# Supporting Information

# An orthogonal biocatalytic approach for the safe generation and use of HCN in a multi-step continuous preparation of chiral *O*-acetylcyanohydrins.

**Aischarya Brahma[a, b#], Biagia Musio[b#], Uliviya Ismayilova[c], Nikzad Nikbin[b], Sonja B. Kamptmann[b,1], Petra Siegert[c], Günter E. Jeromin[c], Steven V. Ley[b]*, Martina Pohl[a]**

[a] IBG-1: Biotechnology, Forschungszentrum Jülich, 52425 Jülich, Germany.
[b] Department of Chemistry, University of Cambridge, Cambridge, CB21EW, UK.
[c] Institute of Nano- and Biotechnologies (INB), Aachen University of Applied Sciences, 52428 Jülich, Germany.

*svl1000@cam.ac.uk

**Content**

## S1. General

$^{1}$H-NMR spectra were recorded on either Bruker Avance DPX-400 or Bruker Avance DPX-600 spectrometer, as specified, with the residual solvent peak as the internal reference ($CDCl_3$ = 7.26 ppm). $^{1}$H resonances are reported to the nearest 0.01 ppm. $^{13}$C-NMR spectra were recorded on the same spectrometer, as specified, with the central resonance of the solvent peak as the internal reference ($CDCl_3$ = 77.00 ppm). All $^{13}$C resonances are reported to the nearest 0.01 ppm. The multiplicity of $^{1}$H signals are indicated as: s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, t = triplet, q = quadruplet, sext = sextet, m = multiplet, br = broad, or combinations of thereof. Coupling constants (*J*) are quoted in Hz and reported to the nearest 0.1 Hz. Where appropriate, averages of the signals from peaks displaying multiplicity were used to calculate the value of the coupling constant. Infrared spectra were recorded neat on a PerkinElmer Spectrum One FT-IR spectrometer using Universal ATR sampling accessories. The removal of solvent under reduced pressure was carried out on a standard rotary evaporator. High resolution mass spectrometry (HR-MS) was performed using a Waters Micromass LCT Premier™ spectrometer using time of flight with positive ESI, or a Bruker BioApex 47e FTICR spectrometer using (positive or negative) ESI or EI at 70 eV to within a tolerance of 5 ppm of the theoretically calculated value. Melting points were uncorrected. TLC was performed on Merck silica gel plates with F-254 indicator; detection was accomplished by UV light (254 nm), by exposing to I2 vapours and spraying a solution of (5% w/v) ammonium molybdate and 0.2% w/v cerium(III)sulphate in 100 ml 17.6% aq. sulphuric acid and heating to 200 °C for some time until blue spots appear. The reactions (conversions and *ee*) were analysed by a gas chromatography (Agilent Technologies 6890N) equipped with a beta-cyclodextrin column (CP-Chiralsil-Dex CB 25 m, 0.25 mm) and an FID detector. For the determination of enzyme activity, measurements were carried out at 280 nm with a spectrophotometer (Shimadzu UV-1600).

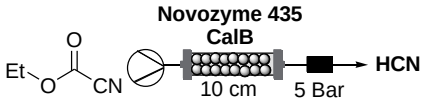
Micro-aqueous MTBE was prepared by mixing pure MTBE and 50 mM potassium phosphate buffer with pH 6.5 (in the ratio 3:1). The mixture was allowed to settle till the phases separate and the organic phase (saturated with the buffer with approx. 10% v/v) was used for all consequent reactions.

In the batch protocols a Wheaton sample glass vial clear, volume 4 mL, screw-cap size, 13 - 425, diam. × H 15 mm × 46 mm.

## S2. General batch procedure for the CalB-catalyzed hydrolysis of ethyl cyanoformate (ECF).

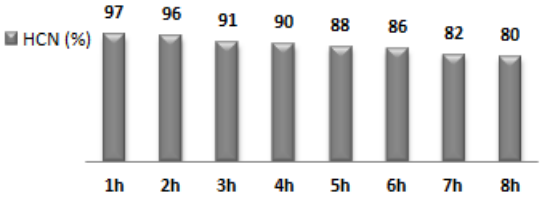
A “tea bag” (1.5 cm x 1 cm) was prepared by sealing three sides of a folded nylon membrane of pore size 43 µm with heat (polystar 100 GE-GS heating unit). The commercially available CalB Novozyme 435 (40 mg) and a small magnetic bar were introduced in and the fourth side was sealed to form a “tea bag” filled with the catalyst. It was introduced in a glass vial containing 1 ml of a 1 M solution of ECF in micro-aqueous MTBE. The vial was closed and a gentle stirring was applied at room temperature for 120 min. The conversion of ECF hydrolysis has been assessed by GC analysis, considering the depletion of ECF. For the calibration curve solutions of ECF in MTBE (0.0 M, 0.25 M, 0.5 M, 0.8 M, 1.0 M) and tetradecane as internal standard (1 M in MTBE) have been used. The following method was applied for the GC analysis: initial temperature 40 ºC, ramp 15 ºC/min to 180 ºC hold for 0 min, post run 180 ºC for 2 min.

## S3. General flow procedure for the CalB-catalyzed hydrolysis of ethyl cyanoformate (ECF).




An omnifit column (Kinesis, Benchmark microbore column 3 MM / 100 MM 2 X F) was loaded with CalB Novozyme 435 (277 mg). It was connected to the pump (Syrris Asia Syringe Pumps, equipped with Asia Blue Syringes of 500 µl / 1 ml)[2] and by PTFE tubing and end fittings. A back pressure regulator (Kinesis, Replacement Cartridge, 5 bar) was connected at the end side of the reactor. A 1 M solution of ECF was pumped continuously at a 0.04 ml·min$^{-1}$ flow rate. The formation of HCN was assessed by GC analysis, considering the ECF depletion (see **S2** for details). To check the system stability a 1 M solution of ECF in micro-aqueous MTBE was passed continuously (0.04 ml·min$^{-1}$) through the CalB-reactor, showing only minor inactivation of the biocatalyst from 97% after 1 h to 80% after 8 h (Figure 1).

**Figure 1. CalB-catalyzed hydrolysis of ECF in a continuous flow set-up.**




## S4. General procedure for the immobilization of *At*HNL on Celite.

Purified lyophilized $His_6$-*At*HNL (25 mg) was dissolved in potassium phosphate buffer (10 mM, pH 6.0, 300 µl) and was carefully pipetted over Celite R-633 (100 mg) on a glass plate (enzyme/Celite ratio is 1:4). This preparation was dried for at least 12 hours under in a desiccator at 20 mbar vacuum with silica gel and molecular sieves.[3] The Celite-*At*HNL was then stored in screw capped glass vials at 4 °C.

## S5. General procedure for the preparation of *At*HNL-*E.coli* and purification.

Preparation of *At*HNL-*E. coli*[4]

To produce *At*HNL with an N-terminal hexahistidine fusion, chemically competent *E. coli* BL21(DE3) cells (50 µl) were thawed on ice and pET28a-*At*HNL plasmid (1 µl) was transformed into it by a heat shock (30 min, 4 °C; 90 s, 42 °C; 5 min, 4 °C). LB medium (500 µl) was added in each vial and subsequently incubated at 37 °C for 1 hour. A sample (200 µl) from these vials was plated on an agar plate containing kanamycin (50 µg/ml). As a negative control, competent cells without the plasmid were plated on a separate agar plate. The plates were incubated overnight at 37 °C.

In order to obtain large amounts of protein, the gene must be over-expressed in a bacterial host, which is *E. coli* in our case. A single colony was picked from the transformed cells with a pipette tip and inoculated into a test tube with LB medium (5 ml) containing kanamycin (50 µg/ml). This pre-culture was then incubated at 37 °C overnight. For the preparation of the main/primary culture, autoinduction medium (1 L) was freshly prepared (Table 1).

**Table 1. Autoinduction medium preparation.**

| Components | Composition | Volume (ml) |
|---|---|---|
| media component | 15 g/L peptone, 30 g/L yeast extract | 80 |
| Potassium phosphate buffer | 1 M, pH 7.0 | 9 |
| Lactose | 20 g/L | 10 |
| Glucose | 50 g/L | 1 |
| Kanamycin | 50 µg/µL | 0.1 |
| sterilized by autoclaving at 121 °C, 2 bar for 3 h | | |

A sample of the pre-culture (1 mL) was inoculated into the main culture and incubated at 37 °C at 120 rpm for the first 3 h, and subsequently at 17 °C at 120 rpm for next 69 h. In order to harvest the cells, the culture broth was centrifuged at 10,000 rpm for 50 minutes at 4 °C. The over-expression of the protein was then determined by a Sodium Dodecyl Sulfate- Poly Acrylamide Gel Electrophoresis (SDS-PAGE).

The gel and the electrophoresis unit were setup as described in the manual from supplier (Life Technologies, Article no- NP0321BOX). Samples were prepared as mentioned in the supplier manual. The optical density ($OD_{600nm}$ = 0.15) of the main culture was adjusted in order to load 10 µg protein per well. Electrophoresis was carried out at 100 mA, 200 V and 150 W for 35 minutes. The gel was carefully removed from the electrophoresis unit and dyed with Coomassie brilliant blue at room temperature for 1 hour.[5] The gel was then washed twice with Millipore water. The over-expression of protein could be visualized by the naked eye as a prominent dark band at 29 kDa, which corresponds to the molecular weight of *At*HNL.

Enzyme purification

The cells were disrupted by ultrasonication. Cells were weighed (9 g) and resuspended in lysis buffer (Table 2) under ice. The sonotrode S14D (Dr. Hielscher GmbH) was used and the sonicator was adjusted to an amplitude of 40% and a cycle of 0.5 minutes. The disruption lasted for 28 minutes, with alternating pulses of ultrasonic waves (70 $W/cm^2$) and a break of 4 minutes each to avoid overheating. The system was cooled to avoid denaturation due to heat. The cell debris was then removed by centrifuging it at 35000 rpm for 45 minutes at 4 °C. The supernatant (lysate) was collected and was used as crude cell extract for further purification.

The freshly prepared crude cell extract was stored on ice and passed through a filter of pore size 0.45 µm to remove left over cell debris. Nitrolotriacetic acid (NTA) complexed with nickel ions ($Ni^{2+}$) was used as the column material. The Ni-NTA column (Qiagen diameter 1.6 cm, volume 17 ml) was equilibrated with 3 column volumes of equilibration buffer (300 mM NaCl, 10 mM Kpi, pH 7.5). The filtered crude cell extract was loaded onto the column with a flow of 2 ml/min. The absorption was monitored via a UV detector at 280 nm. The non-bound proteins were eluted with the equilibration buffer and the equilibration buffer was flowed through the column till the baseline was reached. On applying a low concentration of imidazole (50 mM), the non-specifically bound proteins were eluted. Next, a high concentration of imidazole (200 mM) was applied to elute the target protein.

The purified lysate from the Immobilized Metal Ion Affinity Chromatography (IMAC) contains a high concentration of imidazole and buffer salts which might be harmful for the stability of the protein. In order to separate the salts from the target protein, gel-filtration was used. The column (Sephadex G-25 column, volume 800 ml, diameter 5 cm) was connected to an Äkta-Purifier and equilibrated for 2 column volumes with desalting buffer (Table 2) with a flow rate of 10 ml/min. The protein fraction was monitored by measuring the UV absorption at 280 nm.

Quantification of the protein concentration was performed by the Bradford assay, which is based on the shift in absorption maxima of Coomassie brilliant blue from 465 nm to 565 nm on binding to a protein.[6] A calibration curve was made with defined protein concentrations of bovine serum albumin (BSA) of 0.01 mg/ml to 0.1 mg/ml and was plotted against the respective absorption maxima at 595 nm. Bradford reagent (900 µl) was added to of the protein sample (100 µl) diluted with the respective buffer in a quartz cuvette. As a control, Bradford reagent (900 µl) was added to 100 µl of buffer (without protein). The cuvettes were incubated at RT in the dark for 10 minutes to allow the binding of the dye to the protein. The absorbance was then measured at 595 nm and the protein concentration was determined by referring to the calibration curve.

**Table 2. Protocol for the purification of *At*HNL.**

| | **Buffer** | **Component** |
|---|---|---|
| Lysis buffer for cell disruption | Potassium phosphate buffer<br>Lysozyme | 50 mM, pH 7.5<br>1 mg/ml |
| Purification by affinity chromatography | Equilibration buffer | 300 mM NaCl<br>10 mM potassium phosphate buffer<br>In Millipore water, pH 7.5 |
| | Washing buffer | 300 mM NaCl<br>50 mM potassium phosphate buffer<br>50 mM imidazole<br>In Millipore water, pH 7.5 |
| | Elution buffer | 300 mM NaCl<br>50 mM potassium phosphate buffer<br>300 mM imidazole<br>In Millipore water, pH 7.5 |
| Gel filtration | Desalting buffer | 10 mM potassium phosphate buffer<br>In Millipore water, pH 7.5 |

## S6. Estimation of *At*HNL content in lyophilized *E. coli*cells

The *At*HNL content was estimated from the total amount of purified enzyme attained from the *E.coli* cells following the protocol described in S5. From 9 g of lyophilized *E. coli* BL21 (DE3) cells, 300 mg of purified *At*HNL was obtained. Thus, 1 g of recombinant lyophilized cells contains about 33 mg *At*HNL.

## S7. Gene/amino acid sequence of $His_6$-*At*HNL

Gene sequence

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGAGAG
GAAACATCACTTCGTGTTAGTTCACAACGCTTATCATGGAGCCTGGATCTGGTACAAGCTCAAGCCCCTC
CTTGAATCAGCCGGCCACCGCGTTACTGCTGTCGAACTCGCCGCCTCCGGGATCGACCCACGACCAATC
CAGGCCGTTGAAACCGTCGACGAATACTCCAAACCGTTGATCGAAACCCTCAAATCTCTTCCAGAGAAC
GAAGAGGTAATTCTGGTTGGATTCAGCTTCGGAGGCATCAACATCGCTCTCGCCGCCGACATATTTCCG
GCGAAGATTAAGGTTCTTGTGTTCCTCAACGCCTTCTTGCCCGACACAACCCACGTGCCTTCTCACGTTC
TGGACAAGTATATGGAGATGCCTGGAGGTTTGGGAGATTGTGAGTTTTCATCTCATGAAACAAGAAATG
GGACGATGAGTTTATTGAAGATGGGACCAAAATTCATGAAGGCACGTCTTTACCAAAATTGTCCCATAG
AGGATTACGAGCTGGCAAAAATGTTGCATAGGCAAGGGTCATTTTTCACAGAGGATCTATCAAAGAAAG
AAAAGTTTAGCGAGGAAGGATATGGTTCGGTGCAACGAGTTTACGTAATGAGTAGTGAAGACAAAGCC
ATCCCCTGCGATTTCATTCGTTGGATGATTGATAATTTCAACGTCTCGAAAGTCTACGAGATCGATGGCG
GAGATCACATGGTGATGCTCTCCAAACCCCAAAAACTCTTTGACTCTCTCTCTGCTATTGCCACCGATTA
TATGTAA

Amino acid sequence

MGSSHHHHHHSSGLVPRGSHMERKHHFVLVHNAYHGAWIWYKLKPLLESAGHRVTAVELAASGIDPRPIQ
AVETVDEYSKPLIETLKSLPENEEVILVGFSFGGINIALAADIFPAKIKVLVFLNAFLPDTTHVPSHVLDKYME
MPGGLGDCEFSSHETRNGTMSLLKMGPKFMKARLYQNCPIEDYELAKMLHRQGSFFTEDLSKKEKFSEEGY
GSVQRVYVMSSEDKAIPCDFIRWMIDNFNVSKVYEIDGGDHMVMLSKPQKLFDSLSAIATDYM

The area marked in red shows the gene/protein sequence for the hexahistidine tag at the *N*-terminal end; the area marked in green shows the gene/protein sequnce for the linker between the hexahistidine-tag and the native gene/protein sequence of *At*HNL marked in blue.

## S8. General batch procedure for the synthesis of (*R*)-mandelonitrile.

(*R*)-Mandelonitrile was synthesized in batch as described in literature.[2] A mixture of benzaldehyde (0.5 M) and HCN solution (1.5 M) in micro-aqueous MTBE was filled into a glass vial, containing *At*HNL immobilized on Celite (20 mg, corresponding to 5 mg of pure enzyme). The mixture was stirred at room temperature until complete conversion, and after 125 minutes (*R*)-mandelonitrile **1a** with high conversion (99 %) and high *ee* (98 %) were attained (Figure 2).

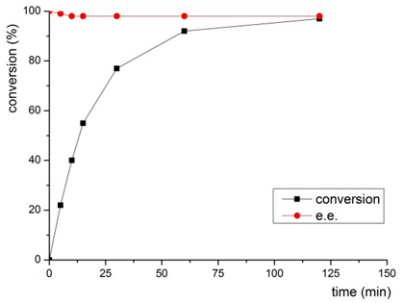
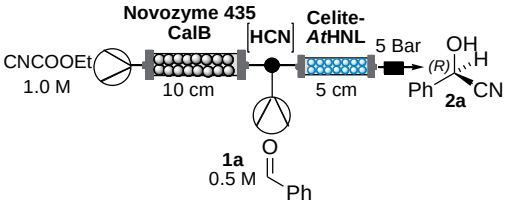


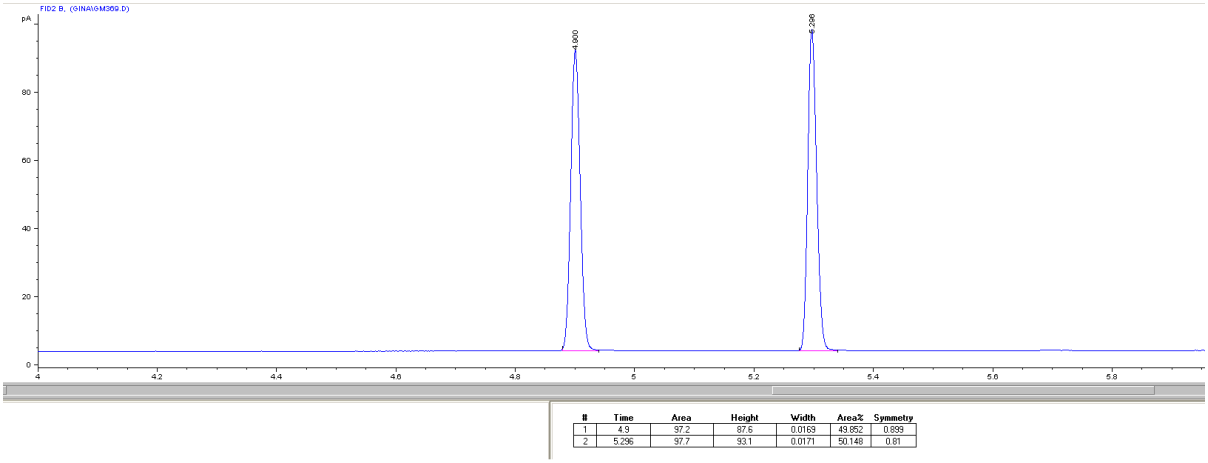

**Figure 2. Synthesis of (*R*)-mandelonitrile in a glass vial.**

## S9. Synthesis of (*R*)-mandelonitrile 2a by two-step (CalB-Celite-*At*HNL) procedure in flow.



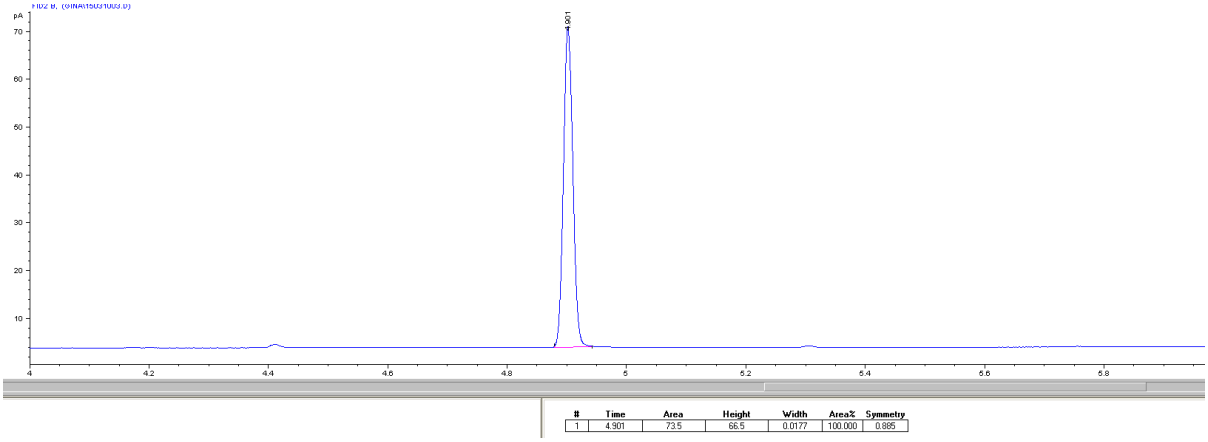

A 1 M solution of ECF in micro-aqueous MTBE was pumped (0.04 ml·min$^{-1}$) through a 10 cm packed-bed reactor containing 277 mg of CalB. This first output, consisting in 1 M solution of HCN, was mixed with a 0.5 M solution of benzaldehyde in micro-aqueous MTBE (0.04 ml·min$^{-1}$) by means of a tee piece assembly. The resulting mixture passed through a second bioreactor (Kinesis, Benchmark microbore column 3MM/50MM 2 X F), containing 100 mg of Celite-*At*HNL, prepared as described in section S4. The entire system was kept pressurized at 5 Bar by a back pressure regulator. The out coming solution was collected and a sample (50 μL in 500 μL of $CDCl_3$) was analyzed by NMR to check the formation of (*R*)-2**a** (97%). The excess of HCN (1 Eq) was treated with an aqueous solution of sodium hypochlorite (10 – 15%) until neutralization. In order to assess the optical purity of **2a** a sample (0.05 ml) from the collected solution was diluted in 1 ml of DCM and poured in a vial. A mixture of pyridine (0.05 ml) and acetic anhydride (0.06 ml) was added. The resulting mixture was stirred for 180 min, affording (*R*)-*O*-acetylcyanohydrin **3a**. The optical purity of **3a** (*ee* > 99%) was assessed by GC analysis. The following method has been used for the analysis: initial temperature 110 ºC, ramp 15 ºC/min to 180 ºC hold for 0 min, post run 180 ºC for 2 min.

**(±)-3a: $t_1$ = 4.9 min, $t_2$ = 5.3 min**



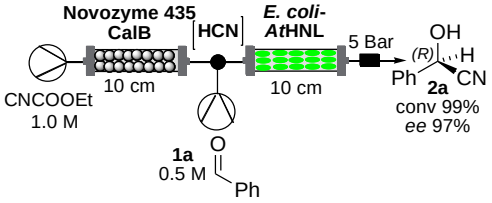

| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 4.9 | 97.2 | 87.6 | 0.0169 | 49.852 | 0.899 |
| 2 | 5.296 | 97.7 | 93.1 | 0.0171 | 50.148 | 0.81 |

**(*R*)-3a: $t_1$ = 4.9 min**



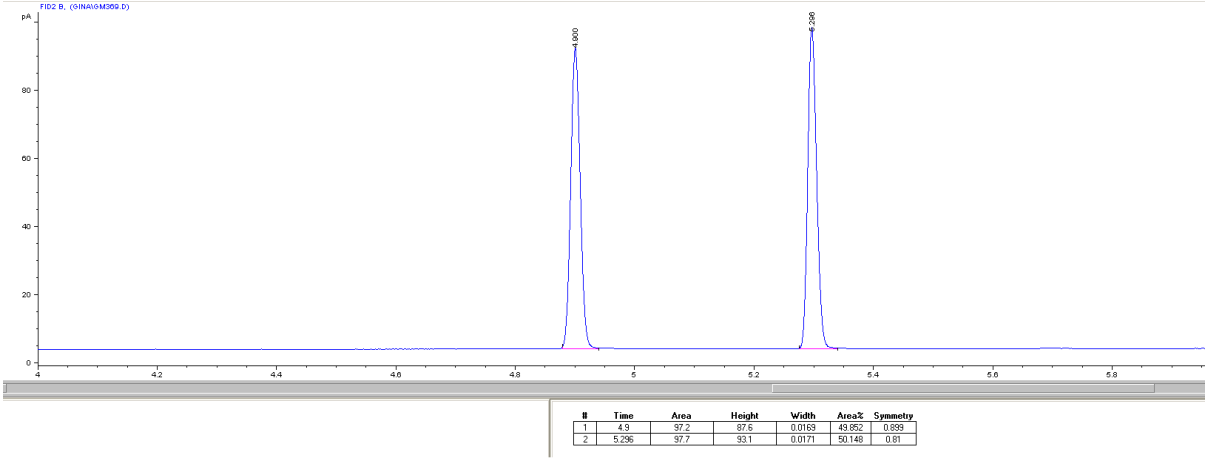

| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 4.901 | 73.5 | 66.5 | 0.0177 | 100.000 | 0.885 |

## S10. Synthesis of (*R*)-mandelonitrile 2a by two-step (CalB-*E. coli*-*At*HNL) procedure in flow.



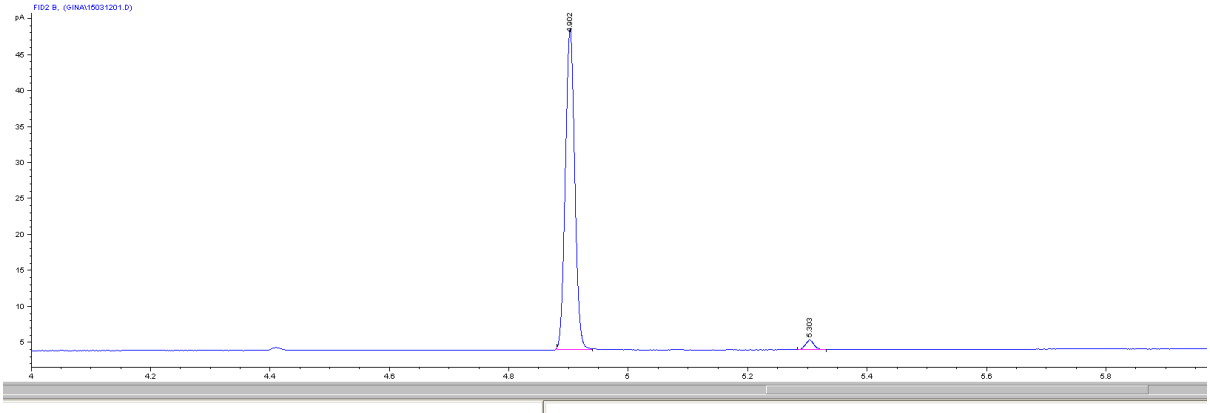

A 1 M solution of ECF in micro-aqueous MTBE was pumped (0.04 ml·min$^{-1}$) into a 10 cm packed-bed reactor containing 277 mg of CalB. This first output, consisting in 1 M solution of HCN, was mixed with a 0.5 M solution of benzaldehyde **1a** in micro-aqueous MTBE (0.04 ml·min$^{-1}$) by means of a tee piece assembly. The resulting mixture passed through a second bioreactor (Kinesis, Benchmark microbore column 3 MM / 100 MM 2 X F), containing 250 mg of lyophilized *E. coli*-*At*HNL, prepared as described in section S5. The entire system was kept pressurized at 5 Bar by a back pressure regulator. The out coming solution was collected and analyzed by NMR to check the formation of (*R*)-mandelonitrile **2a** (97%). In order to assess the optical purity of **2a** a sample (0.05 ml) from the collected solution was diluted in 1 ml of dichloromethane and poured in a glass vial. A mixture of pyridine (0.05 ml) and acetic anhydride (0.06 ml) was added. The resulting mixture was stirred for 180 min, affording (*R*)-*O*-acetylcyanohydrin **3a**. The optical purity of **3a** (*ee* 95%) was assessed by GC analysis ($t_1$ = 4.9, $t_2$ = 5.3). The following method has been used for the analysis: initial temperature 110 ºC, ramp 15 ºC/min to 180 ºC hold for 0 min, post run 180 ºC for 2 min.

**(±)-3a: $t_1$ = 4.9 min, $t_2$ = 5.3 min**




| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 4.9 | 97.2 | 87.6 | 0.0169 | 49.852 | 0.899 |
| 2 | 5.296 | 97.7 | 93.1 | 0.0171 | 50.148 | 0.81 |

**(*R*)-3a Major (97%): $t_1$ = 4.9 min; (*S*)-3a Minor (3%): $t_2$ = 5.3 min**

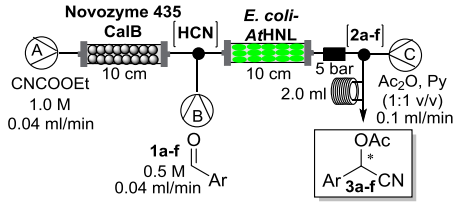


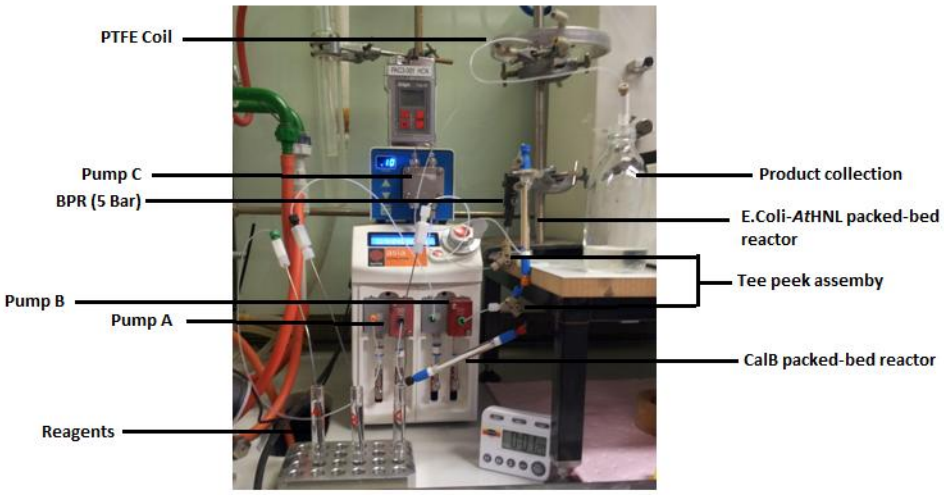

| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 4.902 | 48.8 | 44.4 | 0.0176 | 97.325 | 0.947 |
| 2 | 5.303 | 1.3 | 1.3 | 0.0158 | 2.675 | 0.918 |

## S11. General batch procedure for the synthesis of racemic *O*-acetylcyanohydrins.

A mixture of aldehyde **1b** – **1f** (1 mmol) and ethyl cyanoformate (2 mmol) in MTBE was poured in a glass vial. CalB Novozyme 435 (50 mg) and a KPi (pH = 8.5) were added consecutively. The vial was closed and the resulting mixture was stirred gently for 5 hours. A sample (0.05 ml) was taken from the reaction mixture and diluted in dichloromethane (1 ml) in a clean vial. A mixture of pyridine (0.05 ml) and acetic anhydride (0.06 ml) was added and the resulting mixture was stirred at room temperature for 180 minutes, affording racemic *O*-acetylcyanohydrins **3b** – **3f**.

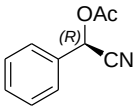
## S12. General flow procedure for the three-step cascade synthesis of (*R*)-*O*-acetylcyanohydrins.



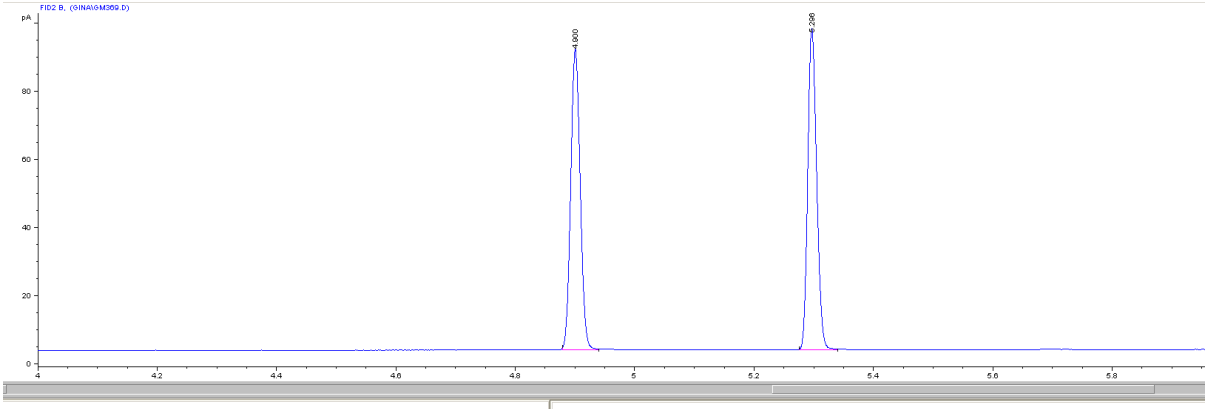

A 1 M solution of ECF in micro-aqueous MTBE was pumped (0.04 ml·min$^{-1}$) into a 10 cm packed-bed reactor containing 277 mg of CalB. This first output, consisting in 1 M solution of HCN, was mixed with a 0.5 M solution of aldehyde **1a** – **1f** in micro-aqueous MTBE (0.04 ml·min$^{-1}$) by means of a tee piece assembly. The resulting mixture was passed through a second bioreactor (Kinesis, Benchmark microbore column 3 MM / 100 MM 2 X F), containing 250 mg of lyophilized *E. coli*-*At*HNL, prepared as described in section S5. A back pressure regulator (5 Bar) was introduced after the packed bed reactor. The out coming solution was mixed with a mixture of pyridine/acetic anhydride (1:1, V/V) pumped by a compact HPLC pump (Knauer) and the resulting solution was passed through a PTFE coil (2 ml). The layers were collected and a sample (50 µL in 500 µL of $CDCl_3$) was analyzed by NMR to check the formation of (*R*)-**3a**, (*R*)-**3b**, (*R*)-**3c**, (*R*)-**3d**, (*S*)-**3e** and (*R*)-**3f** (75 - 99%). The optical purity of the products (40 - 96%) was assessed by GC analysis.

## S13. Enantiomeric excess (*ee*) determination of compounds 3a – 3f prepared by the three-step cascade flow process.



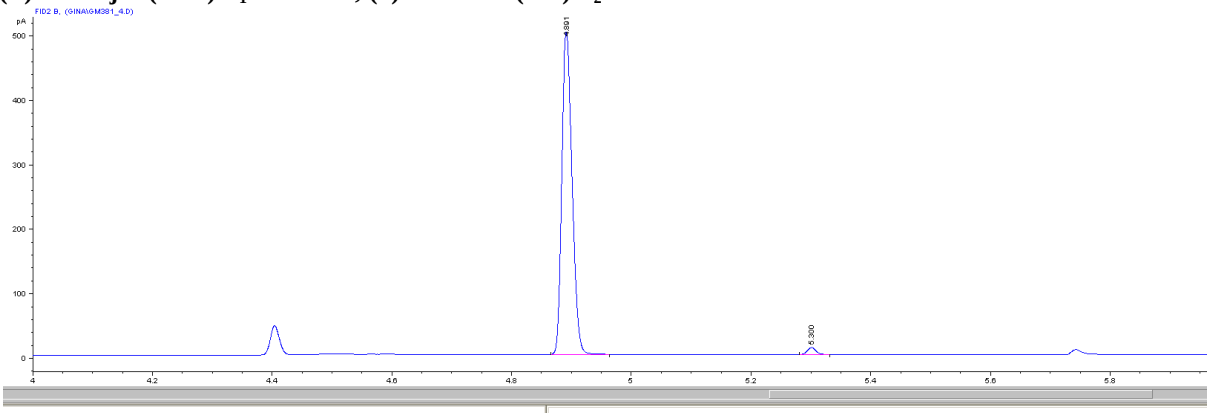

**(*R*)-2-*O*-Acetyl-2-phenyl acetonitrile (3a).** The optical purity of **3a** (*ee* 96%) was assessed by GC analysis ($t_1$ = 4.9, $t_2$ = 5.3). The following method has been used for the analysis: initial temperature 110 ºC hold for 1 min, ramp 15 ºC/min to 180 ºC hold for 0 min, post run 180 ºC for 2 min.

**(±)-3a: $t_1$ = 4.9 min, $t_2$ = 5.3 min**

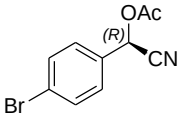



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 4.9 | 97.2 | 87.6 | 0.0169 | 49.852 | 0.899 |
| 2 | 5.296 | 97.7 | 93.1 | 0.0171 | 50.148 | 0.81 |

**(*R*)-3a Major (98%): $t_1$ = 4.9 min; (*S*)-3a Minor (2%): $t_2$ = 5.3 min**

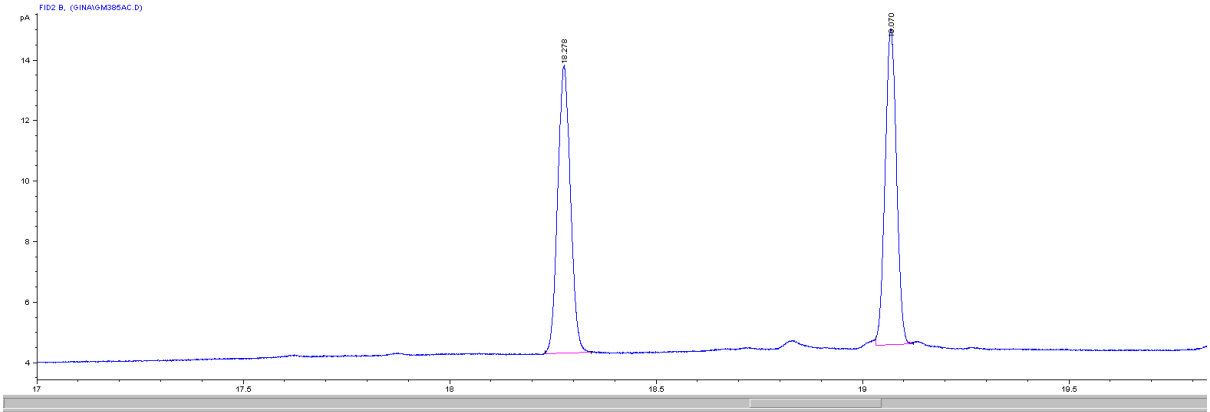



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 4.891 | 595.3 | 494.8 | 0.0188 | 98.213 | 0.756 |
| 2 | 5.3 | 10.8 | 10.8 | 0.0157 | 1.787 | 0.919 |



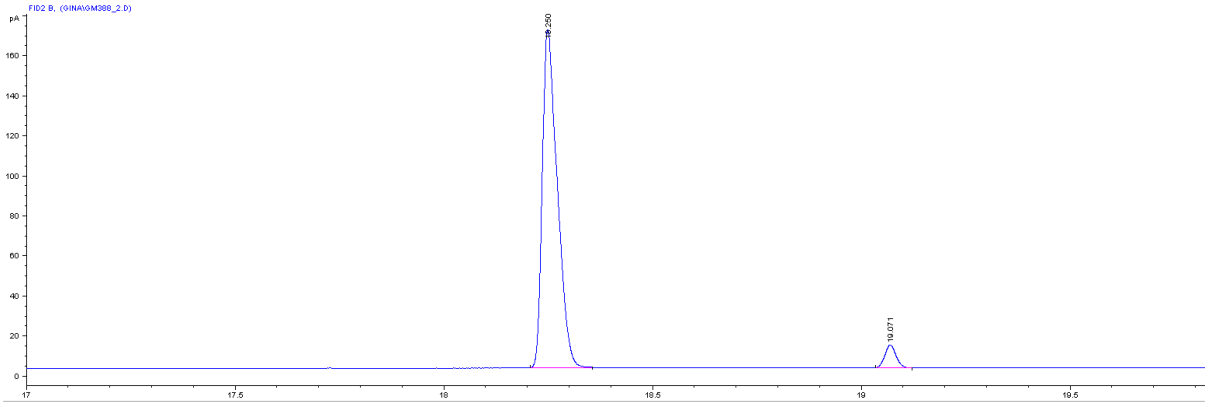

**(*R*)-2-*O*-Acetyl-2-(4-bromophenyl) acetonitrile (3b).** The optical purity of **3b** (*ee* 90%) was assessed by GC analysis ($t_1$ = 18.28, $t_2$ = 19.07). The following method has been used for the analysis: initial temperature 110 ºC hold for 5 min, first ramp 5 ºC/min to 140 ºC hold for 5 min, second ramp 15 ºC/min to 180 ºC hold for 10 min, post run 180 ºC for 2 min.

**(±)-3b: $t_1$ = 18.3 min, $t_2$ = 19.1 min**

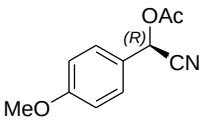



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 18.278 | 20.4 | 9.5 | 0.0348 | 50.469 | 0.919 |
| 2 | 19.07 | 20.1 | 10.4 | 0.0304 | 49.531 | 0.964 |

**(*R*)-3b Major (95%): $t_1$ = 18.2 min; (*S*)-3a Minor (5%): $t_2$ = 19.1 min**

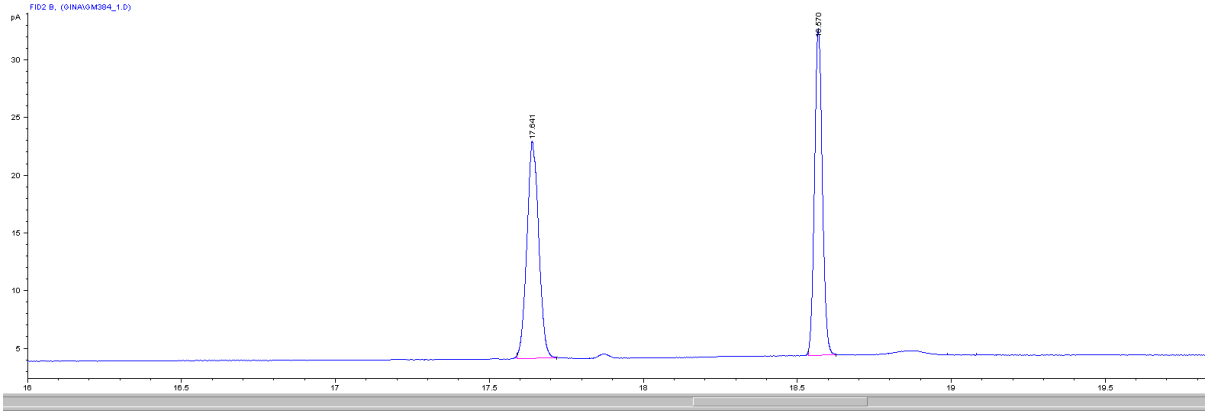



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 18.25 | 421.9 | 168.6 | 0.0372 | 95.207 | 0.544 |
| 2 | 19.071 | 21.2 | 11.4 | 0.0282 | 4.793 | 0.92 |



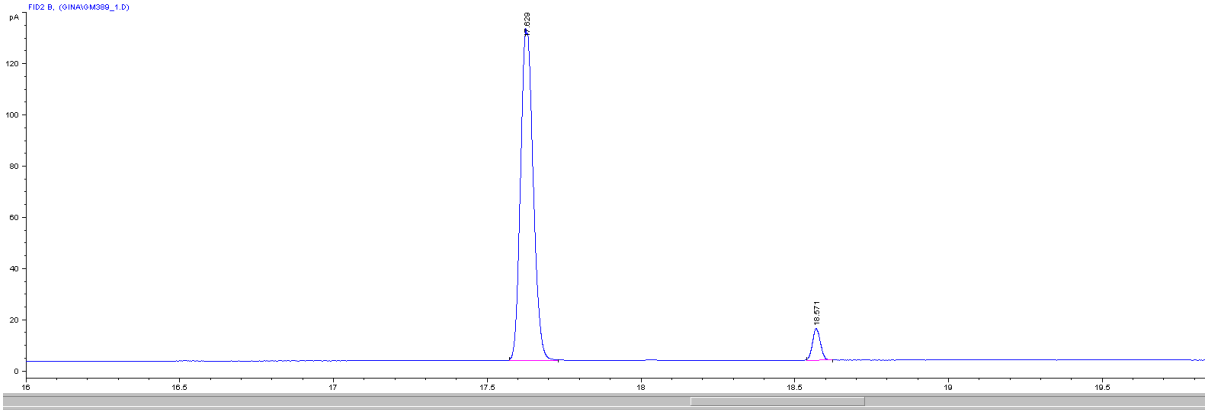

**(*R*)-2-*O*-Acetyl-2-(4-methoxyphenyl) acetonitrile (3c).** The optical purity of **3c** (*ee* 89%) was assessed by GC analysis ($t_1$ = 17.64, $t_2$ = 18.57). The following method has been used for the analysis: initial temperature 110 ºC hold for 5 min, first ramp 5 ºC/min to 140 ºC hold for 5 min, second ramp 15 ºC/min to 180 ºC hold for 10 min, post run 180 ºC for 2 min.

**(±)-3c: $t_1$ = 17.6 min, $t_2$ = 18.6 min**

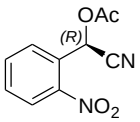



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 17.641 | 50.3 | 18.8 | 0.0408 | 50.236 | 0.816 |
| 2 | 18.57 | 49.8 | 28.2 | 0.027 | 49.764 | 0.883 |

**(*R*)-3c Major (94%): $t_1$ = 17.6 min; (*S*)-3c Minor (6%): $t_2$ = 18.6 min**

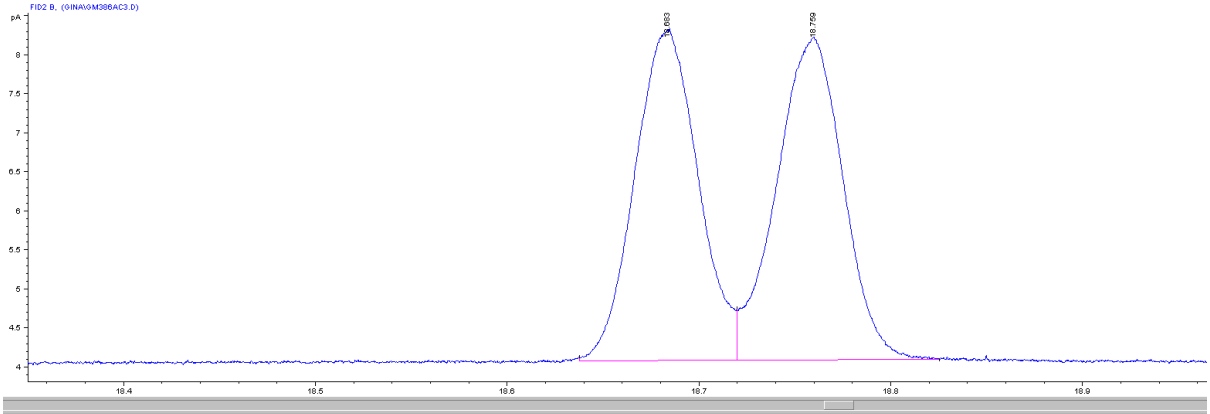



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 17.629 | 369.6 | 128.4 | 0.0448 | 94.374 | 0.806 |
| 2 | 18.571 | 22 | 12.3 | 0.0281 | 5.626 | 0.815 |



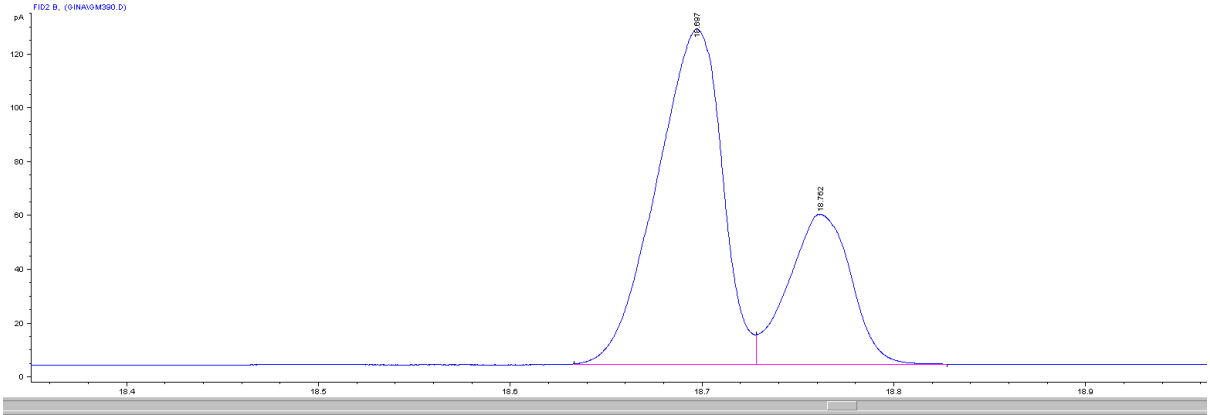

**(*R*)-2-*O*-Acetyl-2-(2-nitrophenyl) acetonitrile (3d).** The optical purity of **3d** (*ee* 40%) was assessed by GC analysis ($t_1$ = 18.68, $t_2$ = 18.76). The following method has been used for the analysis: initial temperature 110 ºC hold for 5 min, first ramp 5 ºC/min to 140 ºC hold for 5 min, second ramp 20 ºC/min to 180 ºC hold for 10 min, post run 180 ºC for 2 min.

**(±)-3d: $t_1$ = 18.7 min, $t_2$ = 18.8 min**

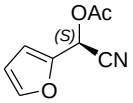



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 18.683 | 9.8 | 4.2 | 0.0365 | 49.380 | 0.952 |
| 2 | 18.759 | 10 | 4.1 | 0.0372 | 50.620 | 1.111 |

**(*R*)-3d Major (70%): $t_1$ = 18.7 min; (*S*)-3c Minor (30%): $t_2$ = 18.8 min**

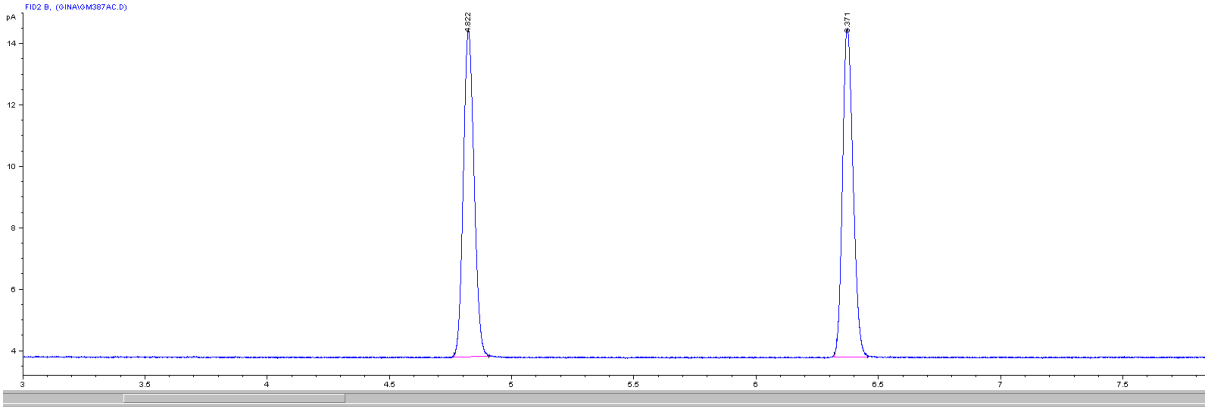



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 18.697 | 295.6 | 124.4 | 0.0373 | 69.975 | 1.556 |
| 2 | 18.762 | 126.8 | 55.8 | 0.0353 | 30.025 | 1.037 |



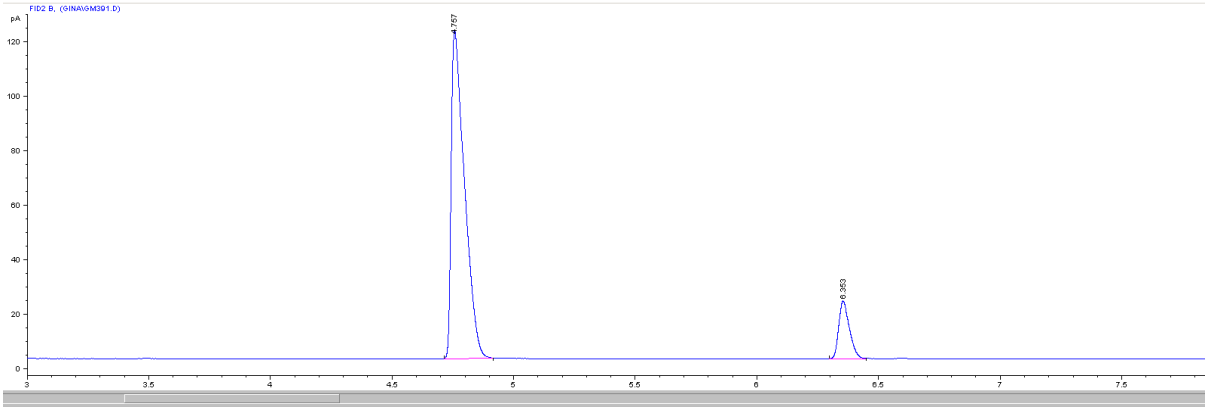

**(*S*)-2-*O*-Acetyl-2-(furan-2-yl) acetonitrile (3e).** Colourless oil, conversion 75%. $[\alpha]_{589}^{25}$ = -21.8 º ($CHCl_3$, c = 1.25). The optical purity of **3e** (*ee* 88%) was assessed by GC analysis ($t_1$ = 4.82, $t_2$ = 6.37). The following method has been used for the analysis: initial temperature 110 ºC hold for 5 min, first ramp 5 ºC/min to 140 ºC hold for 5 min, second ramp 15 ºC/min to 180 ºC hold for 10 min, post run 180 ºC for 2 min.

**(±)-3e: $t_1$ = 4.8 min, $t_2$ = 6.4 min**

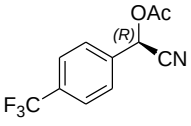



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 4.822 | 32.5 | 10.6 | 0.0477 | 50.302 | 0.848 |
| 2 | 6.371 | 32.1 | 10.7 | 0.0464 | 49.698 | 0.763 |

**(*S*)-3e Major (88%): $t_1$ = 4.8 min; (*R*)-3e Minor (12%): $t_2$ = 6.35 min**

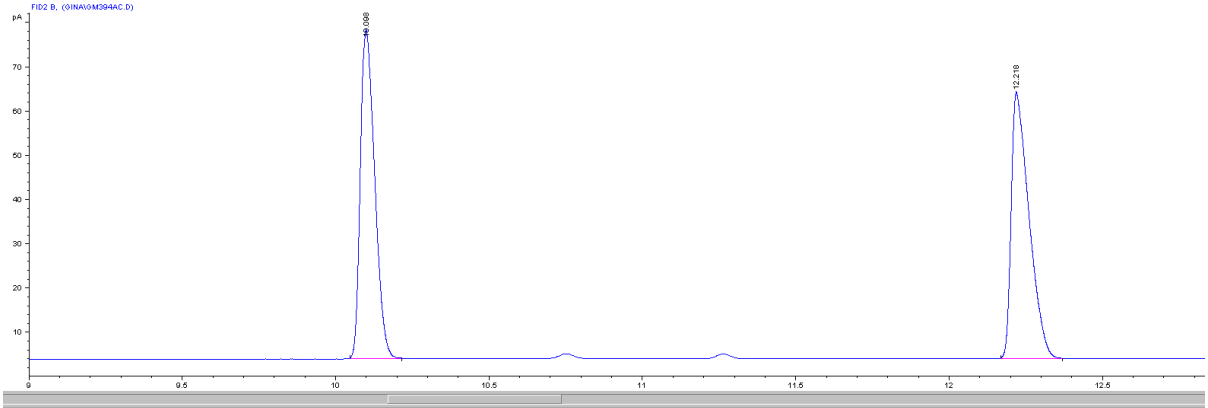



| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 4.757 | 455.1 | 120.4 | 0.0545 | 87.669 | 0.332 |
| 2 | 6.353 | 64 | 21.1 | 0.045 | 12.331 | 0.656 |



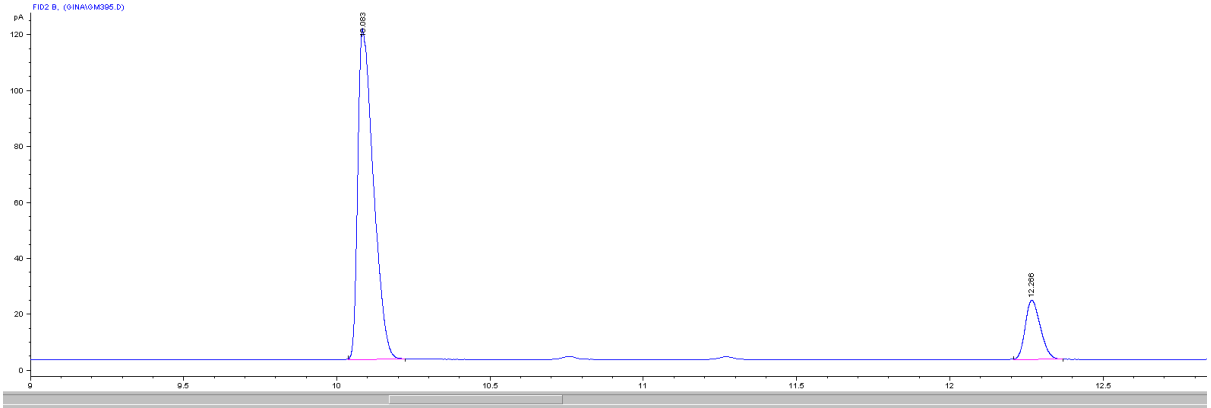

**(*R*)-2-*O*-Acetyl-2-[4-(trifluoromethyl)phenyl] acetonitrile (3f).** The optical purity of **3f** (*ee* 70%) was assessed by GC analysis ($t_1$ = 10.1, $t_2$ = 12.22). The following method has been used for the analysis: initial temperature 110 ºC hold for 5 min, first ramp 5 ºC/min to 140 ºC hold for 5 min, second ramp 15 ºC/min to 180 ºC hold for 10 min, post run 180 ºC for 2 min.

**(±)-3f: $t_1$ = 10.1 min, $t_2$ = 12.2 min**




| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 10.098 | 242.1 | 74.4 | 0.0508 | 49.964 | 0.628 |
| 2 | 12.218 | 242.5 | 60.3 | 0.0565 | 50.036 | 0.393 |

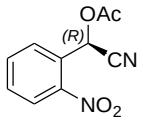
**(*R*)-3f Major (85%): $t_1$ = 10.1 min; (*S*)-3f Minor (15%): $t_2$ = 12.26 min**




| # | Time | Area | Height | Width | Area% | Symmetry |
|---|---|---|---|---|---|---|
| 1 | 10.083 | 417.3 | 117.5 | 0.0534 | 85.197 | 0.432 |
| 2 | 12.266 | 72.5 | 21.2 | 0.0527 | 14.803 | 0.726 |

## S14. Spectroscopic characterization of compound 3a – 3f.

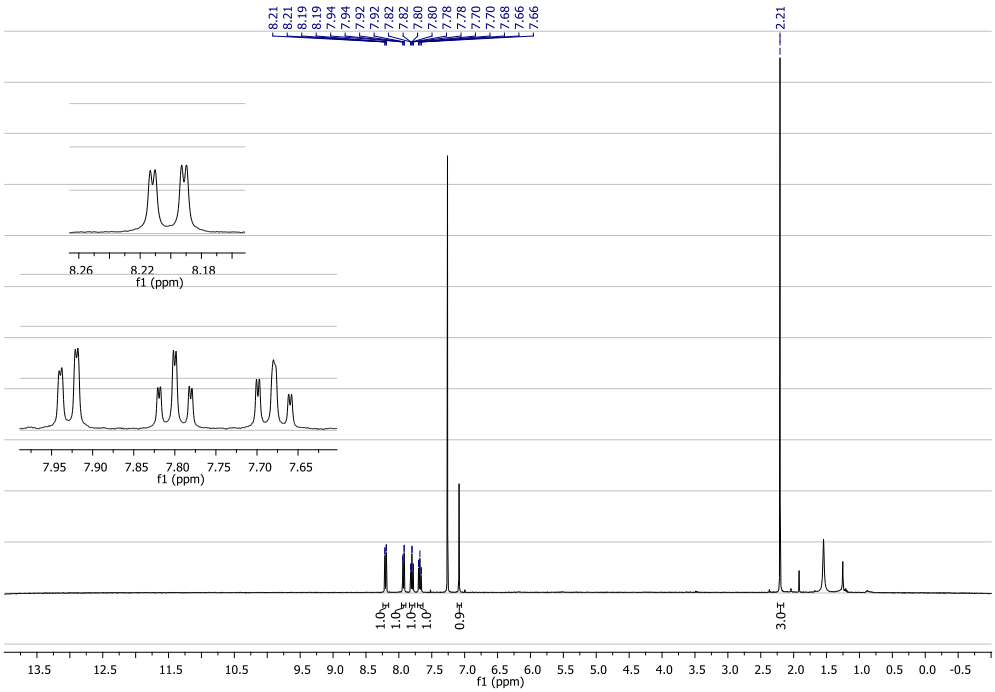
The spectroscopic data of **3a**,[7] **3b**,[8] **3c**,[3] **3e**,[9] **3f**[5] are in accordance with literature.

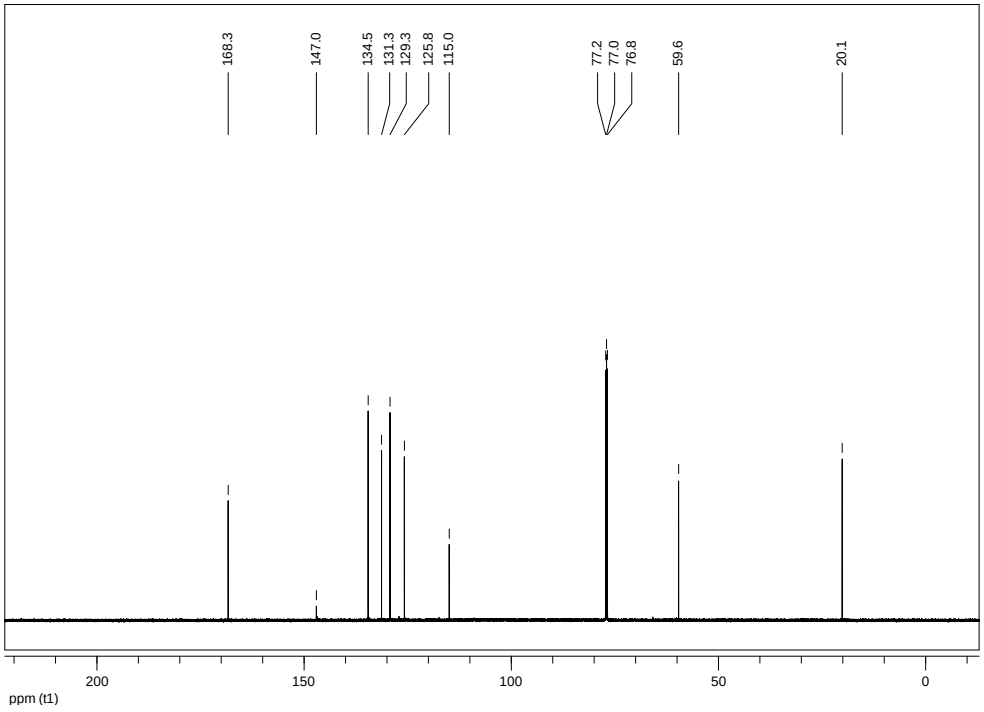



**(*R*)-2-*O*-Acetyl-2-(2-nitrophenyl) acetonitrile (3d).**

A 1 M solution of ECF in micro-aqueous MTBE was pumped (0.04 ml·min$^{-1}$) into a 10 cm packed-bed reactor containing 277 mg of CalB. This first output, consisting in 1M solution of HCN, was mixed with a 0.5 M solution of aldehyde **2d** in micro-aqueous MTBE (0.04 ml·min$^{-1}$) by means of a tee piece assembly. The resulting mixture was passed through a second bioreactor (Kinesis, Benchmark microbore column 3 MM / 100 MM 2 X F), containing 250 mg of lyophilized *At*HNL-*E. coli*, prepared as described in section S5. A back pressure regulator (5 Bar) was introduced after the packed bed reactor. The out coming solution was mixed with a mixture of pyridine/acetic anhydride (1:1, V/V) pumped by a compact HPLC pump (Knauer) and the resulting solution was passed through a PTFE coil (2 ml). The solution was collected and washed with a saturated aqueous solution of $NH_4Cl$ (10 ml x 3). The organic layer was then dried over $MgSO_4$ and concentrated to dryness. The pure product has been achieved by a flash column chromatography (stationary phase : silica, eluent: hexane / AcOEt, 6 : 4).

$^1$H NMR (400 MHz, $CDCl_3$) δ 2.21 (s, 3H), 7.09 (s, 1H), 7.68 (dt, *J* = 8.2, 1.4 Hz, 1H), 7.80 (dt, *J* = 7.7, 1.3 Hz, 1H), 7.93 (dd, *J* = 7.8, 1.2 Hz, 1H), 8.20 (dd, *J* = 8.2, 1.2 Hz, 1H). $^{13}$C NMR (150 MHz, $CDCl_3$) δ 20.1, 59.6, 115.0, 125.8, 129.3, 131.3, 134.5, 147.0, 168.3. IR (Neat, cm$^{-1}$) 3123, 3036, 2333, 1756, 1399, 1116, 1064. HRMS *m/z* calculated for $C_{10}H_9O_4N_2$ $[M + H]^+$ 221.0557, found 221.0547.


8.21
8.21
8.19
8.19
7.94
7.94
7.92
7.92
7.82
7.82
7.80
7.80
7.78
7.78
7.70
7.70
7.68
7.66
7.66
2.21
8.26
8.22
8.18
f1 (ppm)
7.95
7.90
7.85
7.80
7.75
7.70
7.65
f1 (ppm)
1.0
1.0
1.0
1.0
0.9
3.0
13.5
12.5
11.5
10.5
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)


168.3
147.0
134.5
131.3
129.3
125.8
115.0
77.2
77.0
76.8
59.6
20.1
200
150
100
50
0
ppm (t1)

**S15. References.**


[1] Present address: Novartis Pharma, AG WSJ-42.2.08 CH-4002, Basel, Switzerland.
[2] http://syrris.com/flow-products/asia-modules/asia-syringe-pump
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[9] Sakai, T.; Wang, K.; Ema, T. *Tetrahedron* **2008**, *64*, 2178 – 2183.