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Immobilization of chiral enzyme inhibitors on solid supports by amide-forming coupling and olefin metathesis

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Abstract—The question whether phage display can be used as a selection method in the directed evolution of enantioselective enzymes has not been answered satisfactorily to date. In order to be able to test this in a specific case, namely in the hydrolytic kinetic resolution of the acetate derived from α,β-isopropylideneglycerol (IPG) catalyzed by the lipase from *Bacillus subtilis*, suicide enzyme inhibitors anchored on porous glass or polymer beads were designed and synthesized. These are immobilized phosphonates, which bear a leaving group and also contain the chiral substrate (D) and (L)-IPG. Modified SIRAN® (porous glass) and Tentagel® (polymer) were chosen as carriers, attachment occurring via amide-forming coupling or Ru-catalyzed olefin metathesis. Initial lipase inhibition studies are also reported.

1. Introduction

We have previously shown that the methods of directed evolution[1–4] can be used successfully in the quest to create enantioselective enzymes for use in organic chemistry.[5] The combination of random mutagenesis and gene-expression coupled with high-throughput screening for enantioselectivity of thousands of enzyme mutants forms the basis of a fundamentally new concept in the area of asymmetric catalysis. Specifically, the lipase from *Pseudomonas aeruginosa*, which catalyzes the hydrolytic kinetic resolution of a certain chiral ester with a selectivity factor E of only 1.1 (wild-type), was evolved into a variant displaying an E-value of 11.[5] By applying recombinant methods such as DNA shuffling,[3] enantioselectivity was recently increased further to E=51.[6] These efforts involved the screening of about 40,000 enzyme variants. Since one of the problems in this new field of asymmetric catalysis concerns the question of efficient screening-systems for enantioselectivity in a given reaction of interest, a great deal of effort has gone into developing high-throughput *ee*-assays.[7,8] Although none of these assays are universally applicable, in optimal cases 1000–20,000 enzyme variants can be screened per day.

A possible alternative to screening is selection by phage display.[9] Potentially it offers a means to 'evaluate' millions of enzyme variants with respect to a given property within a short time. As originally proposed by Smith[9] the method allows the selection of proteins on filamentous phages by inserting the gene of interest into the phage gene g3p sequence. The encoded enzyme ('phage enzyme') is displayed on its surface. Consequently, proteins with desirable binding properties can be selected from a randomly constructed library of mutants.[10,11] In the field of antibodies new variants have been obtained by affinity maturation, yielding selections of 1 out of 10^9.[12] Moreover, phage display has been used recently to select enzyme variants with improved biophysical properties and/or enhanced catalytic activities. Specifically, the techniques of phage display were utilized to select enzymes on suicide inhibitors,[10] transition state analogs[13] or substrates anchored to the phage.[10]

Enantioselectivity in the present context is a particularly difficult parameter to deal with. Indeed, the idea of applying the technique of phage display in order identify the most enantioselective enzyme or group of enzymes in a super-large library of variants currently does not have an undisputably sound theoretical basis. It may be argued that a search based on maximal enantioselective binding is predestined to lead to the identification of an enzyme displaying unacceptably low activity (in the worst case a single turnover only). On the other hand one can speculate that the process of phage display will reveal enzymes displaying synthetically sufficient but not overpowering differentiation between the (*R*) and (*S*)-enantiomeric forms of the chiral substrate under study, while allowing for acceptable levels of activity. Irrespective of these uncertainties we decided to embark on a long-term project directed towards testing phage display in identifying enantioselective 'hits' in libraries of millions of enzyme variants.

Keywords: biotransformations; olefin metathesis; asymmetric catalysis; lipase; phage display.

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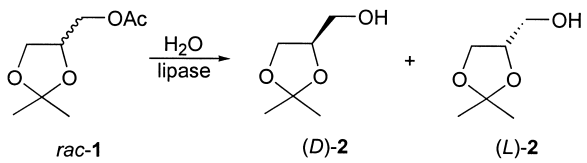
The first phase of our study entails two problems. Firstly, it needs to be demonstrated that the enzyme of interest can in fact be displayed on bacteriophages. Several cases have been reported.[9–11] We ourselves were recently successful in demonstrating this for the lipase from *Bacillus subtilis*.[14] Secondly, since enantioselectivity is the parameter of interest, incorporation of appropriate chirality into the phage display system is necessary. More specifically it requires the synthesis of a chiral enzyme inhibitor, preferably a suicide inhibitor, on a solid phase. In this publication we focus on this synthetic challenge, describing the preparation of appropriate chiral suicide inhibitors anchored on porous glass as well as on polymer beads, designed for a specific enzymatic transformation. Our efforts originate from the notion that phage display may possibly be used in the directed evolution of enantioselective variants of the lipase from *B. subtilis*,[14–16] specifically in the hydrolytic kinetic resolution of the chiral acetate **1** derived from α,β-isopropylideneglycerol (IPG; **2**); the wild-type enzyme shows a selectivity factor of only E=2.1 in slight favor of the alcohol (L)-**2**.[15] This means that the relevant suicide inhibitor needs to be designed so as to mimic the geometric parameters of (L)-**1**.

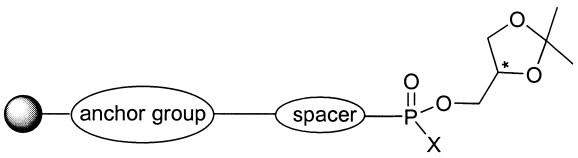


Due to their tetrahedral geometry, bond distances and charge distribution, phosphonic acid esters are generally viewed as acceptable mimics of the transition state of enzyme-catalyzed ester hydrolysis. For example, this idea has been exploited numerous times in the production of catalytic antibodies.[17] It also forms the basis of our own strategy. In our case the purpose of the phosphonate is to function as a suicide inhibitor. This means that it needs to contain a leaving group, which can be displaced by the primary alcohol function of serine, which is at the active site of the lipase. The irreversible formation of a stable enzyme–phosphonate complex is expected to destroy the catalytic property of the lipase.

2. Results and discussion

2.1. Synthesis of immobilized suicide enzyme inhibitors

Our strategy centered around the preparation of solid materials of the type **3**, composed of an insoluble carrier, an anchor-group, a spacer and a phosphonate moiety which contains a leaving group as well as the chiral substrate (D) or (L)-**2**, depending upon whether a (D) or (L)-selective enzyme is to be evolved in future work.



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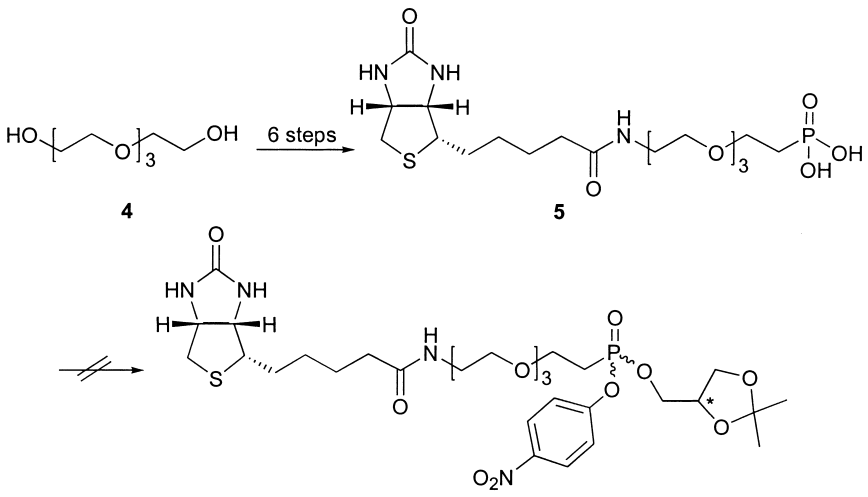
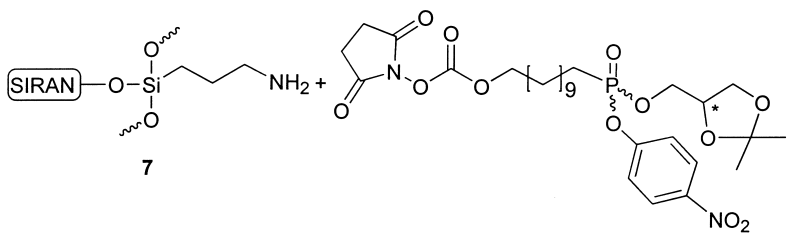
In order to put this into practice we first considered the utilization of the biotin/avidin interaction which is known to be exceedingly strong. Therefore the biotin-modified phosphonic acid **5** was synthesized from commercially available diol **4**, hoping to esterify it with formation of the desired target molecule **6**. However, numerous attempts to affect this seemingly trivial transformation failed.[15]

Since our initial and most obvious approach failed, we changed the synthetic strategy. This time the idea was to anchor the suicide inhibitor on SIRAN® (a porous glass) via an appropriate spacer. Therefore, the activated diastereomeric carbonates **8a**,**b** were chosen as the target molecules because they can be expected to undergo amidation with amino-modified SIRAN® of the type **7** affording the immobilized enzyme inhibitors **9a**,**b**. During our study a related strategy was described by Deussen and Borchert in their efforts to prepare enzyme inhibitors, although in their case these are linked to biotin via spacers and disulfide bridges.[18]

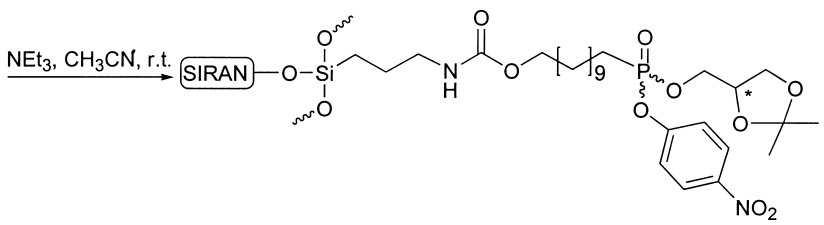
This approach proved to be successful. SIRAN® was first modified chemically with formation of amino-modified beads **7**.

The synthesis of the phosphonates **8a**,**b** was carried out as

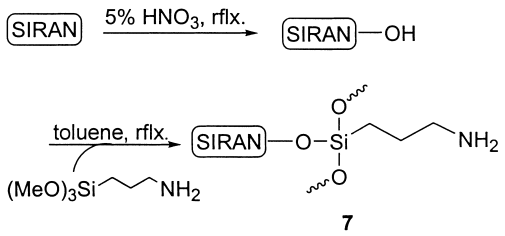




8 a (*D*)-configuration at IPG
b (*L*)-configuration at IPG



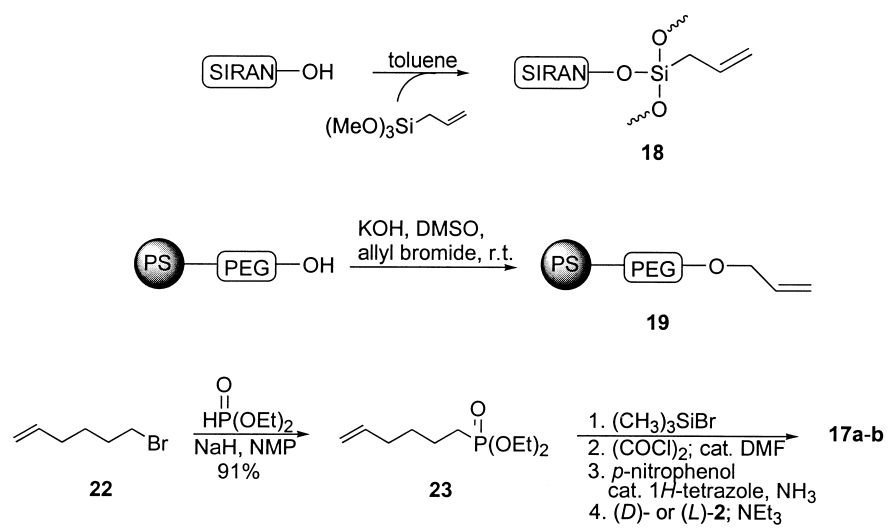
9 a (*D*)-configuration at IPG
b (*L*)-configuration at IPG

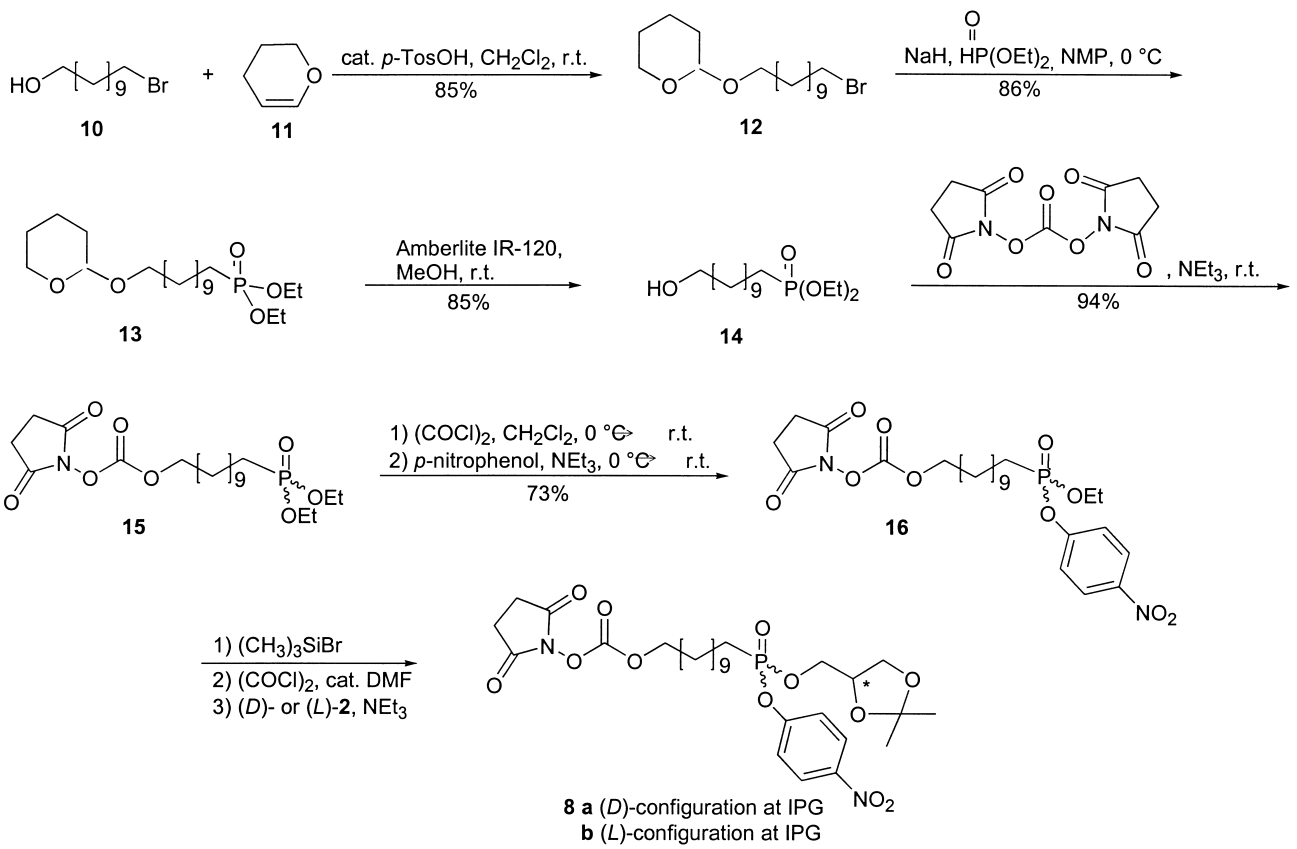


shown in Scheme 1. Compounds **8a** and **8b** each exist as a 1:1 mixture of diastereomers. Finally, reaction of beads **7** with activated carbonates **8a**,**b** afforded the immobilized suicide inhibitors **9a**,**b**.

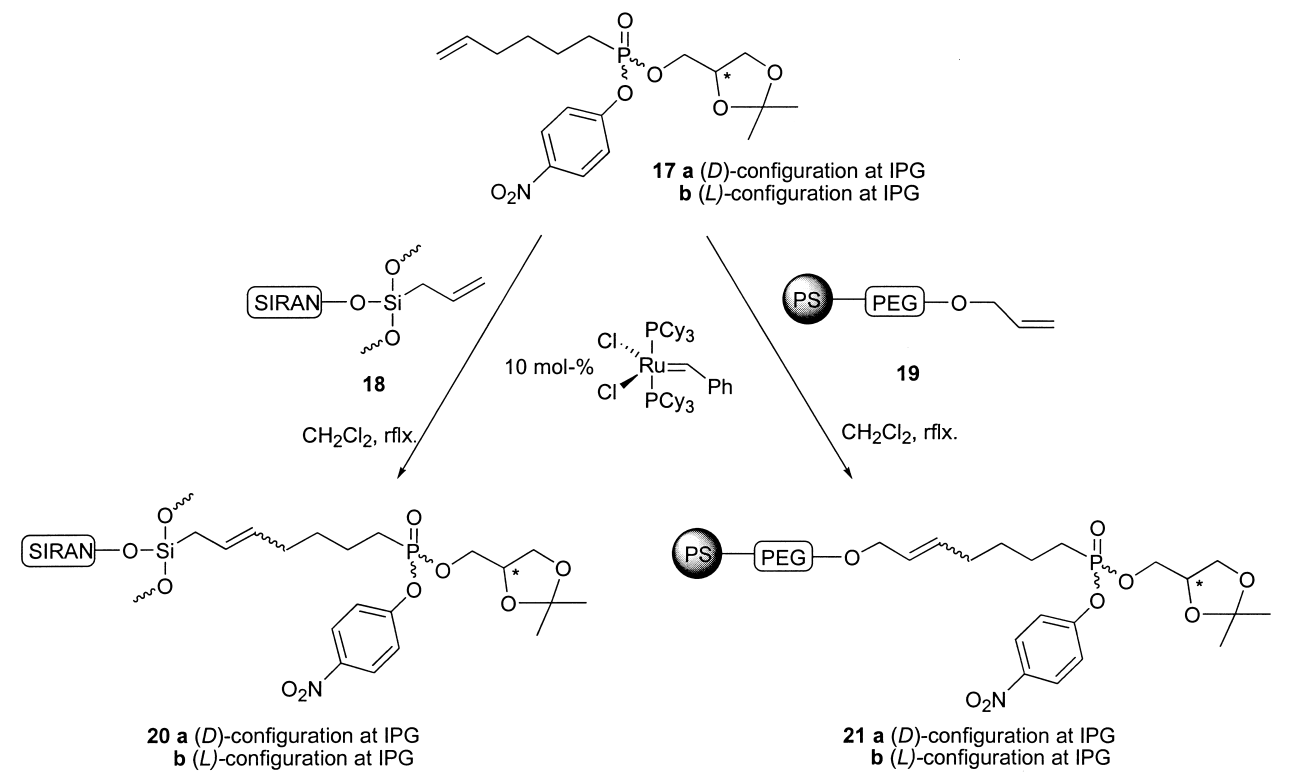
The primary synthetic goal was therefore reached. Nevertheless, we also took a parallel approach and prepared a different type of immobilized enzyme inhibitor. In this case we envisioned that olefin metathesis would provide a means to anchor appropriate soluble enzyme inhibitors on solid carriers. Olefin metathesis reactions, especially when mediated by Grubbs or Schrock catalysts, have been applied successful in a wide variety of synthetic problems.[19] However, attaching enzyme inhibitors or transition state analogs to solid carriers by this method has not been described so far. We hoped to immobilize suicide inhibitors of the type **17a**,**b** on SIRAN® or Tentagel® beads, the latter being appropriately modified as **18** and **19**, respectively (Scheme 2).

The synthesis of the respective chemically modified beads **18** and **19** as well as the phosphonates **17a**,**b** (each as 1:1 diastereomer mixture) proceeded as planned. Moreover, olefin metathesis of these building blocks using the Grubbs catalyst[19] was carried out according to Scheme 2, providing a second set of chiral immobilized suicide inhibitors **20a**,**b** and **21a**,**b**. It should be noted that materials of the type **9a**,**b** and **20a**,**b**/**21a**,**b** are not easy to characterize.





Scheme 1. Synthesis of the activated enzyme inhibitors **8a**,**b**.



Scheme 2. Preparation of the carrier-fixed phosphonates **20a**,**b** and **21a**,**b** by olefin metathesis.

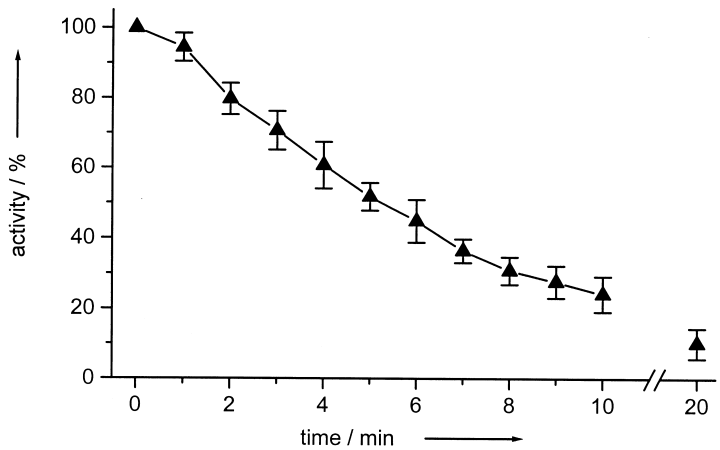


Figure 1. Residual enzyme-activity of the lipase from *B. subtilis* during incubation with the SIRAN®-immobilized suicide inhibitor **9a**.

2.2. Binding studies of the immobilized suicide enzyme inhibitors

Having prepared different types of immobilized enzyme inhibitors **9a**,**b**, **20a**,**b** and **21a**,**b**, it was now possible to carry out binding studies.[14] Note that the potential suicide inhibitors are enantiopure at the stereocenter of the IPG molecule ((D) or (L)-configuration). We hope that in later studies this will impart (D) or (L)-inhibitor properties, i.e. be useful in the selection (D) or (L)-specific enzymes. However, the materials are 'racemic' at the phosphorus atom. Since separation of the respective diastereomers was not possible, we used the mixtures in binding studies. In doing so the residual catalytic activity of the lipase during incubation with the suicide inhibitors was measured.

Experiments involving the incubation of the lipase from *B. subtilis* with the SIRAN®-based phosphonate **20a** showed no significant deactivation of the enzyme within a time range of 5 h. In sharp contrast appreciable deactivation occurred with the other SIRAN®-immobilized suicide inhibitor **9a** within minutes (Fig. 1). The $t_{1/2}$-value turned out to be 5.2 min. This pronounced difference in behavior can be traced to the difference in the length of the respective spacers. In the case of materials **20a**,**b**, the alkenyl-spacer appears to be too short, not allowing the enzyme to undergo phosphorylation at the hydroxy-group of serine. Indeed, X-ray crystallographic investigations of the enzyme with active site bound phosphonates suggest a minimum distance of at least six carbon atoms between phosphorus and the appropriate solid carrier.[20] At shorter distances steric interactions prevent reaction and therefore no deactivation is observed. An alternative explanation is inefficient immobilization of the inhibitors. A similar trend was observed upon comparing **20b** with **9b** ($t_{1/2}$=4.4 min). However, it is not meaningful to compare immobilized inhibitor **9a** with its diastereomeric form **9b** in a quantitative way due to uncertainties in the degree of loading. It needs to be pointed out that this uncertainty is of no relevance to future experiments in the selection step of directed evolution, since only one of the materials will be used (**9a** or **9b** in the evolution of a (D) or (L)-selective mutant enzyme).

Interestingly, the results of the incubation experiments using the Tentagel®-based inhibitor **21a** show that 50% deactivation requires more than 2 h (Fig. 2). Such lower reactivity relative to **9a** can be explained on the basis of reduced swelling ability in aqueous medium.[15] Alternatively, the orientation of the spacer onto the active site of the lipase may be unfavorable resulting in a lower reactivity.

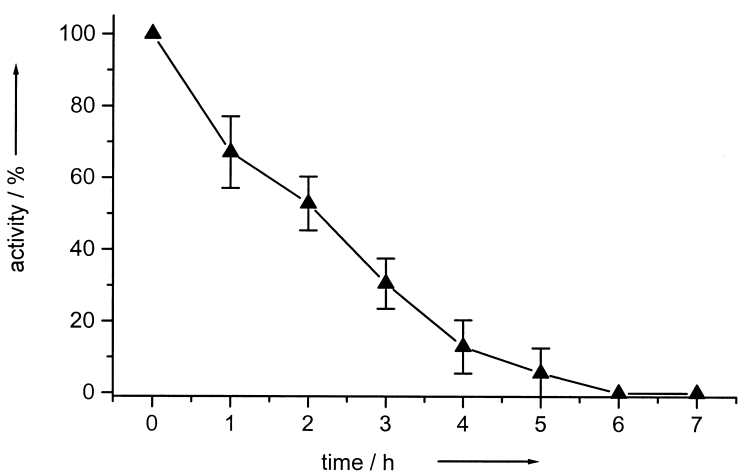


Figure 2. Residual enzyme-activity of the lipase from *B. subtilis* during incubation with the tentagel-based suicide inhibitor **21a**.

3. Conclusion

We have succeeded in attaching chiral phosphonate-based enzyme inhibitors covalently to solid supports. In one approach a soluble chiral phosphonate connected to an activated carbonate via a spacer was prepared and reacted with amino-modified SIRAN®, resulting in a smooth coupling reaction. In the second approach to immobilization a similar chiral enzyme inhibitor connected to an olefinic moiety via an appropriate alkyl chain was synthesized, in this case covalent attachment to allyl-modified SIRAN® or Tentagel® occurring by Ru-catalyzed olefin metathesis. We expect that olefin metathesis as described here will be useful in the immobilization of other suicide inhibitors and/or transition state analogs in general, adding to the power of this olefin-forming process.[19]

The stage is now set for the implementation of a selection system based on phage display for the identification of enantioselective lipase variants from *B. subtilis* in the model reaction **1→2**. The results of these upcoming studies will enable us to decide whether the idea of utilizing phage display in the directed evolution of enantioselective enzymes is viable, or whether only enantioselective binding phenomena will be revealed. Irrespective of the outcome, the synthetic strategies described in the present paper may be of use in other applications as well.

4. Experimental

Unless otherwise noted, all starting materials were obtained from commercial suppliers and were used without further purification. Solvents were dried according to established procedures by distillation from an appropriate drying agent under an inert atmosphere of argon in glassware that has been flame-dried. Column chromatography was performed on Merck silica gel 60 (260–400 mesh). Chemical shifts are quoted in parts per million (ppm, δ) using either the solvent as internal standard (^{1}H NMR, ^{13}C NMR) or phosphoric acid as external standard (^{31}P NMR). In the case of the immobilized inhibitors the amount of phosphorus (or nitrogen) relative to the whole material is very small, which means that the uncertainty in the elemental analysis can be large.

4.1. Enzyme activity

The lipase from *B. subtilis* was purified as described by Lesuisse et al.[21] Enzymatic activity was determined spectrophotometrically by the *p*-nitrophenylcaprylate assay, since a C8 alkyl chain revealed optimal activity for the *Bacillus* lipase.[22] A 10 mM solution *p*-nitrophenylcaprylate in methanol was prepared. 0.5 mM *p*-nitrophenylcaprylate was added to 900 μL assay buffer, containing 50 mM phosphate buffer (pH 8), 0.36% Triton X100 (v/v) and 0.1% gum arabic. The assay buffer was preincubated at 30°C. Fifty microliters purified lipase (1 μg lipase) were added and the absorbance was measured at 410 nm.

4.2. Binding to the suicide inhibitor

The inactivation of lipase by the phosphonate inhibitors was determined by incubation of the enzyme and the appropriate phosphonate inhibitor in assay buffer at room temperature. 10 mg of the inhibitor was washed three times and pre-equilibrated for 10 min using 500 μL assay buffer. Note that the inhibitor does not dissolve in this solution. Therefore the reaction mixture was vertically rotated during incubation. Subsequently, purified lipase (1 μg) was added to the inhibitor. As control, lipase was also incubated in assay buffer without the inhibitor for the same time period. During incubation of the lipase, aliquots of 10 μL were collected to determine the residual lipase activity using the *p*-nitrophenylcaprylate assay, as described above. No inactivation of lipase in the controls could be detected. All results are expressed as mean±SEM. The statistical significance of differences was tested at a significance level of $P<0.05$ using a two-tailed Student's *t*-test.

4.3. Surface modification of SIRAN®

A suspension of SIRAN® (SIKUG/012/XX/300/A; 100 g) in 5% nitric acid (200 mL) was heated under reflux for 8 h. After filtration the glass beads were washed with water (500 mL) and further purified by dialysis for 48 h. The resulting SIRAN® was then washed with 500 mL of acetone followed by removal of solvent in vacuo at 150°C. To a suspension of the dried SIRAN® in toluene (300 mL) allyltrimethoxysilane or 3-aminopropyltrimethoxysilane (9.6 mmol) was added und heated under reflux for 20 h. The modified SIRAN® was filtered off and washed with MeOH (750 mL). After a drying period of 8 h in vacuo at 80°C the SIRAN® was ready to use. *n*-Aminopropyl-modified SIRAN® *7*: elemental analysis: C 0.05, N 0.03. Allyl-modified SIRAN® **18**: elemental analysis (%): C 0.03.

4.3.1. General procedure for the preparation of phosphonates 8a,b. A solution of phosphonate **16** (3.69 g, 16.7 mmol, 20 mL CH_2Cl_2) was cooled to 0°C and bromotrimethylsilane (6.39 g, 41.8 mmol) was added. The mixture was stirred for 40 h at room temperature. All volatile components were removed under reduced pressure and the orange residue was dissolved in CH_2Cl_2 (35 mL). After the addition of oxalyl dichloride (2.43 g, 19.1 mmol) and DMF (0.1 mL) at 0°C the mixture was heated under reflux for 18 h. All volatile components were then removed in vacuo and the residue was dissolved in CH_2Cl_2 (40 mL). To this solution (D) or (L)-**2** (0.84 g, 6.4 mmol) and triethylamine (0.77 g, 7.6 mmol) was added at 0°C. After 2 h at room temperature the solution was concentrated. The resulting black oil was purified by flash chromatography eluting with CH_2Cl_2/EtOAc (3:1). **8a** (2.84 g, 71%): ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=8.23–8.18 (m, 2H), 7.40–7.36 (m, 2H), 4.31–4.00 (m, ^3J(H,H)=6.7 Hz, 6H), 3.73 (dt, ^2J(H,H)=8.6 Hz, ^3J(H,H)=6.1 Hz, 1H), 2.81 (s, 4H), 2.04–1.90 (m, 2H), 1.73–1.60 (m, 4H), 1.42–1.21 (m, 20H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=168.5, 155.4, 151.4, 144.4, 125.9, 120.9, 109.8, 74.1 (d, ^3J(C,P)=7 Hz), 71.6, 66.5, 65.8 (d, ^4J(C,P)=5 Hz), 30.3 (d, ^3J(C,P)=17 Hz), 29.3, 29.2, 29.1, 29.0, 28.9, 28.3, 26.7, 25.8 (d, ^1J(C,P)=140 Hz), 25.4 (d, ^2J(C,P)=7 Hz), 25.1, 22.1 (d, ^6J(C,P)=2 Hz), 22.0 (d, ^6J(C,P)=2 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ=32.0, 31.7; IR (neat): $\tilde{\nu}$=3114, 3080, 2931, 2856, 1812, 1790, 1744, 1592, 1523, 1492, 1347, 1257, 1227, 1048, 862 cm^{-1}; MS (70 eV): *m/z* (%): 525 (3), 404 (69), 384 (100), 360 (11),

263 (32), 217 (37), 139 (10), 69 (14), 55 (27), 41 (13); elemental analysis calcd (%) for $C_{28}H_{41}N_2O_{12}P$ (628.6): C 53.50, H 6.57, N 4.46, P 4.93; found C 53.50, H 6.62, N 4.53, P 4.89. **8b** (2.68 g, 67%): ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=8.19–8.16 (m, 2H), 7.36–7.33 (m, 2H), 4.28–3.97 (m, ^3J(H,H)=6.6 Hz, 6H), 3.71 (dt, ^2J(H,H)=8.7 Hz, ^3J(H,H)=6.0 Hz, 1H), 2.79 (s, 4H), 1.99–1.87 (m, 2H), 1.73–1.58 (m, 4H), 1.36–1.23 (m, 20H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=168.7, 155.5, 151.5, 144.5, 125.6, 120.9, 109.9, 74.1 (d, ^3J(C,P)=7 Hz), 71.6, 66.5, 65.8 (d, ^4J(C,P)=5 Hz), 30.3 (d, ^3J(C,P)=17 Hz), 29.3, 29.2, 29.1, 29.0, 28.9, 28.3, 26.7, 25.8 (d, ^1J(C,P)=140 Hz), 25.4 (d, ^2J(C,P)=7 Hz), 25.1, 22.1 (d, ^6J(C,P)=2 Hz), 22.0 (d, ^6J(C,P)=2 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ=31.9, 31.7; IR (neat): $\tilde{\nu}$=3114, 3080, 2931, 2856, 1812, 1789, 1744, 1592, 1523, 1492, 1347, 1258, 1226, 1049, 862 cm^{-1}; MS (70 eV): *m*/*z* (%): 613 (100), 490 (18), 412 (47), 356 (14), 115 (16), 101 (32), 55 (21), 43 (35); elemental analysis calcd (%) for $C_{28}H_{41}N_2O_{12}P$ (628.6): C 53.50, H 6.57, N 4.46, P 4.93; found C 53.38, H 6.52, N 4.49, P 4.85.

4.4. Attachment of phosphonates 8a, b to SIRAN® 7

To a suspension of SIRAN® **7** (43.3 g, ~0.9 mmol) in CH_3CN (150 mL) **8a** or **8b** (877 mg, 1.40 mmol) and triethylamine (236 mg, 2.33 mmol) were added. The mixture was shaken for 24 h at room temperature. Filtration, subsequent washings with CH_3CN (3×70 mL) and acetone (3×70 mL) and drying in vacuo overnight provided the SIRANS® **9a** and **9b**, respectively. Phosphonate modified SIRAN® **9a**: elemental analysis: N 0.03, P 0.55. Phosphonate modified SIRAN® **9b**: elemental analysis: N 0.03, P 0.03.

4.4.1. 2-[(11-Bromoundecyl)oxy]tetrahydro-2*H*-pyran (12). 11-Bromoundecanol (**10**) (7.45 g, 29.7 mmol, 30 mL CH_2Cl_2) was added dropwise to a solution of 3,4-dihydro-2*H*-pyran (**11**) and toluene-4-sulfonic acid (56 mg, 0.3 mmol) in CH_2Cl_2 (50 mL) at room temperature. The mixture was stirred overnight. After dilution with ether (150 mL) the organic layer was washed with saturated aqueous Na_2CO_3 (2×80 mL), water (3×80 mL) and brine (1×80 mL). Drying over $MgSO_4$, concentration and purification via flash chromatography eluting with EtOAc/hexane (10:90) gave **12** (8.46 g, 85%) as a colorless liquid. ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=4.54 (dd, ^3J(H,H)=2.6, 4.5 Hz, 1H), 3.95–3.64 (m, 2H), 3.52–3.29 (m, ^3J(H,H)=6.8 Hz, 4H), 1.89–1.25 (m, 25H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=98.8, 67.6, 62.3, 34.0, 32.8, 30.7, 29.7, 29.5, 29.4, 28.7, 28.1, 26.2, 25.5, 19.6; IR (neat): $\tilde{\nu}$=2928, 2854, 1034, 645, 563 cm^{-1}; MS (70 eV): *m*/*z* (%): 335 (2) [M$^+$], 261 (1), 151 (1), 135 (1), 115 (3), 101 (8), 85 (100), 69 (7), 56 (16), 41 (11); elemental analysis calcd (%) for $C_{16}H_{31}BrO_2$ (335.3): C 57.31, H 9.32, Br 23.83; found C 57.42, H 9.26, Br 23.67.

4.4.2. Diethyl 11-[(2-tetrahydro-2*H*-pyranyl)oxy]undecylphosphonate (13). Diethyl phosphite (4.84 g, 35.1 mmol, 5 mL NMP) was added dropwise to a ice-cooled suspension of sodium hydride (0.92 g, 38.3 mmol) in NMP (20 mL). After 1 h at 0°C and 1 h at room temperature the solution was again cooled to 0°C and alkyl bromide **12** (10.69 g, 31.9 mmol, 8 mL NMP) was added slowly. The mixture was allowed to warm up to room temperature overnight and was diluted with ether (200 mL). The organic layer was washed with water (2×50 mL) and brine (1×50 mL). Drying over $MgSO_4$, evaporation of the solvent and purification via flash chromatography eluting with EtOAc/hexane (90:10) gave **13** (7.42 g, 91%) as a colorless oil. ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=4.52 (dd, ^3J(H,H)=2.8, 4.5 Hz, 1H), 4.11–3.98 (m, 4H), 3.98–3.86 (m, 1H), 3.68 (dt, ^3J(H,H)=9.8, 6.8 Hz, 1H), 3.48–3.41 (m, 1H), 3.33 (dt, ^3J(H,H)=9.4, 6.8 Hz, 1H), 1.82–1.47 (m, 12H), 1.30–1.22 (m, 20H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=99.2, 68.0, 62.7, 61.7 (d, ^2J(C,P)=7 Hz), 31.2, 31.0 (d, ^3J(C,P)=17 Hz), 30.1, 29.9, 29.9, 29.8, 29.7, 29.4 (d, ^4J(C,P)=1 Hz), 26.6, 26.0 (d, ^1J(C,P)=140 Hz), 25.9, 22.7 (d, ^2J(C,P)=5 Hz), 20.1, 16.9 (d, ^3J(C,P)=6 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ=33.2; IR (neat): $\tilde{\nu}$=2928, 2854, 1247, 1060, 1032 cm^{-1}; MS (70 eV): *m*/*z* (%): 392 (1) [M$^+$], 363 (10), 307 (18), 291 (42), 278 (61), 263 (21), 235 (13), 179 (22), 165 (45), 152 (100), 125 (15), 85 (37); elemental analysis calcd (%) for $C_{20}H_{41}O_5P$ (392.5): C 61.20, H 10.53, P 7.89; found C 61.11, H 10.68, P 7.86.

4.4.3. Diethyl (11-hydroxyundecyl)phosphonate (14). A solution of phosphonate **13** (12.5 g, 31.0 mmol, 120 mL MeOH) was treated with Amberlite IR-120 (19.0 g). The resulting mixture was gently shaken at room temperature for 16 h. The ion-exchange resin was filtered off and evaporation of the solvent gave a crude orange product which was purified by flash chromatography eluting with CH_2Cl_2/MeOH (95:5) to yield **14** (8.11 g, 85%) as a pale yellow solid. ^{1}H NMR (200.1 MHz, $CDCl_3$): δ=4.12–3.97 (m, 4H), 3.58 (t, ^3J(H,H)=6.5 Hz, 2H), 1.86 (broad s, 1H), 1.76–1.24 (m, ^3J(H,H)=7.0 Hz, 26H); ^{13}C NMR (50.3 MHz, $CDCl_3$): δ=62.9, 61.4 (d, ^2J(C,P)=7 Hz), 32.7, 30.5 (d, ^3J(C,P)=17 Hz), 29.5, 29.4, 29.3, 29.2, 29.0, 25.7, 25.6 (d, ^1J(C,P)=140 Hz), 22.3 (d, ^2J(C,P)=5 Hz), 16.4 (d, ^3J(C,P)=6 Hz); ^{31}P NMR (81.0 MHz, $CDCl_3$): δ=33.3; IR (neat): $\tilde{\nu}$=3420, 2927, 2854, 1228, 1059, 1029 cm^{-1}; MS (70 eV): *m*/*z* (%): 308 (1) [M$^+$], 363 (10), 290 (18), 278 (38), 165 (50), 152 (100), 138 (27), 125 (41), 55 (30), 41 (25); elemental analysis calcd (%) for $C_{15}H_{33}O_4P$ (308.4): C 58.42, H 10.79, P 10.04; found C 58.42, H 10.74, P 9.92.

4.4.4. Preparation of activated carbonate 15. To a solution of phosphonate **14** (6.02 g, 19.5 mmol) and triethylamine (5.92 g, 58.5 mmol) in CH_3CN (50 mL) was added di(*N*-succinimidyl) carbonate (10.0 g, 39.1 mmol). The resulting mixture was stirred overnight. After dilution with EtOAc (150 mL) the organic layer was washed with water (3×80 mL) and dried over $MgSO_4$. The solvent was then evaporated and the residue was purified by flash chromatography eluting with EtOAc (100%) to afford **15** (8.28 g, 94%) as a yellow oil. ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=4.26 (t, ^3J(H,H)=6.8 Hz, 2H), 4.24–4.09 (m, 4H), 2.78 (s, 4H), 1.73–1.46 (m, 6H), 1.32–1.22 (m, 20H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=169.1, 152.0, 72.0, 61.7 (d, ^2J(C,P)=7 Hz), 30.9 (d, ^3J(C,P)=17 Hz), 29.7, 29.7, 29.6, 29.4, 28.7, 26.0 (d, ^1J(C,P)=140 Hz), 25.8, 25.7, 22.7 (d, ^2J(C,P)=5 Hz), 16.8 (d, ^3J(C,P)=6 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ=33.5; IR (neat): $\tilde{\nu}$=2923, 2854, 1812, 1785, 1740, 1233, 1027 cm^{-1}; MS (70 eV): *m*/*z* (%): 449 (1) [M$^+$], 404 (2), 307 (3), 291 (100), 263 (12), 165 (24), 152 (43), 125 (15), 55 (13); elemental analysis calcd

(%) for $C_{20}H_{36}NO_8P$ (449.5): C 53.44, H 8.07, N 3.12, P 6.89; found C 53.65, H 8.02, N 3.09, P 7.03.

4.4.5. Preparation of *p*-nitrophenyl substituted phosphonate 16. To a solution of phosphonate **15** (8.00 g, 17.8 mmol, 50 mL CH_2Cl_2) was added oxalyl dichloride (4.52 g, 35.6 mmol) and DMF (0.1 mL) at 0°C. The resulting mixture was stirred for 45 min at 0°C and was allowed to warm up to room temperature overnight. All volatile components were removed under reduced pressure und the brown residue was dissolved in CH_2Cl_2 (60 mL). After the addition of *p*-nitrophenol (2.48 g, 17.8 mmol) the solution was cooled to 0°C and triethylamine (3.60 g, 35.6 mmol) was added. The mixture was stirred for 1 h at 0°C and overnight at room temperature. All volatile components were removed in vacuo and the black residue was purified by flash chromatography eluting with CH_2Cl_2/EtOAc (7:1) to afford **16** (7.05 g, 73%) as a yellow solid. ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=8.23–8.20 (m, 2H), 7.38–7.32 (m, 2H), 4.28 (t, ^3J(H,H)=6.7 Hz, 2H), 4.25–4.10 (m, 2H), 2.81 (s, 4H), 1.96–1.85 (m, 2H), 1.73–1.58 (m, 4H), 1.38–1.25 (m, 17H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=168.7, 155.8, 151.6, 144.4, 125.6, 120.9, 71.6, 62.9 (d, ^2J(C,P)=7 Hz), 30.4 (d, ^3J(C,P)=17 Hz), 29.4, 29.3, 29.2, 29.0, 29.0, 28.3, 26.0 (d, ^1J(C,P)=140 Hz), 25.4, 25.3, 22.2 (d, ^2J(C,P)=5 Hz), 16.3 (d, ^3J(C,P)=6 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ=31.6; IR (KBr): $\tilde{\nu}$=3114, 3081, 2929, 2855, 1812, 1789, 1743, 1592, 1522, 1491, 1347, 1258, 1226, 1037, 913, 862 (m) cm^{-1}; MS (70 eV): *m*/*z* (%): 525 (3), 404 (69), 384 (100), 360 (11), 263 (32), 217 (37), 139 (10), 69 (14), 55 (27), 41 (13)); elemental analysis calcd (%) for $C_{24}H_{35}N_2O_{10}P$ (542.5): C 53.13, H 6.50, N 5.16, P 5.71; found C 53.03, H 6.53, N 5.23, P 5.79.

4.4.6. General procedure for the synthesis of phosphonates 17a,b. A solution of diethyl 6-hexenylphosphonate (**23**) (6.61 g, 30.0 mmol) in CH_2Cl_2 (15 mL) at 0°C was treated with bromotrimethylsilane (11.50 g, 75.1 mmol). After 48 h at room temperature the solvent and excess of silane was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (15 mL) and oxalyl dichloride (11.44 g, 90.1 mmol) as well as DMF (0.1 mL) was added at 0°C. The mixture was allowed to warm up to room temperature and was then heated under reflux for 1 h. All volatile components were removed in vacuo and the residue was dissolved in toluene (150 mL). The resulting mixture was cooled to 0°C and treated with *p*-nitrophenol (3.76 g, 27.0 mmol), triethylamine (3.80 g, 37.6 mmol) and 1*H*-tetrazole (0.21 g, 3.0 mmol). After 1 h at 0°C and 4 h at room temperature the solution was again cooled to 0°C and (D) or (L)-**2** (3.97 g, 30.0 mmol, 15 mL THF) and triethylamine (3.80 g, 37.6 mmol) were added dropwise. The mixture was stirred overnight at room temperature. Filtration, concentration in vacuo and purification via flash chromatography eluting with EtOAc/hexane (1:1) gave **17a** and **17b**, respectively, as yellow oils. **17a** (3.86 g, 32%): ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=8.22–8.17 (m, 2H), 7.39–7.34 (m, 2H), 5.80–5.66 (m, 1H), 5.00–4.91 (m, 2H), 4.31–3.99 (m, 4H), 3.72 (dt, ^2J(H,H)=8.5 Hz, ^3J(H,H)=5.8 Hz, 1H), 2.08–1.90 (m, 4H), 1.76–1.62 (m, 2H), 1.49 (tt, ^3J(H,H)=7.3 Hz, 2H), 1.40–1.31 (m, 6H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=155.3, 144.6, 137.7, 125.6, 121.0, 115.1, 110.0, 73.9 (d, ^3J(C,P)=7 Hz), 66.4, 65.5 (d, ^2J(C,P)=5 Hz), 32.8 (d, ^4J(C,P)=1 Hz), 29.3 (d, ^3J(C,P)=17 Hz), 25.5 (d, ^1J(C,P)=143 Hz), 24.9 (d, ^2J(C,P)=2 Hz), 21.4 (d, ^6J(C,P)=2 Hz), 21.3 (d, ^6J(C,P)=2 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ=31.7, 31.4; IR (neat): $\tilde{\nu}$=3115, 3079, 2987, 2936, 1641, 1592, 1523, 1492, 1347, 1225, 1055, 918, 754 cm^{-1}; MS (70 eV): *m*/*z* (%): 399 (5) [M$^+$], 384 (100), 341 (13), 286 (21), 268 (17), 203 (11), 101 (41), 81 (11), 57 (15), 43 (41); elemental analysis calcd (%) for $C_{18}H_{26}NO_7P$ (399.4): C 54.13, H 6.56, N 3.51, P 7.76; found C 54.20, H 6.65, N 3.54, P 7.61. **17b** (3.47 g, 29%): ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=8.23–8.18 (m, 2H), 7.40–7.33 (m, 2H), 5.81–5.67 (m, 1H), 5.01–4.92 (m, 2H), 4.31–4.00 (m, 4H), 3.73 (dt, ^2J(H,H)=8.7 Hz, ^3J(H,H)=5.8 Hz, 1H), 2.09–1.91 (m, 4H), 1.77–1.63 (m, 2H), 1.50 (tt, ^3J(H,H)=7.3 Hz, 2H), 1.39–1.31 (m, 6H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=155.4, 144.6, 137.7, 125.7, 121.0, 115.2, 110.1, 73.9 (d, ^3J(C,P)=7 Hz), 66.4, 65.5 (d, ^2J(C,P)=5 Hz), 32.8 (d, ^4J(C,P)=1 Hz), 29.3 (d, ^3J(C,P)=17 Hz), 25.5 (d, ^1J(C,P)=143 Hz), 24.9 (d, ^2J(C,P)=2 Hz), 21.4 (d, ^6J(C,P)=2 Hz), 21.3 (d, ^6J(C,P)=2 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ=31.7, 31.4; IR (neat): $\tilde{\nu}$=3114, 3079, 2987, 2937, 1641, 1592, 1523, 1492, 1347, 1225, 1055, 917, 754 cm^{-1}; MS (70 eV): *m*/*z* (%): 399 (5) [M$^+$], 384 (100), 341 (13), 286 (21), 268 (17), 203 (10), 101 (42), 81 (11), 57 (15), 43 (40); elemental analysis calcd (%) for $C_{18}H_{26}NO_7P$ (399.4): C 54.13, H 6.56, N 3.51, P 7.76; found C 54.18, H 6.47, N 3.58, P 7.80.

4.5. Allyl modified Tentagel HL-OH 19

To a suspension of swollen Tentagel HL-OH (600 mg dry weight, ~0.24 mmol) in 30 mL of DMSO allyl bromide (230 mg, 1.90 mmol) and powdered potassium hydroxide (160 mg, 2.85 mmol) were added. The mixture was shaken overnight. After filtration and washings with water (3×30 mL), acetone (3×30 mL), ether (2×30 mL) and pentane (30 mL) the resulting resin was dried in vacuo to afford allyl modified tentagel **19** as yellow beads.

4.5.1. General procedure for the preparation of carrier-fixed phosphonates 20a,b and 21a,b via olefin metathesis. To a suspension of olefin modified carrier (**18** or **19**; ~0.2 mmol) in 30 mL of CH_2Cl_2 phosphonate **17a** or **17b** (160 mg, 0.40 mmol) and Grubbs' catalyst[19] (33 mg, 0.04 mmol) was added. The mixture was heated under reflux for 24 h and filtered off. Subsequent washings with CH_2Cl_2 (200 mL), acetone (250 mL) and ether (250 mL) and drying in vacuo at 40°C gave the appropriate carrier-fixed phosphonates. Tentagel HL fixed phosphonate **21a**: ^{31}P NMR (81.0 MHz, $CDCl_3$): δ=32.3; elemental analysis (%): N 0.11, P 0.19. Tentagel HL fixed phosphonate **21b**: ^{31}P NMR (81.0 MHz, $CDCl_3$): δ=32.0; elemental analysis (%): N 0.11, P 0.14. SIRAN® fixed phosphonate **20a**: elemental analysis (%): C 0.02, N 0.01, P 0.05. SIRAN® fixed phosphonate **20b**: elemental analysis (%): C 0.02, N 0.01, P 0.01.

4.5.2. Diethyl 6-hexenylphosphonate (23). Diethyl phosphite (5.61 g, 40.1 mmol, 20 mL NMP) was added dropwise to a ice-cooled suspension of sodium hydride (1.06 g, 36.9 mmol) in NMP (30 mL). After 1 h at 0°C and 1 h at room temperature the solution again was cooled to 0°C and 6-bromo-hex-1-ene (**22**) (6.02 g, 36.9 mmol) was added

slowly. The mixture was allowed to warm up to room temperature overnight and diluted with ether (200 mL). The organic layer was washed with water (2×50 mL) and brine (1×50 mL). Drying over $MgSO_4$ and evaporation of the solvent gave **23** (7.42 g, 91%) as a yellow liquid. The product was used without further purification. ^{1}H NMR (300.1 MHz, $CDCl_3$): δ=5.76–5.67 (m, 1H), 4.96–4.86 (m, 2H), 4.07–3.96 (m, 4H), 1.97 (dt, ^3J(H,H)=7.1 Hz, 2H) 1.69–1.53 (m, 6H), 1.25 (t, ^3J(H,H)=7.1 Hz, 6H); ^{13}C NMR (50.3 MHz, $CDCl_3$): δ=138.1, 114.7, 61.3 (d, ^2J(C,P)=7 Hz), 33.1 (d, ^4J(C,P)=1 Hz), 29.6 (d, ^3J(C,P)=17 Hz), 25.4 (d, ^1J(C,P)=141 Hz), 21.8 (d, ^2J(C,P)=5 Hz), 16.3 (d, ^3J(C,P)=6 Hz); ^{31}P NMR (81.0 MHz, $CDCl_3$): δ=32.9; IR (neat): $\tilde{\nu}$=3077, 2980, 2932, 2869, 1641, 1246, 1058, 1030, 960 cm^{-1}; MS (70 eV): *m/z* (%): 220 (19) [M^+], 179 (26), 165 (33), 152 (100), 138 (41), 125 (78), 111 (32), 97 (42), 81 (51), 41 (42), 29 (40); elemental analysis calcd (%) for $C_{10}H_{21}O_3P$ (220.3): C 54.53, H 9.61, P 14.06; found C 54.18, H 9.78, P 13.53.

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