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Preparation of enantiomerically enriched aromatic β-hydroxynitriles and halohydrins by ketone reduction with recombinant ketoreductase KRED1-Pglu

Martina L. Contente [a], Immacolata Serra [a], Francesco Molinari [a], Raffaella Gandolfi [b], Andrea Pinto [b,*], Diego Romano [a,*]

[a] Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Via Mangiagalli 25, 20133 Milan, Italy
[b] Department of Pharmaceutical Sciences (DISFARM), University of Milan, Via Mangiagalli 25, 20133 Milan, Italy

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ABSTRACT

A NADPH-dependent benzil reductase (KRED1-Pglu) was used as recombinant enzyme for catalysing the reduction of different functionalised ketones. The reactions were carried out in the presence of a catalytic amount of $NADP^+$ and an enzyme-coupled transformation (oxidation of glucose catalysed by glucose dehydrogenase), for regenerating the cofactor and thus driving the reaction to completion. KRED1-Pglu showed remarkable versatility, being able to reduce different β-ketonitriles and α-haloketones at different pHs; notably, depending on the nature of the substrate, KRED1-Pglu can be used for efficient and clean enzymatic reduction, avoiding side-reactions due to the pH of the medium. The reduction generally occurred with high enantioselectivity, allowing the preparation of enantiomerically enriched β-hydroxynitriles and halohydrins in high yields; the stereochemical outcome of the reduction followed in all the cases the so-called Prelog's rule.

1. Introduction

Asymmetric hydrogenation of functionalized aromatic ketones for preparing optically active secondary alcohols is widely used in the production of drugs.[1] Biocatalysis offers an efficient alternative to conventional chemical methods, ever since enzymes can be produced in large amounts as recombinant proteins and improved by enzyme engineering.[2] Ketoreductases (KREDs), sometimes also described as carbonyl reductases (CR) or alcohol dehydrogenases (ADH), are known to efficiently catalyse the enantiospecific delivery of a hydride to prostereogenic carbonyls, thus producing the desired optically pure alcohols.[3–6]

Stereoselective reduction of aromatic ketones functionalized with β-cyano or β-halo groups are of particular interest, since the corresponding chiral β-hydroxynitriles[7–9] and halohydrins[10,11] (β-halo alcohols) are key-intermediates in the synthesis of a number of biologically active compounds.

The most popular example of the utilization of optically pure β-hydroxynitriles is the synthesis of serotonin/norepinephrine reuptake inhibitors for the treatment of depressive or sleep disorders, anxiety, alcoholism, chronic pain, obesity and anorexia or bulimia.[9,12] Although reduction of aromatic β-ketonitriles can be obtained by chemical chiral catalysts,[13,14] biocatalysis has proved to be an efficient and green alternative; biocatalytic reduction is mostly performed with isolated enzymes,[15–18] since the use of whole cells is often limited by the occurrence of a—competitive ethylation, resulting from non-enzymatic aldol condensation between aromatic β-ketonitriles and acetaldehyde (formed by the whole cells in the presence of glucose or ethanol), followed by reduction of the activated C=C by enoate reductases.[19–23]

Optically pure aromatic halohydrins are versatile synthons that have been used for the synthesis of many pharmaceuticals, including drugs for psychiatric disorders, such as (*R*)-Fluoxetine,[24–27] (*R*)-Duloxetine,[27,28] or (*R*)-Ibutilide, an antiarrhythmic agent.[29] Preparation of aromatic halohydrins by enzymatic reduction of the corresponding aromatic α-haloketones is also a valid option. Both microbial cells[30–34] and isolated enzymes[10,35–37] have proven effective, although sometimes low yields are observed due to

* Corresponding authors. Tel.: +39 0250319323 (A.P.); tel.: +39 0250319134 (D.R); e-mail addresses: andrea.pinto@unimi.it (A. Pinto), diego.romano@unimi.it (D. Romano).

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possible side-reactions, with formation of 1-phenylethanol (as result of reductive dehalogenation, followed by reduction) and 1-phenylethane-1,2-diol (as result of substitution and reduction).[34,38] Reductive dehalogenation has been detected using whole microbial cells as an effect of unwanted enzymatic reactions,[38] whereas substitution can be avoided by tuning the pH of the biotransformation (i.e., slightly acidic conditions).[34]

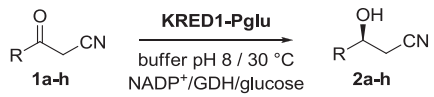
Whole cells of the non-conventional yeast *Pichia glucozyma* CBS 5766 have been thoroughly exploited for the reduction of aromatic ketones, often showing good yields and enantioselectivity.[39–43] A versatile benzil ketoreductase (KRED1-Pglu), with high activity and enantioselectivity towards various functionalized aromatic ketones,[44–46] was identified in the genome of *P. glucozyma* and expressed in *Escherichia coli*.[44] In this work we investigated the reduction of aromatic β-ketonitriles and α-haloketones catalysed by recombinant KRED1-Pglu for checking its potential as stereoselective biocatalyst.

2. Results and discussion

2.1. Reduction of aromatic β-ketonitriles

The recombinant ketoreductase KRED1-Pglu was firstly used to asymmetrically reduce different aryl β-ketonitriles. Biotransformations were performed at 0.25 mmol-scale, in the presence of a catalytic amount of $NADP^+$ and an enzyme-coupled system (glucose/glucose dehydrogenase, *Bm*GDH) for the regeneration of the cofactor. Yield and stereoselectivity were determined after purification of products (Table 1). Reduction followed the Prelog rule,[47] giving the corresponding (*S*)-β-hydroxy nitrile; all the substrates were reduced affording the desired alcohols with high molar conversions and excellent enantiomeric excess after 24 h.

Table 1
Reduction of aromatic β-ketonitriles with KRED1-Pglu

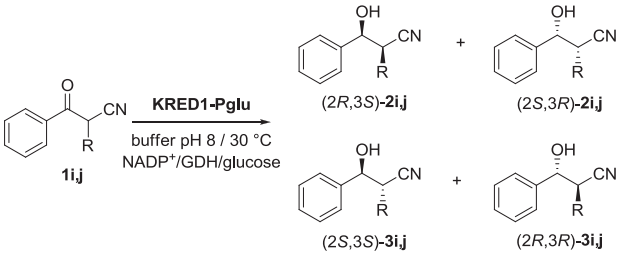


Entry	Substrate	Ar	Yield (%)[a]	ee (%)[b,c]
1	**1a**	Ph	95	>98
2	**1b**	2-Fur	95	>98
3	**1c**	2-Th	94	>98
4	**1d**	4-CH_3Ph	93	>98
5	**1e**	4-OCH_3Ph	70	97
6	**1f**	4-FPh	90	95
7	**1g**	3-CH_3Ph	90	95
9	**1h**	3-ClPh	85	94

[a] Reaction conditions: 10 mM substrate, 0.1 mM $NADP^+$. KRED1-Pglu (20 mU/mL), GDH (1 U/mL), glucose 40 mM in Tris/HCl buffer pH 8.0 (0.05 M, pH 8.0). Reaction time 24 h. Yield and enantioselectivity determined after purification of the product.
[b] Determined by chiral HPLC analysis.
[c] Absolute configuration was assigned by comparison of the specific rotations with the literature values.[22]

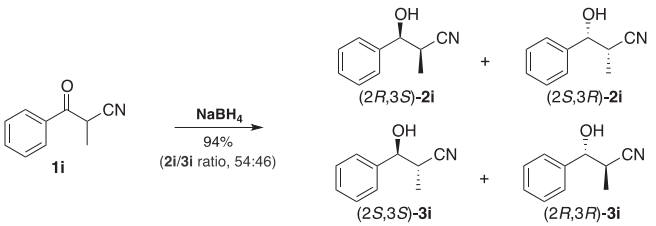
The results observed with α-unsubstituted aromatic β-ketonitriles led us to investigate more hindered substrates such as 2-methyl-3-oxo-3-phenylpropanenitrile **1i** and 2-ethyl-3-oxo-3-phenylpropanenitrile **1j** (Table 2). Since no detailed stereochemical characterization of the reduction products of **1i** has been reported in the literature, compound **1i** was reduced with $NaBH_4$ and the four stereoisomers obtained were isolated and fully characterized (Scheme 1).

Table 2
Reduction of 2-alkyl-3-oxo-3-phenylpropanenitriles with KRED1-Pglu



Entry	Substrate	R	Yield (%)[a]	ee (%)	de (%)[b]
1	**1i**	Me	95	>98 (2*R*,3*S*)[c]	>98 (2*R*,3*S*)
2	**1j**	Et	90	66 (2*S*,3*S*)[d]	46 (2*S*,3*S*)

[a] Reaction conditions: 10 mM substrate, 0.1 mM $NADP^+$, KRED1-Pglu (20 mU/mL), GDH (1 U/mL), glucose 40 mM in Tris/HCl buffer pH 8.0 (0.05 M, pH 8.0). Reaction time 24 h. Yield and enantioselectivity determined after purification of the product.
[b] Determined by HPLC analysis.
[c] Absolute configuration was assigned by a comparative study of its MTPA derivatives (see Experimental section).
[d] Absolute configuration was assigned by comparison of the specific rotations with the literature values (see Experimental section).

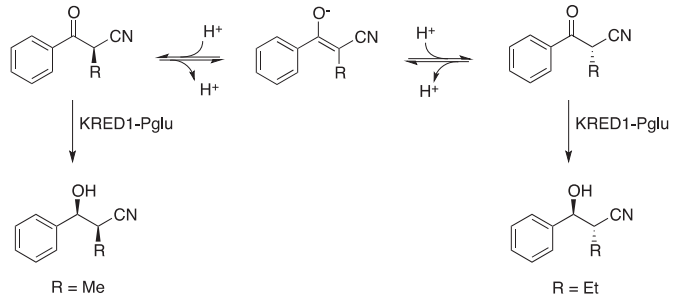


Scheme 1. Reduction of **1i** using $NaBH_4$.

Firstly, *syn* racemate (±)-**2i** was separated from the *anti* racemate (±)-**3i** by semi-preparative silica HPLC and their relative configurations were assigned by comparison of the ^1H NMR coupling constants with the ones already reported in the literature.[48] The racemates (±)-**2i** and (±)-**3i** were, in turn, independently resolved into their pure enantiomers by semi-preparative chiral HPLC (see Experimental section). The absolute configuration of each enantiomer was assigned by Mosher's ester derivatization (see Experimental section).

Enzymatic reduction of **1i** and **1j** took place with high yields, but with different stereoselectivity (Table 2).

The acidity of the α-hydrogen causes spontaneous racemisation of the substrate even at pH 8.0, thus favouring a dynamic resolution process (Scheme 2).[49] Reduction of **1i** proceeded with outstanding stereoselectivity, furnishing *syn* (2*R*,3*S*)-**2i** as the only detectable stereoisomer with 95% yield after 24 h. α-Ethyl derivative **1j** was reduced with low stereoselectivity and formation of the *anti* stereoisomer (2*S*, 3*S*)-**3j** as the main product. The different stereochemical outcome is due to a different enzymatic recognition of the enantiomers of the substrates, depending on the nature of the α-substituent. Namely, reduction of α-methyl substituted **1i** occurs on the *R*-enantiomer furnishing (2*R*,3*S*)-**2i**, whereas (*S*)-**1j** is the preferred substrate, giving (2*S*,3*S*)-**3j**, although with moderate overall stereoselectivity (Scheme 2).

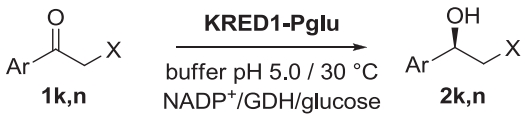


Scheme 2. Enzymatic reduction of α-alkyl-β-ketonitriles with dynamic resolution caused by racemisation of the substrate.

2.2. Reduction of aromatic α-haloketones

The substrate scope of KRED1-Pglu was also studied using the 2-halo-1-arylethanones **1k**–**l** (Table 3). The biotransformation carried out at pH 8.0 furnished low yields. Previous reports show that enzymatic reduction of 2-halo-1-phenylethanones **1k** and **1l** proceeded with high yields only at lower pH, since side-reactions like halo substitution may occur at higher pHs.[34] Thus, the biotransformations were carried out in buffers at lower pH; a Na-acetate buffer (pH 5.0, 0.1 M) proved suited for achieving (*R*)-**2k** and (*R*)-**2l** with high enantioselectivity and excellent yields (Table 3); good results were also observed with 2-thienyl derivatives **1m** and **1n**. It should be remarked that formation of aromatic halohydrins with *R*-configuration follows Prelog rule (as observed in all the reductions carried out in this study), and the *R*-configuration of the products is merely due to different CIP priority.

Table 3
Reduction of 2-halo-1-arylethanones with KRED1-Pglu at pH 5.0



Entry	Substrate	Ar	X	Yield (%)[a]	ee (%)[b,c]
1	**1k**	Ph	Br	95	>98
2	**1l**	Ph	Cl	94	95
3	**1m**	2-Th	Br	92	90
4	**1n**	2-Th	Cl	87	92

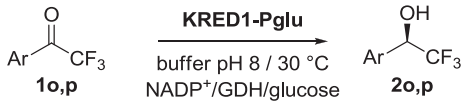
[a] Reaction conditions: 10 mM substrate, 0.1 mM $NADP^+$, KRED1-Pglu (20 mU/mL), GDH (1 U/mL), glucose 40 mM in acetate buffer (0.1 M, pH 5.0). Reaction time 24 h. Yield and enantioselectivity determined after purification of the product.
[b] Determined by chiral HPLC analysis.
[c] Absolute configuration was assigned by comparison of the specific rotations with the literature values.[27,51,52]

Finally, 2,2,2-trifluoro-1-phenylethanone (**1o**) and 2,2,2-trifluoro-1-(thiophen-2-yl)ethanone (**1p**) were also tested (Table 4), showing high conversion and enantioselectivity. Again the reduction of the compound **1o** gave the corresponding (*R*)-alcohol because of the different CIP priority of the $-CF_3$ group.

3. Conclusion

The recombinant ketoreductase KRED1-Pglu isolated from the genome of the non-conventional yeast *P. glucozyma* CBS 5766 proved to be a versatile biocatalyst for the asymmetric reduction of aromatic functionalised aromatic ketones. The versatility and enantioselectivity of KRED1-Pglu is noteworthy, since different β-ketonitriles and α-haloketones are reduced to the corresponding secondary alcohols with high enantiomeric excesses. Moreover, the possibility to use this enzyme at different pHs allowed for efficient reduction of substrates whose reactivity strongly depends on the acidity of the medium. Previously poorly studied substrates, such as 2-alkyl-3-oxo-3-phenylpropanenitriles **1i**–**j**, were reduced at pH 8.0 with concurrent racemization of the α-stereocenter, thus allowing for the preferential obtainment of (2*R*,3*S*)-**2i-j** as the major stereoisomer produced. Reduction of 2-halo-1-arylethanones **1-k**–**l** could be carried out at pH 5.0, avoiding side-reactions. The use of a cheap and easy-to-use system for co-factor regeneration (glucose dehydrogenase/glucose) allowed for high yields in all the biotransformations studied. Enzymatic reduction occurred with excellent enantioselectivity with all the substrates, with the exception of **1j**, for which the occurrence of an α-ethyl group limits the enantioselectivity and stereoselectivity.

Table 4
Reduction of 2,2,2-trifluoro-1-arylethanones with KRED1-Pglu



Entry	Substrate	R	Yield (%)[a]	ee (%)[b,c]
1	**1o**	Ph	94	>98 (*R*)
2	**1p**	2-Th	95	>98 (*S*)

[a] Reaction conditions: 10 mM substrate, 0.1 mM $NADP^+$, KRED1-Pglu (20 mU/mL), GDH (1 U/mL), glucose 40 mM in Tris/HCl buffer pH 8.0 (0.05 M, pH 8.0). Reaction time 24 h. Yield and enantioselectivity determined after purification of the product.
[b] Determined by chiral HPLC analysis.
[c] Absolute configuration was assigned by comparison of the specific rotations with the literature values.[53,54]

4. Experimental part

4.1. General procedures

All reagents and solvents were obtained from commercial suppliers and used without further purification. Merck SilicaGel 60 F254plates were used for analytical TLC; ^1H and ^{13}C NMR spectra were recorded on a Varian-Gemini 200 spectrometer. Flash column chromatography was performed on Merck SilicaGel (200–400 mesh). ^1H and ^{13}C chemical shifts are expressed in (ppm) and coupling constants (*J*) in Hertz (Hz). Rotary power determinations were carried out using a Jasco P-1010 spectropolarimeter, coupled with a Haake N3-B thermostat. Elemental analyses were carried out on a Carlo Erba Model 1106 (Elemental Analyzer for C, H, and N), and the obtained results are within 0.4% of theoretical values. HPLC analyses were performed with a Jasco PU-980 pump equipped with a UV/Vis detector Jasco UV-975 and a OJ-H column (4.6×250 mm, Daicel), OD-H column (4.6×250 mm, Daicel) or Lux Amylose-2 column (4.6×150 mm, Phenomenex). Preparative HPLC was performed with a 1525 Extended Flow Binary HPLC pump equipped with a Waters 2489 UV/Vis detector and Luna 5 μm C18 (2) 100 Å column (21.2×220 mm; Phenomenex), Lux Amylose-2 column (21.2×220 mm, 5 μm, Phenomenex) or Kromasil 5-AmyCoat column (21.2×250 mm, AkzoNobel) at a flow rate of 15 mL/min.

4.2. Preparation of pure KRED1-Pglu

KRED1-Pglu was prepared as recombinant protein in *E. coli*.[25] KRED1-Pglu gene was amplified from the genomic DNA of *P. glucozyma* by PCR using the following primers, carrying *Nde*I and *Hind*III restriction sites:

Forward: 5′-ATAC**CATATG**ACGAAGGTGACTGTTGTGAC-3′
Reverse: 5′-AGAG**AAGCTT**GGCGTACTCCTTCAACTCTG-3′

The amplified gene was then cloned into a pET26b(+) vector using the *Nde*I and *Hind*III restriction sites. With this cloning strategy, the resulting protein is expressed with a terminal His_6 tag.

The correct construction of the expression plasmid was confirmed by direct sequencing.

Cultures of *E. coli* BL21(DE3)Star transformed with the resulting plasmid were grown overnight at 37 °C in LB medium supplemented with 25 μg/mL kanamycin. The seed culture was then diluted into a stirred fermenter containing 4.0 L of cultivation medium (Terrific Broth, 12 g/L bacto-tryptone, 24 g/L yeast extract, 4 g/L glycerol, 2.3 g/L KH_2PO_4, 9.4 g/L K_2HPO_4, pH 7.2) to an initial OD_{600nm} of 0.05. Cultivation was carried out in batch-mode at 37 °C, 250 rpm stirring and 250 L/h aeration rate. Cells were grown until OD_{600nm} reached the value of 0.8. The cultures were induced for 20 h with IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.5 mM. Cells were then harvested by centrifugation at 4500 rpm for 30 min, washed once with 20 mM phosphate buffer at pH 7.0 and stored at −20 °C. Protein purification was carried out with cells suspended in 50 mM Tris−HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0 buffer. Proteins were extracted by sonication (5 cycles of 30 s each, in ice, with 1 min interval) and cell debris were harvested by centrifugation at 15,000 rpm for 30 min at 4 °C. The enzyme was purified by affinity chromatography with HIS-Select® Nickel Affinity Gel. Briefly, the column was equilibrated with 50 mM Tris−HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0 and the crude extract loaded; column was then washed with 50 mM Tris−HCl, 100 mM NaCl, 6 mM imidazole; finally, the adsorbed enzyme was eluted with 50 mM Tris−HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0.

4.3. General method for the enzymatic reduction

The enzymatic reaction was performed biotransformations at 25 mL-scale, using an enzyme-coupled system (glucose and glucose dehydrogenase from *Bacillus megaterium*) for cofactor recycling. Biotransformations were carried out by addition of 10 mM substrate dissolved in 250 μL of DMSO, 0.1 mM $NADP^+$, KRED1-Pglu (20 mU/mL), GDH (1 U/mL), glucose 40 mM in Tris/HCl buffer pH 8.0 (0.05 M, 25 mL) at 30 °C. Biotransformations of **1k**−**n** were also performed also in acetate buffer (NaAB 0.1 M, pH 5.0). The reaction mixture was kept under stirring at 30 °C until completion and then extracted with 20 mL of EtOAc; the aqueous phase was extracted twice more with 15 mL of EtOAc. The organic phases were collected and dried over Na_2SO_4 and the solvent was evaporated. The crude residue was purified by flash chromatography.

*4.3.1. (S)-3-Hydroxy-3-phenylpropanenitrile (**2a**).* Oil. R_f=0.62 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.74 (d, *J*=6.1 Hz, 2H), 5.01 (t, *J*=6.1 Hz, 1H), 7.36−7.39 (m, 5H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ=27.9, 70.0, 117.4, 125.5, 128.8, 128.9, 141.1 ppm. Elemental analysis: calcd (%) for C_9H_9ON (147.07): C 73.45, H 6.16 N 9.52; found: C 73.41; H 6.16, N 9.45. The ee was measured by chiral HPLC on an OJ-H column (*n*-hexane/2-propanol=90:10, 1.0 mL/min, 220 nm), *t*(*S*)=24.5, *t*(*R*)=31.1 min; $[\alpha]_D^{20}$=−58.5 (*c*=1.0, EtOH) [lit. $[\alpha]_D^{20}$=−57.7 (*c*=2.6, EtOH)].[22]

*4.3.2. (S)-3-(2-Furyl)-3-hydroxypropanenitrile (**2b**).* Oil. R_f=0.49 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.57 (s, br s, OH), 2.88 (d, *J*=6.2 Hz, 2H), 5.05 (t, *J*=6.2 Hz, 1H), 6.37−6.41 (m, 2H), 7.41−7.42 (m, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ=24.9, 63.8, 107.5, 110.6, 116.9, 142.9, 152.8 ppm. Elemental analysis: calcd (%) for $C_7H_7ON_2$ (137.05): C 61.31; H 5.14, N 10.21; found: C 61.24; H 5.52, N 10.58. The ee was measured by chiral HPLC on an OJ-H column (*n*-hexane/2-propanol=90:10, 1.0 mL/min, 220 nm), t(*S*)=21.9, t(*R*)=25.6 min; $[\alpha]_D^{20}$=−40.2 (*c*=1.0, EtOH) [lit. $[\alpha]_D^{20}$=−38.6 (*c*=1.3, $CHCl_3$)].[22]

*4.3.3. (S)-3-(2-Thieyl)-3-hydroxypropanenitrile (**2c**).* Oil. R_f=0.52 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.85 (d, *J*=6.2 Hz, 2H), 3.04 (s, br s, OH), 5.26 (t, *J*=6.2 Hz, 1H), 6.98−7.00 (m, 2H), 7.06−7.07 (d, *J*=3.5 Hz, 1H), 7.29−7.32 (m, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ=28.2, 66.2, 117.1, 124.7, 125.7, 127.1, 144.5 ppm. Elemental analysis: calcd (%) for C_7H_7ONS (153.02): C 54.88; H 4.61, N 9.14; found: C 54.77; H 4.96, N 8.77. The ee was measured by chiral HPLC on an OJ-H column (*n*-hexane/2-propanol=90:10, 1.0 mL/min, 220 nm), *t*(*S*)=27.0, *t*(*R*)=32.3 min; $[\alpha]_D^{28}$=−19.4 (*c*=1.0, $CHCl_3$) [lit. $[\alpha]_D^{28}$=−16.71 (*c*=1.01, $CHCl_3$)].[50]

*4.3.4. (S)-3-Hydroxy-3-(p-tolyl)propanenitrile (**2d**).* Oil. R_f=0.58 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.32 (s, 3H), 2.74 (d, *J*=6.2 Hz, 2H), 2.12 (s, br s, OH), 4.94 (t, *J*=6.2 Hz, 1H), 7.19 (d, *J*=7.9 Hz, 2H), 7.27 (d, *J*=7.9 Hz, 2H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ=21.1, 27.9, 69.4, 117.5, 125.5, 129.4, 138.3, 138.4 ppm. Elemental analysis: calcd (%) for $C_{10}H_{11}ON$ (161.08): C 74.51; H 6.88, N 8.69; found: C 74.24; H 7.21, N 8.66. The ee was measured by chiral HPLC on an OJ-H column (*n*-hexane/2-propanol=90:10, 1.0 mL/min, 220 nm), *t*(*S*)=19.1, *t*(*R*)=22.3 min; $[\alpha]_D^{29}$=−58.3 (*c*=1.0, $CHCl_3$) [lit. $[\alpha]_D^{28}$=−55.68 (*c*=0.85, $CHCl_3$)].[50]

*4.3.5. (S)-3-Hydroxy-3-(4-methoxyphenyl)propanenitrile (**2e**).* Oil. R_f=0.50 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.56 (s, br s, OH), 2.71−2.74 (m, 2H), 3.80 (s, 3H), 4.96 (t, *J*=6.2, 1H), 6.90 (m, 2H), 7.30 (m, 2H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=27.9, 55.3, 69.7, 114.3, 117.5, 126.9, 133.2, 159.9 ppm. Elemental analysis: calcd (%) for $C_{10}H_{11}O_2N$ (177.08): C 67.68; H 6.26, N 7.90; found: C 66.89; H 6.44, N 7.67. The ee was measured by chiral HPLC on an OJ-H column (*n*-hexane/2-propanol=90:10, 1.0 mL/min, 220 nm), *t*(*S*)=43.7, *t*(*R*)=45.2 min; $[\alpha]_D^{28}$=−50.5 (*c*=1.0, EtOH) [lit. $[\alpha]_D^{28}$=−41.34 (*c*=0.55, EtOH)].[50]

*4.3.6. (S)-3-Hydroxy-3-(4-fluorophenyl)propanenitrile (**2f**).* Oil. R_f=0.50 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.70 (d, *J*=6.2 Hz, 2H), 2.97 (s, br s, 1H, OH), 4.99 (t, *J*=6.2 Hz, 1H), 7.02−7.08 (m, 2H), 7.32−7.37 (m, 2H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ=28.0, 69.2, 115.8 (d, *J*=21.9 Hz), 117.3, 127.4 (d, *J*=8.1 Hz), 136.8 (d, *J*=2.3 Hz), 162.7 (d, *J*=246.4 Hz) ppm. Elemental analysis: calcd (%) for C_9H_8ONF (165.06): C 65.45; H 4.88, N 8.48; found: C 65.26; H 5.19, N 8.01. The ee was measured by chiral HPLC on an OJ-H column (*n*-hexane/2-propanol=90:10, 1.0 mL/min, 220 nm), *t*(*S*)=20.4, *t*(*R*)=24.8 min; $[\alpha]_D^{24}$=−35.5 (*c*=1.0, $CHCl_3$) [lit. $[\alpha]_D^{24}$=−34.83 (*c*=0.9, $CHCl_3$)].[50]

*4.3.7. (S)-3-Hydroxy-3-(m-tolyl)propanenitrile (**2g**).* Oil. R_f=0.58 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.36 (s, 3H), 2.71 (d, *J*=6.2 Hz, 2H), 2.85 (s, br s, OH), 4.95 (t, *J*=6.2, 1H), 7.13−7.19 (m, 3H), 7.24−7.29 (m, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=21.4, 27.9, 70.0, 117.5, 122.6, 126.2, 128.8, 129.5, 138.6, 141.1 ppm. Elemental analysis: calcd (%) for $C_{10}H_{11}ON$ (161.08): C 74.51; H 6.88, N 8.69; found: C 74.24; H 7.21, N 8.66. The ee was measured by chiral HPLC on an OJ-H column (*n*-hexane/2-propanol=90:10, 1.0 mL/min, 220 nm), *t*(*S*)=18.9, *t*(*R*)=23.1 min; $[\alpha]_D^{20}$=−62.5 (*c*=1.0, $CHCl_3$) [lit. $[\alpha]_D^{20}$=−59.3 (*c*=0.8, $CHCl_3$)].[22]

*4.3.8. (S)-3-Hydroxy-3-(3-chlorophenyl)propanenitrile (**2h**).* Oil. R_f=0.53 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.72 (d, *J*=6.2 Hz, 2H), 3.09 (s, br s, OH), 4.98 (t, *J*=6.2, 1H), 7.25−7.28 (m, 1H), 7.29−7.30 (m, 2H), 7.31−7.37 (m, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=27.9, 69.2, 117.2, 123.8, 125.8, 128.8, 130.2, 134.8, 143.2 ppm. Elemental analysis: calcd (%) for C_9H_8ONCl (181.03): C 59.52; H 4.44, N 7.71; found: C 59.65, H 4.74, N 7.32. The ee was measured by chiral HPLC on an OJ-H column (*n*-hexane/2-

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propanol=90:10, 1.0 mL/min, 220 nm), $t(S)$=20.0, $t(R)$=23.1 min; $[\alpha]_D^{20}$=−55.5 (c=1.0, $CHCl_3$) [lit. $[\alpha]_D^{20}$=−56.8 (c=1.3, $CHCl_3$)].[22]

*4.3.9. (2R,3S)-3-Hydroxy-2-methyl-3-phenylpropanenitrile [(2R,3S)-**2i**].* Oil. R_f=0.58 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.25 (d, J=6.6 Hz, 3H), 2.93 (dq, J=6.6, 6.6 Hz, 1H), 4.73 (d, J=6.6 Hz, 1H), 7.26–7.44 (m, 5H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=14.7, 34.6, 75.1, 121.1, 126.3, 128.8, 140.1 ppm. Elemental analysis: calcd (%) for $C_{10}H_{11}ON$ (161.08) C 74.51, H 6.88, N 8.69; found: C 74.65, H 7.22, N 8.46. The ee and de were measured by chiral HPLC on Lux Amylose-2 column (*n*-hexane/isopropanol=95:5, 1.0 mL/min, 216 nm), t(2*R*,3*S*)=16.8 min. $[\alpha]_D^{20}$=−12.0 (c=0.2 in $CHCl_3$).

*4.3.10. (2S,3S)-2-(1-Hydroxy-1-phenylmethyl)butanenitrile [(2S,3S)-**3j**].* Solid; mp 68–70 °C. R_f=0.59 (CH_2Cl_2/*n*-hexane/EtOAc=4:1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.05 (t, J=7.7 Hz, 3H), 1.51–1.70 (m, 2H), 2.76–2.83 (m, 1H), 4.79 (d, J=6.2 Hz, 1H) 7.33–7.56 (m, 5H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=10.4, 24.9, 76.6, 127.0, 128.1, 128.7, 140.7 ppm. Elemental analysis: calcd (%) for $C_{11}H_{13}ON$ (175.10) C 75.40, H 7.48, N 7.99; found: C 75.23, H 7.32, N 7.89. The ee and de were measured by chiral HPLC on OD-H column (*n*-hexane/isopropanol=95:5, 0.8 mL/min, 216 nm), t(2*R*,3*S*)=26.9 min, t(2*S*,3*S*)=28.6 min, t(2*S*,3*R*)=34.2 min, t(2*R*,3*R*)=36.4 min. $[\alpha]_D^{20}$=−32.5 (c=1.0, $CHCl_3$) [lit. $[\alpha]_D^{28}$=−46.4 (c=0.5, $CHCl_3$)].[51]

*4.3.11. (R)-2-Bromo-1-phenylethanol (**2k**).* Oil. R_f=0.57 (*n*-hexane/EtOAc=7:3). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.70 (s, br s, OH), 3.52–3.67 (m, 2H), 4.92 (d, J=8.5 Hz, 1H), 7.38–7.32 (m, 5H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ=40.2, 73.8, 125.9, 128.4, 128.7, 140.3 ppm. Elemental analysis: calcd (%) for C_8H_9OBr (197.97) C 47.79, H 4.51; found: C 47.47, H 4.76. The ee was measured by chiral HPLC on an OD-H column (*n*-hexane/isopropanol=97:3, 1.0 mL/min, 220 nm), $t(S)$=16.9, $t(R)$=19.3 min; $[\alpha]_D^{25}$=−45.5 (c=1.0, $CHCl_3$) [lit.[29] $[\alpha]_D^{20}$=−39.5 (c=0.592, $CHCl_3$)].[52]

*4.3.12. (R)-2-Chloro-1-phenylethanol (**2l**).* Oil. R_f=0.56 (*n*-hexane/EtOAc=7:3). ^{1}H NMR (300 MHz, CDCl3): δ=2.65 (d, J=3.0 Hz, 1H), 3.65 (dd, J=11.0, 8.5 Hz, 1H), 3.76 (dd, J=11.0, 3.0 Hz, 1H), 4.90 (dt, J=8.5, 3.0 Hz, 1H), 7.30–7.40 (m, 5H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 50.9, 74.1, 126.1, 128.5, 140.0 ppm. Elemental analysis: calcd (%) for C_8H_9OCl (156.61) C 61.35, H 5.79; found: C 61.40, H 6.11. The ee was measured by chiral HPLC on an OD-H column (*n*-hexane/isopropanol=97:3, 1.0 mL/min, 220 nm), $t(S)$=18.9, $t(R)$=20.3 min; $[\alpha]_D^{20}$=−48.5 (c=1.0, cyclohexane) [lit. $[\alpha]_D^{20}$=−50.7 (c=0.225, cyclohexane)].[52]

*4.3.13. (R)-2-Bromo-1-thienylethanol (**2m**).* Oil. R_f=0.49 (*n*-hexane/EtOAc=7:3). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.64 (s, br s, OH), 3.66–3.80 (m, 2H), 4.65–4.69 (m, 1H), 6.94–7.01 (m, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 39.5, 67.2, 124.7, 125.4, 126.7, 142.5 ppm. Elemental analysis: calcd (%) for C_6H_7OBrS (205.94) C 34.80, H 3.41; found: C 34.90, H 3.75. The ee was measured by chiral HPLC on an OD-H column (*n*-hexane/isopropanol=98:2, 1.0 mL/min, 220 nm), $t(S)$=29.8, $t(R)$=31.7 min; $[\alpha]_D^{20}$=+30.1 (c=1.0, $CHCl_3$) [lit. $[\alpha]_D^{20}$=−32.3 (c=1.0, $CHCl_3$; *S*-enantiomer)].[27]

*4.3.14. (R)-2-Chloro-1-thienylethanol (**2n**).* Oil. R_f=0.46 (*n*-hexane/EtOAc=7:3). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.75 (s, br s, OH), 3.70–3.83 (m, 2H), 5.12–5.20 (m, 1H), 6.96–7.06 (m, 1H), 7.28–7.33 (m, 2H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ=50.9, 70.7, 125.2, 125.8, 127.3, 143.6 ppm. Elemental analysis: calcd (%) for C_6H_7OClS (161.99) C 44.31, H 4.34; found: C 43.99, H 4.73. The ee was measured by chiral HPLC on an OD-H column (*n*-hexane/isopropanol=98:2, 1.0 mL/min, 220 nm), $t(S)$=30.1, $t(R)$=33.5 min; $[\alpha]_D^{20}$=+32.1 (c=1.0, $CHCl_3$) [lit. $[\alpha]_D^{20}$=+28.5 (c=0.53, $CHCl_3$)].[53]

*4.3.15. (R)-2,2,2-Trifluoro-1-phenylethanol (**2o**).* Oil. R_f=0.48 (*n*-hexane/EtOAc=7:3). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.59 (s, br s, OH), 5.0 (q, J=6.6 Hz, 1H), 7.42–7.47 (m, 5H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 72.6 (q, J=32.3 Hz), 123.9 (q, J=282.2 Hz), 127.4, 128.6, 129.6, 133.9 ppm. Elemental analysis: calcd (%) for $C_8H_7OF_3$ (176.04) C 54.55, H 4.01; found: C 54.17, H 4.20. The ee was measured by chiral HPLC on an OD-H column (*n*-hexane/isopropanol=98:2, 0.8 mL/min, 220 nm), $t(S)$=35.4, $t(R)$=45.2 min; $[\alpha]_D^{21}$=−26.5 (c=1.0, $CHCl_3$) [lit. $[\alpha]_D^{21}$=−20.41 (c=0.48, $CHCl_3$)].[54]

*4.3.16. (S)-2,2,2-Trifluoro-1-thienylethanol (**2p**).* Oil. R_f=0.45 (*n*-hexane/EtOAc=7:3). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.87 (s, br s, OH), 5.26 (q, J=6.15 Hz, 1H), 7.03–7.09 (m, 1H), 7.20 (d, J=3.50), 7.34–7.42 (m, 1H) ppm. ^{1}H NMR (300 MHz, $CDCl_3$): δ=69.5 (q J=34.6 Hz) 123.7 (q, J=282.2 Hz), 127.0, 127.1, 127.5, 136.1 ppm. Elemental analysis: calcd (%) for $C_6H_5OF_3S$ (182.00) C 39.56, H 2.77; found: C 39.49, H 3.10. The ee was measured by chiral HPLC on an OD-H column (*n*-hexane/isopropanol=98:2, 0.8 mL/min, 220 nm), $t(S)$=36.4, $t(R)$=47.2 min; $[\alpha]_D^{25}$=+24.2 (c=1.0, $CHCl_3$) [lit. $[\alpha]_D^{25}$=+27.6 (c=0.88, CH_3OH)].[55]

4.4. Chemical preparation of (2*R**,3*S**)-3-hydroxy-2-methyl-3-phenylpropanenitrile [(±)-2i] and (2*S**,3*S**)-3-hydroxy-2-methyl-3-phenylpropanenitrile [(±)-3i]

To a solution of 2-methyl-3-oxo-3-phenylpropanenitrile **1i** (100 mg, 0.63 mmol) in EtOH (4 mL) was added $NaBH_4$ (6.0 mg, 0.16 mmol) and the reaction mixture was kept under agitation at room temperature. After 1 h, the reaction was evaporated under vacuum and the crude residue was purified by flash chromatography (*n*-hexane/EtOAc 9/1) to give a mixture of (±)-**2i** and (±)-**3i** (95 mg, 0.59 mmol). (±)-**2i** and (±)-**3i** were separated by preparative HPLC under the following conditions: Luna 5 μm C18 (21.2×250 mm, Phenomenex); *n*-hexane/isopropanol=95:5; 15 mL/min; 220 nm. Retention times of (±)-**2i** and (±)-**3i** were 12.5 and 13.5 min, respectively.

(±)-**2i**: ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.25 (d, J=6.6 Hz, 3H), 2.93 (dq, J=6.6, 6.6 Hz, 1H), 4.73 (d, J=6.6 Hz, 1H), 7.26–7.44 (m, 5H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=14.7, 34.6, 75.1, 121.1, 126.3, 128.8, 140.1 ppm.

(±)-**3i**: ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.28 (d, J=6.6 Hz, 3H), 3.02 (dq, J=5.7, 6.6 Hz, 1H), 4.80 (d, J=5.7 Hz, 1H), 7.34–7.43 (m, 5H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=13.5, 34.0, 74.5, 120.8, 126.3, 128.7, 139.6 ppm.

*4.4.1. Preparative chiral HPLC resolution of (±)-**2i**.* Preparative chiral HPLC conditions: Lux Amylose-2 column (21.2×250 mm, Phenomenex); *n*-hexane/isopropanol 9:1; 15 mL/min 220 nm; Retention times of (−)-**2i** and (+)-**2i** were 19.9 and 21.2 min, respectively.

(−)-**2i**: $[\alpha]_D^{20}$=−12.0 (c=0.2 in $CHCl_3$);
(+)-**2i**: $[\alpha]_D^{20}$=+12.0 (c=0.2 in $CHCl_3$).

*4.4.2. Preparative chiral HPLC resolution of (±)-**3i**.* Preparative chiral HPLC conditions: Kromasil 5-AmyCoat column (21.2×250 mm, AkzoNobel); *n*-hexane/isopropanol 95:5; 15 mL/min, 220 nm; Retention times of (+)-**3i** and (−)-**3i** were 18.2 and 22.0 min, respectively.

(+)-**3i**: $[\alpha]_D^{20}$=+18.5 (c=0.2 in $CHCl_3$);
(−)-**3i**: $[\alpha]_D^{20}$=−18.5 (c=0.2 in $CHCl_3$).

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4.4.3. General procedure for the synthesis of Mosher's esters. (*S*)-MTPA (31.5 mg, 0.13 mmol) was added to a solution of the alcohol (20 mg, 0.12 mmol) in anhydrous CH_2Cl_2 at 4 °C. The reaction mixture was kept at 4 °C under stirring and DCC (29.2 mg, 0.14 mmol) and DMAP (4.7 mg, 0.038 mmol) were added; the reaction was left at room temperature for 6 h, then filtered and the filtrate was concentrated under vacuum. The crude residue was purified by flash chromatography (*n*-hexane/EtOAc 9:1).

***(S)*-MTPA ester of (−)-2i**: ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.20 (d, *J*=6.6, 3H), 3.09 (dq, *J*=6.6, 6.6 1H), 3.47 (s, 3H), 6.00 (d, *J*=6.6, 1H), 7.30–7.50 (m, 10H) ppm.

***(S)*-MTPA ester of (+)-2i**: ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.20 (d, *J*=6.6, 3H), 3.09 (dq, *J*=6.6, 6.6, 1H), 3.64 (s, 3H), 5.85 (d, *J*=6.6, 1H), 7.16–7.20 (m, 2H), 7.27–7.41 (m, 8H) ppm.

In (*S*)-MTPA ester of (−)-**2i** the $-OCH_3$ signal (3.48 ppm) is further upfield respect to the $-OCH_3$ signal (3.64 ppm) of (*S*)-MTPA ester of (+)-**2i**. Based upon the already assigned relative configurations of **2i**, these data are in agreement with an absolute configuration of (2*R*,3*S*) for enantiomer (−)-**2i** and (2*S*,3*R*) for enantiomer (+)-**2i**.

***(S)*-MTPA ester of (+)-3i**: ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.29 (d, *J*=6.6, 3H), 3.19 (dq, *J*=5.7, 6.6, 1H), 3.58 (s, 3H), 5.99 (d, *J*=5.7, 1H), 7.22–7.26 (m, 2H), 7.31–7.45 (m, 8H) ppm.

***(S)*-MTPA ester of (−)-3i**: ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.26 (d, *J*=6.6, 3H), 3.17 (dq, *J*=5.7, 6.6, 1H), 3.46 (s, 3H), 5.96 (d, *J*=5.7, 1H), 7.32–7.44 (m, 10H) ppm.

In (*S*)-MTPA ester of (−)-**3i** the $-OCH_3$ signal (3.46 ppm) is further upfield respect to the $-OCH_3$ signal (3.58 ppm) of (*S*)-MTPA ester of (+)-**3i**. Based upon the already assigned relative configurations of **3i**, these data are in agreement with an absolute configuration of (2*S*,3*S*) for enantiomer (−)-**3i** and (2*R*,3*R*) for enantiomer (+)-**3i**.

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