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ORIGINAL ARTICLE

Organic Solvent-Tolerant Marine Microorganisms as Catalysts for Kinetic Resolution of Cyclic β-Hydroxy Ketones

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Abstract Chiral cyclic β-hydroxy ketones represent key motifs in the production of natural products of biological interest. Although the molecules are structurally simple, they require cumbersome synthetic steps to get access to them and their synthesis remains a challenge in organic chemistry. In this report, we describe a straightforward approach to enantiomerically enriched (*R*)- and (*S*)-3-hydroxycyclopentanone **2a**, (*R*)- and (*S*)-3-hydroxycyclohexanone **2b**, and (*R*)- and (*S*)-3-hydroxycycloheptanone **2c** involving a transesterification resolution of the racemates using whole cells of marine microorganisms as catalysts and vinyl acetate the acyl donor and solvent. Twenty-six strains from a wide collection of isolates from marine sediments were screened, and seven strains were found to markedly catalyze the resolution in an asymmetric fashion. Using the strain *Serratia* sp., (*R*)-**2a** was isolated in 27% yield with 92% *ee* and (*S*)-**2a** in 65% yield with 43% *ee*, corresponding to an *E*-value of 37; (*R*)-**2b** was isolated in 25% yield with 91% *ee* and (*S*)-**2b** in 67% yield with 39% *ee*, corresponding to an *E*-value of 40; and (*R*)-**2c** was isolated in 30% yield with 96% *ee* and (*S*)-**2c** in 63% yield with 63% *ee*, corresponding to an *E*-value of 75.

Keywords Cyclic β-hydroxy ketones · Kinetic resolution · Biocatalysis · Marine microorganisms · *E*-value

Introduction

Cyclic β-hydroxy ketones represent an important class of compounds often found as valuable building blocks in the production of natural products of biological interest (Hertweck 2009; Acetti et al. 2010). Their simple structures belie challenging multi-step syntheses, especially in the production of optically active variants (Mestres 2004; Karmee and Hanefeld 2011a; Bisogno et al. 2009). Lipase-catalyzed kinetic resolution is a potentially useful method for obtaining enantiopure compounds (de Miranda et al. 2015). This process presents a broad substrate scope with no expensive cofactors, excellent stereoselectivity, environmentally benign conditions, and organic solvent-tolerance, which has resulted in its application in academic and industrial chemistry. Indeed, many microbial-derived biocatalysts, including isolated enzymes and whole cells, may serve as candidates for the kinetic resolution of cyclic β-hydroxy ketones (Baxter et al. 2012; Busto et al. 2010; Hsu et al. 2006; Kapoor and Gupta 2012; Shakeri et al. 2010; Zhang et al. 2013). However, in a kinetic resolution process, the ideal biocatalyst would only convert one enantiomer to the product and leave the antipode mostly unreacted. The preparation of the ideal biocatalyst, as well as the reaction itself, should be simple and cheap.

In our previous work (Chen and Hanefeld 2013a; Karmee et al. 2011b), we screened nine different isolated lipases (CAL-A, CAL-B, CRL, PCL, Amano PSD-I, *Pseudomonas stutzeri*, *Alcaligenes* sp., PFL, and pancreatic porcine lipase (PPL)) from commercial sources for the kinetic resolutions of *rac*-3-hydroxycylopentanone **2a** and *rac*-3-hydroxycyclohexanone **2b** and obtained enantiomerically enriched (*R*)-**2a** in 21% yield and 83% *ee*, corresponding to an *E*-value (enantiomeric ratio) of

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20, and (*R*)-**2b** in 30% yield and 91% *ee*, corresponding to an *E*-value of 32, respectively. The modest *E*-values in both cases were due to the small size of target molecules, which renders the regio- and stereoinductions difficult. Still, the kinetic resolution via lipase-catalyzed transesterification is rather straightforward compared to the laborious multi-step synthesis of chiral cyclic β-hydroxy ketones. For example, the (*S*)-3-hydroxycyclohexanone (*S*)-**2b** was previously synthesized via a seven-step pathway involving a β-boration oxidation reaction sequence using cyclohexenone as a substrate and bis(pinacolato)diboron as a reagent (Arai et al. 2005; Feng and Yun 2009).

New robust biocatalysts able to improve the efficiency of kinetic resolutions of racemic cyclic β-hydroxy ketones such as **2a-2c** are in great demand. Marine microorganisms produce novel enzymes with high stability and activity at extreme conditions (i.e., extreme temperature, pressure, pH, or organic solvent) compared to enzymes isolated from conventional sources (Antranikian et al. 2005; Dionisi et al. 2012; Ferrer et al. 2012). These advantages, in addition to the availability of marine microorganisms from various sources such as seaweeds, sponges, mollusks, and marine sediments, make marine enzymes ideal biocatalysts for broad application in organic synthesis (Trincone 2011; Rocha et al. 2010, 2012, 2015; de Oliveira et al. 2014; De Vitis et al. 2015; Sarkar et al. 2010). Nevertheless, of the enzymes currently applied as catalysts for the kinetic resolution of optically active compounds, few are derived from marine microorganisms or their purified enzymes (Trincone 2010; Veberlen et al. 2006).

Continuing our long-standing interest in the application of marine microorganisms, and in conjunction with our recent interest in the synthesis of cyclic β-hydroxy ketones, we engaged in the preparation of (*R*)- and (*S*)-**2a**, (*R*)- and (*S*)-**2b**, and (*R*)- and (*S*)-**2c** using marine strain-catalyzed transesterification. We screened 26 organic solvent-tolerant strains isolated from marine sediments for the activities of enantioselective transesterification, using *rac*-**2b** as a test substrate and vinyl acetate as the acyl donor and solvent. Of these strains, *Bacillus* sp. BT05 and BT03, *Serratia* sp. BOC07 and BOC45, and *Pseudomonas* sp. BT08, BOC06, and BOC09 showed remarkable enzymatic activities and were selected for a detailed investigation of the transesterification resolutions of racemic **2a**-**2c**.

Materials and Methods

General Methods

All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) and were used without further purification unless otherwise specified. The culture media components were obtained from BD (Becton, Dickinson and Company, Germany).

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Advance 400 instrument (400 and 100 MHz, respectively) and were internally referenced to residual solvent signals. Data for ^{1}H NMR are reported as followings: chemical shift (d ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration, coupling constant (Hz), and assignment. Data for ^{13}C NMR were reported in terms of chemical shift. Optical rotations were obtained at 20 °C with a PerkinElmer 241 polarimeter (sodium D line). Column chromatography was carried out with silica gel (0.060–0.200 mm, pore diameter ca. 6 nm) and with mixtures of petroleum ether (PE) and ethyl acetate (EtOAc) as solvents. Thin-layer chromatography (TLC) was performed on 0.20 mm silica gel 60-F plates. Organic solutions were concentrated under reduced pressure with a rotary evaporator.

The enantiomeric excess (*ee*) of acetates **3a-3c** were determined using a Shimadzu type GC 2012 equipped with a Chiradex GTA column (50 m × 0.25 mm × 0.12 μm) using helium as the carrier gas. The following conditions were used for the chiral separation: injector 200 °C, detector 220 °C, split 60, flame ionization detector (FID) hydrogen 30, oxygen 300, column flow: 0.49 mL/min, maximum temp: 175 °C. Temperature program: start 150 °C, hold time 10 min; rate 25 °C/min to 170 °C, hold time 5 min. The retention times of acetates **3a-3c** were as follows: (*R*-**3a**: 4.70 min; *S*-**3a**: 4.89 min), (*R*-**3b**: 6.30 min; *S*-**3b**: 6.90 min), (*R*-**3c**: 8.05 min; *S*-**3c**: 8.75 min). Stereochemical assignment was investigated in previous reports (Chen and Hanefeld 2013a; Chen et al. 2015).

The enantiomeric excess (*ee*) of alcohols **2a-2c** were determined using a Shimadzu type GC 2012 equipped with a chiral Ivadex7/PS086 column (50 m × 0.25 mm × 0.25 μm) using helium as the carrier gas. The following conditions were used for the chiral separation: injector 200 °C, detector 330 °C, split 60, FID hydrogen 30, oxygen 300, column flow 0.20 mL/min, and maximum temp 220 °C. Temperature program were as follows: start 80 °C, hold time 3 min; rate 5 °C/min to 100 °C, hold time 1 min; and rate 1 °C/min to 160 °C, hold time 1 min. The retention times of alcohols **2a-2c** were as follows: (*R*-**2a** 30.1 min; *S*-**2a** 31.2 min), (*R*-**2b** 33.5 min; *S*-**2b** 34.4 min), and (*R*-**2c** 36.7 min; *S*-**3c** 37.5 min). Stereochemical assignment was investigated in the previous report (Chen et al. 2015).

The quantification of alcohols **2a-2c** and acetates **3a-3c** was performed using calibration curves with decane as an internal standard. In each case, seven standard samples were prepared and each sample contained 0.125 mmol/mL decane and 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.015625 mmol/mL alcohols and acetates in ethyl acetate, respectively.

Synthesis of Racemic Cyclic β-Hydroxy Ketones 2

In a round-bottom flask (100 mL) fitted with a mechanical stirrer, cyclopentane-1,3-diol **1a** (3.52 g, 34.44 mmol) was dissolved in 40 mL of acetone. The solution was cooled in an ice bath, and a solution of $Na_2Cr_2O_7{\cdot}2H_2O$ (3.49 g, 11.72 mmol), concentrated H_2SO_4 (2 mL), and H_2O (14 mL) was added over 25 min. The green-blue solution was allowed to warm to room temperature over 15 min. The reaction mixture was filtered through Celite, the solid was washed by acetone, and the solvent was evaporated. The residue was purified by flash column chromatography (eluent: PE/EtOAc 1:2) to give *rac*-**2a** (2.36 g, 23.57 mmol, 68%) as a colorless oil.

Using the procedure described above, reaction of cyclohexane-1,3-diol **1b** (4.00 g, 34.44 mmol) gave *rac*-**2b** (3.14 g, 27.51 mmol, 80%) as a colorless oil.

2-Cyclohepten-1-one **4c** (10.30 g, 93.50 mmol) was dissolved in 350 mL sodium phosphate buffer (pH 7, 250 mM) and 150 mL of acetonitrile. L-Lysinemonohydrochloride (4.51 g, 25.02 mmol) was added and the reaction was stirred for 24 h at 40 °C. Due to the high solubility of the resulting alcohols in water, the workup was performed by continuous extraction with ethyl acetate (500 mL) overnight. The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The crude product mixture was purified using flash chromatography on silica gel (eluent: PE/EtOAc 1:1) to give *rac*-**2c** (1.17 g, 9.13 mmol, 10%) as a yellowish oil.

Synthesis of Acetates

Rac-**2a** (700 mg, 6.99 mmol) obtained above was dissolved in pyridine (0.5 mL), and acetic anhydride (2.0 mL, 60.00 mmol) was added. The reaction was allowed to proceed overnight at room temperature. The reaction was then quenched by extraction with acidified water (3 mL) and Et_2O (3 × 10 mL). The combined organic layers were washed with water and brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude product mixture was purified using flash chromatography on silica gel (eluent: PE/EtOAc 1:1) to give *rac*-**3a** (923 mg, 6.50 mmol, 93%) as yellowish oil. *Ee* was measured on GC (see the "General Methods" section).

Using the procedure described above, *rac*-**2b** (400 mg, 3.50 mmol) gave *rac*-**3b** (449 mg, 2.88 mmol, 82%) as a colorless oil. *Ee* was measured on GC (see the "General Methods" section).

Due to the low production, the acylation of *rac*-**2c** was not performed in a preparative scale; instead, it was performed in a 2-mL Eppendorf tube. DMAP (5 mg) was dissolved in 5 mL of ethyl acetate. From this mixture, 100 μL was added to 3 mg of *rac*-**2c** in 2 mL ethyl acetate, followed by the addition of 100 μL acetic anhydride. The reaction was allowed to proceed for 1 h at room temperature and was quenched by the addition of 0.5 mL water. After shaking for an additional hour, the phases were separated and the organic layer was dried over Na_2SO_4 to obtain *rac*-**3c**. *Ee* was measured on GC (see the "General Methods" section).

Determination of the *ee* of Alcohols 2

Cultivation of *Rhodococcus rhodochrous* ATCC 17895 was previously described (Chen et al. 2015). Whole cells were used for the reaction to prepare reference compounds (*R*)-**2a**, (*R*)-**2b**, and (*R*)-**2c** for chiral GC measurements. Approximately 330 mg/mL of the cells was resuspended in the potassium phosphate buffer (100 mM, pH 6.2, 15 mL) containing 33 mM substrates **4a-4c**. The resting cell reactions were carried out in screw-capped Erlenmeyer flasks. Reactions were shaken at 28 °C overnight (17 h). For the workup, the cells were removed by centrifugation and 1 mL of the supernatant was saturated with NaCl followed by extraction with 2 × 0.5 mL isoamyl alcohol by shaking for 5 min. The combined organic layers were dried over Na_2SO_4 and measured on GC for retention times of (*R*)-**2a**, (*R*)-**2b**, and (*R*)-**2c** (see the "General Methods" section).

Screening of Marine Microorganisms

Samples from marine sediments are collected and stored in our laboratory. Since the kinetic resolution proceeded using vinyl acetate as an acyl donor and solvent, the target strains also had to be tolerant of organic solvents. Thus, the marine microorganisms with organic solvent-tolerance were screened using a minor modification of a known procedure (Cao et al. 2012) using enriched medium of 0.1% tryptone, 0.18% corn steep liquor, 0.35% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.25% NaCl, 0.1% $MgSO_4{\cdot}7H_2O$, and 5% sunflower oil. Vinyl acetate was added to the medium at a concentration of 25% (*v*/*v*). The cultures were then acclimated by repeated transfer to sterile enrichment medium incubated at 30 °C. Samples of the repeated batch cultures were diluted and spread on tributyrin agar plates (0.3% yeast extract, 1.0% tryptone, 0.25% NaCl, 0.5% tributyrin, and 1.8% agar). Colonies exhibiting a clear zone were picked and screened for their transesterification activities on rhodamine B agar plates (0.1% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 1.5% agar, 0.0024% rhodamine B, 0.5% (*v*/*v*) corn steep liquor, 6% (*v*/*v*) olive oil, and 1.8% agar). The microbes showing high ratios of the orange fluorescent halo clear zone diameter to the colony diameter under UV light at 350 nm were selected for their potentially high esterification activities for subsequent experiments.

The strains with remarkable vinyl acetate tolerance (25%, *v*/*v*) and transesterification activities were selected for further research. Extraction of the genomic DNA was performed individually on the selected seven strains with high solvent-

tolerance and lipase activity by the standard phenol/chloroform precipitation method (Chen et al. 2013b). Genomic DNA libraries for the Illumina platform were generated and sequenced at BaseClear BV (Leiden, the Netherlands). The seven strains were identified as *Bacillus* sp. (two isolates), *Serratia* sp. (two isolates), and *Pseudomonas* sp. (three isolates) based on a molecular biological protocol by DNA amplification and sequencing of the ITS region together with 16S rRNA sequence BLAST in the GenBank database.

Microorganisms and Culture Conditions

The strains were routinely maintained on marine broth agar slants at 4 °C and were subcultured at regular intervals. The medium used for cultivation contained 3 g/L peptone, 8 g/L glucose, 15 g/L yeast extract, 0.05% (*v*/*v*) Triton X-100, and 0.5% (*v*/*v*) sunflower oil and artificial seawater (1.36 g/L $CaCl_2{\cdot}2H_2O$, 9.68 g/L $MgCl_2{\cdot}6H_2O$, 0.61 g/L KCl, 30 g/L NaCl, 14×10^{-6} g/L Na_2HPO_4, 3.47 g/L Na_2SO_4, 0.17 g/L $NaHCO_3$, 0.1 g/L KBr, 0.04 g/L $SrCl_2{\cdot}6H_2O$, 0.03 g/L H_3BO_3) at pH 7.0 and was sterilized at 121 °C in an autoclave. A loop of single colony was cut from the agar stock cultures and inoculated in 1 L medium in a 2-L Erlenmeyer flask. This culture was shaken reciprocally at 28 °C for 2–5 days on an orbital shaker (150 rpm) to an optical density (OD_{600}) of approximately 10. The cells of different strains were harvested by centrifugation at 10,000 rpm and 4 °C for 20 min. The supernatant was removed and the cells were rinsed with potassium phosphate buffer (100 mM, pH 7.0) and centrifuged again. The wet pellets were lyophilized and preserved at −20 °C prior to use. All reactions were performed using lyophilized cells unless otherwise specified.

Assay of Enzymatic Activity of Lyophilized Cells

Enzyme activities of the lyophilized cells were measured by a modified lipase assay method (Winkler and Stuckmann 1979) with *p*-nitrophenyl butyrate (*p*NPB) as the substrate. A resuspension of lyophilized cells or a blank solution (100 μL) was added to 2.870 mL 100 mM potassium phosphate buffer (KPB, pH 7.0). After pre-incubation at the reaction temperature of 30 °C for 3 min, the reaction was initiated by a quick mixing of the reaction mixture with 30 μL 100 mM *p*NPB solution in dimethyl sulfoxide (DMSO), and the change in absorbance at 405 nm was recorded. One unit of enzyme activity was defined as the amount of lyophilized cells that liberated 1 μmol *p*NPB per minute under these standard conditions.

Procedure for the Kinetic Resolution of *rac*-2 Using Whole Cells

The screening of lipase activities using whole cells from different strains for kinetic resolution was performed at 25 °C in 2 mL freshly distilled vinyl acetate (**5**) containing 0.5 mmol substrate (*rac*-**2a**, *rac*-**2b** or *rac*-**2c**) and 450 U (or 112 U) enzyme. After 16 h (or 4 h), a 100-μL sample was taken from the reaction mixture and added to 100 μL internal standard solution (0.25 mmol/mL decane in ethyl acetate) and the mixture was centrifuged to separate the whole cells. The obtained samples were analyzed by GC to determine the yield and *ee* of acetates **3a-3b** and the *ee* of substrates **2a-2c**. Each experiment was performed in duplicate. The enantiomeric ratio (*E*-value) was calculated using the literature equation below (Chen et al. 1982).

$$E = \frac{ln\left[1-c\left(1+ee_p\right)\right]}{ln\left[1-c\left(1-ee_p\right)\right]}$$

Preparative-Scale Resolution of Racemic 2a-2c

To obtain both (*R*)- and (*S*)-enantiomers of the resulting alcohols, the kinetic resolution was carried out on a preparative scale. To a 100-mL Erlenmeyer flask was added *rac*-**2a** (700 mg, 6.99 mmol) [or *rac*-**2b** (800 mg, 7.00 mmol) or *rac*-**2c** (900 mg, 7.02 mmol)], freshly distilled vinyl acetate (**5**, 26 mL), and whole cells of *Serratia* sp. BOC45 (1568 U, 5 g). The reaction was incubated at 25 °C and shaken at 150 rpm for 4 h. The cells were removed by centrifugation, the supernatant was concentrated under reduced pressure, and the residue was purified by chromatography on silica gel (eluent: EtOAc/PE 2:1) to give the corresponding *R*-acetates and *S*-alcohols: (*R*)-**3a** (298 mg, 2.09 mmol, 30% yield, 92% *ee*) $[\alpha]_D^{20}$ + 46.4 (*c* 0.60, CH_2Cl_2), (*S*)-**2a** (454 mg, 4.54 mmol, 65% yield, 43% *ee*) $[\alpha]_D^{20}$–18.3 (*c* 0.50, CH_2Cl_2); (*R*)-**3b** (306 mg, 1.96 mmol, 28% yield, 91% *ee*) $[\alpha]_D^{20}$ + 45.8 (*c* 0.57, CH_2Cl_2), (*S*)-**2b** (535 mg, 4.69 mmol, 67% yield, 39% *ee*) $[\alpha]_D^{20}$–16.6 (*c* 0.50, CH_2Cl_2); (*R*)-**3c** (406 mg, 2.38 mmol, 34% yield, 92% *ee*) $[\alpha]_D^{20}$ + 46.7 (*c* 0.50, CH_2Cl_2), (*S*)-**2c** (566 mg, 4.42 mmol, 63% yield, 63% *ee*) $[\alpha]_D^{20}$–26.8 (*c* 0.50, CH_2Cl_2).

Ethanolysis of (*R*)-Acetates 3a-3c

To a round-bottom flask, *R*-acetate **3** was added, obtained by preparative-scale kinetic resolution (*R*)-**3a** 160 mg, 1.13 mmol; (*R*)-**3b** 160 mg, 1.02 mmol; or (*R*)-**3c** 160 mg, 0.94 mmol), ethanol (1 mL), MTBE (1 mL), and CAL-B (500 U, 83 mg). The reaction mixture was stirred at 30 °C for 30 h. The enzyme (CAL-B) was

filtered away and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (eluent: EtOAc/PE 2:1) to yield (*R*)-**2a** (101 mg, 1.01 mmol, 89% yield, 92% *ee*) $[\alpha]_D^{20}$ + 38.7 (*c* 0.50, CH_2Cl_2); (*R*)-**2b** (105 mg, 0.92 mmol, 90% yield, 91% *ee*) $[\alpha]_D^{20}$ + 39.5 (*c* 0.50, CH_2Cl_2); and (*R*)-**2c** (106 mg, 0.83 mmol, 88% yield, 92% *ee*) $[\alpha]_D^{20}$ + 39.3 (*c* 0.50, CH_2Cl_2).

Results and Discussion

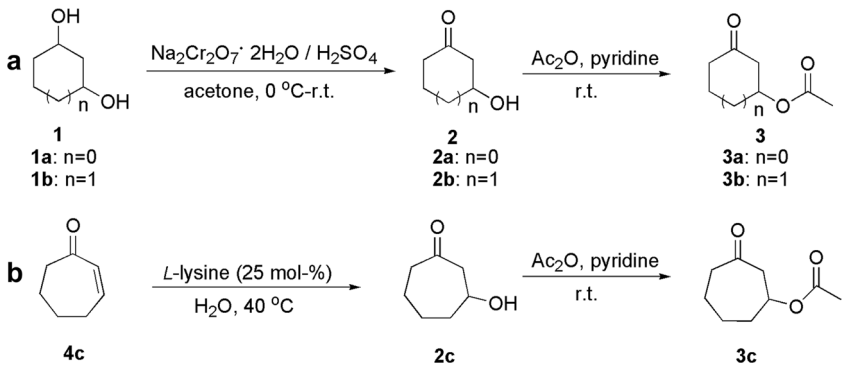
Substrate Preparation

The racemic starting materials 3-hydroxycyclopentanone **2a** and 3-hydroxycyclohexanone **2b** were readily synthesized from commercial cyclopentane-1,3-diol **1a** and cyclohexane-1,3-diol **1b**, with freshly prepared Jones reagent (1.6 M in acetone) through a minor modification of a known method (Scheme 1A) (Seo et al. 2006). Because cycloheptane-1,3-diol is not commercially available, the racemic starting material 3-hydroxycycloheptanone **2c** was prepared by the direct Michael addition of water to 2-cycloheptenone **4c** using L-lysine as a non-chiral catalyst (Scheme 1B) (Resch et al. 2013). The compounds *rac*-**2a-c** were obtained in 68, 80, and 10% isolated yields, respectively, after column chromatography purification. The spectroscopic data (^{1}H and ^{13}C NMR) of the racemic cyclic 3-hydroxy ketones **2a-c** were in agreement with those reported in the literature (Chen and Hanefeld 2013a; Chen et al. 2015; Karmee et al. 2011b; Resch et al. 2013). In the next step, *rac*-**2a-c** were further converted to the corresponding acetates via acylation. In general, the faster reacting secondary alcohol enantiomer could be predicted according to Kazlauskas' Rule (Kazlauskas et al. 1991). However, since it is difficult to determine which groups at the chiral center of a cyclic compounds are considered "large" versus "medium," Kazlauskas' Rule may not apply. Therefore, it was necessary to determine the absolute stereochemistry of the favored enantiomer for each tested substrate. To this end, racemic alcohols **2a-c** and acetates **3a-c** were subjected to chiral GC to separate the enantiomers. Enantiomerically enriched *R*-**2a-c** were obtained in 18% yield with 22% *ee*, 22% yield with 65% *ee*, and 15% yield and 20% *ee*, respectively (Scheme 2), by enantioselective Michael addition of water to enones **4a-c** using whole cells of *R. rhodochrous* ATCC 17895 as described previously (Chen et al. 2015). The poor conversions and enantioselectivities were due to ene-reductases also present in the *Rhodococcus* cells (Chen et al. 2015), which competed with the Michael hydratase, so that most of the substrates **4a-c** were reduced to saturated ketones. Co-injection of *R*-**2** to the chiral GC showed the stereoselectivities of the transesterification resolution using whole cells of marine microorganisms.

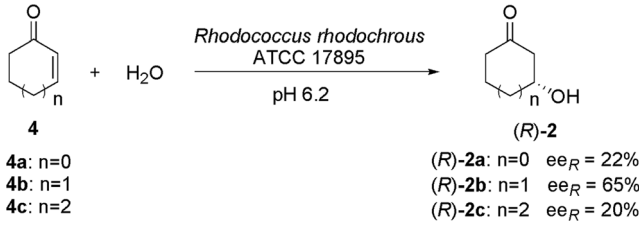
Bioresolution

Because the resulting chiral alcohols **2** are highly hydrophilic and difficult to extract from water, kinetic resolution via hydrolysis of the acetates is an ineffectual process. Instead, the kinetic resolution using vinyl acetate, for which the green properties were recently evaluated (Paravidino and Hanefeld 2011), as the acyl donor and the solvent was performed. In the first step, microorganisms from a wide collection of marine sediments were screened for organic solvent tolerance by the addition of vinyl acetate (25% *v*/*v*) to the culture. As a result, 26 strains were identified as vinyl acetate tolerant microorganisms. Of these strains, 15 were found to be potential biocatalysts for the transesterification resolution via enzymatic activity assay screening on rhodamine B agar plates.

The 15 strains that exhibited potential enzymatic activity were grown in liquid cultures using marine broth (lipase-producing medium), and the resting cells were collected and freeze-dried. The lyophilized cells were then subjected to a lipase activity test using a standard assay. Enzyme activities of 450 U were used for the bioresolution of *rac*-**2b** (0.5 mmol) as a test substrate due to its rigid character and its use in earlier studies (Resch et al. 2013; Feng and Yun 2009). Screening reactions were carried out using the same activity of all 15 strains at 25 °C, although different lipases can have different optimal temperatures. For example, the lipase from *Serratia*



Scheme 1 Preparation of *rac*-3-hydroxycyclopentanone **2a**, *rac*-3-hydroxycyclohexanone **2b,** and *rac*-3-hydroxycycloheptanone **2c** for kinetic resolution and the corresponding acetates racemic **3a-3c** as reference compounds for chiral GC analysis. **a** Selective oxidation of diols to prepare *rac*-**2a** and *rac*-**2b**. **b** Direct water addition of kenone to prepare *rac*-**2c**



Scheme 2 Preparation of enantiomerically enriched (*R*)-3-hydroxycyclopentanone (*R*)-**2a**, (*R*)-3-hydroxycyclohexanone (*R*)-**2b,** and (*R*)-3-hydroxycycloheptanone (*R*)-**2c** as reference compounds for stereochemical assignment

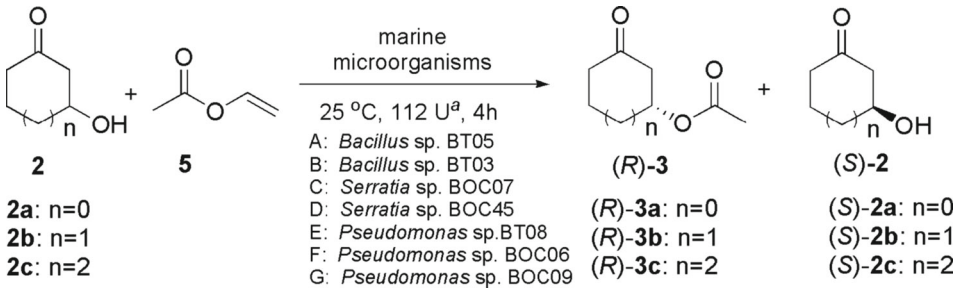
marcescens ECU1010 shows optimum activity at 45 °C according to reported guidelines (Gardossi et al. 2010). Blank reactions containing only substrate and vinyl acetate, without the addition of whole cells, were performed in parallel. After 16 h reactions, it was found that seven strains exhibited any degree of stereoselection, manifesting as a higher activity of esterification toward (*R*)-**2b**. Five strains showed detectable biotransformation but no stereoselectivity (data not shown). As a result, the seven organic solvent-tolerant strains with noted enantioselective esterification activities toward *rac*-**2b** were chosen for further investigation. The strains were identified as *Bacillus* sp. (two isolates), *Serratia* sp. (two isolates), and *Pseudomonas* sp. (three isolates) according to DNA amplification and sequencing of the ITS region. This result is in agreement with the previous observation that most reported solvent-tolerant lipases are obtained from *Pseudomonas* and *Bacillus* genus (Cao et al. 2012).

Most organic reactions are performed in organic solvent instead of water, but many enzymes are inactivated or denatured in the presence of organic solvent. Organic solvent-tolerant enzymes applicable as catalysts for organic synthesis have attracted the attention of many researchers (Doukyu and Ogino 2010). For example, nine different isolated lipases were screened for the kinetic resolution of **2a** with vinyl acetate as acyl donor and solvent as we reported previously (Chen and Hanefeld 2013a). However, few organic solvent-tolerant bacteria have been identified, and an insufficient number of solvent-stable lipases have been isolated to satisfy the diverse substrates used in organic chemistry. The search for organic solvent-tolerant microorganisms that secrete solvent-stable lipases can expand the application of enzyme catalysis in organic synthesis. It needs to be emphasized that the presence of organic solvent could influence the multiple metabolic pathways and the strains that did not grow could still have solvent-resistant hydrolases. Regarding this point, there might be more than 15 strains possessing solvent-resistant lipases by our screening assay.

Different genus, species and even strains may produce different enzymes with different substrate specificity, enantioselectivity, as well as solvent stability. Therefore, the esterification activity of the selected seven strains toward *rac*-**2a** and *rac*-**2c** should be examined independently. The first screening reaction, which was performed using 450 U activity with 0.5 mmol of *rac*-**2b**, proceeded at 25 °C for 16 h and did not result in significant stereoselectivity (data not shown). Attempts to increase the enantioselectivity by reducing the amount of resting cells from 450 U activity to 112 U and shortening the reaction time from 16 to 4 h, using the same amount of substrate (0.5 mmol of *rac*-**2b**), increased the resulting stereoselectivity in all cases, as shown in Table 1 (the data for the resolution using 450 U activity and 16 h reaction time are not shown). The transesterifications of *rac*-**2a** and *rac*-**2c** were also performed using the ideal optimized reaction conditions of 112 U activity and 4 h reaction time. As shown in Table 1, all seven selected strains (A-G) showed higher esterification activity toward (*R*)-enantiomers of the tested substrates *rac*-**2**, leading to the formation of (*R*)-**3**, while the antipode (*S*)-enantiomers were left unreacted. For substrate *rac*-**2a**, strains A, B, C, and D gave 38, 36, 31, and 32% yield of (*R*)-**3a** with 87, 89, 92, and 92% *ee*, respectively (entries 1, 4, 7, and 10). The calculated *E*-values were between 24 and 37. Strain D gave the highest *E*-value at 37. In contrast, for strains E, F, and G, high yields of (*R*)-**3a** were achieved (89, 94, and 92%) but with less than 10% *ee* (entries 13, 16, 19). For the substrate *rac*-**2b**, strains A-G gave 30–39% yield of (*R*)-**3b** with 80–92% *ee* (entries 2, 5, 8, 11, 14, 17, and 20). The *E*-values obtained were between 14 and 40; the highest *E*-value of 40 was achieved with strain D. For the substrate *rac*-**3c**, strains A-G gave 31–38% yield of (*R*)-**3c** with 91–95% *ee* (entries 3, 6, 9, 12, 15, 18, and 21). The *E*-values were between 36 and 75; again, the *E*-value of 75 was achieved with strain D.

Comparing at the *E*-values between the tested substrates, it was clear that all strains accepted *rac*-**2c** as the best substrate and showed poorer esterification activity toward *rac*-**2a**, which is in line with previous observations that cyclic ketones with large or many substituents are readily resolved, while simple compounds tend to give less enantiopure products (Joly and Nair 2001; Demir and Sesenoglu 2002; Klomklao et al. 2003; Pinot et al. 2005; Etheridge and Caddick 2004; Doussot et al. 2000; Galano et al. 2000; Nakashima et al. 2000; Chen et al. 2004). Among the seven screened marine strains, *Serratia* sp. BOC45 (strain D) showed the highest activity of esterification toward the three tested substrates, although for other strains, there is no clear relationship between the biocatalyst enantioselectivity and the substrate structures. For example, the *Pseudomonas* sp. strains showed modest enantioselectivities toward *rac*-**2b** (*E*-value of 14–16) and high enantioselectivity toward *rac*-**2c** (*E*-value of 40–45) but a very low *E*-value of 2 for *rac*-**2a**.

Table 1 Kinetic resolution of cyclic β-hydroxy ketones **2a-c** using marine bacteria



Entry	Strain	Substrate	Yield % (*R*)-3[b]	ee % (*R*)-3[c]	ee % (*S*)-2[c]	E[d]	Enantio Preference[e]
1	A	*rac*-**2a**	38	87	53	24	*R*
2	A	*rac*-**2b**	30	90	39	28	*R*
3	A	*rac*-**2c**	31	92	41	36	*R*
4	B	*rac*-**2a**	36	89	50	29	*R*
5	B	*rac*-**2b**	31	91	41	32	*R*
6	B	*rac*-**2c**	32	92	43	37	*R*
7	C	*rac*-**2a**	31	92	41	36	*R*
8	C	*rac*-**2b**	36	92	52	40	*R*
9	C	*rac*-**2c**	38	95	58	70	*R*
10	D	*rac*-**2a**	32	92	43	37	*R*
11	D	*rac*-**2b**	30	91	39	40	*R*
12	D	*rac*-**2c**	36	92	63	75	*R*
13	E	*rac*-**2a**	89	7	56	2	*R*
14	E	*rac*-**2b**	35	80	43	14	*R*
15	E	*rac*-**2c**	30	91	39	40	*R*
16	F	*rac*-**2a**	94	<1	78	2	*R*
17	F	*rac*-**2b**	39	80	57	15	*R*
18	F	*rac*-**2c**	35	93	50	45	*R*
19	G	*rac*-**2a**	92	<1	86	2	*R*
20	G	*rac*-**2b**	37	82	48	16	*R*
21	G	*rac*-**2c**	36	92	52	40	*R*

Enzyme activities were determined by a modified lipase assay method (see the "General Methods" section)

[a] Yield of acetates was determined by GC analysis using a CP WAS 52 CB column with decane as an internal standard (see the "General Methods" section)

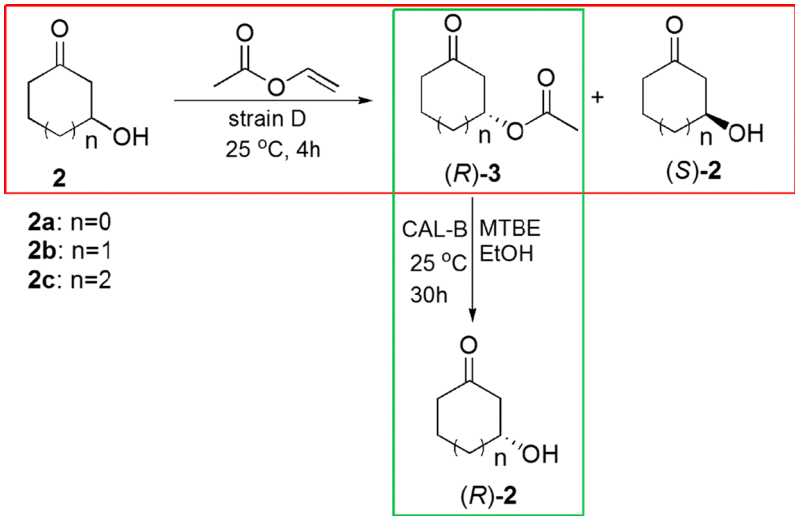
[b] *ee* of acetates and remaining alcohols were determined by chiral GC analysis using Chiradex GTA column (see the "General Methods" section)

[c] E was calculated by using the equation $E = \frac{\ln[1-c(1+ee_p)]}{\ln[1-c(1-ee_p)]}$

[d] Stereochemical assignment was determined by using (*R*)-**2** obtained in Scheme 2 as reference compounds on chiral GC analysis

A previous transesterification resolution of *rac*-**2a,** catalyzed by *Pseudomonas cepacia* lipase immobilized on diatomite (Amano lipase PS-DI), obtained an *E*-value of 20 (Chen and Hanefeld 2013a). The kinetic resolution of *rac*-**2b** by a lipase-like enzyme purified from the pancreatic porcine lipase (PPL) gave an *E*-value of 32 (Karmee et al. 2011b). For both substrates, the wild-type whole cells of strain *Serratia* sp. showed higher enantioselectivities, with *E*-values of 37 and 40, respectively. Essentially, the use of whole cells as the biocatalyst is preferable for industrial application. The efficiency of the kinetic resolution of **2a-c** as reported here may be further increased by careful optimization of the reaction and operation conditions. Remarkably, this study is the first example of the kinetic resolution of *rac*-**2c**, affording enantiomerically enriched (*R*)-**3c** and (*S*)-**2c** with the high *E*-value of 75. Further studies are being conducted to analyze and annotate the genome sequences of the seven strains, with the goal of isolating the putative lipases for application in bioresolution.

Table 2 Preparative-scale synthesis of the target compounds (*R*)- and (*S*)-**2**



	(*R*)-**2a**	(*S*)-**2a**	(*R*)-**2b**	(*S*)-**2b**	(*R*)-**2c**	(*S*)-**2c**
Yield %	27	65	25	67	30	63
Ee %	92	43	91	39	92	63

Ethanolysis of (*R*)-Acetates to Obtain the Corresponding (*R*)-Alcohols

To isolate the two enantiomers, the transesterification resolution reactions were carried out on a preparative scale using whole cells of strain D. As a result, (*R*)-acetates (*R*)-**3a-c** were isolated in 30% yield with 92% *ee*, 28% yield with 91% *ee* and 34% yield with 92% *ee*, respectively, after simple purification by column chromatography. At the same time, the unreacted alcohols (*S*)-**3a-c** were obtained in 65% isolated yield with 43% *ee*, 67% isolated yield with 39% *ee*, and 63% isolated yield with 63% *ee*, respectively. The acetate groups of (*R*)-**3** were deprotected using the non-selective enzyme CAL-B (data not shown) in ethanol/MTBE at 25 °C. After 30 h, the enzyme was filtered off and the enantiomerically enriched (*R*)-alcohols (*R*)-**3a-c** were isolated in 27% yield with 92% *ee*, 25% yield with 91% *ee*, and 30% yield with 92% *ee*, respectively. The isolation yields and enantioselectivities of the target compounds (*R*)- and (*S*)-**2a**, (*R*)- and (*S*)-**2b**, and (*R*)- and (*S*)-**2c** are summarized in Table 2.

Conclusions

After screening for organic solvent tolerance, 26 marine microorganisms were selected as potential catalysts for the transesterification resolution of racemic cyclic β-hydroxy ketones **2a-c**, which are essential building blocks used in drugs synthesis. For example, 25-hydroxy-19-norvitamin D3 analogues, which possess antiproliferative activities toward prostate cells, have been prepared from 3-hydroxycyclohexanone **2b** (Arai et al. 2005), and **2a** is a valuable building block for the prostaglandins (Nicolaou and Sorensen 1996). Of these strains, seven showed significantly higher esterification activity toward one enantiomer (*R*)-**2**, affording acetates (*R*)-**3,** and the unreacted substrate (*S*)-**2** which were easily separated by column chromatography. The obtained acetates (*R*)-**3** were further treated with the non-selective enzyme CAL-B to afford the antipode (*R*)-**2**. Although wild-type whole cells, not isolated enzymes, were used in this study, the excellent results obtained demonstrate the potential of marine bacteria for asymmetric kinetic resolution. *Serratia* sp. BOC45 gave the highest *E*-values of 37, 40, and 75 toward the tested substrates *rac*-**2a**, *rac*-**2b**, and *rac*-**2c**, respectively. The enantiomerically enriched (*R*)-**2a** was obtained in 27% yield with 92% *ee* and (*S*)-**2a** in 65% yield with 43% *ee*; (*R*)-**2b** was obtained in 25% yield with 91% *ee* and (*S*)-**2b** in 67% yield with 39% *ee*; and (*R*)-**2c** was obtained in 30% yield with 96% *ee* and (*S*)-**2c** in 63% yield with 63% *ee*. This method is an essential improvement on the resolution of **2a** and **2b** by conventional isolated lipases, and represents the first synthesis of optically active (*R*)- and (*S*)-3-hydroxycycloheptanone **2c**.

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