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Rapid screening of hydrolases for the enantioselective conversion of 'difficult-to-resolve' substrates

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Abstract

Hydrolases showing high enantioselectivity towards three racemic alcohols (1-methoxy-2-propanol, 3-hydroxy-tetrahydrofuran, 3-butyn-2-ol) and pantolactone were identified by a step-wise screening procedure. Initially, those biocatalysts, which exhibited hydrolytic activity towards the corresponding acetates or butyrates, were selected out of >100 enzymes. Here, rapid screening was performed in a pH-indicator-based format in microtiter plates. Subsequently, enantioselectivity of active hydrolases was determined in small scale reactions ($\sim$1 mg substrate per reaction) by means of gas chromatography using chiral columns. Enzymes exhibiting highest enantioselectivities were then chosen for preparative scale resolution. Using this strategy, at least one suitable hydrolase was found for 3 out of the 4 model compounds examined, allowing efficient kinetic resolution. Moreover, in all cases enantiocomplementary enzymes were identified thus enabling access to both enantiomers of all substrates.

1. Introduction

The application of hydrolases, especially lipases and esterases for the synthesis of enantiomerically pure compounds is well documented.[1–4] Lipases and esterases have been used for the resolution of >1000 alcohols, carboxylic acids and lactones by means of hydrolysis or transesterification. For the latter, reactions are usually performed in organic solvents using activated acyl donors, i.e. vinyl acetate for the resolution of alcohols. Several biotransformations including use of lipases are already performed on an industrial scale.[5] The majority of compounds investigated so far are secondary alcohols, because most hydrolases show sufficient enantioselectivity E only for them. In order to rationalize this observation, an empirical model ('Kazlauskas rule') has

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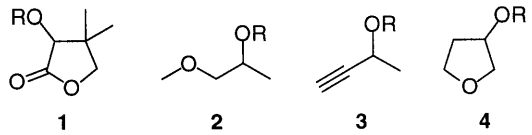
been proposed, which is mainly based on the size of substituents attached to the stereogenic center.[6] High *E*-values[7] are achieved, when a small sized (i.e. methyl-) and a large substituent (i.e. phenyl-) are present. On the other hand, secondary alcohols with residues having only small differences in their size are difficult-to-resolve. The same holds true for primary alcohols, where much less examples for efficient enzymatic resolution or desymmetrization have been reported mostly using lipases from porcine pancreas and *Pseudomonas* sp.[1,4,8]

The vast progress in genetic engineering over the last decades facilitated the production of enzymes. As a consequence, a considerable number of hydrolases is currently available. However, the appropriate choice of useful enzymes requires a rapid screening technology in order to save time and chemicals. In this study, we have selected four model compounds (one lactone, **1a**, three racemic alcohols, **2a**–**4a**, Scheme 1), which so far could not be resolved with sufficiently high *E*-values allowing the isolation of enantiomerically pure substrate and product. All compounds are important building blocks for organic synthesis. For instance, 3-butyn-2-ol **3a** can be used for the synthesis of Fenleuton, the sex pheromone of a male mountain pine beetle,[9] and the synthesis of (*S*)-(−)-Austrocorticin, the major pigment in the fruit bodies of an Australian toadstole.[10] Pantolactone **1a** is the precursor of D-pantothenic acid.[11] 3-Hydroxy-tetrahydrofuran **4a** and derivatives possess biological activity exhibiting anaesthetic qualities and are reported as HIV I protease inhibitors.[12,13]



1 **2** **3** **4**

a, R=H; **b**, R=acetate; **c**, R=butyrate

Scheme 1. Structures of racemic compounds resolved in this study

Identification of enantioselective biocatalysts followed a step-wise screening procedure, in which first active enzymes were targeted, followed by determination of their enantioselectivity in small-scale reactions using gas chromatographic analysis and finally preparative hydrolyses or acylations.

2. Results and discussion

In the first screening round we employed 114 enzymes[†] out of which 110 were obtained from commercial producers and four esterases made in our laboratory by recombinant expression in *E.coli*. Here, we selected for biocatalysts capable of hydrolyzing the corresponding acetates **1b**–**4b** or the butyrate **3c** using a pH-indicator-based screen in microtiter plates. A color change from blue to yellow using bromothymol blue (at pH = pKs 7.3) indicated active enzymes. A representative example is shown in Fig. 1. Compounds **2b**–**4b** and **3c** exhibited sufficient stability under assay conditions whereas **1b** decomposed partially by incubation. Experiments with tributyrin as test substrate before and after a measurement session (6 h) showed that all enzymes kept their activity while storing them in solution. Furthermore they were all active against tributyrin with the exception of Diversa BD051, BD094 and BD151.

[†] A complete list of enzymes used and details for GC analysis is available from the corresponding author, but also can be downloaded as a PDF file at http://www.chemie.uni-greifswald.de/~biotech/publicat.html.

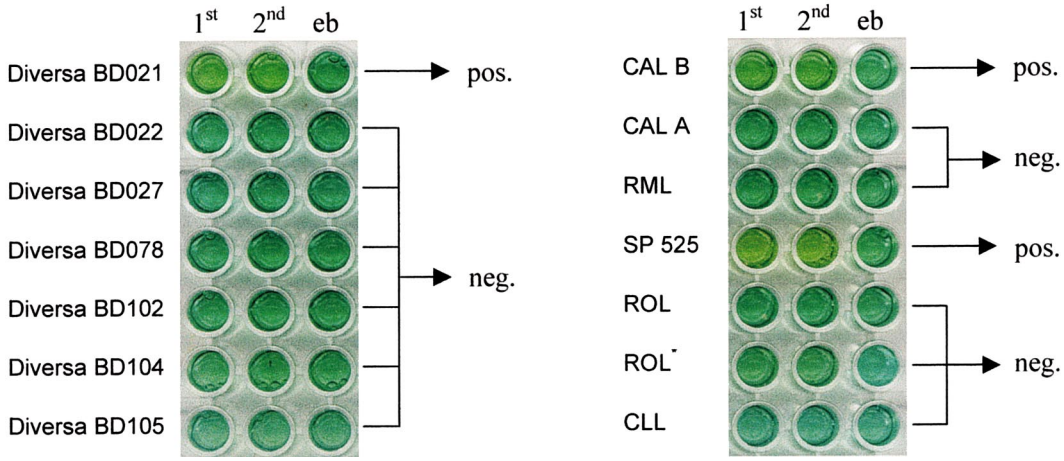


Figure 1. Examples of the selective screen using (*R*,*S*)-**2b**. Enzyme reactions are performed twice (1st; 2nd). Color change compared to enzyme blank (eb) after 3 h at 37°C indicates cleavage of the ester. Sources of hydrolases: CAL B: *Candida antarctica* lipase B (Roche); CAL A: *Candida antarctica* lipase A (Roche); RML: *Rhizomucor miehei* lipase (Roche); SP 525: *Candida antarctica* lipase B (Novo); ROL: *Rhizopus arrhizus* lipase (Fluka); ROL*: *Rhizopus niveus* lipase (Fluka); CLL: *Candida lipolytica* lipase (Fluka); pos., active enzyme; neg., no reaction

By this strategy, in total approx. 20 lipases or esterases and one protease were identified showing sufficiently high activity. These enzymes were then subjected to small scale reactions ($\sim$1 mg substrate per reaction) to determine enantioselectivities. This required a rapid and reliable separation of enantiomers of the corresponding esters and alcohols. We found that all enantiomers could easily be base-line separated using a range of cyclodextrin-based gas chromatography columns with analysis times below 20 min. Representative examples for efficient GC separation and high enantioselectivity are shown in Fig. 2A–D. The results of this screening are summarized in Table 1.

For the resolution of pantolactone acetate **1b**, four enzymes were found showing *E*-values >100. One Diversa-hydrolase (BD423) gave opposite (*R*) enantiopreference albeit an *E*-value of only 5. For 1-methoxy-2-propyl acetate **2b**, one *Pseudomonas* and a *Candida antarctica* lipase were highly (*R*)-selective and again a Diversa enzyme produced the other enantiomer. The resolution of 3-butyn-2-ol was studied using the acetate and the butyrate. Here, the extremely short reaction time in the reactions using esterases from *Pseudomonas fluorescens* (PFE I), from *Streptomyces diastatochromogenes* (SDE) and the lipase A from *Candida antarctica* of only two minutes are noteworthy. Nine enzymes preferentially hydrolyzed the (*S*)-enantiomer, whereas three preferred the (*R*)-configuration. Satisfactory *E*-values ($E\sim$47–75) were achieved with PFE-I, SDE and PCL–AH. Only three hydrolases showed enantioselectivity towards 3-acetoxy-tetrahydrofuran, and enantiomeric excesses were rather low (Table 1).

Despite the investigation of >100 hydrolases, only approximately 15 enzymes exhibited sufficiently high enantioselectivity for preparative purposes.

Six of these selective enzymes were then used for preparative reactions. In addition, some less-selective hydrolases were included, because they showed opposite stereopreference. The results are summarized in Table 2. Going from the one milligram to the 2–20 mmol range caused several effects, which are more or less pronounced for different substrates and enzymes: The *E*-values sometimes changed considerably, for instance, $E=29$ was determined for the

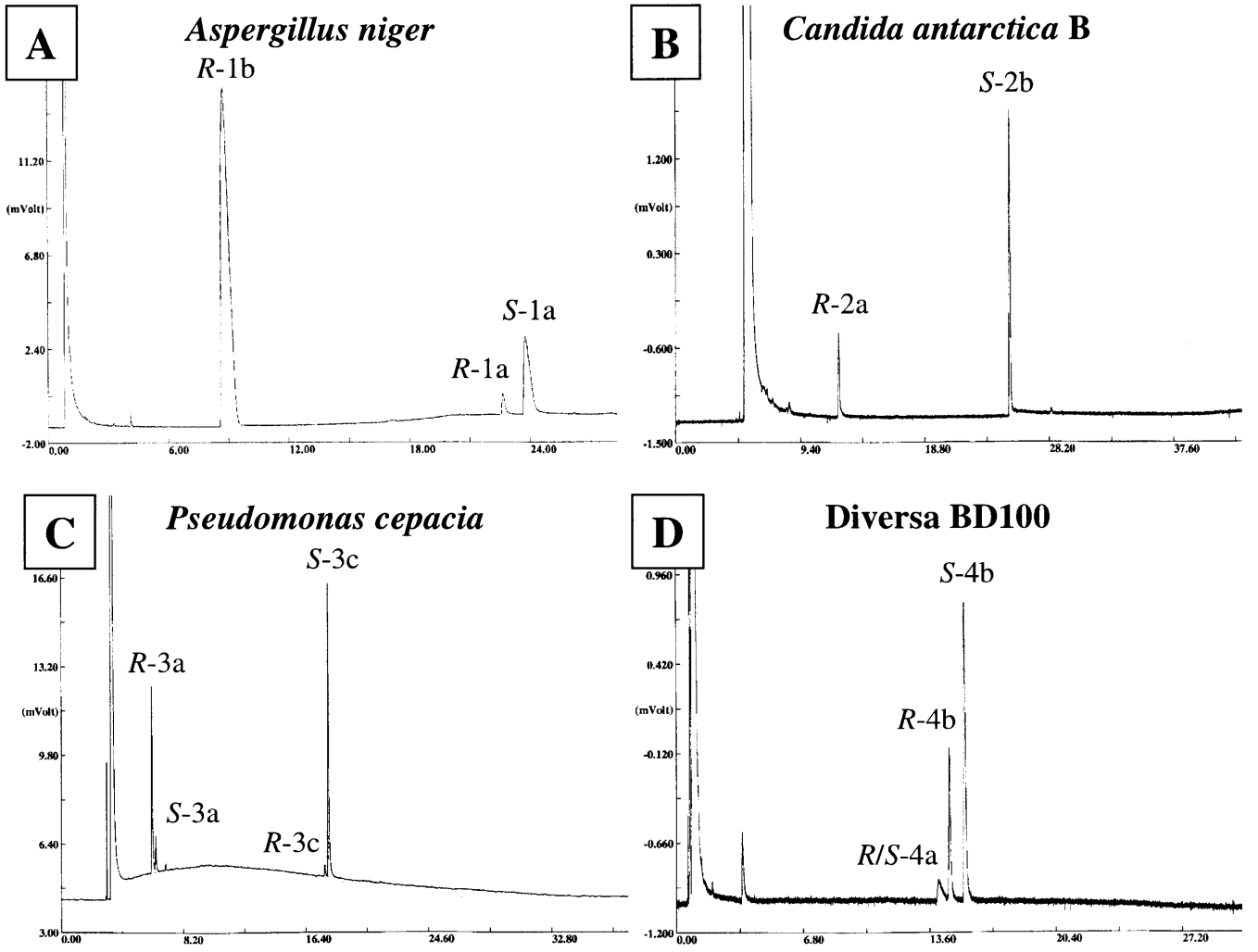


Figure 2. Representative examples of gas chromatographic analyses of reaction mixtures from hydrolyses using lipase from *Aspergillus niger* for the resolution of **1b** (**A**), lipase B from *Candida antarctica* for the resolution of **2b** (**B**), lipase from *Pseudomonas cepacia* for the resolution of **3c** (**C**) and the hydrolase Diversa BD100 for the resolution of **4b** (**D**; only the separation of the acetate is shown)

Table 1
Results of the small-scale reactions using hydrolases identified by the microtiter plate/pH-indicator assay

Sub.	Source of hydrolase	Conditions [°C/h]	Ester [% ee_S]	Alcohol [% ee_P]	Conversion [%]	E-value[a]
1b	*Aspergillus niger* (ANL)[b]	37/17	>99(*R*)	78(*S*)	56	>100
	Aspergillus oryzae[c,d]	37/39	83(*R*)	95(*S*)	47	>100
	Bac. stearothermophilus (BSE)[e]	40/24	84(*R*)	82(*S*)	51	26
	Strept. diastatochromogenes[e]	37/23	20(*R*)	94(*S*)	18	23
	E018b[f]	40/24	86(*R*)	82(*S*)	51	29
	BD073[g]	80/6	68(*R*)	94(*S*)	42	66
	BD094[g]	rt/39	49(*R*)	>99(*S*)	33	>100
	BD405[g]	37/17	25(*R*)	>99(*S*)	20	>100
	BD423[g]	rt/17	25(*S*)	59(*R*)	30	5
2b	*Candida antarctica* B (CAL B)[h]	37/1	>99(*S*)	>99(*R*)	50	>100
	Pseudomonas species[h,i]	37/1	17(*S*)	>99(*R*)	15	>100
	Pseudomonas cepacia[b]	37/23	62(*S*)	62(*R*)	50	8
	BD021[g]	37/1	27(*R*)	79(*S*)	26	11
3b	*Aspergillus nige* (ANL)[b]	37/26	98(*R*)	44(*S*)	69	11
	Ps. fluorescens I (PFE I)[e]	37/2 min	>99(*R*)	69(*S*)	59	75
	Bac. stearothermophilus (BSE)[e]	37/1	45(*R*)	84(*S*)	35	18
	Strept. diastatochromogenes[e]	37/2 min	49(*R*)	93(*S*)	34	47
	BD021[g]	37/26	99(*R*)	58(*S*)	63	19
	BD213[g]	37/26	80(*R*)	85(*S*)	48	30
	Alcaligenes species[h]	37/26	>99(*S*)	33(*R*)	75	20
3c	*Ps. cepacia* (PCL–AH)[b,j]	37/20 min	>99(*S*)	67(*R*)	60	68
	Ps. cepacia (PCL)[h,j]	37/20 min	>99(*S*)	25(*R*)	80	15
	Candida antarctica A (CAL A)[h]	37/2 min	>99(*R*)	50(*S*)	67	30
	Ps. fluorescens I (PFE I)[e]	37/10 min	75(*R*)	87(*S*)	46	33
	Strept. diastatochromogenes[e]	37/25 min	67(*R*)	84(*S*)	44	23
4b	*Candida antarctica* B[h]	37/30 min	90(*R*)	37(*S*)	69	6
	Ps. fluorescens I (PFE I)[e]	37/22	31(*R*)	40(*S*)	43	3
	BD100[g]	37/22	34(*S*)	33(*R*)	51	3

[a] Calculated according to Chen et al.[7]
[b] Amano Pharmaceutical Co., Ltd (Nagoya, Japan).
[c] Protease 2A from *Aspergillus oryzae*.
[d] Fluka Chemie AG (Buchs, Switzerland).
[e] Self-produced recombinant esterases according to Krebsfänger et al.[14] (PFE-I), Khalameyzer and Bornscheuer[15] (SDE) and Amaki et al.[16] (BSE).
[f] ThermoGen (USA, Chicago, IL).
[g] Diversa Corporation (USA, San Diego).
[h] Roche Molecular Biochemicals (Mannheim, Germany).
[i] It is not clear from the product literature which of the *Pseudomonas* lipases it corresponds to.
[j] Lipase from microorganism ATCC21808. Early reports classified this microorganism as *Pseudomonas fluorescens*, later as *Pseudomonas cepacia*, most recently as *Burkholderia cepacia*. Neither the microorganism nor the lipase has changed by the change in name.

Table 2
Preparative-scale resolution of **1a**–**4a** using selected hydrolases. Most reactions were performed as hydrolysis of corresponding esters and conversion was monitored by pH-stat. Enantiomeric excesses were determined by GC analysis after extraction of the compounds

Substr. [mmol]	Hydrolase	[mg]	Conditions [°C/min]	Conversion [%][a]	Ester		Alcohol		*E*-value[a]
					Yield [%]	[% *ee*]	Yield [%]	[% *ee*]	
1b/2	ANL	20	37/382	32	47	45(*R*)	13	94(*S*)	78
1b/2	BSE	20	37/214	53	30	97(*R*)	21	87(*S*)	60
1b/2	E018b	20	40/13	79	15	92(*R*)	23	25(*S*)	5
1b/2	BD423	15	rt/9	75	21	>99(*S*)	27	28(*R*)	5
2b/1	CAL B	10	37/205	50	23	>99(*S*)	N.d.[b]	>99(*R*)	>100
2b/20	CAL B	50	37/210	50	55	>99(*S*)	N.d.	>99(*R*)	>100
2a/1.5	CAL B[c]	25	37/20[d]	47	20	98(*R*)	N.d.	86(*S*)	>100
2b/20	BD021	20	37/383	7	N.d.	3(*R*)	N.d.	28(*S*)	2
3c/20	PFE I	50	37/150	46		73(*R*)	N.d.	85(*S*)	26
3c/1	PCL	10	37/70	69	4	79(*S*)	N.d.	35(*R*)	5
3c/20	PCL–AH	100	37/170	60	N.d.	93(*S*)	N.d.	63(*R*)	15
4b/2	CAL B	20	37/63	75	8	83(*R*)	N.d.	28(*S*)	4
4a/1.5	CAL B[c]	25	37/5[d]	83	31	15(*S*)	7	71(*R*)	3
4b/2	BD100	15	80/123	70	4	49(*S*)	N.d.	24(*R*)	3

[a] Calculated according to Chen et al.[7]
[b] N.d. = not determined.
[c] The lipase B of *Candida antarctica* immobilized on carrier C3 supplied by Roche Molecular Biochemicals (Mannheim, Germany).
[d] Enantioselective syntheses of the acetates (**2b**; **4b**) in hexane using vinyl acetate as acyl donor.

Thermogen enzyme E018b in the small-scale resolution of **1b**, but decreased to only 5 on a preparative scale. On the other hand, *E*-values calculated for **1b**/ANL, **2b**/CAL-B or **3c**/PFE-I remained the same for both reactions including also acylation of **2a** with CAL-B (Fig. 3). In case of **1b**/BSE we observed higher *E*-values in the preparative-scale reaction ($E=60$ versus 26). Reaction times were in the same range despite a considerable increase in substrate concentration. Thus, enzymes showing sufficiently high selectivity were found for substrates **1**–**3**. The only exception is the tetrahydrofuran derivative **4**. Although we screened >100 hydrolases, none was proven to be selective enough for an efficient kinetic resolution.

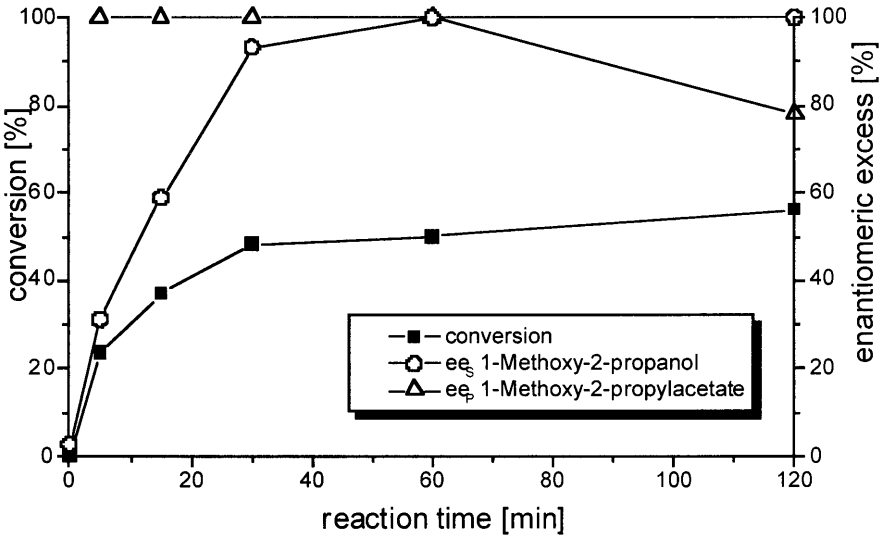


Figure 3. Time course for the acetylation of **2a** (1.5 mmol) with vinyl acetate (7.5 mmol) using 25 mg immobilized *Candida antarctica* lipase B in 5 ml hexane at 37°C

3. Experimental

Racemic and enantiomerically pure alcohols **1a**–**4a** were obtained from common suppliers at the highest purity available. Acetates **1b** and **4b** and the butyrate **3c** were synthesized from the corresponding acid chloride in pyridine using standard procedures. All organic solvents were analytical grade or higher and dried overnight over activated molecular sieves (3 Å) prior to use. Some enzyme suppliers are included in the footnotes of Table 1. The enzyme preparations were used 'straight from the bottle'. Production of the recombinant esterases has already been described.[14–17]

^{1}H NMR und ^{13}C NMR spectra were recorded at 250/500 and 63/126 MHz, respectively, in $CDCl_3$ on Bruker (Karlsruhe, Germany) spectrometers. Chemical shifts (δ) are given in ppm relative to internal TMS, coupling constants (J) in Hz. Enantiomeric excess values (% ee) were calculated from gas chromatographic analysis.

Conversions in hydrolysis reactions were determined by a pH-stat device (Metrohm, Herisau, Switzerland) using 0.1 or 1.0N NaOH solution. Conversion in acylations were calculated from GC analysis as described by Chen et al.[7]

3.1. (RS)-2-Acetoxy-3,3-dimethyl-γ-butyrolactone **1b**

To a solution of (*RS*)-2-hydroxy-3,3-dimethyl-γ-butyrolactone (9.8 g, 75 mmol) in 75 ml pyridine, acetyl chloride (7.5 ml, 105 mmol) was added, while cooling with iced water, and stirred at rt for 19 h. Then water (50 ml) was added, extracted with diethyl ether and the organic layer was washed with hydrochloric acid (0.1 mol), saturated sodium carbonate solution and water. After drying and removal of solvent, **1b** was purified by silica gel column chromatography to give 6.4 g (50%) of pure **1b**: ^{1}H NMR spectra were identical to literature data;[18] δ_C (126 MHz): 19.89, 20.55, 23.03, 40.16, 75.06, 76.21, 169.79, 172.46; Anal. calcd for $C_8H_{12}O_4$: C 55.81, H 7.02. Found: C 55.69, H 7.11.

3.2. (RS)-3-Butyryloxy-1-butyne **3c**

Synthesis was performed according to Nakamura et al.[9] starting with 3-butyne-2-ol (10.5 g; 150 mmol), pyridine (60 ml; 750 mmol), butyryl chloride (78 ml; 750 mmol) and 150 ml dichloromethane as solvent. After extraction, crude **3c** was purified by distillation yielding 12.3 g (59%) of pure **3c**. The substance was identical to literature data.[9]

3.3. (RS)-3-Tetrahydrofuryl-acetate **4b**

To a solution of **4a** (45 mmol; 4.0 g) in 75 ml dichloromethane a five times molar excess of pyridine (16 ml) and acetyl chloride (200 mmol; 14.2 ml) was added, while cooling with iced water. After 24 h, the reaction mixture was worked up as described for **1b** yielding 2.7 g (46%) pure **4b**. δ_H (500 MHz): 2.02 (1H, m, 4-H), 2.07 (3H, s, $COCH_3$), 2.17 (1H, m, 4-H), 3.88 (4H, m, 2-H/5-H), 5.29 (1H, m, 3-H); δ_C (126 MHz): 21.13, 32.69, 67.00, 73.14, 74.83, 170.91; Anal. calcd for $C_6H_{10}O_3$: C 55.37, H 7.74. Found: C 55.41, H 7.79.

3.4. Selective screen

Screening reactions were performed in 96-well microtiter plates operated by a pipetting robot system (Beckman 2000, Unterschleißheim, Germany). Sodium phosphate buffer (180 μl; 10 mM; pH 7.3) with an enzyme concentration of ~1 mg hydrolase/ml buffer was mixed with 20 μl DMSO containing 20 mM **1b**–**4b** or **3c** and bromothymol–blue (0.75 mM). Then the microtiter plates were covered and incubated at rt, 37, 50 or 80°C until a visually monitored color change indicated active enzymes. Analogous set ups without substrates or enzymes served as blanks.

3.5. Small-scale reactions for the determination of enantioselectivities

Acetates **1b**–**4b** or butyrate **3c** (50 mM) were added to an enzyme solution (~1 mg hydrolase/ml) in sodium phosphate buffer (10 mM; pH 7.3). After shaking at rt, 37, 40, 50 or 80°C (reaction times are indicated in Table 1), an aliquot of 500 μl was taken, extracted with 250 μl dichloromethane **1b** or 250 μl ethyl acetate **3b**; **3c**; **4b**. The organic layer was separated, dried and used directly for GC analysis after dilution. In case of **2b** the aliquot was extracted with 500 μl dichloromethane and derivatized with trifluoroacetic acid anhydride prior to GC analysis.

3.6. General procedure for preparative enantioselective hydrolyses

Preparative reactions were performed similar to the small-scale reactions, but in a pH-stat device to maintain the pH at 7.3 and monitor the conversion by addition of 0.1 or 1.0N NaOH solution. Enzyme amounts, substrate concentrations and reaction temperatures are indicated in Table 2. After the desired conversion has been achieved, the reaction was stopped by extraction with diethyl ether. The combined organic layers were dried and the solvent was removed by evaporation in vacuum. Alcohol and non-reacted ester were separated by silica gel column chromatography. Enantiomeric excess and conversion were determined by GC, chemical identity was confirmed by NMR spectroscopy.

3.7. General procedure for enzyme-catalyzed transesterifications in organic solvent

To a solution of 1.5 mmol of **2a** or **4a**, respectively, in 5 ml hexane, activated molecular sieves (3 Å), 25 mg *Candida antarctica* lipase B (Chirazyme® L-2; c.f.; lyo; C3) and vinyl acetate (463 μl; 7.5 mmol) were added. The reactions were performed at 37°C (reaction times are indicated in Table 2). The reactions were stopped by centrifugation to remove the enzyme, and the solvent was removed by evaporation in vacuo. Ester and non-reacted alcohol were separated by silica gel column chromatography. Enantiomeric excess and conversion were determined by GC, chemical identity was confirmed by NMR spectroscopy.

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