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Novel Sol-Gel Lipases by Designed Bioimprinting for Continuous-Flow Kinetic Resolutions

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Abstract: The bioimprinting effect in sol-gel immobilization of lipases was studied to develop efficient novel immobilized biocatalysts with significantly improved properties for biotransformations in continuous-flow systems. The bioimprinting candidates were selected systematically among the substrate mimics already found in the active site of experimental lipase structures. Four lipases (Lipase AK, Lipase PS, CaLB and CrL) were immobilized by a sol-gel process with nine bioimprinting candidates using various combinations of tetraethoxysilane (TEOS), phenyltriethoxysilane (PhTEOS), octyltriethoxysilane (OcTEOS) and dimethyldiethylsilane (DMDEOS) as silica precursors. The biocatalytic properties of the immobilized lipases were characterized by enantiomer selective acylation of various racemic secondary alcohols in two different multisubstrate systems (mixture A: a series of alkan-2-ols *rac*-**1a–e** and mixture B: heptan-2-ol *rac*-**1f** and 1-phenylethanol *rac*-**1g**). Except with Lipase AK, the most significant activity enhancement was found with the imprinting molecules already found as substrate mimics in X-ray structures of various lipases. The synthetic usefulness of the best biocatalysts was demonstrated by the kinetic resolution of racemic 1-(thiophen-2-yl)ethanol (*rac*-**1h**) in batch and continuous-flow systems.

Keywords: continuous flow technique; enzyme catalysis; immobilization; imprinting; lipases; sol-gels

Introduction

Synthetic application of novel biocatalytic methods is a continuously growing area of chemistry, microbiology and genetic engineering, due to the fact that biocatalysts are selective, easy-to-handle and environmentally friendly.[1–3] The broad substrate tolerance and unique catalytic performance of lipases (glycerol ester hydrolases, triacylglycerol hydrolases EC 3.1.1.3.) have attracted growing interest. Lipases are characterized by the phenomenon called interfacial activation. The active site is covered by a lid when lipases are dissolved in water. This closed form is inactive for many lipases. When lipases are in contact with an interface between water and an apolar phase, the lid opens allowing access to the active site.[4–7]

Lipases under reverse hydrolytic conditions are able to form ester bonds which enables them to catalyze various other types of reactions such as esterification, transesterification. Furthermore, lipases are enantioselective catalysts useful in the synthesis of natural products, pharmaceutical intermediates, fine chemicals, food ingredients and bio-lubricants.[7,8,9,10]

Although the flow-through approach could provide several advantages such as facile automation, reproducibility, safety, and process reliability, it is still quite usual that chemists are working on a fixed, batch-based infrastructure, especially in research and development. There are only a few examples of hydrolase-catalyzed enantioselective processes carried out in continuous-flow systems.[11–13] Most of the continuous-mode biocatalytic syntheses of chiral pharmaceutical intermediates are performed on a relatively large scale using immobilized lipases in a packed-bed reactor.[13,14] Recently, continuous-flow packed-bed bioreactors were used to study the effects of tempera-

ture, pressure and flow rate on lipase-catalyzed kinetic resolutions.[15,16]

The catalytic activity, selectivity, specificity and enzyme stability are key factors affecting the efficiency of biocatalysts.[17,18] Immobilization can enhance the key properties of the biocatalysts such as stability and convenience of recovery and reuse.[19–24]

Entrapment of enzymes in inorganic/organic hybrid polymer matrices (sol-gel encapsulation) has proven to be an easy and effective way to immobilize enzymes, whole cells, antibodies and other proteins.[25,26] The seminal works of Avnir,[26] Reetz[27] and their coworkers led to the generalization of this technique involving the acid- or base-catalyzed hydrolysis of tetraalkoxysilanes $Si(OR)_4$[25] in the presence of an enzyme. Variation of the silane precursors of different hydrophilicity enabled further alteration of enzyme performance such as activity, stability or selectivity.[27,28] Moreover, additives such as polyethylene glycol (PEG), crown ethers or solid supports were applied to modulate the pore size and increase the permeability of the substrate through the pores and the accessibility of the enzyme.[20,21,27]

One of the most successful strategies for enhancing enzyme activity in organic solvents involves tuning the enzyme active site by molecular imprinting with substrates or their analogues.[29] The history of molecular imprinting is traced back to the work of Dickey in the 1940s who was inspired by Pauling's theory on the formation of antibodies.[30] Combining imprinting with protein surface coating and salt activation was reported as dual bioimprinting.[31] Pretreatment of a lipase with chiral template substrates such as (*R*)-(−)-2-octanol resulted in enantioselective activation.[32] However, there are only a few publications on the mutual effects of sol-gel entrapment and bioimprinting for lipases.[27,33] Imprinting effects of *ad hoc* selected molecules involving lauric acid (LA),[33] Tween 80[27] and other molecules (18-crown-6,[27] methyl-β-cyclodextrin[27]) were investigated so far.

Our goal is to investigate the imprinting effect of rationally selected molecules (compounds which were found as substrate mimics in experimental X-ray structures of lipases and their structural analogues) in sol-gel process resulting in robust immobilized biocatalysts for continuous-flow kinetic resolutions.

Results and Discussion

Rational Selection of the Imprinting Molecules for Lipases

In 1990, the first 3D structures of lipases from *Rhizomucor miehei*[34] and human pancreatic lipase[35] were elucidated. In 1993, the interfacial activation of the lipase from *Rhizomucor miehei* was rationalized on a structural basis with substrate-like inhibitors.[36] Since then 121 lipase structures have been deposited in the Protein Data Bank (Brookhaven PDB). Among the 76 lipase structures containing ligands, those structures which included non-covalently bound ligands mimicking the substrates were analyzed (Figure 1).[37–42]

The non-covalently bound ligands mimicking the arrangement of substrates in the experimental structures can be divided into three categories. As expected, free fatty acids were found within lipases in the close proximity of the catalytically active Ser (stearic acid in Figure 1 (**A**) and oleic acid in Figure 1 (**B**). Non-ionic surfactants occupying the active site were also found in lipases [hydroxy(ethyloxy)-(triethyloxy)octane in Figure 1 (**C**) and methylpenta-(oxyethyl) heptadecanoate in Figure 1 (**D**)]. Only the polyethylene glycol part of the non-ionic surfactant Triton X-100 was present in a thermoalkalophilic lipase [Figure 1 (**E**)]. Pentaethylene glycol was also found as a substrate mimic in lipase A from *Candida antarctica* [Figure 1 (**F**)].

Because it was expected that the best imprinting molecule may vary from one lipase to another, nine imprinting candidates were selected for this systematic study including substrate [olive oil (OA)], products [lauric acid (LA) and oleic acid (OA)], non-ionic surfactants [polyethylene glycol dodecyl ether (BRIJ-30) and polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100)] and polyethylene glycol derivatives [tetraethylene glycol (TEG), polyethylene glycol 400 (PEG 400) and 1000 (PEG 1000), and the dilaurate of tetraethylene glycol (L-TEG-L)].

The imprinting properties of the rationally selected additives were tested with four different lipases of well documented properties such as CaLB and CrL from yeasts and Lipase AK and Lipase PS from bacterial sources. Whereas CrL is the more sensitive to conformation changes, Lipase AK, Lipase PS and especially CaLB are more heat resistant and, respectively, less mobile. The most dramatic imprinting effects were expected in the case of the conformationally sensitive lipases.

Studies on subtilisin revealed that the decreased flexibility of the enzyme relative to the aqueous situation in an apolar solvent resulted in "ligand-induced memory".[43] It was expected also that encapsulation of the lipases can "freeze" their conformation and therefore an encapsulated lipase can "remember" the imprinting effect even after removal of the template molecule. Thus, selection of suitable imprinting additives might be crucial to improve the activity of the immobilized enzyme.

In a study on sol-gel immobilization of lipases, enhancing effects of various small molecules (Tween 80, 18-crown-6 and methyl-β-cyclodextrin) and supporting materials such as Celite were investigated simulta-

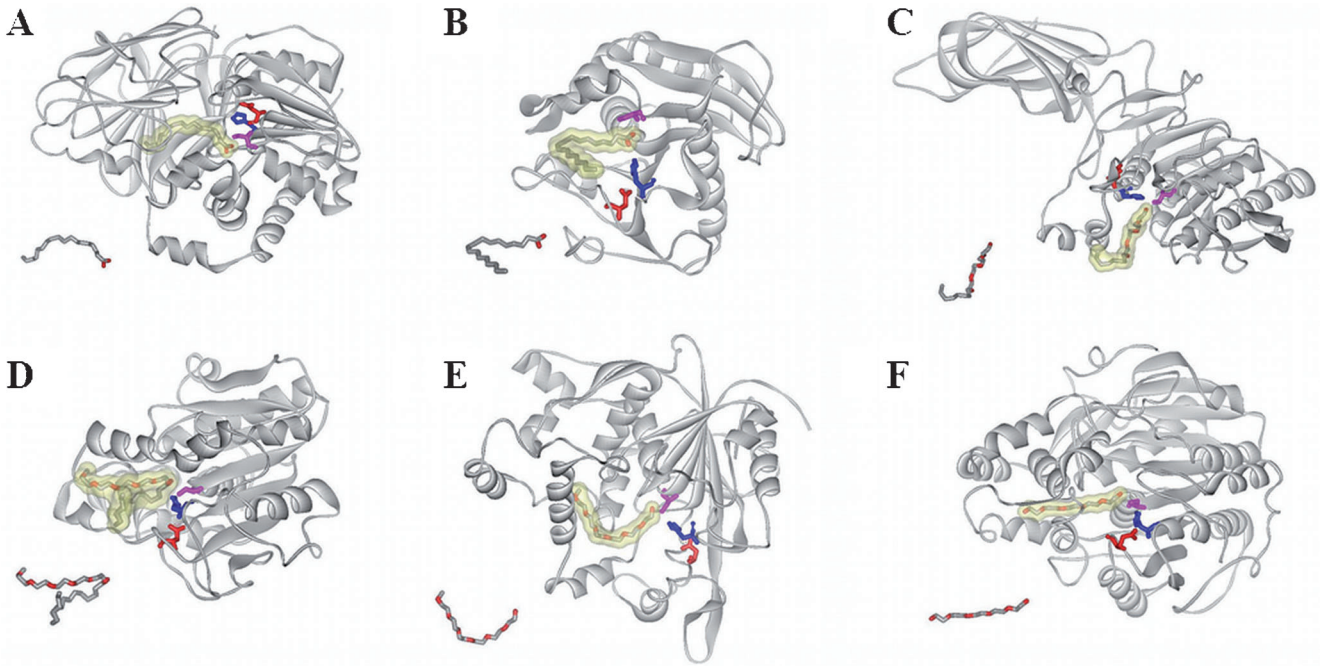

Figure 1. A–F: Crystal structures of lipases including substrate analogues within the active site. For better visibility, the catalytic triad is coloured (Ser: magenta, His: blue, Asp/Glu: orange) and the amphiphile present in the active site is repeated in the left down corner; **A**) *Archaeoglobus fulgidus* lipase with stearic acid (PDB code: 2ZYI)[37]; **B**) *Thermomyces lanuginosa* lipase with oleic acid (PDB code: 1GT6)[38]; **C**) porcine pancreatic lipase-colipase with hydroxy(ethyloxy)(triethyloxy)octane (PDB code: 1ETH)[39]; **D**) *Candida antarctica* lipase B with methylpenta(oxyethyl) heptadecanoate (PDB code: 1 LBT)[40]; **E**) bacterial thermoalkalophilic lipase with Triton X-100 (PDB code: 2W22)[41]; **F**) *Candida antarctica* lipase A with pentaethylene glycol (PDB code: 3GUU).[42]

neously.[27] However, the enhancing effects by small molecules and solid supports in sol-gel immobilization systems may be different. The enhancing effect in supported sol-gel techniques is mainly due to formation of a thin enzyme layer on the large surface of the supporting material, so lowering the diffusion limits which are present in large enzyme aggregates.[28] Small molecules, as additives, can induce conformational changes contributing mostly to the enhancement of enzyme activity. The additives may also improve the porosity of the forming sol-gel matrix thus mitigating diffusion constraints.[24]

This study was designed in non-supported, binary sol-gel systems (TEOS:PhTEOS 1:1)[28] solely to detect the imprinting effect of the additives (Table 1).

Multisubstrate Kinetic Resolution as Test System for the Preliminary Investigations

In order to distinguish effects due to induced conformation changes of the enzyme and the various hydrophobicities of encapsulating matrices, tests of the imprinted biocatalysts were designed with multisubstrate mixtures consisting of a series of racemic, aliphatic alcohols of various chain lengths (Figure 2). Another mixture was applied to compare the behaviour of an

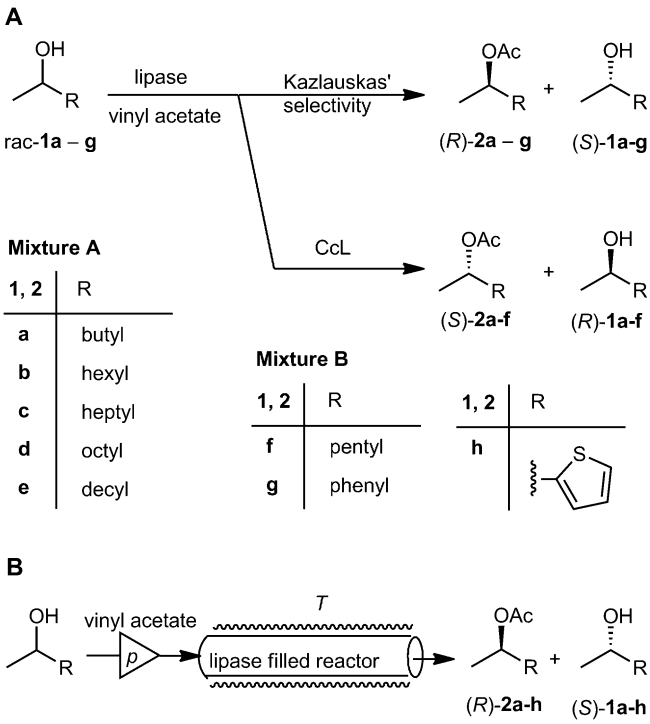

Figure 2. Normal and multiple kinetic resolutions with substrate mixtures for testing the various biocatalysts in batch (**A**) and continuous-flow (**B**) mode [▷ = pump; ~ ~ = temperature control unit]

Table 1. Screening the imprinting effect of additives in sol-gel immobilization of lipases using binary sol-gel system (TEOS: PhTEOS = 1:1). Specific activity (U_B) and enantiomer selectivity (E) of the free and immobilized lipases are shown with octan-2-ol *rac*-**1b** (Panel A) and with 1-phenylethanol *rac*-**1g** (Panel B). Reaction times were 2 h for Lipase AK, Lipase PS and CaLB and 8 h for CrL (for further reaction details, see Experimental Section).

Form (Additive)[a]	Lipase AK		Lipase PS		CaLB		CrL	
	U_B [U g^{-1}]	E[b]	U_B [U g^{-1}]	E[b]	U_B [U g^{-1}]	E[b]	U_B [U g^{-1}]	E[b]
Panel A (*rac*-**1b**)								
native[c]	23	7.8	3	7.1	48	>100	2.2	1.9[d]
SG (−)	23	6.2	23	10.7	9	≫200	0.7	1.8[d]
SG (LA)	12	4.0	25	10.4	11	>200	0.4	2.2[d]
SG (OA)	20	5.1	21	9.2	15	>200	0.7	2.0[d]
SG (TEG)	17	4.6	20	9.0	10	>200	0.4	1.9[d]
SG (PEG 400)	19	5.0	23	11.3	12	>200	0.5	1.8[d]
SG (PEG 1000)	20	5.2	19	9.2	16	>200	0.5	2.0[d]
SG (BRIJ)	11	4.2	11	5.7	22	≫200	2.3	1.7[d]
SG (L-TEG-L)	5	2.1	24	10.2	17	>200	0.3	1.9[d]
SG (TRX100)	17	4.4	6	5.7	21	>200	2.4	1.6[d]
SG (olive oil)	15	4.3	11	6.4	18	>200	0.5	1.8[d]
Panel B (*rac*-**1g**)								
native[c]	31	>200	13	>200	126	≫200	1.6	2.4
SG (−)	31	>200	31	≫200	16	>200	0.7	3.7
SG (LA)	30	>100	32	≫200	14	≫200	0.2	3.8
SG (OA)	26	>200	30	≫200	19	≫200	0.5	3.6
SG (TEG)	26	>100	31	≫200	8	>200	0.3	3.9
SG (PEG 400)	32	>200	31	≫200	20	>200	0.5	3.1
SG (PEG 1000)	29	>100	31	≫200	13	>200	0.5	3.1
SG (BRIJ)	30	>100	25	>200	25	≫200	2.2	3.5
SG (L-TEG-L)	29	>100	31	≫200	10	>200	0.3	3.5
SG (TRX100)	30	>100	17	>200	17	≫200	2.4	3.2
SG (olive oil)	14	>100	26	≫200	15	≫200	0.5	3.7

[a] Sol-gel immobilized enzyme with or without additive (0.5 v/v%).
[b] If not stated otherwise, formation of the (*R*)-esters were preferred.
[c] The corresponding commercially available native lipase powder for Lipase AK, Lipase PS and CrL. For CaLB, the commercial liquid enzyme (Novozym CaLB L) was immobilized on Novozym carrier (1 mg carrier to 8 μL enzyme solution).
[d] The formation of (*S*)-**2b** is preferred.

aliphatic and aromatic secondary alcohols directly. According to our best knowledge, no investigations on the biocatalytic properties of sol-gel entrapped enzymes have been performed with multisubstrate systems.

Because the lipases may be sensitive to variations in the nature and area of the forming aqueous-organic interface, the sol-formation steps (mixing the silane precursors with the aqueous phase, time of enzyme addition and NaF initiator, ultrasonic parameters) were varied with two enzymes (Lipase PS and CrL) in a series of preliminary experiments.

In the case of CrL, intensive emulsion formation was crucial and thus ultrasonication had a beneficial effect on the activity of the resulting biocatalyst. In contrast, variations in the sequence of sol-formation resulted in no significant alterations the properties of the immobilized biocatalysts from Lipase PS.

Initial experiments were then performed to investigate the effect of the amount of additive in the sol-gel formation on the properties of the encapsulated lipases. For this study, imprinting Lipase PS and CrL with PEG400 as the additive was selected (Figure 3).

Depending on the nature of the lipase and substrate, the Michaelis constants for various substrates for lipases are in the 0.1–40 mM range (K_m = 2.2 mM and K_m = 5.1 mM for lipase from porcine pancreas with sunflower oil and palm oil, respectively;[44] K_m = 0.15 mM for lipase from *Pseudomonas aeruginosa* on *p*-nitrophenyl palmitate;[45] K_m = 38 mM for lipase A from *Candida antarctica* with *p*-nitrophenyl butyrate;[8] K_m = 0.22 mM for pancreatic lipase on *p*-nitrophenyl acetate[4,7]). Comparing the concentration of the PEG 400 with the K_m values of the substrates leads to the hypothesis that the first increase in activity for both sol-gel entrapped lipases is related with the substrate analogue-like behaviour. The second increase in the specific activity (K_m = 5–20 mM interval) can be related to the mutual effects of the substrate analogue-like habitat and the impact of the additive on the quality of matrix.

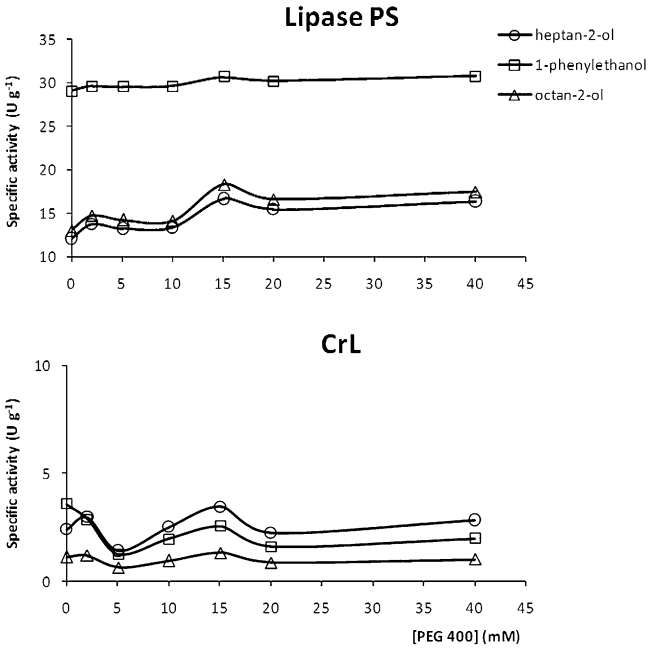

Figure 3. Dependence of the specific activity (U_B, U g^{-1}) on the concentration of PEG 400 as imprinting additive in TEOS:PhTEOS 1:1 system (Lipase PS: 2 h; and CrL: 8 h).

In a work on porcine pancreatic phospholipase A2 imprinted with octyl-β-D-glucopyranoside, a similar two-stage activation was observed.[30] The second activation was attributed to the appearance of interfaces, and activity increase was also saturable at higher concentrations.

Based on these considerations, the further investigations have been performed at 10–20 mM concentrations of the designed imprinting molecules.

Imprinting Effect of the Selected Additives on Lipases Entrapped in a Sol-Gel System

To evaluate the imprinting effects in this sol-gel immobilization of lipases, specific activities (U_B),[28] activity yields (Y_A)[28] and enantiomer selectivities (E)[46] from multisubstrate test reactions were compared (for U_B and E see Table 1; further data such as activity yields (Y_A) are given in the Supporting Information). It is worth noting that our sol-gel entrapped biocatalysts even without imprinting additives were superior to the corresponding commercially available sol-gel preparations (data not shown).

In the case of Lipase AK, the sol-gel entrapment resulted in robust immobilized biocatalysts with the same effective specific activity as the free lipase powder. This represents more than 2.2-fold increase in the activity of Lipase AK upon immobilization ($Y_A = 224\%$, in sol-gel matrix without additive). Whereas the sol-gel immobilization of Lipase AK led to a much large activity enhancement, none of the imprinting additives could achieve further improvements.

This result can be explained in two ways. The first being that Lipase AK is not sensitive to imprinting. Another explanation – which we believe more plausible – is that PhTEOS or its partially hydrolyzed derivatives play the same role [Figure 4 (**B**)] as the imprinting additives [Figure 4 (**A**)]. This assumption is supported by the fact that lipases can accept alkoxysilanes as substrates as indicated by the study on a lipid-coated lipase which was able to catalyze the oligomerization of DMDEOS.[47] Accordingly, phenyltriethoxysilane at high concentrations can occupy the active site of the lipase [Figure 4 (**B**)] and additives at lower concentrations could not achieve further imprinting effects on this enzyme.

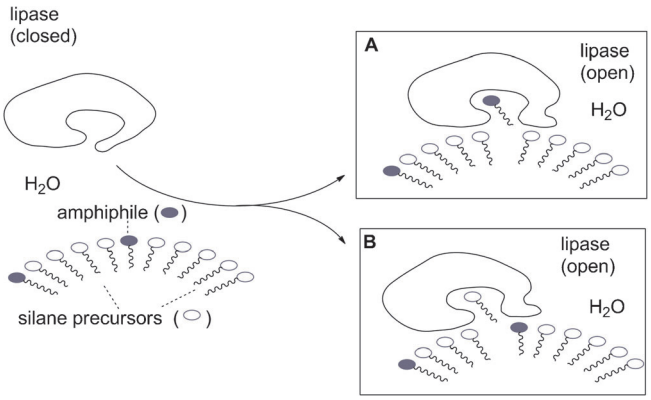

Figure 4. Illustration of interfacial activation based molecular imprinting of lipase with amphiphile (**A**) or with silane precursors (**B**)

The sol-gel entrapment resulted in very efficient immobilized Lipase PS biocatalysts [SG (−)] with remarkably higher effective specific activity than the free Lipase PS [$U_B = 31$ U g^{-1} vs. 13 U g^{-1}, respectively, with *rac*-**1g**]. This remarkable efficiency increase is even more pronounced when the activity yields are taken into account [e.g., 25-fold increase for SG (−) with *rac*-**1b**, see Supporting Information]. Similarly to Lipase AK, it might be hypothesized that phenyltriethoxysilane or its derivatives possess an imprinting effect on Lipase PS during the sol-gel entrapment. However, a slight additional imprinting effect on Lipase PS was observed with lauric acid as additive [$U_B = 25$ U g^{-1} for SG (LA) *vs.* $U_B = 23$ U g^{-1} for SG (−), with *rac*-**1b**]. This is in agreement with the previous results from the *Burkholderia cepacia* lipase in a sol-gel system from methyltrimethoxysilane (MTMOS) and tetramethoxysilane (TMOS) precursors, yielding an LA-imprinted entrapped lipase with 47.9- and 2.5-fold increases in specific activity over

Table 2. Comparison of lipases imprinted with the best performing additives as immobilized biocatalysts in binary and ternary sol-gel matrices. Specific activity (U_B), activity yield (Y_A) and enantiomer selectivity (E) of the free and immobilized forms of lipases in multiple kinetic resolutions are shown in Panel A (for octan-2-ol *rac*-**1b**) and Panel B (for 1-phenylethanol *rac*-**1g**). The reaction times were 0.5 h for Lipase AK, Lipase PS and CaLB and 4 h for CrL in hexane:THF 2:1 solvent (for further reaction details, see Experimental Section).

Silane matrix[a] (enzyme amount)	Lipase AK[b]			Lipase PS[b]			CaLB[b]			CrL[b]		
	U_B [U g^{-1}]	Y_A [%]	E[c]	U_B [U g^{-1}]	Y_A [%]	E[c]	U_B [U g^{-1}]	Y_A [%]	E[c]	U_B [U g^{-1}]	Y_A [%]	E[c]
Panel A (*rac*-**1b**)												
–[d]	34	100	5.2	4	100	4.3	186	100	>200	10.5	100	2.3[e]
Ph (1×)[f]	42	274	4.3	30	1854	7.1	63	2571	≫200	2.8	37	2.0[e]
OcPh (1×)[f]	111	835	9.4	54	3741	6.7	85	2829	>200	0.4	43	1.5[e]
OcPh (2×)[g]	118	409	12.0	120	3760	11.0	92	1459	≫200	0.9	49	1.8[e]
PhDM (1×)[f]	12	145	5.8	59	6063	8.3	79	4611	>200	0.6	481	1.3[e]
PhDM (2×)[g]	58	327	5.6	101	5222	11.0	91	2095	>200	1.1	119	1.7[e]
Panel B (*rac*-**1g**)												
–[d]	109	100	>200	23	100	>200	507	100	≫200	4.4	100	2.3
Ph (1×)[f]	98	198	>200	94	1080	≫200	86	1150	≫200	2.3	75	3.1
OcPh (1×)[f]	127	295	>200	118	1520	≫200	76	843	>200	0.1	14	5.3
OcPh (2×)[g]	127	136	>200	128	737	>200	125	652	≫200	0.8	165	4.6
PhDM (1×)[f]	74	283	>200	125	2363	≫200	117	2263	≫200	0.8	681	9.2
PhDM (2×)[g]	126	217	≫200	126	1210	≫200	113	857	≫200	1.2	95	6.1

[a] Ph: TEOS:PhTEOS 1:1; OcPh: TEOS:OcTEOS:PhTEOS 10:7:3; PhDM: TEOS:PhTEOS:DMDEOS 4:1:1.
[b] Imprinting additives: no additive for Lipase AK, LA (0.5 v/v%) for Lipase PS, BRIJ (0.5 v/v%) for CrL, BRIJ for (0.5 v/v%) for CaLB.
[c] If not stated otherwise, formation of the (*R*)-esters were preferred.
[d] The corresponding commercially available native lipase powder for Lipase AK, Lipase PS and CrL. For CaLB, the commercial liquid enzyme (Novozym CaLB L) was immobilized on Novozym carrier (1 mg carrier to 8 μL enzyme solution).
[e] The formation of (*S*)-**2b** is preferred.
[f] Normal (single) enzyme loading (for further details, see Experimental Section).
[g] Double enzyme loading (for further details, see Experimental Section).

the free and non-imprinted sol-gel lipases, respectively.[32]

In the sol-gel entrapment of lipases of a yeast origin (CaLB and CrL), BRIJ and TRX100 had remarkable imprinting effects on the specific activity of the immobilized enzymes [Table 2: $U_B = 9$ U g^{-1} *vs.* 22 U g^{-1} for SG (–) and SG (BRIJ), respectively, for CaLB with *rac*-**1b**; or $U_B = 0.7$ U g^{-1} *vs.* 2.4 U g^{-1} for SG (–) and SG (TRX100), respectively, for CrL with *rac*-**1b**]. The polyethylene glycol ether structures of BRIJ 30 and Triton X-100 are highly reminiscent of the imprinting molecules found in the related experimental lipase structures [Figure 1 (**C**), (**D**), (**F**)]. Interestingly, not only the specific activity but the enantiomer selectivity of the sol-gel entrapped Lipase PS, CaLB and CrL were enhanced to some extent.

It is worthwhile to note that CrL preferred the acylation of (*S*)-enantiomers of the aliphatic secondary alcohols *rac*-**1a–f**. Earlier it was found that coating of CrL with surfactant molecules affects the recognition of an alcohol substrate and changes the stereopreference.[30c]

Scaling-Up the Imprinted Sol-Gel Lipases for Continuous-Flow Applications

Lipases can be applied advantageously in continuous-flow systems.[11–16] Although the free native lipases behave as enzyme powders in organic media and can be used as fillings in packed-bed bioreactors,[15] one problem to overcome is the relatively low activity and stability of the non-immobilized enzyme aggregates. Therefore, a packed-bed reactor filled with robust sol-gel immobilized enzyme is an appealing alternative.

A recent study indicated that lipases entrapped in sol-gel matrices from ternary silane precursor systems surpassed the catalytic properties of corresponding immobilized biocatalysts from binary silane precursor systems.[28] It was also found that lipases in ternary sol-gel systems with DMDEOS as one of the silane precursors had even more beneficial properties.[48] Therefore, the potential imprinting candidates exhibiting superior properties in the binary sol-gel system were investigated further in ternary sol-gel systems. The properties of the best imprinted ternary sol-gel lipases as biocatalysts were determined in batch and in continuous-flow mode kinetic resolutions with multi-

ple substrates and with a single substrate on a preparative scale.

First, scaling-up the sol-gel immobilization of the lipases with the best performing imprinting additives from sub-gram scale to multi-gram scale was performed with the earlier found best ternary compositions TEOS:OcTEOS:PhTEOS 10:7:3[28] and TEOS:OcTEOS:DMDEOS 4:1:1[48] (Table 2). Expectedly, the lipases with the same additive which proved to be the most effective in the preliminary tests performed better in the ternary sol-gel systems than in the binary system (Table 2: no additive for Lipase AK, LA for Lipase PS, BRIJ for CrL and CaLB). Although the two-fold amounts of the entrapped lipases usually resulted in higher specific activity (U_B) of the forming sol-gel biocatalysts, the activity enhancement was not proportional with the enzyme loading. In all cases, the higher activity yields (Y_A) were observed for the ternary sol-gel systems utilizing a lower lipase loading. Taking the specific activity (U_B) and enantiomer selectivity (E) together into account, the most efficient systems were obtained from the TEOS:PhTEOS:DMDEOS 4:1:1 silane precursors with high lipase loading.

It is also obvious that the microenvironment of the resulting matrix has to be taken into consideration. Correlation was found between the order of specific activity (U_B) for the various members of the homologous series *rac*-**1a–e** and the hydrophobicity of the silane precursors (see Supporting Information).

After selecting the TEOS:PhTEOS:DMDEOS 4:1:1 silane precursor system with high lipase loading and with the best imprinting molecules (no additive for Lipase AK, LA for Lipase PS, BRIJ for CrL and CaLB), the productivity (r) and enantiomer selectivity (E) of the resulting imprinted lipases in kinetic resolutions with the sol-gel lipases were compared in batch mode and in a continuous-flow reactor. The tests were performed with multisubstrate systems (mixtures A and B in Figure 2) and with the single racemate *rac*-**1h** (Figure 2). This series of investigations was performed in hexane:methyl *tert*-butyl ether (MTBE):vinyl acetate 6:3:1, which is more compatible with the usual pump sealings than system containing THF.

The comparison of the four selected sol-gel lipases in batch mode and continuous-flow mode with the multisubstrate systems revealed that irrespective of the substrate (*rac*-**1a–g**) or enzyme (Lipase AK, Lipase PS, CaLB or CrL), the productivity (specific reaction rate, r) of the continuous-flow system always exceeded the corresponding value for the batch mode (Figure 5). This outcome was in agreement with previous results in kinetic resolutions of single racemates with immobilized lipases in continuous-flow reactors.[15,16]

To demonstrate the synthetic capabilities of the imprinted sol-gel lipases in a real kinetic resolution, the enzymatic acylation of the less examined racemic 1-(thiophen-2-yl)ethanol *rac*-**1h** was chosen (Figure 2). The acylation of *rac*-**1h** was investigated with the four imprinted lipases (Lipase AK without additive, Lipase PS with LA, CrL and CaLB with BRIJ) in two ternary sol-gel systems at two lipase loadings. As the CrL preparations performed with low selectivity, only other three lipases were characterized further (Figure 6 and Table 3).

The productivity (specific reaction rate, r) of these biocatalysts was consistently higher in the continuous-flow systems (empty bars in Figure 6) than in the corresponding batch mode reactions (black bars in Figure 6).

The results with the lipases of sufficient enantiomer selectivity (E) in the kinetic resolution of *rac*-**1h** indicated also that a higher enzyme loading resulted in

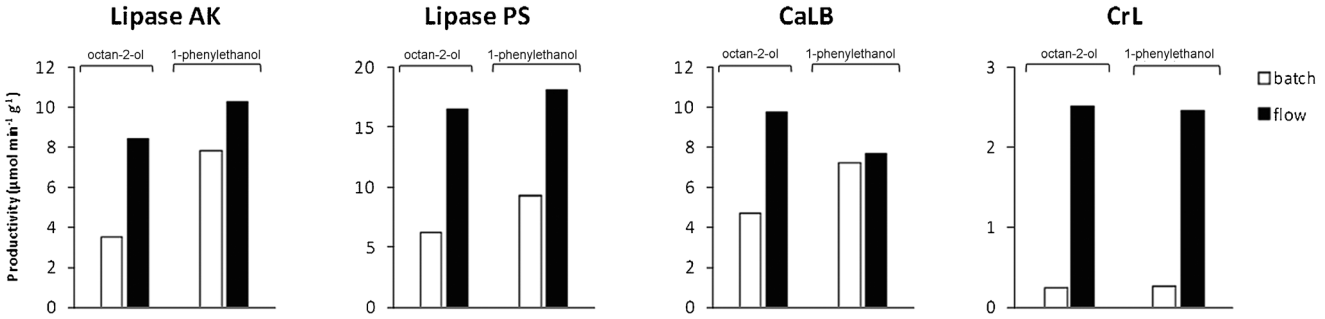

Figure 5. Comparison of the productivity (specific reaction rate, r) for the best performing imprinted sol-gel lipases in batch mode and in continuous-flow packed-bed reactor using multiple kinetic resolutions as test reactions. Representative data for octan-2-ol, *rac*-**1b** and 1-phenylethanol, *rac*-**1g** with each biocatalysts are shown. [Lipase AK (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, no additive); Lipase PS (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% LA); CaLB (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% BRIJ); CrL (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% BRIJ); solvent: hexane:MTBE 2:1, reaction times: 1 h for Lipase AK, Lipase PS and CaLB, 4 h for CrL (batch), flow rate: 0.2 mL min^{-1} (flow); for further reaction details, see Experimental Section and Supporting Information].

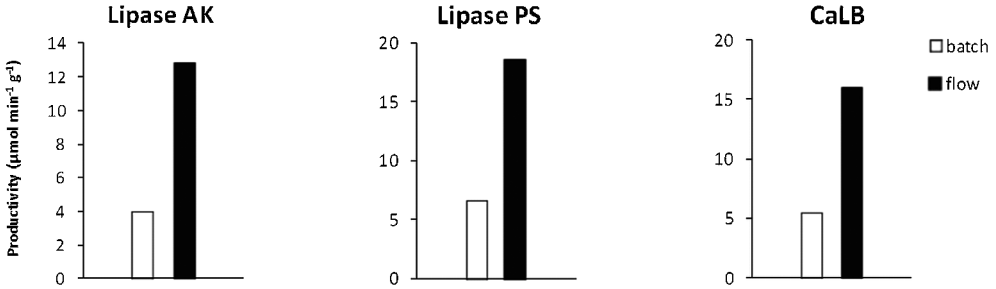

Figure 6. Comparison of the productivity (specific reaction rate, *r*) for the best performing imprinted sol-gel lipases in batch mode and in continuous-flow packed-bed reactor in kinetic resolution of 1-(thiophen-2-yl)ethanol *rac*-**1h**. [Lipase AK (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, no additive); Lipase PS (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% LA); CaLB (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% BRIJ); reaction time: 1 h (batch), flow rate: 0.2 mL min^{-1} (flow) ; for further reaction details, see Experimental Section].

more active immobilized biocatalysts (Table 3). Predictably, the same ternary sol-gel biocatalysts proved to be the most efficient in the single racemate-based kinetic resolutions (*rac*-**1h**) which were the best in the multiple substrate tests (TEOS:PhTEOS:DMDEOS 4:1:1, high lipase loading; Table 2).

Finally, the two most selective sol-gel entrapped lipases (Lipase AK, 2×loading in TEOS:PhTEOS:DMDEOS 4:1:1 system without additive and CaLB, 2×loading in TEOS:PhTEOS:DMDEOS 4:1:1 system with BRIJ) were applied to perform the kinetic resolutions of 1-(thiophen-2-yl)ethanol *rac*-**1h** in continuous-flow reactors on preparative scale [Figure 2 (**B**); Table 4]. Analysis of the products at various times in the 24-h-long runs indicated the operational stability of both sol-gel biocatalysts (see Supporting Information).

Although the enantiomer selectivity for both biocatalysts was reasonably high in the batch mode tests ($E > 200$, see Table 3), the enantiomeric excess of the isolated acetate (*R*)-**2h** from the preparative-scale continuous-flow experiment was not as high as in the test reactions. In a next experiment, however, the (*S*)-**1h** of 99.2% *ee* was acetylated quantitatively to (*S*)-**2h** using the non-selective CaLA in a continuous-flow reaction. The fact that (*S*)-**2h** had only 92.0% *ee* after the same chromatographic purification indicated that the low enantiomeric excesses of the acetates (*R*)- and (*S*)-**2h** are due to the sensitivity of the product to racemization rather than the insufficient selectivity of the lipases in the continuous-flow system.

Conclusions

The study on bioimprinting effects of substrate-mimicking molecules in sol-gel immobilization of lipases with binary and ternary silane precursor compositions indicated that independently from the nature of sol-gel matrix, the most pronounced imprinting effects were found with such additives which were found mimicking the substrates in the experimental structures of the lipases.

This case study revealed lauric acid as being the most effective imprinting additive for lipase PS (the crystal structure of lipase from *Burkholderia cepacia* included stearic acid) while in the cases of *Candida* species (CaLB, CrL) tetraethylene glycol dodecyl

Table 3. Comparison of lipases imprinted with the best performing additives as immobilized biocatalysts in ternary sol-gel matrices in batch mode kinetic resolutions of 1-(thiophen-2-yl)ethanol *rac*-**1h**.

Silane precursors (enzyme amount)	Lipase AK[a]		Lipase PS[a]		CaLB[a]	
	U_B [U° g^{-1}]	E	U_B [U g^{-1}]	E	U_B [U g^{-1}]	E
_[b]	70	>100	28	>100	70	≫200
TEOS:OcTEOS:PhTEOS 10:7:3 (1×)[c]	23	90	44	>100	25	>100
TEOS:OcTEOS:PhTEOS 10:7:3 (2×)[d]	71	89	73	73	66	>200
TEOS:PhTEOS:DMDEOS 4:1:1 (1×)[c]	31	81	33	>100	23	>100
TEOS:PhTEOS:DMDEOS 4:1:1 (2×)[d]	70	≫200	70	>100	54	>200

[a] Imprinting additives: no additive for Lipase AK, LA (0.5 v/v%) for Lipase PS, BRIJ for (0.5 v/v%) for CaLB; solvent: hexane:MTBE 2:1, reaction time 1 h; for further reaction details, see Experimental Section.
[b] The corresponding commercially available native lipase powder for Lipase AK and Lipase PS. For CaLB, the commercial liquid enzyme (Novozym CaLB L) was immobilized on Novozym carrier (1 mg carrier to 8 μL enzyme solution).
[c] Normal (single) enzyme loading (for further details, see Experimental Section).[d] Double enzyme loading (for further details, see Experimental Section).

Table 4. (*S*)-1-(Thiophen-2-yl)ethanol (*S*)-**1h** and (*R*)-1-(thiophen-2-yl)ethyl acetate (*R*)-**2h** prepared by kinetic resolutions with sol-gel immobilized imprinted lipases in continuous-flow reactors. [*rac*-**1h**, 5 mg mL^{-1} in hexane:MTBE:vinyl acetate 6:3:1, 0.2 mL min^{-1}, 30 °C, 24 h flow time].

Compound	Lipase (precursors, additive, enzyme amount)	Y[a] [%]	ee[b] [%]
(*S*)-**1h**	Lipase AK (TEOS:PhTEOS:DMDEOS 4:1:1, -, 2×)	35.0	99.2
(*R*)-**2h**	CaLB (TEOS:PhTEOS:DMDEOS 4:1:1, BRIJ, 2×)	20.8	88.8

[a] Yields refer to isolated products after chromatographic separation.
[b] The *ee* values were determined by enantioselective GC.

ether (BRIJ 30) exhibited the most significant imprinting effect [the porcine pancreatic lipase resembling similarity to CrL included tetraethylene glycol octyl ether and the crystal structure of CaLB included methylpenta(oxyethyl)-heptadecanoate].

The high efficiency of sol-gel systems containing trialkoxysilanes OcTEOS or PhTEOS may be explained by assuming that these silanes or their partially hydrolyzed forms induce an imprinting effect as well. This assumption can explain why Lipase AK (lipase from *Pseudomonas fluorescens*) entrapped in OcTEOS- or PhTEOS-based sol-gel matrices required no imprinting additives for maximal performance.

The rational selection of imprinting molecules can be combined with proper compositions of silane precursors in large-scale production of sol-gel immobilized lipases for various applications. The robust sol-gel entrapped forms of the selected four lipases (from Lipase AK, Lipase PS, lipase B from *Candida antarctica* and lipase from *Candida rugosa*) proved to be ideal biocatalysts in kinetic resolution of a racemic alcohols in batch and continuous-flow systems.

Experimental Section

Materials

Details on enzymes, chemicals and solvents are given in Supporting Information.

General Procedure for Sol-Gel Immobilization of Lipases (using Binary and Ternary Silane Precursor Systems)

IPA (200 μL), TRIS-HCl buffer (0.1 M, pH 7.5, 390 μL), the imprinting additive [0.5% v/v, 5 μL: lauric acid (LA), oleic acid (OA), polyethylene glycol 400 (PEG 400), polyethylene glycol 1000 (PEG 1000), Triton X-100 (polyethylene glycol tert-octylphenyl ether, TRX100), BRIJ 30 (polyethylene glycol dodecyl ether, BRIJ), tetraethylene glycol (TEG), tetraethylene glycol dilauryl ester (L-TEG-L) or olive oil] and NaF solution (1 M, 100 μL) were mixed in a 5-mL glass vial and the resulting solution was shaken at 1000 rpm at room temperature for 10 min. The corresponding silane precursors [TEOS:PhTEOS (1:1), TEOS:OcTEOS:PhTEOS (10:7:3) or TEOS:PhTEOS:DMDEOS (4:1:1); 3 mmol (in the given molar ratio)] and enzyme (50 mg or 50 μL for liquid CaLB) were added to the vial while continuously shaking. To complete the polymerization of the product in a sol suspension, the mixture was shaken for 12 h at room temperature. The resulting solid was washed with IPA (7 mL), distilled water (5 mL), IPA (5 mL) and *n*-hexane (5 mL). The residual white powder was dried in a vacuum desiccator for 3 h (until 0.4 mmHg final level of vacuum). Experiments with Lipase PS and CrL were carried out in triplicates. The sol-gel lipase preparations were stored at room temperature.

Scaling up the Sol-Gel Immobilization of Lipases using Ternary Silane Precursor Systems

Scale-up with the general enzyme loading (1×): The general sol-gel production procedure with 20-fold enzyme amounts (1 g of Lipase AK, Lipase PS and CrL; and 1 mL of CaLB L) using ternary silane precursors [TEOS:OcTEOS:PhTEOS (10:7:3) or TEOS:PhTEOS:DMDEOS (4:1:1); 60 mmol (in the given molar ratio)] was performed with the selected imprinting additives [no additive for Lipase AK, LA (0.5 v/v%) for Lipase PS, BRIJ (0.5 v/v%) for CrL, BRIJ for (0.5 v/v%) for CaLB]. All the other components of the general procedure were added in 20-fold amounts.

Scale-up with doubled enzyme loading (2×): The 20-fold amount of enzyme (1 g of Lipase AK, Lipase PS and CrL; and 1 mL of CaLB L) was added to a system containing 10-fold amounts of the components of the general procedure, the ternary silane precursors [TEOS:OcTEOS:PhTEOS (10:7:3) or TEOS:PhTEOS:DMDEOS (4:1:1); 30 mmol (in the given molar ratio)] and the selected imprinting additives [no additive for Lipase AK, LA (0.5 v/v%) for Lipase PS, BRIJ (0.5 v/v%) for CrL, BRIJ for (0.5 v/v%) for CaLB].

Characterization of the Sol-Gel Lipase Preparations Using Multisubstrate Test Systems

The biocatalyst (native lipase or sol-gel immobilized lipase; 50 mg in each case) was added to the solution of multisubstrate mixture A (50 μL; equimolar mixture of hexan-2-ol *rac*-**1a**, octan-2-ol *rac*-**1b**, nonan-2-ol *rac*-**1c**, decan-2-ol *rac*-**1d** and dodecan-2-ol *rac*-**1e**) or multisubstrate mixture B (50 μL; equimolar mixture of heptan-2-ol *rac*-**1f** and 1-phenylethanol *rac*-**1g**) in hexane:THF 2:1 (1 mL) or in hexane:MTBE 2:1 (1 mL) containing vinyl acetate (100 μL). The resulting mixture was shaken at 30 °C in a sealed glass vial at 1200 rpm. Samples were taken directly from the reaction mixture (sample size: 50 μL, diluted with hexane to 500 μL) at 0.5, 1, 2, 4, 8, 24 h, and analyzed by GC (for details see Supporting Information).

The specific activity of the biocatalyst [U_B (μmol × min^{-1} g^{-1})] could be determined in the test reaction from the

amount of the racemic alcohol [n_{rac} (μmol)], the conversion (c), the reaction time [t (min)] and the total mass of the native free enzyme or the sol-gel immobilized biocatalyst [m_B (g)] using the $U_B = (n_{rac} \times c)/(t \times m_B)$ equation.[28] Because the activities in the present work were calculated from single time-point data, the term "activity" must be considered only for comparative evaluation of the catalytic efficiencies, and not for the real kinetic behaviour of the biocatalyst.

The activity yield [YA (%)] can be calculated from the effective specific activity of the immobilized biocatalyst ($U_{E,imm}$) compared to the specific activity of the native enzymes ($U_{B,native}$).[28]

Enantiomer selectivity (E) was calculated from c and $ee_{(R)-2}$.[46] Due to the sensitivity to experimental errors, E values calculated in the 100–200 range are reported as >100, values in the 200–500 range are reported as >200 and values calculated above 500 are given as $\gg 200$.

Characterization of the Sol-Gel Lipase Preparations Using Multisubstrate Test Systems in Continuous-Flow Reactors

The continuous-flow reactions were performed in CatCart® columns[15] (stainless steel, inner diameter: 4 mm; total length: 70 mm; packed length: 65 mm; inner volume: 0.816 mL) filled with the immobilized sol-gel lipases (the filling mass of the enzymes was 170–440 mg) by a laboratory flow reactor (an HPLC pump and a column thermostat). Prior to use, the freshly filled columns were washed with hexane:MTBE 2:1 mixture (50 mL). The multisubstrate mixture A or B (5 mg mL^{-1}) in hexane:MTBE:vinyl acetate 6:3:1 mixture was pumped through the lipase-filled column (30 °C, 0.2 mL min^{-1}). Samples were collected and analyzed during stationary operation (GC at every 10 min between 30–90 min after changing the conditions).

Preparative Scale Kinetic Resolution in Continuous-Flow Mode

The solution of 1-(thiophen-2-yl)ethanol (*rac*-**1h**, 5 mg mL^{-1}, 0.039 mmol mL^{-1}) in hexane:MTBE:vinyl acetate 6:3:1 mixture was pumped through the lipase-filled column [filled with sol-gel preparations of Lipase AK, Lipase PS, CaLB]. The reaction mixture was collected for 24 h, and then the solvent was removed from the resulting reaction mixture under vacuum. The residue containing the mixture of the forming acetate [(*R*)-**2h**] and unreacted alcohol [(*S*)-**1h**] was separated by column chromatography (Geduran® Si 60, dichloromethane). For data on the products see Supporting Information.

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