediate occuring during ester hydrolysis. A direct covalent adduct between nucleophilic hydroxyl group of active site serine residue in the catalytic triad and the phosphorus atom of the inhibitor

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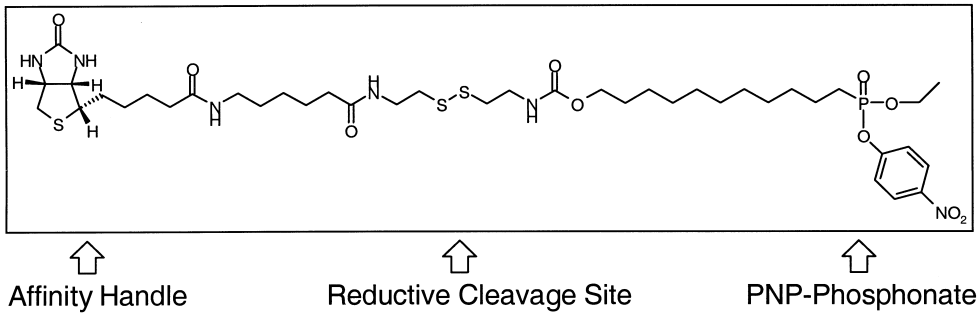


Figure 1. Structure of the affinity label (**1**): a 4-nitrophenol activated phosphonate, i.e., a suicide substrate of lipolytic enzymes is connected to a biotin moiety (affinity handle) through a spacer containing a disulfide bridge (reductive cleavage site).[7]

is formed releasing the nucleofuge 4-nitrophenolate from the phosphonate.[11] 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 allowing to fit into the hydrophobic cleft of lipase variants leaving the cleavage site outside.[12] The leaving group PNP ensures efficient reactivity towards the active site together with the necessary stability towards hydrolysis with water.[13] The suicide substrate design of **(1)** was chosen on the basis of information about lipase-substrate interaction at a submolecular level gained earlier through X-ray crystallography of crystals of simple organophosphonates conjugated with various lipases.[11b,c,d,12,14] However, the inhibitors used in these studies showed only little similarity to the natural lipase substrates, i.e., triacylglycerols. Accordingly the suicide inhibitor part of **(1)** only resembles the transition state intermediate of ester hydrolysis and not the additional structural features of triacylglycerols, which are important for specific substrate-enzyme interactions apart from the catalytic triad. The most important rule of screening is 'You get what you screen for', so the ability to screen for the closest structural analogue of the target compound is an important advantage. More recently, dicarmoyloxypropanol,[15] diacylglycerol,[16] and dialkylglycerol[17] esters of alkylphosphonic acid have been reported as lipase inhibitors, containing alkyl chains bound to a glycerol backbone, thus structurally resembling triacylglycerols. In order to obtain triglyceride analogue biotinylated suicide inhibitors useful for the selection of lipases with improved catalytic activity towards triacyl glycerols, we synthesized **(2)** and **(3)**.[18] There the phosphonate unit is attached at the *sn*-1 position (the most general approach for 1,3- or unspecific lipases) of the triglyceride mimicking moiety (Fig. 2).

The additional glycerol-like moieties providing more natural lipase–substrate interactions due to the alkyl chains in the *sn*-2 and *sn*-3 positions are thought to result in a more targeted screening: **(2)** mimics a short chain triglyceride with C_9 at *sn*-2 and C_{10} at *sn*-3 without, however, the features of the two ester bonds at those positions. **(3)** corresponds to a long-chain ($C_{18}N$) analogue, where the hydrolyzable ester bonds are replaced

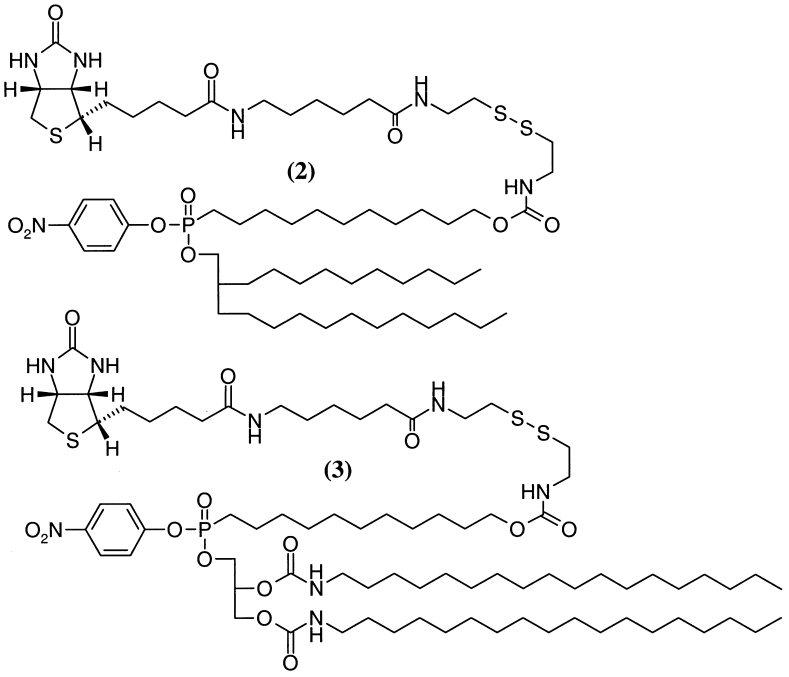


Figure 2. Structures of the triglyceride analogue affinity labels (**2**) and (**3**): the 4-nitrophenol activated phosphonate is attached at the *sn*-1 position to two different glyceride-like analogues. The remaining design (biotin moiety, spacer, disulfide bridge) remained unchanged in comparison to (**1**).

by carbamoyl functions.[15] As the PNP-phosphonate ester is the only moiety, which is susceptible towards nucleophilic attack by the serine residue in the active site, undesirable side reactions by lipases are avoided. Hence, improved tools for directed evolution of lipases towards triglyceride activity have been designed.

Lipase Inhibition

The two affinity labels **(2)** and **(3)** were tested for their ability to inactivate Lipolase® according to method used previously for **(1)**.[19] Like **(1)** do both new labels irreversibly inactivate native Lipolase® in a pseudo-first-order kinetics. However, the half-life of inactivation was found to be 65 min for **(2)** and 85 min for **(3)** (Fig. 3) in comparison to the earlier determined 12 min for **(1)**.[7] As all three labels consist of the same type of suicide inhibitor, these differences can only be attributed to the additional steric requirements of the glycerol-like molecular units attached to the phosphorus atom, or due to chiral discrimination at one of the stereocenters.[17a] Accordingly, the least sterically requiring label **(1)** has the shortest t-half, while **(3)**, which is the most bulky, has the longest t-half. The slow rate of inhibition observed for **(2)** and **(3)** might provide an interesting opportunity for selection of mutant lipases having acquired increased activity towards these inhibitors.

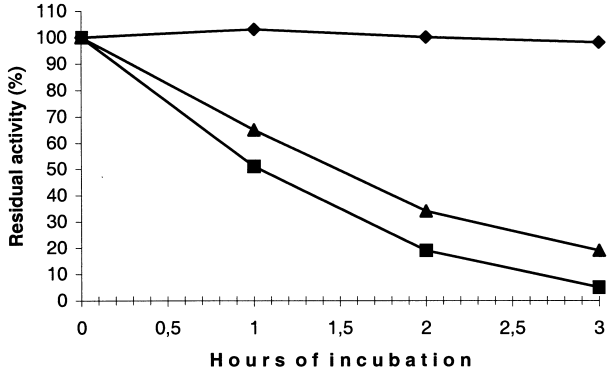
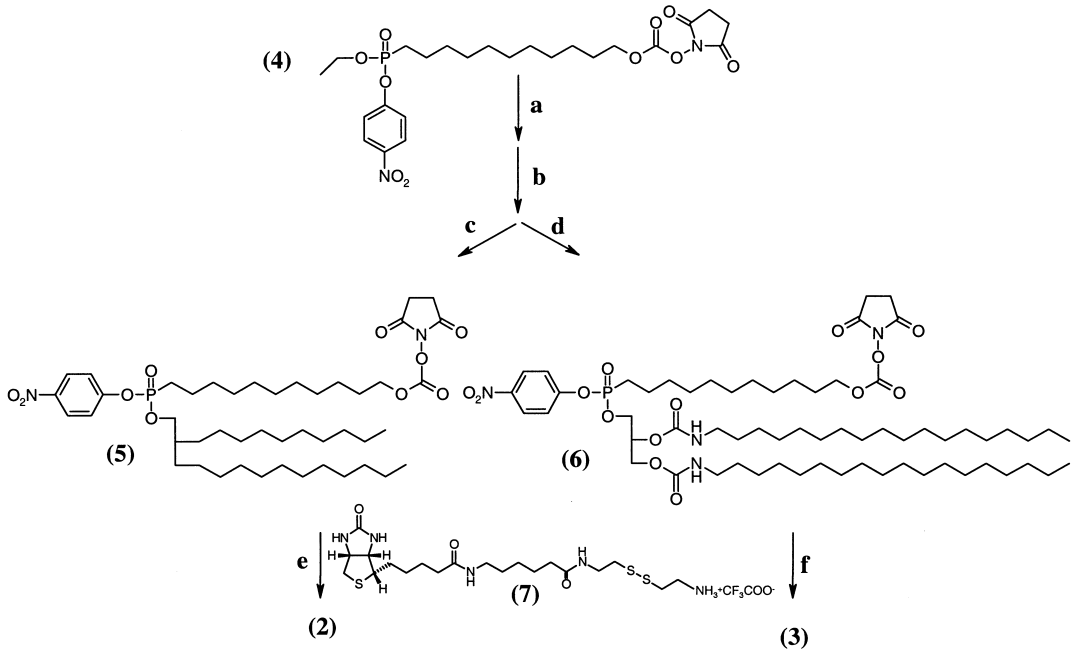


Figure 3. Time dependent inhibition of purified lipolase.[19] ◆: Control (no inhibitor); ■: affinity label/inhibitor (2); ▲: affinity label/inhibitor (3).

Synthesis (Scheme 1)

Ethyl (4-nitrophenyl)[11-([(2,5-dioxotetrahydro-1*H*-1-pyrrolyl)oxy]carbonyloxy)undecyl] phosphonate **(4)** was synthesized in a five step synthesis as described ealier.[7] **(4)** was dealkylated by bromotrimethylsilane (a) to give a trimethylsilyl phosphonate,[20] which was transformed to the PNP-phosphonochloridate by oxaloyl chloride (b).[21] The PNP-phosphonochloridate was esterified either with commercially available racemic 2-decyl-1-tetradecanol (c), or with (*R*,*S*)-1,2-dioctadecylcarbamoyl-glycerol (prepared from octadecyl isocyanate and (*R*,*S*)-1-*O*-benzylglycerol[15a]) (d) to give 2-decyltetradecyl (4-nitrophenyl) [11-([(2,5-dioxotetrahydro-1*H*-1-pyrrolyl)oxy] carbonyloxy)undecyl] phosphonate **(5)**[22] or 2,3-di[(octadecylamino) carbonyl] oxypropyl (4-nitrophenyl) [11-([(2,5-dioxotetrahydro-1*H*-1-pyrrolyl) oxy] carbonyloxyundecyl] phosphonate **(6)**.[23] This reaction sequence of successive dealkylation and esterification of the phosphonate was found superior to the method described by Marguet et al.[13b] using a phosphonodichloridate and *N*-methylimidazole. **(5)** and **(6)** were finally coupled with biotinyl-*N*-ε-aminocaproyl-*N*-cystamine-*N*′- cystaminammonium



Scheme 1. Synthesis of (**2**) and (**3**): (**a**) 3 equiv Me_3SiBr, rt, CH_2Cl_2, 2 days; (**b**) 1.5 equiv $(COCl)_2$, 0°C-rt, CH_2Cl_2, 2h; (**c**) 1 equiv (*R*,*S*)-2-decyl-1-tetradecanol, 1.2. equiv Et_3N, CH_2Cl_2, rt, 4h, chromatography on silica: CH_2Cl_2:AcOEt 25:1, (v:v), 57%; (**d**) 1 equiv (*R*,*S*)-1,2-dioctadecylcarbamoylglycerol, 1.2 equiv Et_3N, CH_2Cl_2, reflux, overnight, recryst. from EtOH, 66%; (**e**) 1 equiv **(7)**, 3 equiv Et_3N, DMF, rt, 3h, chromatography on silica: CH_2Cl_2:MeOH 13:1, (v:v), 39%; (**f**) 1 equiv **(7)**, 3 equiv Et_3N, DMF, 40°C, 3h, chromatography on silica: CH_2Cl_2:MeOH 12:1, (v:v), 36%.

trifluoroacetate **(7)** (prepared in a six step synthesis according to ref 4d), to give the final biotinylated suicide inhibitors 2-decyltetradecyl (4-nitrophenyl) (11-[(2-[(2-[6-(5-[(3a*R*,4*S*,6a*S*)-2-oxoperhydrothieno[3,4-*d*]imidazol-4-yl]pentanoylamino)hexanoyl]aminoethyl)disulfanyl] ethylamino)carbonyl] oxyundecyl)phosphonate **(2)**[24] (e) and 2,3-di[(octadecylamino)carbonyl]oxypropyl (4-nitrophenyl) 11-[([2-(2-[(6-[5-((3a*R*,4*R*,6a*S*)-2-oxoperhydrothieno [3,4-*d*]imidazol-4-yl) pentanoyl] aminohexanoyl) amino] ethyldisulfanyl) ethyl]aminocarbonyl)oxy]undecylphosphonate **(3)**[25] (f).

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18. As neither the stereochemistry at the phoshorus, nor at the chiral C-2 of the glycerol-like backbone is important for our selection purpose, **(2)** and **(3)** were prepared as racemic mixtures.
19. Briefly, 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 the suicide inhibitors (5 mM in chloroform) to a final inhibitor concentration of 50 μM). At t = 1, 2, 3 and 4 h, 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 spectrophotometer.
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22. Data for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz): 0.88 (t, 6H), 1.2–1.3 (m, ca. 52H), 1.39 (m, 3H), 1.65 (m, 2H), 1.74 (m, 2H), 1.94 (m, 2H), 2.84 (s, 4H), 4.00 (m, 2H), 4.32 (t, 2H), 7.39 (d, 2H), 8.23 (d, 2H); FAB$^+$MS: 851.6 (MH^+); mp: 58 °C Anal. $C_{46}H_{79}N_2O_{10}P$: C 65.08 (calc. 64.92), H 9.48 (9.36), N 3.30 (3.29).
23. Data for **6**: ^{1}H NMR ($CDCl_3$, 400 MHz): 0.88 (t, 6H), 1.2–1.3 (m, ca. 66 H), 1.40 (m, 2H), 1.47 (m, 4H), 1.68 (m, 2H), 1.73 (m, 2H), 1.96 (m, 2H), 2.84 (s, 4H), 3.15 (m, 4H), 4.19 (m, 1H), 4.20 (m, 1H), 4.28 (m, 1H), 4.30 (m, 1H), 4.32 (t, 2H), 4.70 (m, 2H), 5.09 (m, 1H), 7.38 (d, 2H), 8.24 (d, 2H); FAB$^+$MS: 1179.8 (MH^+); mp: 67–69 °C. Anal. $C_{63}H_{111}N_4O_{14}P$: C 64.75 (calc. 64.15), H 9.98 (9.48), N 4.46 (4.75).
24. Data for **2**: ^{1}H NMR ($CDCl_3$, 400 MHz): 0.89 (t, 6H), 1.2–1.8 (m, ca. 71H), 1.94 (m, 2H), 2.21 (t, 2H), 2.23 (t, 2H), 2.74

(d, 1H), 2.83 (t, 2H), 2.85 (t, 2H), 2.93 (dd, 1H), 3.16 (m, 1H), 3.24 (br. q, 2H), 3.49 (br. q, 2H), 3.55 (q, 2H), 3.96 (m, 1H), 4.05 (m, 2H), 4.06 (m, 1H), 4.34 (m, 1H), 4.52 (m, 1H), 5.18 (br. s, 1H), 5.39 (br. t, 1H), 6.05 (br. s, 1H), 6.13 (br. t, 1H), 6.67 (br. t, 1H), 7.38 (d, 2H), 8.23 (d, 2H); FAB$^+$MS: 1227.7 (MH^+). Anal. $C_{62}H_{111}N_6O_{10}PS_3$: C 61.83 (calc. 60.65), H 9.42 (9.11), N 6.31 (6.85).

25. Data for **3**: ^{1}H NMR ($CDCl_3$, 400 MHz): 0.88 (t, 6H), 1.2–1.8 (m, ca. 94H), 1.96 (m, 2H), 2.22 (t, 2H), 2.23 (t, 2H), 2.74 (d, 1H), 2.83 (t, 2H), 2.85 (t, 2H), 2.93 (dd, 1H), 3.15 (m, 4H), 3.17 (m, 1H), 3.24 (br. q, 2H), 3.48 (br. q, 2H), 3.55 (q, 2H), 4.05 (t, 2H), 4.19 (m, 2H), 4.20 (m, 1H), 4.30 (m, 1H), 4.34 (dd, 1H), 4.52 (m, 1H), 4.81 (br. s, 2H), 5.05 (br. s, 1H), 5.10 (m, 1H), 5.41 (br. t, 1H), 5.82 (br. s, 1H), 6.06 (br. t, 1H), 6.67 (br. t, 1H), 7.38 (d, 2H), 8.23 (d, 2H); ESMS: 1555.8 (MH^+); mp: 134–135 °C. Anal. $C_{79}H_{143}N_8O_{14}PS_3$: C 61.24 (calc. 60.97), H 9.79 (9.26), N 6.41 (7.20).