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Article

Chemo-Enzymatic Synthesis of Synthons as Precursors for Enantiopure Clenbuterol and other β2-agonists

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Abstract: (*R*)-1-(4-Amino-3,5-dichlorophenyl)-2-bromoethan-1-ol has been synthesised in 93% enantiomeric excess (*ee*) by asymmetric reduction of the corresponding ketone catalysed by a ketoreductase and NADPH as the co-factor in DMSO. (*S*)-*N*-(2,6-Dichloro-4-(1-hydroxyethyl)phenyl)acetamide has been synthesised in >98% *ee* by the same system. Both synthons are potential precursors for clenbuterol enantiomers. Clenbuterol is a β2-agonist used in veterinary treatment of asthma in several countries. The drug is listed on the World Anti-doping Agency´s Prohibited list due to its effect on increased protein synthesis in the body. However, racemic clenbuterol has recently been shown to reduce the risk of Parkinson's disease. In order to reveal which one (or both) of the enantiomers that cause this effect, the pure enantiomers need to be studied separately. Our biocatalytic approach in order to obtain enantiopure clenbuterol should be applicable to industrial scale.

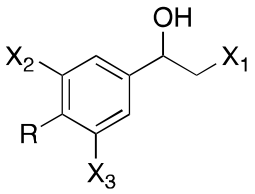
Keywords: (*R*)-1-(4-Amino-3,5-dichlorophenyl)-2-bromoethan-1-ol, (*S*)-*N*-(2,6-dichloro-4-(1-hydroxyethyl)phenyl)acetamide, clenbuterol, ketoreductase, chiral chromatography

1. Introduction

The need for enantiopure compounds for treatments of human diseases and combatting microbial attacks on crop etc. can be explained by the fact that all living organisms in Nature are chiral. Protein biosynthesis and most of the metabolic processes are mediated by enzymes which are specific for a particular isomeric form of a certain substrate, and this is essential for ensuring the high degree of three-dimensional organization which is found in structures within cells. It is presumably evolutionary chance which has determined that life is based on L- rather than D-amino acids [1].

When drug-receptor interactions are considered it is postulated that "the lower the effective dose of a drug, the greater the difference in the pharmacological effect of the optical isomers" [2]. The ratio of the more active enantiomer (eutomer) compared to the less active enantiomer (distomer) is defined as the eudismic ratio, and the higher the eudismic ratio, the higher the effectiveness of the drug [3]. The β2-agonists clenbuterol and salbutamol are drugs used in the treatment of f. inst. angina pectoris, asthma and other disorders related to the sympathetic nervous system. β2-Receptors are found

mainly in the bronchi, bronchioles and gastrointestinal tract, and one of their main actions is the relaxation of smooth muscles (bronchodilation). Racemic clenbuterol and salbutamol are showed in Fig. 1. Clenbuterol is marketed with racemic active pharmaceutical ingredient (API), while salbutamol is marketed both as the racemate and as Xopenex®, with the *R*-enantiomer as the API. The alcohols (*R*)-1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol ((*R*)-**2a**) and *S*)-*N*-(2,6-dichloro-4-(1-hydroxyethyl)phenyl)acetamide ((*S*)-**6a**) are also shown in Fig. 1.



Clenbuterol: X_1 = *t*-BuNH, X_2 = Cl, X_3 = Cl, R = NH_2
Salbutamol: X_1 = *t*-BuNH, X_2 = CH_2OH, X_3 = H, R =OH
(*R*)-**2**: X_1 = Br, X_2 = Cl, X_3 = Cl, R = NH_2
(*S*)-**6**: X_1 = H, X_2 = Cl, X_3 = Cl, R = NHAc

Figure 1. Structures of the racemic β_2-agonists clenbuterol and salbutamol and (*R*)-1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol ((*R*)-**2a**) and (*S*)-*N*-(2,6-dichloro-4-(1-hydroxyethyl)phenyl)acetamide ((*S*)-**6a**).

Clenbuterol (Ventipulmin®, Boehringer Ingelheim Vetmedica, Inc.) is a long acting, selective β_2-agonist with anabolic properties used in the treatment of shock and airway obstructing diseases in veterinary medicine [4,5]. It is not approved by the FDA for human use or for food producing animals, although it is illegally added to livestock feed to promote growth of lean meat [6]. Clenbuterol can reduce body fat, and is extensively used by athletes and body-builders, even though it is listed on the World Anti-Doping Agency's prohibited list [7]. In addition to the desired effects of smooth muscle relaxation, clenbuterol can cause several side effects such as heart palpitations, muscle tremors, and nervousness [5].

Previously it has been reported that (*S*)-clenbuterol had neuroprotective properties, caused reduced blood pressure and enhanced blood glucose levels in rats. While (*R*)-clenbuterol did not promote these properties, it was found that it caused decreased motor activity, head twitches and tremors [8,9]. Upon examination of human urine of participants who ingested racemic clenbuterol, it was found that the *S*-enantiomer is longer retained in the body [10]. Recently, Mittal *et al.* found that the brains of patients with Parkinson´s disease contained accumulations of α-synuclein protein, so-called Lewy bodies. The β_2-agonists metaproterenol, clenbuterol and salbutamol lowered the levels of α-synuclein gene mRNA and α-synuclein protein levels in human SK-N-MC neuroblastoma cells [11]. Only the racemic compounds were studied, however, pure enantiomers of the drugs should be studied independently in order to reveal if the enantiomers would show different effects.

Racemic clenbuterol has been synthesised through the incorporation of carbon monoxide in aryl iodides [12], while racemic ^{13}C-labeled clenbuterol has been synthesized from acetanilide [13]. Enantiopure (*R*)-1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol, a precursor for (*R*)-clenbuterol, through an asymmetric reduction of the parent ketone, catalysed by an alcohol dehydrogenase from *Rhodococcus erythropolis*. No enantiomeric ratio was reported [14].

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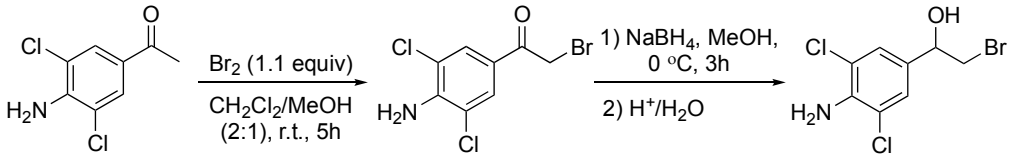
Our attempts to synthesise enantiopure precursors for (*R*)-clenbuterol include both lipase catalysed kinetic resolutions of racemates and asymmetrisations of substituted acetophenones catalysed by ketoreductases. Previously, we have utilised chemo-enzymatic methods in syntheses of enantiopure building blocks for compounds which are related to clenbuterol [15,16].

2. Results

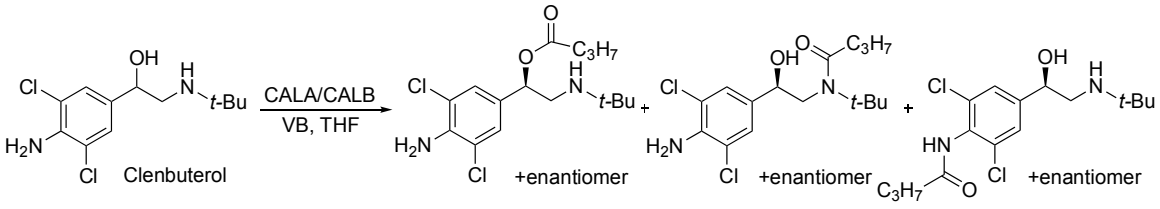
In order to obtain a precursor for enantiopure (*R*)-clenbuterol, the bromo alcohol 1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol (**3**) was synthesised in 45% yield (Scheme 1) for the purpose of performing lipase catalysed kinetic resolution with lipases suitable for industrial scale production. Bromination of substrate ketone **1** produced bromoketone **2** in 86% yield. Various methods for α-bromination of ketone **1** were performed, where bromine in dichloromethane and methanol gave the highest conversion of **2**. Subsequent reduction of **2** with sodium borohydride gave **3** in 45% yield.



Scheme 1. Synthesis of 1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-one (**2**) and 1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol (**3**) from commercial 1-(4-amino-3,5-dichlorophenyl)ethan-1-one (**1**).

Kinetic resolutions

Kinetic resolutions of **3** were performed in dichloromethane with vinyl butanoate as the acyl donor catalysed by commercial immobilised lipases A and B from *Candida antarctica* (CALA and CALB, Sigma-Aldrich). The reactions showed no conversion after five days. It was also attempted to resolve racemic clenbuterol with CALA and CALB from Sigma-Aldrich and vinyl butanoate as the acyl donor in tetrahydrofuran, Scheme 2. However, these reactions resulted in several acetylation products after five days and the enzyme showed low enantioselectivity.



Scheme 2: Kinetic resolution of clenbuterol catalysed by CALA and CALB in dry THF with vinyl butanoate (VB) as acyl donor, showed several acylation products. The *E*-value for the desired acylation was $E = 1.3$ after 5 days.

The electropherogram from the CALA catalysed kinetic resolution of clenbuterol after 78 hours (Fig. 2), shows the peaks of (*R*)-clenbuterol at t_R 9.70 min and (*S*)-clenbuterol at t_R 11.50 min and the butanoic esters of (*S*)-clenbuterol at t_R 12.52 min and ester of (*R*)-clenbuterol at t_R 12.98 min. Elution orders are consistent with previously reported retention times [17]. The remaining peaks have not been identified, but are most likely the amide products from acylation of the aromatic or aliphatic amines, which would produce two enantiomers each.

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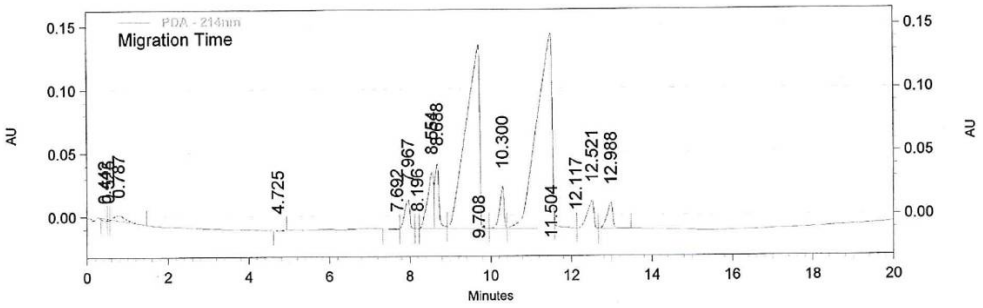
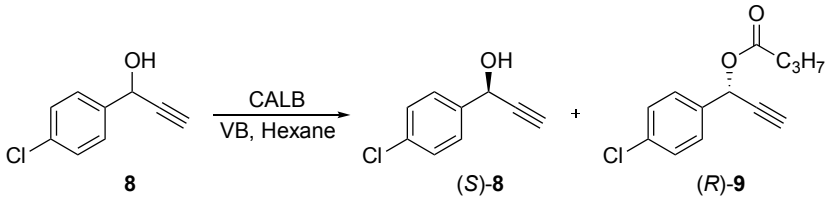


Figure 2: Electropherogram from the kinetic resolution of clenbuterol, catalysed by CALA with vinyl butanoate in tetrahydrofuran. t_R (*R*)-clenbuterol 9.70 min, t_R (*S*)-clenbuterol at 11.50 min, and the butanoic esters (*R*)- and (*S*)-clenbuterol at t_R 12.52 minutes and t_R 12.98 minutes, respectively. For resolutions, see experimental.

We anticipate that the α-bromo substituent in **3** could be one reason for the low selectivity and low reaction rate in the transesterification. In order to reveal the influence of an electron rich substituent next to the stereocenter compared to bromine, transesterification of the propargyl alcohol **8** was performed in dry hexane with vinyl butanoate as the acyl donor and CALB as the catalyst, see Scheme 3. The catalyst showed excellent enantioselectivity towards **8** after 2.5 hours with an *E*-value >200 resulting in conversion of (*R*)-**8** to (*R*)-**9** with no sign of (*R*)-**8** on the chromatogram, giving (*S*)-**8** in 99% *ee*. The reaction progress is shown in Fig. 3. From this test reaction it seems that either the chloro substituents in *meta*-position or the amino substituent in *para*-position on the benzene ring on **3** must be the reason for the low selectivity and slow esterifications of **3** and clenbuterol with CALB.



Scheme 3. Kinetic resolution of **9** with CALB as the catalyst and vinyl butanoate in dry hexane.

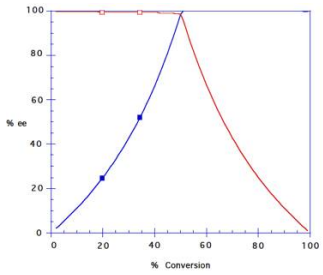


Figure 3. Reaction progress of kinetic resolution of **8** with CALB in dry hexane with vinyl butanoate as acyl donor, *E* = >200. Both the butanoate ester enantiomer of **8**, (*R*)-**9** and the remaining alcohol (*S*)-**8** were obtained in 99% *ee*. Chiral GLC and HPLC analyses gave ee_S- and ee_P-values from which the degree of conversion was calculated according to $c = ee_S/(ee_S + ee_P)$.

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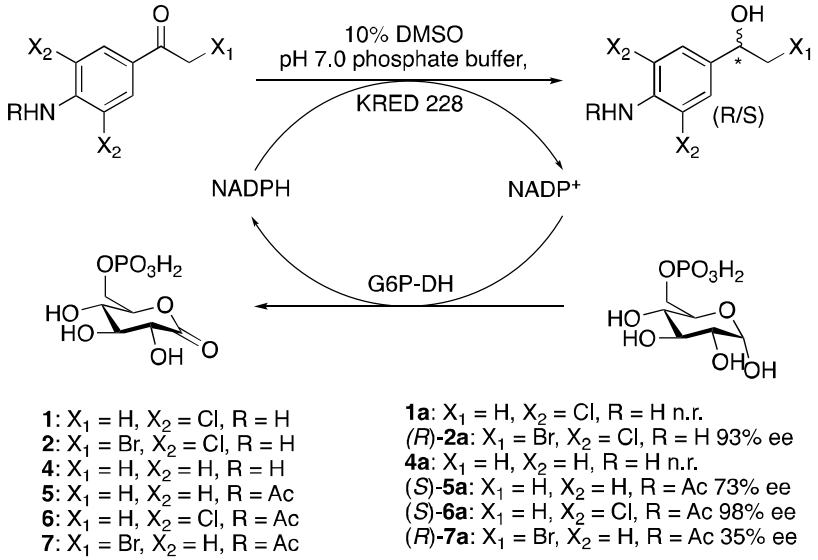
Asymmetrisations

Asymmetrisations of ketones with suitable enzymes is another way of obtaining enantiopure compounds. Depending on the catalysts stereoselectivity towards a chosen substrate, a theoretical yield of 100% might be obtained. Scheme 4 shows asymmetrisation of ketone **1, 2** and **4**-**7** with ketoreductase KRED 228 (Syncozymes Co., Ltd., Shanghai, China). Several regeneration systems were tested in order to find a suitable system to regenerate $NADP^+$, and we were successful with glucose-6-phosphate dehydrogenase with glucose-6-phosphate as the co-substrate [18,19]. The co-solvent was dimethyl sulfoxide (DMSO) in all the asymmetrisation reactions.



1: X_1 = H, X_2 = Cl, R = H
2: X_1 = Br, X_2 = Cl, R = H
4: X_1 = H, X_2 = H, R = H
5: X_1 = H, X_2 = H, R = Ac
6: X_1 = H, X_2 = Cl, R = Ac
7: X_1 = Br, X_2 = H, R = Ac

1a: X_1 = H, X_2 = Cl, R = H n.r.
(*R*)-**2a**: X_1 = Br, X_2 = Cl, R = H 93% ee
4a: X_1 = H, X_2 = H, R = H n.r.
(*S*)-**5a**: X_1 = H, X_2 = H, R = Ac 73% ee
(*S*)-**6a**: X_1 = H, X_2 = Cl, R = Ac 98% ee
(*R*)-**7a**: X_1 = Br, X_2 = H, R = Ac 35% ee

Scheme 4. Reduction of the ketones **1, 2** and **4-7** with ketoreductases (KRED 228 and KRED 238 from Syncozymes Co., Ltd.) and glucose-6-phosphate dehydrogenase (G6P-DH) as the regeneration system for $NADP^+$ with glucose-6-phosphate (G6P) as the co-substrate in DMSO.

Through the reduction of 1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-on (**2**) with KRED 228, (*R*)-**2a** was obtained in 93% *ee*. Reduction of *N*-(4-acetylphenyl)acetamide (**5**) by the same enzyme gave the *S*-alcohol (*S*)-*N*-(4(1-hydroxyethyl)phenyl)acetamide, (*S*)-**5a**, in 73% *ee*. Reduction of *N*-(4-acetyl-2,6-dichlorophenyl)acetamide, **6**, produced the *S*-alcohol (*S*)-1-(4-amino-3,5-dichlorophenyl)ethan-1-ol, (*S*)-**6a**, in 98% *ee*. The reduction of *N*-(4-(2-bromoacetyl)phenyl)acetamide (**7**) gave an *ee* of 35% of the alcohol **7a**, with *R*-configuration. The asymmetrisations of ketones **1** and **4** with use of the same system were not successful. Asymmetrisations of the above-mentioned ketones were also performed with *S. cereviciae* and several alcohol dehydrogenases without any success. Valuable enantiopure synthons could be synthesised from (*S*)-**6a**, see suggestions in Scheme 5.

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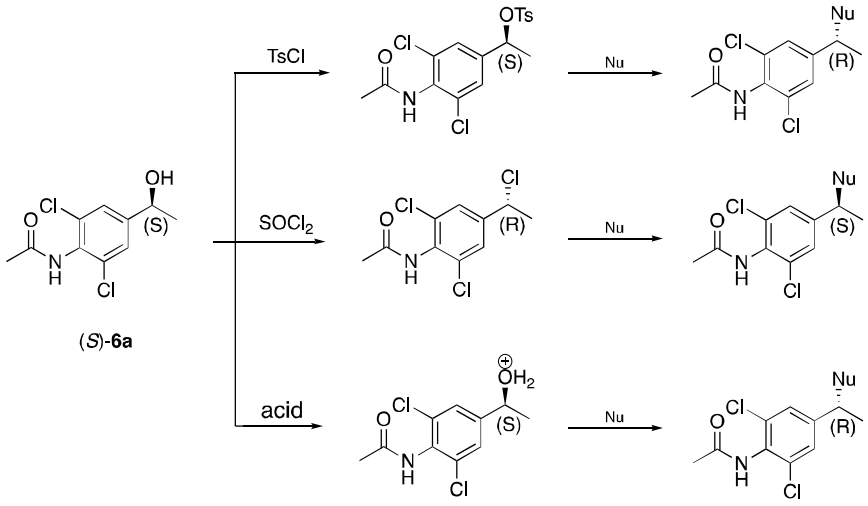

Scheme 5. The stereoconfiguration of products from nucleophilic substitutions of enantiopure (*S*)-**6a** depends on the nucleophile (Nu).

4. Materials and Methods

Analytical grade chemicals and solvents were purchased from Sigma-Aldrich (Oslo, Norway). HPLC grade solvents were purchased from Sigma-Aldrich were used. Dry solvents (THF and CH_2Cl_2) were acquired from a MBraun MB-SPS-800 solvent purification system, (MBraun, München, Germany). *Candida antarctica* lipase A (activity 1612 U/g; lot no. BCBR2259V), lipase B (activity 1800 U/g, lot no BCBP3525V) immobilized on Immobead 150, recombinant from *Aspergillus oryzae* and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (G6P-DH, ≥550 U/mg, lot no. 107K7700V) were also purchased from Sigma-Aldrich. Crystalline KRED 228 (331.9 U/g, lot no. 20160509) and KRED 238 (63.1 U/g, lot no. 20151106) were from Syncozymes Co., Ltd., (Shanghai, China). Flash chromatography was performed with silica gel from Sigma-Aldrich (pore size 60 Å, 230-400 mesh, 40-63 µm particle size). Ketones **1**, **4, 7** and clenbuterol were bought from Sigma-Aldrich (Oslo, Norway).

The enzyme catalysed kinetic resolutions were performed in a New Brunswick G24 Environmental Incubator Shaker (New Brunswick Co. Inc., Edison, NJ, USA) at 30°C and 200 rpm or 300 rpm. Optical rotations ($[\alpha]_D$) were determined at 20°C using a Perkin-Elmer 343 instrument (Perkin Elmer, Waltham AS, USA) concentrations are given in g/100 mL. NMR spectra were recorded with a Bruker Avance DPX 400 instrument (Bruker, Rheinstetten, Germany) operating at 400 MHz for ^1H and 100 MHz for ^{13}C, respectively. Chemical shifts are in ppm rel. to TMS and coupling constants are in Hertz (Hz). Accurate mass determination in positive and negative mode was performed on a WatersSynapt G2-S Q-TOF instrument (Waters UK, Elstree, United Kingdom) with Waters™ Software (Masslynx V4.1 SCN871). Samples were ionized by use of ASAP probe (APCI).

Achiral chromatographic analyses

Achiral GLC-analyses were performed on an Agilent 7890A gas chromatograph, with an Agilent 7890B autosampler, a split injector (280°C), a 4 mm ID tap GW liner (Agilent Technologies, Santa Clara, CA,USA), a flame ionization detector (FID, 280 °C). All analyses were performed on a Restek

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Rtx®-1701 column (Crossbond® 14% cyanopropylphenyl - 86% dimethylpolysiloxane, 30 m x 0.32 mm ID, df 0.25 μm, (Restek Corporation, U.S., Bellefonte, PA, USA), carrier gas He 5.0, temp. prog.: 100-280°C/10°C min^{-1}.

Chiral analyses

Chiral GLC-analyses

Chiral GLC-analyses of **3** and **6a** were performed on an Agilent 7890B gas chromatograph, with an Agilent 7890B autosampler, an Agilent G4513A - 7693A autoinjector, a split injector (225-230 C) and a flame ionization detector (FID, 275 C). The enantiomers of alcohol **3** were separated on a CP-Chirasil-Dex CB column (24.3 x 0.25 mm ID, df 0.25 μm) from Agilent technologies with He 5.0 as the carrier gas, flow 81 min^{-1}, split flow 75 mL min^{-1} (25:1), temp. prog.: 100-150°C/10°C min^{-1}, 150-160°C/3°C min^{-1}, 160-170°C/1°C min^{-1}. Retention times: t_R (*R*)-**3** = 25.363 min, t_R (*S*)-**3**, R_S = 1.82.

Separation of enantiomers of **6a** was performed on the same system: flow 75 mL min^{-1}, split flow 1:30, temp program: 100-160°C/10°C min^{-1}, 160-170°C/1°C min^{-1}, 170-185°C/0.5 °C min^{-1}. Retention times t_R (*S*)-**6a** = 44.467 min, t_R (*R*)-**6a** = 45.255 min, R_S = 2.05.

Chiral GLC-analyses of **8** was performed on a Varian 3380 gas chromatograph, with a Varian CP-8410 autosampler, a split injector (200°C), and a flame ionization detector (FID, 200°C). The enantiomers of alcohol **8** and butanoic ester **9** were separated on a CP-Chirasil-Dex CB column (25 x 0.25 mm ID, d_f 0.25 μm) from Agilent technologies. Carrier gas H_2 5.0, gas pressure 8 psi, split flow 60 mL/min, temp prog 100-140°C/10°C min^{-1} (5), 140-180°C/10°C min^{-1} (7). Retention times t_R (*S*)-**8** = 12.690 min, t_R (*R*)-**8** = 12.901 min, R_S = 3.5, t_R (*S*)-**9** = 13.162 min, t_R (*R*)-**9** = 14.359 min, R_S > 1.5.

Chiral HPLC analyses

HPLC analyses were performed on an Agilent HPLC 1100 system with a manual injector (Rheodyne 77245i/Agilent 20 μL loop), and a variable wavelength detector (VWD) set to 254 nm. Alcohol **5a** was separated on a Chiralcel OD-H column (250 mm L x 4.6 mm ID, 5 μm particle size, Daicel, Chiral Technologies Europe, Illkirch-Graffenstaden, France). Eluent: 7% propan-2-ol/93% n-hexane; 1 mL min^{-1}, 1.5 μL injected. Retention times: t_R (*R*)-**5a** = 84.474 min, t_R (*S*)-**5a** = 91.765 min, R_S =1.56.

Alcohol **7a** was separated on the same column, eluent: 17% propan-2-ol/83% n-hexane; 1 mL min^{-1}, 2 μL injected. Retention times: t_R (*R*)-**7a** = 14.037 min, t_R (*S*)-**7a** = 17.035 min, R_S = 2.55.

Capillary zone electrophoresis

Chiral CZE-analyses of clenbuterol and the corresponding butanoate were performed on a Beckman MDQ electrophotometer in a fused silica capillary (31 cm x 50 μm, 21 cm to the detection window) with a diode array detector (DAD, 214 nm). Running buffer: pH 2.5 phosphate buffer (0.100 M), chiral selector: highly sulfonated β-cyclodextrin (5% w/V). Injection mode: hydrodynamic injection (0.5 psi for 5 seconds), separations voltage: −10 kV. Migration times: t_M (*R*)-clenbuterol 9.708 min, t_M (*S*)-clenbuterol at 11.504 min, t_M butanoates: t_M (*R*)-ester 12.521 min, t_M (*S*)-ester 12.988 min.

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Determination of enantiomeric excess (ee), conversion (c) and the enantiomeric ratio (E)

Chiral GLC and HPLC analyses gave ee_s- and ee_p-values from which the degree of conversion was calculated according to $c = ee_s/(ee_s + ee_p)$. Enantiomeric ratios, *E*, were calculated based on ping-pong bi-bi kinetics using the computer program *E&K Calculator 2.1b0 PPC* [21]. Two or more replicates of the transesterification and asymmetrisation reactions were performed.

Absolute configurations

The absolute configurations of (*R*)-**2a**, (*S*)-**6a** and (*R*)-**9** were determined by comparing the elution orders of the enantiomers with GLC elution orders of similar enantiopure compounds synthesised from (*S*)-epichlorohydrin. Elution orders on Daicel Chiralcel OD-H of the faster reacting enantiomers were the same [15, 22, 23]. Optical rotation values of (*R*)-**2a**, (*S*)-**5**, (*S*)-**6a** and (*R*)-**7a** have not been reported previously.

*Synthesis of 1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-one (**2**)*

To a solution of 1-(4-amino-3,5-dichlorophenyl)ethan-1-one (**1**) (1.10 g, 5.39 mmol) dissolved in CH_2Cl_2 (20 mL) and MeOH (10 mL), a solution of Br_2 (0.167 mL, 3.23 mmol) in CH_2Cl_2 (6 mL) was added at a rate of 40 drops per minute under strong agitation at RT. After an observed color change from bromine-red to pale yellow, two additional portions of Br_2 (0.056 mL, 1.09 mmol) were added as described above. After the observed color change, the product was confirmed by TLC: R_f (**2**) = 0.58 (1:3 *n*-pentane: CH_2Cl_2). The reaction mixture was washed with satd. K_2CO_3 (2x40 mL) and brine (2x40 mL) before the organic layers were dried over $MgSO_4$ and filtered before the solvents were removed under reduced pressure. The crude compound was stirred in EtOH (4.4 mL) at 50°C for 30 min, and for an additional 60 min at RT. The solids were filtered and re-crystallised from EtOAc. The crystals were dried under vacuum overnight to afford **2** in 86% yield (1.04 g, 4.22 mmol) with 98% purity (GLC). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (s, 2H, H_{arom}), 5.05 (s, 2H, N**H**$_2$), 2.31 (s, 2H, C**H**$_2$-Br). ^{13}C NMR (600 MHz, $CDCl_3$): δ 188.0, 144.9, 129.2, 124.0, 188.9, 29.8. MS (TOF-ASAP): $[M+H]^+$ 281.9088 *m/z*.

*Synthesis of 1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol (**3**)*

1-(4-Amino-3,5-dichlorophenyl)-2-bromoethan-1-one (**2**) (0.50 g, 1.77 mmol) dissolved in EtOH (12 mL) at 0°C was added $NaBH_4$ (0.136 g, 3.60 mmol) in 4 portions over 20 min, under stirring. The reaction was slowly heated to RT, and the conversion was monitored by TLC: R_f (**3**) = 0.43 (1:3 *n*-pentane/CH_2Cl_2). After full conversion of the starting material, the reaction was quenched with dilute HCl until gas formation stopped. H_2O and EtOH were removed through azeotropic distillation, and the solids were dissolved in EtOAc (50 mL), and extracted with satd. $NaHCO_3$ (3x30 mL) and brine (3x30 mL). The aqueous layers were back extracted with EtOAc (2x50 mL), and the combined organic layers were dried over $MgSO_4$. The solvent was removed under reduced pressure, and the crude product was purified by flash chromatography (1:3 *n*-pentane/ CH_2Cl_2) to afford **3** as a light-yellow solid in 44% yield (0.22 g, 0.77 mmol) in 95% purity (GLC). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.22 (s, 2H_{arom}), 4.80-4.76 (dd, 1H, C**H**OH, $^3J_{HH}$ = 3.4 Hz, 8.9 Hz), 4.49 (s, 2H, N**H**$_2$), 3.59-3.55 (dd, 1H, C**H**-Br, $^2J_{HH}$ = 10.5 Hz, $^3J_{HH}$ = 3.4 Hz), 3.49-3.45 (dd, 1H, C**H**-Br, $^3J_{HH}$ = 8.9 Hz, $^2J_{HH}$ = 10.4 Hz). ^{13}C NMR (400 MHz, $CDCl_3$): δ 140.1, 130.4, 125.6, 119.6, 72.6, 39.8. MS (TOF-ASAP) $[M+H]^+$ 283.9241 *m/z*.

*Synthesis of N-(4-acetyl-phenyl)acetamide (**5**)*

To a stirred solution of 1-(4-aminophenyl)ethan-1-one (0.53 g, 3.92 mmol) in CH_2Cl_2 (15 mL), a solution of AcCl (0.56 mL, 7.84 mmol) in CH_2Cl_2 (2 mL) was added dropwise at room temperature. After 5 min, a white precipitate started forming, and a solution of Et_3N (1.09 mL, 7.84 mmol) in CH_2Cl_2 (2 mL) was added dropwise, after which the solution cleared and turned a dark yellow color. Full conversion was observed after 24 h by TLC: R_f (**5**) = 0.05 (1:4 EtOAc/n-pentane,), and the solution was extracted with brine (3x15 mL). The aqueous layers were combined and extracted with EtOAc (2x15 mL), before the combined organic layers were dried over MgSO4, filtered, and the solvents were removed under reduced pressure. The crude product was recrystallized from EtOAc to afford **5** in 53% yield (0.37 g, 2.09 mmol) and 98% purity (GLC). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.95-7.93 (m, 2H$_{arom}$, $^2J_{HH}$, ortho = 8.7 Hz, $^3J_{HH}$, meta = 2.5 Hz), 7.62-7.61 (d, 2H, H$_{arom}$, $^2J_{HH}$ = 8.3 Hz), 7.42 (s, 1H, N**H**R), 2.58 (s, 3H, CO-C**H**$_3$), 2.22 (s, 3H, NCO-C**H**$_3$). ^{13}C NMR (600 MHz, $CDCl_3$): δ 196.9, 168.4, 142.2, 132.9, 129.8, 118.8, 26.4, 24.8. MS (TOF-ASAP) $[M+H]^+$ 178.0867 *m/z*.

*Synthesis of N-(4-acetyl-2,6-dichlorophenyl)acetamide (**6**)*

To a stirred solution of 1-(4-amino-3,5-dichlorophenyl)ethan-1-one (**1**) (6.00 g, 29.40 mmol) in CH_2Cl_2 (150 mL) AcCl (10.49 mL, 147.01 mmol) in CH_2Cl_2 (15 mL) was added. To the stirred solution Et_3N (4.29 mL, 30.78 mmol) in CH_2Cl_2 (15 mL) was added dropwise. The reaction was monitored by TLC. R_f (**6**) = 0.64 (1:4 EtOAc/n-pentane). After 48 h, full conversion was observed. The reaction mixture was washed with satd. K_2CO_3 (2x100 mL) and brine (2x100 mL), before the organic layers were collected and dried over anhydrous $MgSO_4$, filtered, and the solvent removed under reduced pressure. The crude product was recrystallized from EtOAc, and the purified compound dried *in vacuo* overnight to afford **6** as white crystals in 58% yield (4.20 g, 17.07 mmol) in 99% purity. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (s, 2H, H$_{arom}$), 7.42 (s, 1H, N**H**R), 2.56 (s, 3H, CO-C**H**$_3$), 2.22 (s, 3H, NCO-C**H**$_3$). ^{13}C NMR (400 MHz, $CDCl_3$): δ 195.0, 168.4, 136.7, 136.4, 133.9, 128.3, 26.6, 23.2. MS (TOF-ASAP) $[M+H]^+$ 246.0089 *m/z*.

*Synthesis of 1-(4-chlorophenyl)prop-2-yn-1-ol (**8**)*

A solution of ethynylmagnesium bromide (0.50 M in THF; 18 mL, 9.0 mmol) was cooled to –20°C. A solution of 4-chlorobenzaldehyde (1.0105 g, 7.19 mmol) in THF (2.5 mL) was added, and the flask washed out with THF (0.5 mL), which was then added to the reaction mixture. The cooling bath was removed, and the reaction mixture was stirred at room temperature for 5 min. The reaction was quenched by addition of satd. aq. NH_4Cl (10 mL), and the mixture was extracted with Et_2O (2x20 mL). The combined organic extracts were dried with Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by flash chromatography (gradient 1:10-1:3 EtOAc/pentane) to give **8** in 90% yield (1.078 g, 6.47 mmol) as a pale yellow oil. R_f = 0.47 (1:3 EtOAc/pentane). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.51-7.48 (m, 2H, H$_{arom}$), 7.38-7.35 (m, 2H, H$_{arom}$), 5.45 (dd, *J* = 6.1, 2.1 Hz, C**H**C≡), 2.68 (d, *J* = 2.2 Hz, ≡C**H**), 2.21 (d, *J* = 6.1 Hz, O**H**) ppm. ^{1}H NMR spectroscopic data are in accordance with literature data [20].

Kinetic resolution of secondary alcohols with lipases

Kinetic resolution of clenbuterol

1-(4-Amino-3,5-dichlorophenyl)-2-(tert-butylamino)ethan-1-ol (clenbuterol, 31.2 mg, 0.11 mmol)

dissolved in dry THF (4.0 mL) was added 3 pellets of molecular sieve, and vinyl butanoate (0.058 mL, 0.55 mmol) before the mixture was incubated at 30°C and 200 rpm and the reaction was started by addition of lipase A from *Candida antarctica* (CALA, 55.4 mg). Aliquots of 100 µL were collected every 30 min for the first 5 h, then every hour for 3 h, and every 24 h for 5 days. The solvent in the aliquots was removed with N_2, before 0.100 M pH 2.5 phosphate buffer (0.5 mL) was added, and the samples were analysed by chiral CE, from which the enantiomeric excess of (*R*)-clenbuterol was calculated to 11% *ee*.

*Kinetic resolution of 1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol (**3**)*

To a solution of racemic 1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol (**3**, 55 mg, 0.19 mmol) and vinyl butanoate (0.1225 mL, 0.97 mmol) in CH_2Cl_2 (3 mL), lipase B from *Candida antarctica* (CALB, 55 mg) was added before the solution was placed in an incubator set to 30°C and 200 rpm. Aliquots of 100 µL were collected every 30 min for the first 4 h, then at every 12 h for 3 days, and finally every 24 h for 4 days. The aliquots were analysed by chiral GLC, and no conversion was observed. A new reaction was performed with Lipase A from *Candida antarctica* (CALA, 60 mg), and aliquots were collected as described above, then analysed by chiral GLC. No conversion was observed.

*Kinetic resolution of 1-(4-chlorophenyl)prop-2-yn-1-ol (**8**)*

To a solution of 1-(4-chlorophenyl)prop-2-yn-1-ol (**8**) (20.0 mg, 0.12 mmol), and vinyl butanoate (0.069 mL, 0.60 mmol) in dry n-hexane (3.0 mL), 5 pellets of molecular sieve wereadded before the mixture was incubated at 40°C and 200 rpm. CALB (22.1 mg) was added to the mixture, and aliquots (0.100 mL) were collected every 30 min for the first 3 h, and finally after 19.5 h, which were analysed by chiral GLC: t_R (*S*)-**8** = 12.880 min, t_R (*R*)-**8** = 13.087 min, R_S = 3.5. After 2.5 h, (*R*)-**8** was not observed by GLC. The ester (*R*)-**9** was produced in 99% *ee*.

Asymmetrisations of ketones by ketoreductases

General procedure

The ketones **1, 2** and **4-7** (2.5-60.8 mg, 0.01-0.16 mmol) were dissolved in DMSO (0.10 mL) and transferred to a solution of ketoreductase 228 (KRED 228), NADPH, glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6P-DH) in pH 7.0 phosphate buffer (0.9 mL). The reactions were incubated at 30°C and 300 rpm for 1-28 days, and were monitored by TLC (1:2 *n*-pentane/EtOAc) every 24 h. After full conversion of the starting material, the mixtures were extracted with EtOAc (4x10 mL) and the organic phases were combined, washed with water (2x 20 mL), dried over $MgSO_4$, and filtered before the organic solvent was removed under reduced pressure. The crude products were either purified by flash chromatography (silica, EtOAc/*n*-pentane) to afford the pure alcohols, or the crude product was analysed by chiral GLC or chiral HPLC.

*Asymmetric synthesis of (R)-1-(4-amino-3,5-dichlorophenyl)-2-bromoethan-1-ol, (R)-**2a***

1-(4-Amino-3,5-dichlorophenyl)-2-bromoethan-1-one (**2**) (28.6 mg, 0.10 mmol) in DMSO (0.10 mL), KRED 228 (10.4 mg), G6P (77.7 mg), G6P-DH (0.1 mL, 20 U) and NADPH (0.3 mg) in pH 7.0 phosphate buffer (0.90 mL). Reaction time: 4 weeks, (*R*)-**2a** was obtained in 93% *ee*. GLC of rac-**2**: t_R (*R*) = 25.363 min, t_R (*S*) = 25.636 min, R_S = 1.9.

*Asymmetric synthesis of (S)-N-(4-acetyl-phenyl)acetamide, (S)-**5a***

N-(4-Acetylphenyl)acetamide (**5**) (20.3 mg, 0.11 mmol) in DMSO (0.10 mL), KRED 228 (7.2 mg), G6P (94.1 mg), G6P-DH (0.1 mL, 20 U) and NADPH (0.3 mg) in pH 7.0 phosphate buffer (0.90 mL) was incubated for 2 weeks. After flash chromatography, (100% EtOAc,), (*S*)-**5a** was obtained in 45% isolated yield, (8.8 mg, 0.05 mmol), 96% purity, 73% *ee*. R_f (*S*)-**5** = 0.42 (EtOAc). HPLC of rac-**5**: t_R (*R*) = 84.474 min, t_R (*S*) = 91.765 min, R_S =1.56. $[\alpha]_D^{20}$= 0.11 (c 0.88, EtOH). ^{1}H NMR (600 MHz, CD3OD): δ 7.50-7.49 (d, 2H, H_{arom}, $^3J_{HH}$ = 8.4 Hz), 7.31-7.30 (d, 2H, H_{arom}, $^3J_{HH}$ = 8.4 Hz), 4.80-4.77 (q, 1H, HO-C**H**, $^3J_{HH}$ = 6.5 Hz), 2.11 (s, 3H, CO-C**H**$_3$), 1.42-1.41 (d, 3H, CH-C**H**$_3$, $^3J_{HH}$ = 6.5 Hz) ^{13}C NMR (600 MHz, MeOD): δ 171.6 (CO), 143.4 (C), 138.8 (C), 126.9 (CH_{arom}), 121.1 (CH_{arom}), 70.5 (CH), 25.4 (CH_3), 23.7 (CH_3). MS (TOF-ASAP): $[M+H]^+$ 180.1025 *m/z*.

*Asymmetric synthesis of (S)-N-(2,6-dichloro-4-(1-hydroxyethyl)phenyl)acetamide, (S)-**6a***

N-(4-Acetyl-2,6-dichlorophenyl)acetamide (**6**) (32.1 mg, 0.13 mmol) in DMSO (0.10 mL), KRED 228 (8.1 mg), G6P (74.4 mg, 0.286 mmol), G6P-DH (0.1 mL, 20 U) and NADPH (0.2 mg) in phosphate buffer (pH 7.0, 0.90 mL). Reaction time 48 h, flash chromatography (1:2 *n*-pentane/EtOAc), isolated yield (*S*)-**6a**: 31% yield (10.1 mg, 0.04 mmol), 99% purity, >98% *ee*. GLC of *rac*-**6**: t_R (*S*)-**6a** = 44.46 min, t_R (*R*)-**6a** = 45.25 min, R_S = 2.05. (*S*)-**6a** $[\alpha]_D^{20}$= −0.21 (c 1.01, EtOH). ^{1}H NMR (600 MHz, MeOD,): δ 7.45 (s, 2H, H_{arom}), 4.82-4.79 (q, 1H, C**H**-OH, $^3J_{HH}$ = 6.3 Hz), 2.17 (s, 3H, CO-C**H**$_3$), 1.42-1.41 (d, 3H, CH(OH)-C**H**$_3$, $^3J_{HH}$ = 6.3 Hz). ^{13}C NMR (100 MHz MeOD,): δ 172 (CO), 150 (C), 135.2 (C), 132.2 (C), 126.5 (CH_{Ar}), 69.4 (CH), 25.4 (CH_3), 22.3 (CH_3). MS: (ASAP-TOF) $[M+H]^+$ 248.0245 *m/z*.

*Asymmetric synthesis of (R)-N-(4-(2-bromo-1-hydroxyethyl)phenyl)acetamide, (R)-**7a***

N-(4-(2-Bromoacetyl)phenyl)acetamide (**7**) (27.1 mg, 0.11 mmol) in DMSO (0.10 mL), KRED 228 (8.7 mg), G6P (82.9 mg, 0,319 mmol), G6P-DH (0.1 mL, 20 U) and NADPH (0.4 mg) in phosphate buffer (pH 7.0, 0.90 mL). Two days reaction time, flash chromatography (100% EtOAc), R_f (*R*)-**7a** = 0.57, isolated yield: 27% (7.3 mg, 0.03 mmol), 98% purity, 35% *ee*. HPLC of rac-**7**: t_R (*R*) = 14.037 min, t_R (*S*) = 17.035 min, R_S = 2.55. $[\alpha]_D^{20}$= +0.07 (c 0.73, EtOH). ^{1}H NMR (600 MHz, CD_3OD): δ 7.56-7.55 (m, 2H, H_{arom}, $^3J_{HH}$ = 8.7 Hz), 7.36-7.35 (m, 2H, H_{arom}, $^3J_{HH}$ = 8.6 Hz), 4.85-4.83 (m, 1H, HO-C**H**, $^3J_{HH}$ = 4.6 Hz, $^3J_{HH}$ = 7.7 Hz), 3.62-3.60 (dd, 1H, C**H**-Br, $^3J_{HH}$ = 4.4 Hz, $^2J_{HH}$ = 10.5 Hz), 3.56-3.53 (dd, 1H, C**H**-Br, $^3J_{HH}$ = 7.7 Hz, $^2J_{HH}$ = 10.5 Hz), 2.13 (s, 3H, CO-C**H**$_3$). ^{13}C NMR (600 MHz, CD_3OD): δ 170.2, 138.2, 137.6, 126.4, 119.6, 73.1, 37.8, 22.4. MS (TOF-ASAP): $[M+H]^+$ 258.0130 *m/z*.

5. Conclusions

Asymmetrisation of ketones **2** and **6** with KRED 228 were the only asymmetrisations in the series of ketones **1**-**2** and **4**-**7** that produced the respective alcohol with high enantiomeric excess, giving (*R*)-**2a** in 93% *ee* (low yield) and (*S*)-**6a** in 98% *ee* and 31% yield. (*R*)-**2a** may be reacted with *t*-butylamine yielding (*R*)-clenbuterol in high enantiomeric excess. (*S*)-**6a** may be a precursor for important enantiopure compounds.

The absolute configuration of the alcohols was determined by comparing elution orders on GLC with similar compounds. KRED 228 from Syncozymes Co., Ltd. was found to be a suitable enzyme for the reduction of substitued arylketones with several substituents on the benzene ring, however when an

α-halogen is present the enzyme is not efficient. In addition, protection of the amino group on the benzene ring seems to be cruicial.

Kinetic resolutions of the racemic bromo alcohol **3** with CALA and CALB were not successful. The transesterification reactions showed low reactivity and low stereoselectivity with both lipases. Due to the excellent selectivity of the CALB catalysed kinetic resolution of propargyl alcohol **8** with vinyl butanoate in hexane it was anticipated that both the amino and the chloro substituents in *meta*-positions of **3** cause the problem. Both steric hindrance and electronic effects of these substituents leading to an irreversible substrate-enzyme complex might be the reason for the low conversion of the two lipases.

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