INVENTION DISCLOSURE –

CHEMISTRY/PHARMACEUTICAL/BIOTECHNOLOGY SUBJECT MATTER

Highly enantioselective CALB-catalyzed kinetic resolution ofbuilding blocks for β-blocker atenolol

1. Introduction

The amount of approved new racemic drugs, so-called NewMolecular Entities (NME's), has decreased in the past 20 years.This can be explained by increased knowledge of the difference in the biological effects of the two enantiomers, in addition to improvedasymmetric synthesis. In the years between 1992 and 2011the amount of racemic NME's approved by The US Food and DrugAdministration (FDA) decreased from 21% to 5%. Globally no racemicNME's were approved in 2011, and almost 70% of the approveddrugs were enantiomerically pure. The rest were achiralcompounds. In the US the number of enantiopure NME's werelower, and a few racemates were also approved by FDA for theAmerican market.[1,2] Although there is an increasing demand forenantiopure drugs, still several drugs are being sold as the racemate.In 2010 there were still more β-blockers sold as the racemate,however, mostly the *S*-enantiomers are the activeenantiomer (eutomer).[3] Some of the *R*-enantiomers can even haveadverse effects, rather than any effect at all.[4] Atenolol is a cardioselectiveβ-blocker, which means that it is selective towards $\beta_1$receptors found in the heart. For a long time atenolol has beena popular choice for treatment of high blood pressure, but hasbecome more controversial in recent years.[5] Atenolol is also usedin the treatment of angina and myocardial infarction.[6] Atenolol issold both enantiomerically pure as the *S*-enantiomer as Atpure®and racemic as Mylan®, but it has been found that only the *S*-enantiomerhas the desired effect.[7] The S-enantiomer has been foundto lack the reported side effect of a lowered heart rate sometimesencountered with the racemate.

Several researchers have used biocatalysis in order to obtainenantiopure atenolol, however *ee's* of the building blocks have notreached 99%.[18,19] A recent paper reports kinetic resolution of *4-(3-chloro-2-hydroxypropoxy)phenyl)acetamide* with lipase A from*Candida antarctica* in toluene with an E-value of 142 producingenantiomers of the compound.[110] However, optical rotations of these enantiomer products have not been reported previously. *(S)*-Atenolol has also been made from *(R)*-epichlorohydrin.[11]

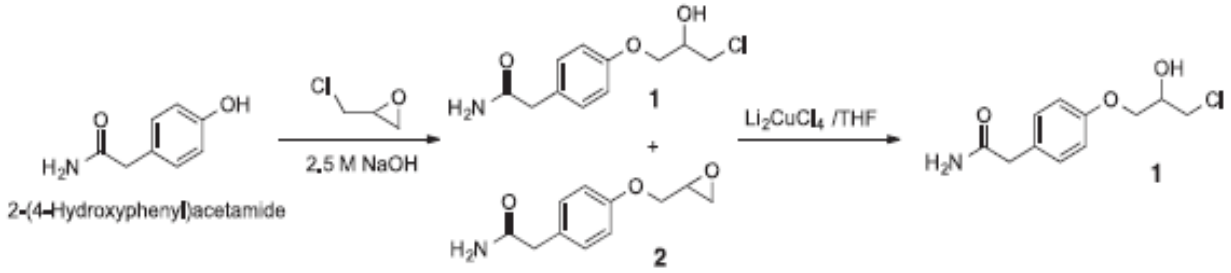
Previously, we have achieved 99% *ee* of product enantiomers ofsecondary phenoxy alcohols with a halogen as a leaving group in 3-position in the side chain in kinetic resolutions using lipase B from*C. antarctica* (CALB),[12,13] and it was desirable to prepare enantiomericallypure building blocks for atenolol using the same enzyme.

We have used the computer program *E&K Calculator 2.1b0 PCC*for calculations of the E-value based on 5e7 *ee*-values for a kineticresolution.[14] Resolutions of racemic compounds catalyzed by lipasesreact by a ping-pong bi-bi mechanism and in this programcalculations of E and K_{eq} based on both the ping-pong bi-bi and uni-unikinetic models can be performed.[15-20]

2. Results and discussion

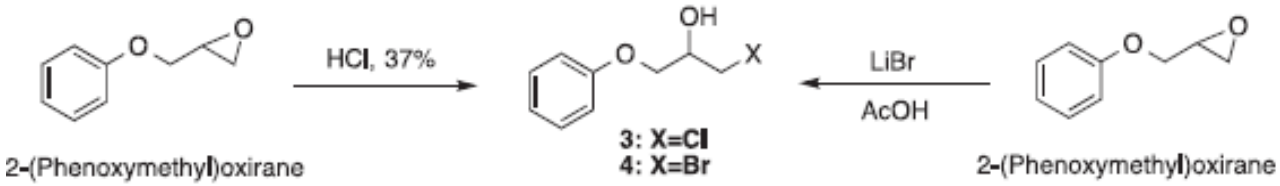
2.1. Synthesis of racemic compounds

Racemic *4-(3-chloro-2-hydroxypropoxy)phenyl)acetamide* (**1**)was synthesized from epichlorohydrin and *2-(4-hydroxyphenyl) acetamide* in 2.5 M NaOH. A mixture of **1** (70%) and the epoxide **2**(30%) was obtained. In order to open the epoxide **2** Li_2CuC_{l4} intetrahydrofuranwas applied as a complexing agent. Scheme 1. NMRspectra were in accordance with previously reported data.[110] Problemswith the extraction resulted in lower total yield than previouslyreported by us in similar reactions.[12]



Scheme 1. Synthesis of 4-(3-chloro-2-hydroxypropoxy)phenyl)acetamide, **1**.

3-Chloro-1-phenoxy-2-propanol (**3**) was synthesized in highyield and purity by opening benzyl glycidyl ether with conc.hydrochloric acid in dichloromethane according to Chini et al.[21] 3-Bromo-1-phenoxy-2-propanol (**4**) was synthesized in high yieldand purity by opening benzyl glycidyl ether by addition of LiBr inacetic acid according to Bajwa et al.[22]Scheme 2. NMR spectra forboth compounds were in accordance with previously reporteddata.[13,21-23]

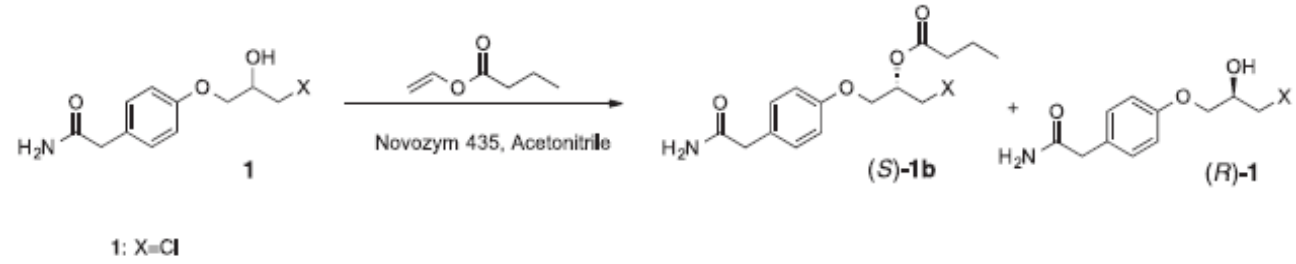


Scheme 2. Synthesis of 3-chloro-1-phenoxy-2-propanol, **3**, and 3-bromo-1-phenoxy-2-propanol, **4**.

2.2. Kinetic resolution of 4-(3-chloro-2-hydroxypropoxy)phenyl)acetamide (1) by CALB

Resolution of **1** with CALB (Novozym 435) was performed in dryacetonitrile with vinyl butanoate as acyl donor. The reaction wasincubated at 30^0C and stirred at 200 rpm for 27 h. (*R*)-**1** was producedin 99% *ee* in 16% yield, and the butanoate (*S*)-**1b**was obtainedin 18% yield. the *E*-value was 238. Scheme 3 and Fig. 1.

The alcohol enantiomers from the transesterification of **1** wasanalyzed on Daicel Chiralcel OD-H HPLC columns with baselineseparation (hexane:2-propanol, 83:17, 1.00 mL min^{-1}, UV254 nm). However, we were not able to separate the butanoateenantiomers of **1b** (or the acetate enantiomers of **1**) on the OD-Hcolumn (one column from 2004 and one from 2014) or otherChiralcel columns.



Scheme 3. CALB catalyzed kinetic resolution of **1** with vinyl butanoate in acetonitrile.

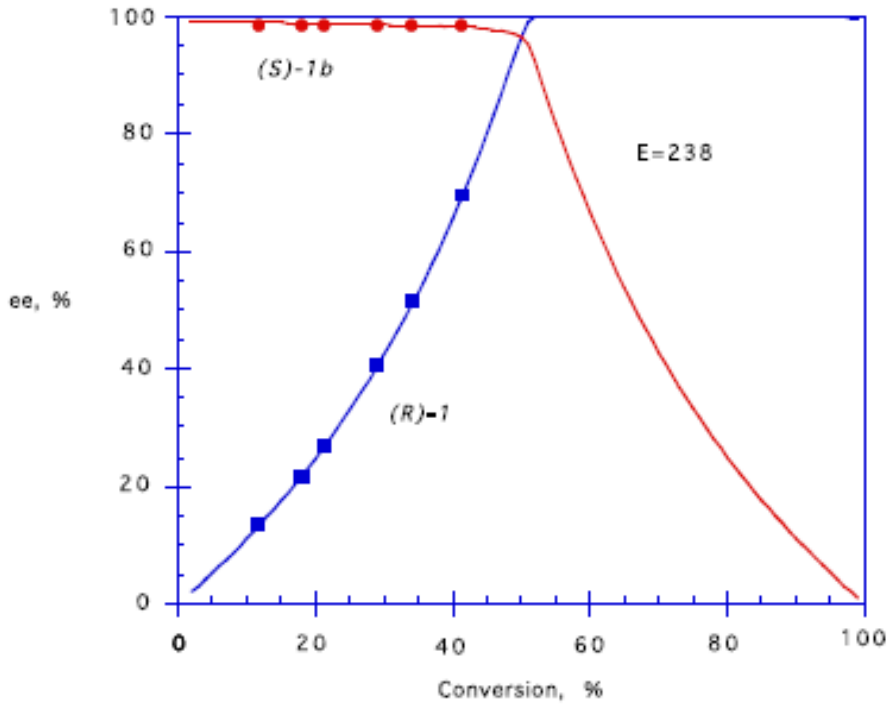
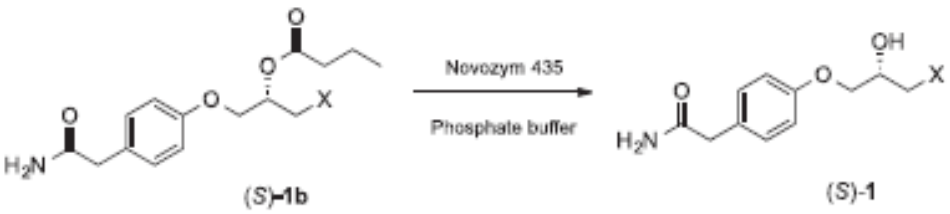


Fig. 1. The enantiomeric excess (*ee*) of kinetic resolution of **1** in acetonitrile with CALB and vinyl butanoate as acyl donor, E=238. Circles: *ee* of butanoate ((*S*)-**1b** in excess). Squares: *ee* of remaining alcohol ((*R*)-**1** in excess).

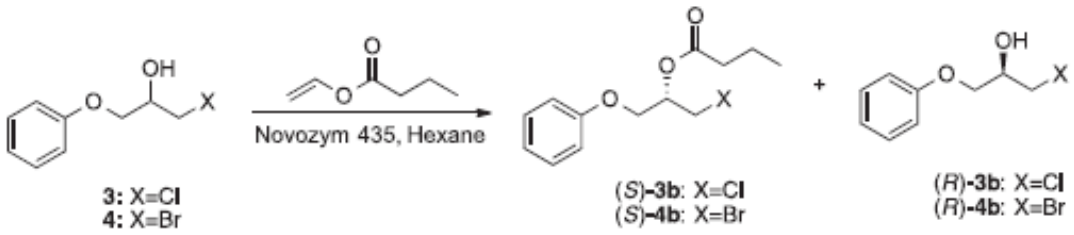
It was obvious from the chromatograms of resolutions of **1** thatonly one alcohol peak was decreasing. The formed butanoate **1b**was hydrolyzed in phosphate buffer by CALB in order to obtaincorrect *ee*-values for calculation of the *E*-value. Scheme 4. The *S*-alcohol(*S*)-**1** was produced in 98.5% *ee*, thus we present **1b** as (*S*)-**1b** in Scheme 4. Optical rotation (o.r.) values of (*R*)-**1**(99.0% *ee*)is$[\alpha]_D^{23}$= -3.0 (*c* 1.0, MeOH). O. r. for (*S*)-**1** (98.5% *ee*) is $[\alpha]_D^{23}$= +3.0 (c1.0, MeOH). Optical rotation values for these compounds have notbeen reported previously.



Scheme 4. CALB catalyzed hydrolysis of (*S*)-**1b** in phosphate buffer.

2.3. Improvement of enantiomeric ratio (*E*) with new CALBpreparation

As model substrates for comparison of *E*-values in CALB resolutionsof the series of secondary halogenated aryl alcohols 3-chloro-1-phenoxy-2-propanol (**3**) and 3-bromo-1-phenoxy-2-propanol (**4**) (Scheme 5), have been synthesized and resolved byanother batch of CALB (with vinyl butanoate as acyl donor in dryhexane) than previously reported by us.



Scheme 5. CALB catalyzed kinetic resolution of **3** and **4**.

The *E*-value in resolution of **3** increased from E=11 with theprevious CALB preparation (PLU 7000 g^{-1}, no lot information)[23] toE=220 with the new enzyme preparation (Novozym 435, PLU10000 g^{-1}, batch no LC200204). The *E*-value in the resolution of **4**increased from E=58 with the previous CALB preparation (PLU7000 g^{-1})[23] to E=278 with the new enzyme preparation. Chromatographicanalyses (GLC) of **3** and **4** have been performed onsimilar Chirasil DEX columns with the same conditions as previouslyreported. Different water content in the reaction mediummay affect the *E*-value.[24,25] The water content of the old enzymepreparation was 2.16%, the water content of the newer preparationis 2.0%. Molecular sieve has been used in all reactions. We havepreviously resolved the secondary alcohols **3** and **4** with the olderCALB preparation with vinyl butanoate as acyl donor in toluenewith fixed water activity (a_w) (a_w=0.18-0.65), resulting in *E*-valuesranging from 33 to 21 for **3** depending on the water activity of themedium, and from 12 to 16 for **4** depending on the a_w.[12]

Optical rotation (*S*)-**3** (96% *ee*) $[\alpha]_D^{22}$=+5.3 (c 1.71, EtOH). Reportedpreviously for 97% *ee* of (*S*)-**3**: $[\alpha]_D^{20}$=+3.6 (c 0.56, $CHCl_3$).[26](*S*)-**4b** (93% *ee*) $[\alpha]_D^{22}$=+14.4 (c 1.71, EtOH). O. r. of (*S*)-**4** (96% *ee*)$[\alpha]_D^{22}$=+5.26 (c 1.71, EtOH) corresponded with previously reportedo. r.[12,22] See Table 1 for *E*-values, *ee*'s and optical rotation values forenantiomers of **1, 3** and **4**.

Table 1
E-values from kinetic resolutions of secondary alcohols **1** and **3**–**4** with CALB (Novozym 435) and vinyl butanoate as acyl donor. For further details, see Experimental section

Substrate, X	*E*-value	Alcohol, % *ee*	$[\alpha]_D^{23}$	Butanoate, % *ee*	$[\alpha]_D^{23}$	Rx time to 50% conv. (h)
1, Cl	238	(*R*)-**1**, 99	−3.0, (MeOH)			27
		(*S*)-**1**, 98.5	+3.0, (MeOH)			
3, Cl	220	(*S*)-**3**, 96	+5.3, (EtOH)			23
		(*S*)-**3**, 97	+3.6 ($CHCl_3$)[26]			
4, Br	278	(*S*)-**4**, 96	+5.26, (EtOH)	(*S*)-**4b**, 93	+14.0 (EtOH)	10

3. Experimental

3.1. General

Analytical grade chemicals and solvents were purchased fromSigma-Aldrich, Oslo, Norway. For HPLC-analyses solvents withHPLC grade from Sigma-Aldrich were used. CALB was a gift fromNovozymes AS, Bagsværd, Denmark. Enzyme specifications:Novozym 435 (CALB) immobilized on macroporous acrylic resin,activity 10000 PLU g^{-1}, batch nr. LC200204. Flash chromatographywas performed with silica gel from Sigma-Aldrich, Oslo, Norway(pore size 60 Å, 230-400 mesh, 40-63 μm particle size).

The enzyme catalyzed kinetic resolutions were performed ina NewBrunswick G24 Environmental Incubator Shaker at 30°C and200 rpm. Optical rotations ($[\alpha]_D$) were determined at 23°C usinga Perkin-Elmer 243 B instrument, concentrations are given in g/100 mL. NMR spectrawere recorded with a Bruker Avance DPX 400instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively.Chemical shifts δ are in ppm rel to TMS and couplingconstants are in Hertz (Hz). Accurate mass determination in positiveand negative mode was performed on aWatersSynapt G2-S QTOFinstrument with Waters™ Software (Masslynx V4.1 SCN871).Samples were ionized by use of ASAP probe (APCI). Infrared spectroscopywas performed on a Nexus FTIR instrument.

3.2. Chiral analyses

3.2.1. *Chiral HPLC-analyses*. Chiral HPLC analyses of **1** were performedon an Agilent 1100 HPLC instrument with manual injectorRheodyne 77245i/Agilent, 10 μL-loop, UV 254 nm. The column wasChiralcel OD-H column (25 cm, i.d 4.6 mm, film density 5 μm) fromDaicel, Chiral Technologies Europe. Eluent **1**: Hexane:2-propanol,83:17, flow rate 1.00 mL min^{-1}. R_t alcohol enantiomers of **1**: R_t (*S*)-**1**=37.70 min, R_t (R)-**1**42.75 min, R_S=1.84. R_t butanoate enantiomersof

1: R_t (*S*)-**1b**=21.83 min, (butanoate enantiomers were not separatedat R_t 23.48 min (from **1** derivatized with butyric anhydride).After hydrolysis of the butanoate **1b** it was shown that only onebutanoate enantiomer had been produced in the transesterificationreaction (giving R_t (*S*)-**1**=36.91).

3.2.2. *Chiral GLC-analyses*. Chiral GLC-analyses of **3** and **4** wereperformed on a Varian 3380 gas chromatograph with a Varian CP-8410 autoinjector and a split injector (200^oC) and flame ionizationdetector (FID, 250^oC). The secondary chloro alcohol **3** and the correspondingbutanoate **3b**were separated on a Varian CP Chirasil DEXcolumn (25m, i.d. 0.25mm, film density 0.25 μm). Carrier gas H_2 5.0,flow 7.5 psi, split flow 60 mL min$^-$1, temp program: 110-120^oC/2^oC min$^-$1, 120-140^oC/1^oC min$^-$1, 140-150^oC/0.5^oC min$^-$1, R_t alcohol(*R*)-**3**=38.13 min, R_t (*S*)-**3**38.67min. R_S=1.50. R_t butanoates: R_t(*R*)-**3b**=63.57 min and R_t (*S*)-**3b**=64.55 min. R_S=2.30.

The bromo alcohol **4** and the corresponding butanoate **4b** wereseparated on the same column with H_2 5.0 carrier gas, flow 9 psi,split flow 80 mL min$^-$1, temp program: 105-115^oC/2^oC min$^-$1,115-130^oC/1^oC min$^-$1, 130-140^oC/0.5^oC min$^-$1 (5 min hold),140-150^oC/0.5^oC min$^-$1. R_t alcohols: R_t (*R*)-**4**=51.10 min Rt (*S*)-**4**=51.60 R_S=1.80. R_t butanoates: R_t (*R*)-**4b**=67.53 min and R_t (*S*)-**4b**=67.92 min. R_S=2.40.

3.3. Assignment of absolute configurations

The absolute configuration of the faster reacting enantiomer inlipase catalyzed resolution was determined by the known enantiopreferenceof CALB[27] and by comparing the elution orders of theenantiomers with GLC elution orders of similar enantiopure compoundssynthesized from (*S*)-epichlorohydrin.[12] In chiral HPLCanalyses, the enantiomers eluted in the opposite order compared tochiral GLC.

3.4. Synthesis of racemic substrates

3.4.1. *4-(3-Chloro-2-hydroxypropoxy)phenyl)acetamide* (***1***).[10,11] 2-(4-Hydroxyphenyl)acetamide (2.52 g, 16.6 mmol) and epichlorohydrin(13 mL) was mixed and transferred to a solution of sodiumhydroxide (0.50 g, 12.3 mmol) and water (5 mL). The reaction wasstirred for 48 h at rt. TLC chromatography (DCM:MeOH, 4:1) showedthat both alcohol **1** and the epoxide **2**was produced. The white solidwas washed with DCM and filtrated, then Li_2CuC_{l4} in THF (0.1 M,30

mL) was added. The reaction mixture was stirred for 24 h underinert atmosphere. Sodium phosphate buffer (0.1 M, 30 mL) wasadded. THF was removed and the residue was extracted with ethylacetate (5×20 mL) andwashed with a satd sodium chloride solution(2×20 mL). Ethyl acetate was removed under reduced pressure and**1**was isolated in 22% yield (0.901 g, 3.70 mmol), mp 129e132^{o}C. ^{1}HNMR (MeOD): 7.23-7.21 (m, 2H, aromatic), 6.92-6.90 (m, 2H, aromatic),4.13-4.10 (m, 1H, -CH-), 4.06-4.02 (m, 2H, -O-CH_2-),3.77-3.66 (m, 2H, -CH_2-C_l), 3.44 (s, 2H, -CH_2-$CONH_2$). ^{13}C NMR:177.4,159.2,131.3 (2C),129.4, 115.7 (2C), 71.0, 70.2, 46.8, 42.5. HRMS(APCI/ASAP, m/z): 244.0744 $[M+H]^+$, (calcd. $C_{11}H_{14}NO_3Cl$, 243.654).IR (cm^-1): 3349, 1633, 1241, 706.

3.4.2. *3-Chloro-1-phenoxy-2-propanol* (***3***).[21]HCl(37%, 50mL) was added to a flask with a solution of 1,2-epoxy-3-phenoxypropane (3.31g, 22.0 mmol) and DCM (25mL). The reaction mixture wasstirred for 0^{o}C. TLC showedfull conversion of the substrate after 30min (DCM:acetonitrile, 40:1, R_f=0.42). The organic phase was concentrated under reduced pressure and purified on a silicagel flash column (DCM: acetonitrile, 40:1). The colorless fluid **3** was isolated in 76% yield (3.11g, 16.7mmol).^{1}H NMR ($CDCl_3$) 7.29-7.27 (m, 2H, aromatic), 6.98-6.96 (m, 1H, aromatic), 6.91- 6.89 (m, 2H, aromatic), 4.21-4.17 (m, 1H, -CH-), 4.08-4.03 (m, 2H, -O-CH_2-), 3.77-3.68 (m, 2H, -CH2-Cl-), 2.81-2.80 (d, 1H, OH; J=6.0). ^{13}C NMR: 158.3, 129.7 (2C), 121.5, 114.6 (2C), 70.0, 68.5, 46.0. HRMS (APCI/ASAP, *m/z*): 169.0424 $[M-OH]^+$, (calcd. $C_9H_{11}O_2Cl$, 186.628). IR (cm^-1): 3405, 751, 690.

3.4.3. *3-Bromo-1-phenoxy-2-propanol* (**4**).[22,23] 3-Bromo-1-phenoxy-2-propanol (**4**) was synthesized and characterized aspreviously described in 99% purity (GLC) and in 82% yield.

3.5. Kinetic resolutions of racemic compounds

3.5.1. Small scale transesterifications. Transesterification reactionswere performed in a New Brunswick Incubator Shaker at 30^{o}Cagitating at 200 rpm. Racemic alcohols **1** and **3-4** (1.31×10^-4 mol)and vinyl butanoate (6.5×10^-4 mol) were mixed in solvent (acetonitrile,hexane and DCM/hexane, respectively) and the reactionsstarted by addition of immobilized lipase (20 mg) and were incubatedfor 10-27 h before the enzyme was filtered off and thesolvent removed. Two replicates of each reaction were performed.The enantiomers were separated by column chromatography.Chiral GLC and HPLC analyses gave ee_S- and ee_P-values from whichthe degree

of conversion was calculated according to $c=ee_S/(ee_S+ee_P)$. The E-values were calculated using the program *E&K Calculator 2.1b0 PCC*.[14] In control experiments under the same reactionconditions but without enzyme, no acylation was observed.

3.6. Large scale transesterifications

3.6.1.(R)-2-(4-(3-Chloro-2-hydroxypropoxy)phenyl)acetamide, (*R*)-**1**. Alcohol **1** (0.56 g, 2.3 mmol) and vinyl butanoate (1.43 g,12.5 mmol) was added to a flask with dry acetonitrile (40 mL) andmolecular sieve. CALB (0.71 g) was added and the reaction wasincubated at 30°C and 200 rpm for 27 h in an incubator shaker. Theenzyme and molecular sieve was filtered off and the solvent wasremoved under reduced pressure. The ester (*S*)-**1b** and the alcohol(*R*)-**1** were separated on a silica column with ethyl acetate as eluent.(*S*)-**1b** was isolated in 99% *ee* and 18% yield (0.13 g, 0.41 mmol). (*R*)-**1** was isolated in 99% *ee* and 16% yield (0.090 g, 0.37 mmol).$[\alpha]_D^{23}$=-3.0 (c 1.0, MeOH).

3.6.2. (S)-2-(4-(3-Chloro-2-hydroxypropoxy)phenyl)acetamide, (*S*)-**1**. Butanoate **1b** (0.092 g, 0.29 mmol) and phosphate buffer (0.1 M,pH=7, 4.0mL)wasmixed in a reaction vessel. CALB (0.8 g)was addedand the reactionwas incubated for 48 h. The enzymewas filtered offand the mixture was extracted with ethyl acetate (5×2 mL). Thesolvent was removed under reduced pressure and gave (*S*)-**1** in 48%yield (0.034 g, 0.14 mmol) 98.5% *ee*, $[\alpha]_D^{23}$=+3.0 (c 1.0, MeOH).

3.6.3. *(S)-3-Chloro-1-phenoxy-2-propanol*, *(S)-3 from butanoate (S)*-**3b**. Alcohol **3** (1.00 g, 4.2 mmol) was dissolved in dry hexane(100 mL) with molecular sieve. Vinyl butanoate (3.02 g, 26.4 mmol)and CALB (1.00 g)was added. The suspension was incubated at 30°Cat 200 rpm for 23 h. The enzyme was filtered off and the mixturewas extracted with ethyl acetate (5×2 mL). The solvent was removedunder reduced pressure and after separation of the enantiomerson silica column (*S*)-**3b** was hydrolyzed by CALB and gave(*S*)-**3** in 96% *ee*, optical rotation $[\alpha]_D^{22}$=+5.3 (c 1.71, EtOH).

3.6.4. *(S)-3-Bromo-1-phenoxy-2-propanol (S)-**4** and butanoate (S)- **4b***. Alcohol **4** (1.26 g, 5.5 mmol) was dissolved in dry hexane(100 mL) with molecular sieve. Vinyl butanoate (3.15 g, 27.5 mmol)and CALB (0.85 g)was added. The suspensionwas incubated at 30°Cat 200 rpm for 10 h. The enzymewas filtered off and the mixturewasextracted with ethyl acetate (5×2 mL). The solvent was removedunder reduced pressure and after separation of the enantiomers onsilica

column (*S*)-**4b** was produced in 57% yield (0.81 g, 3.2 mmol)ee=93%, $[\alpha]_D^{22}$=+14.4 (c 1.71, EtOH). CALB catalyzed hydrolysis of *(S)*-**4b** gave (*S*)-**4**in 96% *ee,* optical rotation $[\alpha]_D^{22}$=+5.26 (c 1.71, EtOH)corresponded with previously determined o. r. of this compound in96% *ee*, which was $[\alpha]_D^{22}$=+5.3 (c 1.71, EtOH).[13,23]

4. Conclusion

An efficient process for synthesis of both enantiomers (*S*)-**1** and(*R*)-**1** (in 98.5 and 99% *ee*, respectively) as building blocks for the β-blocker atenolol has been developed using CALB as catalyst andvinyl butanoate as acyl donor. All enantiopure compounds havebeen extensively characterized. Different preparations of CALB(Novozym 435) show different enantioselectivity in transesterificationreactions of the halo alcohols **3** and **4** with the samereaction conditions as in resolutions previously performed by us,which indicates that model reactions should be performed withnew enzyme preparations.

References and notes

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Note for the Participants in IPDC:

The evaluation of the patent specification and claims would be based on how many additional embodiments are added to make the coverage broad in addition to what is provided in the disclosure. Exact replica of the structures and schemes would affect marking. The participant is expected to read and understand the background of the subject matter himself/herself in order to search for more relevant prior art and formulate the various embodiments of the invention with proper Markush structure coverage etc.