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Emulation of Racemase Activity by Employing a Pair of Stereocomplementary Biocatalysts

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Abstract: Racemization is the key step to turn a kinetic resolution process into dynamic resolution. A general strategy for racemization under mild reaction conditions by employing stereoselective biocatalysts is presented, in which racemization is achieved by employing a pair of stereocomplementary biocatalysts that reversibly interconvert an sp^3 to a sp^2 center. The formal interconversion of the enantiomers proceeds via a prochiral sp^2 intermediate the formation of which is catalyzed either by two stereocomplementary enzymes or by a single enzyme with low stereoselectivity. By choosing appropriate reaction conditions, the amount of the prochiral intermediate is kept to a minimum. This general strategy, which is applicable to redox enzymes (e.g., by acting on R_2CHOH and R_2CHNHR groups) and lyase-catalyzed addition–elimination reactions, was proven for the racemization of secondary alcohols by employing alcohol dehydrogenases. Thus, enantiopure chiral alcohols were used as model substrates and were racemized either with highly stereoselective biocatalysts or by using (rarely found) non-selective enzymes.

Keywords: alcohol dehydrogenase · biotransformations · dynamic resolution · racemization · redox chemistry · secondary alcohols

Introduction

Recently, considerable efforts have been made to improve and develop novel methods for racemization.[1] Racemization is needed in asymmetric synthesis for the recycling of undesired enantiomers that are obtained from kinetic resolution,[2] as well as for the preparation of chiral alcohol and amine functionalities by dynamic resolution.[3,4] The racemization of stereolabile compounds, such as cyanohydrins, hemi(thio)acetals, α-substituted carbonyl compounds or α-aryl-substituted hydantoins, can easily be achieved by mild acid or base catalysis.[1,4c,5] In contrast, stereochemically stable compounds, such as secondary alcohols and chiral amines, are more difficult to racemize. Previously, this has been accomplished by redox processes that are mediated by transition-metal complexes.[3a–d,4] Due to the high specificity of biosynthetic pathways, nature has, in general, little need for racemization and thus the number of "true" racemases is very limited.[3e] Thus only a few specialized enzymes—racemases—are known to catalyze the racemization of α-hydroxycarboxyllic acids (such as mandelate and derivatives), (*N*-acyl) α-amino acids, and hydantoins.[1,3e] However, no defined enzymes have been described for the racemization of secondary alcohols or chiral primary amines.[1,6]

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Results and Discussion

Inspired by the racemization mechanism of chiral primary amines and secondary alcohols by transition-metal catalysis[7] via a prochiral intermediate, as well as a consequence of our own research on biocatalytic racemization,[6] we developed a racemization strategy based on stereoselective (bio)catalysts. We envisaged applying biocatalysts that interconvert a chiral center to a prochiral intermediate in analogy to transition-metal catalyzed racemization (Scheme 1).

As indicated in Scheme 1a, the chiral catalyst has a certain stereopreference to transform the prochiral compound

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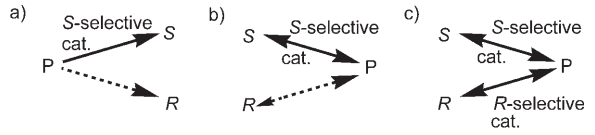


Scheme 1. P = compound with a prochiral center, sp^2 hybrid; *S*, *R* = enantiomers, sp^3 hybrid. a) Desymmetrization of a prochiral compound P by a stereoselective catalyst furnishes a single product enantiomer *S*; b) reversible transformation of enantiomer *S* to the prochiral intermediate P by a stereoselective catalyst "destroying" the chiral center; c) racemization of *R* or *S* either by employing two (bio)catalysts with opposite stereopreference or a single (bio)catalyst with little (or no) stereopreference.

P into enantiomer *S* in a desymmetrization reaction. If the process is reversible, one can start from a single enantiomer (e.g., *S*), of which the chiral centre becomes destroyed during the transformation to prochiral P (Scheme 1b). To achieve racemization, two reversible stereocomplementary transformations (P⇄*S*, and P⇄*R*) have to take place (Scheme 1c). The equilibrium between the substrate enantiomers, *S* and *R*, and the prochiral intermediate P can be kept on the substrate side by choosing the appropriate reaction conditions (e.g., redox-balance, etc.).

On first glimpse, a single chiral catalyst that possesses perfect stereoselectivity is not suitable. Fortunately, no catalyst is absolutely stereoselective and during the interconversion of *S* to P, a small amount of *R* will always be formed due to "selectivity mistakes" that finally lead to racemization.[8] The rate of racemization is a function of the stereoselectivity of the chiral catalyst; in other words, the number of "selectivity mistakes" that occur. However, most biocatalysts show such high stereoselectivity that racemization which is based on a single catalyst would take an inordinately long reaction time; this would be insufficient to allow dynamic kinetic resolution, which requires fast racemization rates.[9]

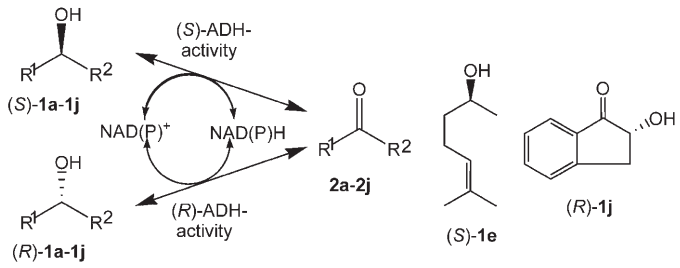
This bottleneck can be overcome by employing two different biocatalysts that possess opposite stereopreferences in order to provide reaction rates of a similar magnitude for both enantiomers (Scheme 1c); this ensures fast racemization. For evident reasons, the fastest rates are achieved by employing either a pair of stereocomplementary biocatalysts with equal activity or a single nonstereospecific catalyst. Due to the intrinsic excellent regio- and chemoselectivity of the enzymes, one can expect that such racemization processes will not affect other functional groups within the molecule, and are therefore "clean"; this avoids the formation of undesired side-products.

This general concept is applicable for all substrates of central chirality, in which an sp^3 center is enzymatically transformed to an sp^2 center in a reversible fashion. Examples for such enzymes are redox enzymes that act on R_2CHOH and R_2CHNHR groups (such as alcohol dehydrogenases and α-amino acid dehydrogenases) and C=C bonds (enoate reductases). Alternatively, lyase-catalyzed addition–elimination reactions that involve C=C bonds (for instance hydratases, ammonia lyases) can be employed. Applications for epimerization of diastereomers represent an extension of the concept.

Overall, the following prerequisites for biocatalytic racemization have to be fulfilled:

1) One must have a matching pair of (highly selective) biocatalysts that possess opposite stereopreferences for the reversible transformation of both enantiomers via the corresponding prochiral intermediate.
2) Alternatively, a single biocatalyst with incomplete stereoselectivity will equally be effective.
3) The equilibrium between substrate enantiomers and prochiral intermediates can be controlled by adjustment of the reaction conditions (e.g., the redox balance, etc.).
4) In case a cofactor is required and two enzymes are employed, free exchange of the (identical) cofactor between the biocatalysts has to be ensured.
5) For redox-mediated racemization with cofactors, external cofactor recycling should not be required, because the net redox balance of this process is zero.[10]

To prove this general strategy, we envisaged employing alcohol dehydrogenases (ADHs) for the racemization of secondary alcohols via the prochiral ketone intermediate.[11] As ADHs are highly enantioselective in general, only enantiomer *S* is oxidized to ketone P (Scheme 1b). After the thermodynamic ketone–alcohol–NAD(P)H–$NAD(P)^+$ equilibrium is reached, the velocity of oxidation and reduction are identical and (in the case that the ADH shows perfect stereoselectivity) the ketone is reduced back to the same substrate enantiomer, *S*. Consequently, by employing a second ADH with the opposite stereopreference, ketone P can be reduced to enantiomer *R*. If both processes are in equilibrium, overall racemization is achieved (Scheme 2).[12] The ketone-to-alcohol ratio depends on the amount and ratio of the oxidized and reduced form of the cofactor added, that is, $NAD(P)^+$ versus NAD(P)H. Hence, the ketone-to-alcohol ratio can be controlled by the amount of cofactors applied to the system. By keeping the concentration of $NAD(P)^+$ to a minimum, the ratio of ketone to alcohol is



	1a	1b	1c	1d	1f	1g	1h	1i
R^1	n-C_6H_{13}	n-C_5H_{11}	n-C_7H_{15}	Ph	n-C_5H_{11}	n-C_5H_{11}	CH_2Ph	$CH_3(C=O)$
R^2	CH_3	CH_3	CH_3	CH_3	$CH=CH_2$	$C\equiv CH$	CH_3	CH_3

Scheme 2. Biocatalytic racemization of secondary alcohols **1a**–**1h** and acyloins **1i** and **1j** via the ketone intermediate by employing alcohol dehydrogenase(s).

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kept on the alcohol side in the ketone–alcohol–NAD(P)H–NAD(P)$^+$ equilibrium.

For the racemization experiments, two commercially available ADHs, ADH-"A" from *Rhodococcus ruber*[13] and ADH from *Lactobacillus kefir* (LK-ADH) were employed.[14] LK-ADH is known[15] to possess opposite (anti-Prelog) stereospecificity to ADH-"A". The racemization of enantiopure (*R*)- and (*S*)-2-octanol (**1a**) was investigated by monitoring the decline of enantiomeric excess over time (Figure 1).

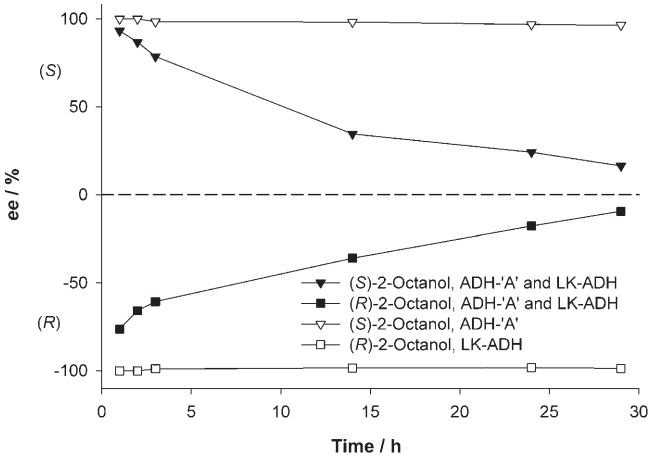

Figure 1. Racemization of (*R*) and (*S*)-2-octanol (**1a**) by employing a combination of two ADHs that possess opposite stereopreference (Prelog-ADH-"A" from *Rhodococcus ruber* and anti-Prelog-LK-ADH from *Lactobacillus kefir*). Experiments with single ADHs served as controls.

After 28 h, an enantiomeric excess (*ee*) of $<15\%$ for both (*S*)- and (*R*)-**1a** was achieved, while the amount of 2-octanone (**2a**) was kept below 5% by choosing an appropriate amount and ratio of NADH/NAD$^+$; this proves that the strategy is successful. As expected, no significant racemization was observed by using only a single stereoselective ADH.

Besides **1a**, a range of aliphatic and aromatic secondary alcohols **1b**–**1g** were successfully racemized by employing this two-enzyme system (Table 1). It is worth noting that substrates that containing carbon–carbon multiple bonds such as sulcatol (**1e**), 1-octene-3-ol (**1f**), and 1-octyn-3-ol (**1g**), which would be susceptible to reduction by using transition-metal-catalyzed racemization, were interconverted in a clean fashion without side-reactions.

Additionally, racemization was proven for a combination of another commercially available ADH, that is, Prelog-ADH from *Rhodococcus erythropolis* (RE-ADH), and anti-Prelog-selective LK-ADH. Only substrate **1h** could not be racemized, either with the combination of ADH-"A"/LK-ADH or RE-ADH/LK-ADH.

Acyloins represent a group of substrates that are rather difficult to racemize, because their chemical racemization is often plagued by elimination as a major side-reaction, due to the ease of formation of a resonance-stabilized conjugated enone.[4c,16] Nevertheless, **1i**,**1j** were successfully racemized by using pairs of isolated ADHs (Table 1). A clean racemization procedure for the cyclic acyloin **1j** is of special interest, because the elimination reaction for this substrate is promoted even by exposure to neutral silica gel during column chromatography.

Table 1. Biocatalytic racemization of secondary alcohols and acyloins by employing pairs of stereocomplementary alcohol dehydrogenases.[a]

Enzymes	Substrate	*ee* [%][b]	(Di)Ketone [%][c]
ADH-"A" and LK-ADH	(*S*)-**1a**	*rac*	2.3
	(*R*)-**1a**	*rac*	1.3
	(*R*)-**1b**	*rac*[d,f]	<0.1
	(*R*)-**1c**	*rac*[d,f]	8
	(*S*)-**1d**	*rac*[f]	0.9
	(*R*)-**1d**	*rac*[f]	3.4
	(*S*)-**1e**	*rac*	2.4
	(*R*)-**1e**	*rac*	5.1
	(*S*)-**1f**	*rac*[f]	2.6
	(*S*)-**1g**	19.2 (*S*)[d]	0.3
	(*R*)-**1g**	25.0 (*R*)[d]	1.3
	(*S*)-**1h**	>99.5 (*S*)[d]	13.9
	(*R*)-**1i**	70.3 (*R*)[d]	n.d.[e]
	(*R*)-**1j**	>99.5 (*R*)[d]	8.6
RE-ADH and LK-ADH	(*S*)-**1a**	*rac*[e]	3.4
	(*R*)-**1a**	*rac*[e]	2.2
	(*R*)-**1b**	40.2 (*R*)[d]	<0.1
	(*R*)-**1c**	32.2 (*R*)[d]	2.6
	(*S*)-**1e**	35.4 (*S*)[d]	3.4
	(*R*)-**1e**	23.9 (*R*)[d]	5.7
	(*S*)-**1h**	>99.5 (*S*)	23.1
	(*R*)-**1i**	91.4 (*R*)	n.d.[e]
	(*R*)-**1j**	30.1 (*R*)[d]	36.2

[a] Reaction conditions: Substrate 2.5 g L^{-1}, 24 h, 30 °C, buffer pH 7.5. [b] The *ee* is based on GC (**1a**–**1h**) and HPLC (**1i**–**1j**); the *ee* of the starting material was $>99.5\%$. *rac* means *ee* $<5\%$. [c] Determined by GC/HPLC. [d] 1/5th of the enzyme was applied. [e] The diketone was not detectable by HPLC. [f] 72 h.

For certain applications, the use of two enzymes might be impossible, most notably if enzymes of opposite stereopreference are not available. To overcome this limitation, we envisaged the use of a single enzyme for racemization. However, as shown in Figure 1 and Table 2 (Entries 1 and 2), no significant racemization was observed when a highly selective single ADH was employed. Only when enantiopure (*R*)-2-octanol (**1a**) was incubated with the stereoselective ADH-"A" for an extended period of 14 days, racemization

Table 2. Racemization of (*R*)-**1a** (*ee* >99.5) by employing single alcohol dehydrogenases.

Entry	Enzyme	*t* [h]	*ee* [%]	Ketone [%][a]
1	ADH-"A"	24	99.2 (*R*)	<0.1
2	LK-ADH	24	98.0 (*R*)	1.1
3	*E. coli* extract[c]	24	>99.5 (*R*)[d]	0.3
4	ADH-"A"	336	82.0 (*R*)	2.1
5	Control	336	99.8 (*R*)	<0.1
6	KRED-118[b]	72	65.5 (*R*)	0.7
7	KRED-119[b]	72	44.3 (*R*)	1.2
8	PF-ADH	24	*rac*	5.3

[a] Determined by GC. [b] NADPH was used instead of NADH. [c] Cell-free extract of *E. coli*. [d] No racemization took place with the (*S*)-enantiomer.

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was observed (Table 2, Entry 4, *ee* 82%), while the control experiment in the absence of enzyme showed no significant decrease of optical purity (Table 2, Entry 5). This demonstrates that even a single highly selective catalyst is effective, albeit at a slow rate.

For fast racemization, a catalyst with low stereoselectivity is required. However, because the primary goal during the last decades was to identify highly selective ADHs for the reduction of ketones, little is published about nonselective enzymes. After testing approximately 60 ADHs, we identified three candidates that reduce 2-octanone (**2a**) with modest or no stereoselectivity. The enzymes of interest were KRED-118, KRED-119[17] and PF-ADH.[18] Due to their low stereoselectivity in the reduction mode, they were chosen for racemization experiments: (*R*)-**1a** was successfully racemized in the presence of limiting amounts of NAD^+/NADH (Table 2, entries 6–8). These data show that racemization is also feasible by employing a single "non-racemase" enzyme that shows imperfect stereoselectivity. A detailed investigation of the racemization of both enantiomers of 2-octanol by employing a single ADH (PF-ADH) was carried out by monitoring the decline of *ee* over a period of 48 h; this showed that (*R*)-**1a** and (*S*)-**1a** were successfully racemized (Figure 2). It is worth noting that both graphs are symmetrical, thus no preference for one enantiomer was observed.

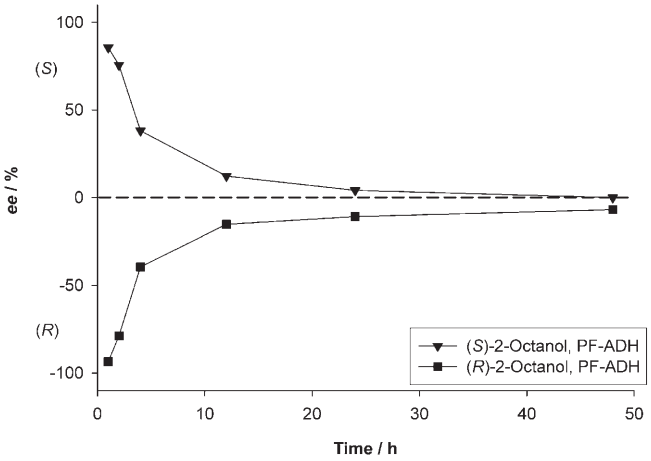

Figure 2. Racemization of (*R*)- and (*S*)-2-octanol (**1a**) by employing a single ADH from *Pseudomonas fluorescen*s (PF-ADH) possesing low stereoselectivity.

Conclusion

A racemization strategy is presented that is based on the reversible interconversion of substrate enantiomers via the corresponding prochiral intermediate by using either a pair of stereocomplementary biocatalysts, or a single enzyme that shows low stereoselectivity. Due to the increasing importance of biocatalytic transformations in asymmetric organic synthesis,[19] novel methods are needed. This general concept is applicable for substrates that bear, for instance, R_2CHOH or R_2CHNHR groups, or in general for substrates in which an sp^3 center can be enzymatically transformed to an sp^2 center in a reversible fashion. Examples include redox enzymes (alcohol dehydrogenases, α-amino acid dehydrogenases, enoate reductases, etc.) and lyases (hydratases, ammonia lyases, etc.). The feasibility of the concept was successfully proven for the racemization of secondary alcohols by employing alcohol dehydrogenases.

This approach to biocatalytic racemization is widely applicable and allows the interconversion of enantiomers under mild (physiological) conditions and thus ensures "clean" and efficient isomerization processes.[20] Due to the recent reports of applications of alcohol dehydrogenases in organic solvents,[21] it can be expected that this racemization protocol can be combined with a lipase to perform a dynamic kinetic resolution. Alternatively, the following two processes are feasible: 1) racemization and resolution can be performed simultaneously in separated vessels that are connected by a membrane or related techniques or 2) a stepwise process, in which the racemization and the resolution step are performed sequentially in cycles.[2]

Experimental Section

Substrates and reference compounds: Enantiopure compounds **1a**, **1d**–**1g** were obtained from Alfa Aesar (Lancaster, Frankfurt/Main Germany), enantiopure **1b**, **1c**, **1h** and ketones **2a**–**2f**, **2h** were purchased from Sigma–Aldrich. Compounds **1i**–**1j** and **2i**–**2j** were synthesized as previously described.[6a] Reference compound **2g** was synthesized by enzymatic oxidation of **1g** by employing whole cells of *E. coli* Tuner™ (DE3)/pET22b+-ADH-"A" with hydrogen transfer by using acetone as a hydrogen acceptor as previously described.[22]

Enzymes: RE-ADH (#68482, alcohol dehydrogenase from *Rhodococcus erythropolis*) and LK-ADH (#05643, alcohol dehydrogenase from *Lactobacillus kefir*) were obtained from Fluka. KRED ketoreductases/alcohol dehydrogenases were obtained from BioCatalytics Inc.[17] Lyophilized ADH from *Pseudomonas fluorescens* (PF-ADH) was prepared from the cell-free extract of *E. coli* that contained the overexpressed enzyme as previously reported.[18] ADH-"A" was commercially available from BioCatalytics Inc. For the experiments described, the following cell-free preparation was employed: *E. coli* Tuner™ (DE3)/pET22b+-ADH-"A" was grown as previously described in LB-amp medium (250 mL) with additional Zn^{2+} (100 mg L^{-1}).[13a] The medium (OD ≈ 3) was centrifuged (Jouan KE22i, AK-500.11, 20 min, 8000 rpm, 4 °C) and the cell debris was resuspended in buffer (50 mL, 50 mM Tris–HCl pH 7.5). The cells were disrupted by ultrasonication (Branson, S250D CE, 200W, 5 mm spike, 50 mL tubes, 1 s impulse, 2 s pause, amplitude 50%, 16 min, 4 °C) and centrifuged (Jouan KE22i, AK-100.21, 20 min, 13000 rpm, 4 °C). The supernatant was transferred to an Erlenmeyer flask (250 mL) and kept at 65 °C for 25 min. After centrifugation (Jouan KE22i, AK-100.21, 20 min, 13000 rpm, 4 °C) the supernatant was used for the experiments.

Representative procedures for racemization employing two enzymes

ADH-'A' and LK-ADH: ADH-"A", isolated from *Rhodococcus ruber* (0.15 U,[23] 5 mg), LK-ADH (0.2 U,[22] 0.5 mg), and a mixture of NAD^+ and NADH (final concentration 0.7 μmol mL^{-1} NAD^+ and 1.2 μmol mL^{-1} NADH) were dissolved in phosphate buffer (50 mM, pH 7.5, total volume 1 mL). The reaction was started by addition of enantiopure (*R*)-**1a** (2.5 μL, 26 μmol mL^{-1}, *ee* > 99.9%). After 24 h of shaking (130 rpm) at 30 °C the mixture was extracted with EtOAc (500 μL) and centrifuged to achieve phase separation.

RE-ADH and LK-ADH: RE-ADH (0.05 U, 2 mg), LK-ADH (0.04 U, 0.1 mg), and a mixture of NAD^+ and NADH (final concentration

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0.7 μmol mL^{-1} NAD$^+$ and 1.2 μmol mL^{-1} NADH) were dissolved in phosphate buffer (50 mM, pH 7.5, total volume 1 mL). The reaction was started by the addition of enantiopure (*R*)-**1a** (2.5 μL, 26 μmol mL^{-1}, *ee* >99.9%). After 24 h of shaking (130 rpm) at 30°C the mixture was extracted with EtOAc (500 μL) and centrifuged to achieve phase separation.

Representative procedures for racemization employing a single enzyme

KRED-118: KRED-118 (0.1 mg) and a mixture of NAD$^+$ and NADH (final concentration 0.7 μmol mL^{-1} NADP$^+$ and 1.2 μmol mL^{-1} NADPH) were dissolved in phosphate buffer (50 mM, pH 7.5, total volume 1 mL). The reaction was started by the addition of enantiopure (*R*)-**1a** (2.5 μL, 26 μmol mL^{-1}, *ee* >99.9%). After 24 h of shaking (130 rpm) at 30°C the mixture was extracted with EtOAc (500 μL) and centrifuged to achieve phase separation.

PF-ADH: Crude PF-ADH preparation (4 mg) and a mixture of NAD$^+$ and NADH (final concentration 0.7 μmol mL^{-1} NAD$^+$ and 1.2 μmol mL^{-1} NADH) were dissolved in phosphate buffer (50 mM, pH 7.5, 1 mL). The reaction was started by the addition of either enantiopure (*S*)-**1a** (2.5 μL, 26 μmol mL−1, *ee* >99.9%) or enantiopure (*R*)-**1a** (2.5 μL, 26 μmol mL^{-1}, *ee* >99.9%). After incubation (130 rpm) at 30°C, the reaction mixture was extracted with EtOAc (500 μL).

General workup procedure: The organic phase was dried (Na_2SO_4) and filtered and the alcohol was acetylated by allowing it to react with acetic anhydride (100 μL) and DMAP (0.5 mg) for 2 h. After aqueous workup, the organic phase was analyzed by GC on a chiral stationary phase.

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