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# Synthesis of ethyl (*R*)-4-cyano-3-hydroxybutyrate at high concentration by using a novel halohydrin dehalogenase HHDH-PL from *Parvibaculum lavamentivorans* DS-1

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## Supporting information

**Contents of experiments**

## 1. Expression of SDR genes

Bacterial Strain *P. lavamentivorans* DS-1 (DSM 13023) was purchased from DSMZ and cultured in the medium containing 1.5% (wt/vol) peptone, 0.5% (wt/vol) NaCl and 0.1% (wt/vol) $CaCl_2$. *Bacillus cereus* ATCC 10876 was screened and deposited in our laboratory, culturing in medium containing 0.5% (wt/vol) peptone, 0.3% (wt/vol) meat extract and 0.001% (wt/vol) $MnSO_4$. The total genomic DNAs of the strains were extracted using a FastDNA® Spin Kit for Soil (MPBio, Santa Ana, CA) following the manual provided by manufacturer. The oligonucleotide sequences for cloning SDR genes were designed according to genes information and listed in Table S1. Six fragments with the length about 750 bp were amplified and inserted into plasmid pGEM-T. The positive clones were successfully verified by sequencing. Then the SDR genes were cloned into corresponding expression plasmids by using restriction enzymes and the ligated products were transformed into *E. coli* BL21 (DE3) to construct the recombinant strains of SDR enzymes. Finally, the expressions of the SDR genes were detected by SDS-PAGE (Figure S1).

Recombinant SDR3, SDR5, SDR6(pET28a) and SDR6(pET32a) were grown at 37 $^{o}$C in LB medium containing Kan (50 μg·mL$^{-1}$) and ampicillin (50 μg·mL$^{-1}$), respectively. Until the value of $OD_{600}$ reached 0.6-0.8, 0.1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added and induced overnight at 28 $^{o}$C. Strains SDR1, SDR2, SDR4 and SDR6(pET20b) were cultured in LB medium at 37 $^{o}$C for 18 h without adding IPTG for induction. The cells were harvested by centrifugation at 8,000×g for 10 min at 4 $^{o}$C and washed once with 50 mM phosphate buffer (pH 8.0). The HHDH activity assay were performed in 20 mL PBS (50 mM, pH 7.5) containing 50 mM 1,3-dichloro-2-propanol and 0.6 g (wet weight) whole cell of SDR enzymes. The reactions were incubated at 40 $^{o}$C and each 800 μL of these mixtures were taken to extract with ethyl acetate for GC analysis. A control reaction with substrate 1,3-dichloro-2-propanol was carried out for comparison. The formed epoxides of epichlorohydrin were determined by BGB-175 column using the following GC program: 90 $^{o}$C for 10min. The inlet and detector temperatures were all set at 220 $^{o}$C. The retention times of (*S*)-epichlorohydrin and (*R*)-epichlorohydrin were 4.38 and 4.56 min, respectively.

**Table S1.** Oligonucleotide primers for SDR sequences cloning

| SDR | organism | Primers |
|---|---|---|
| SDR1 | *Bacillus cereus* ATCC 10876 | F-5'-CATATGGCCGCAGCGTTCGACGG-3' |
| | | R-5'-CTCGAGTTATTGGGCGACATAGCC-3' |
| SDR2 | *Bacillus cereus* ATCC 10876 | F-5'-CATATGACACAAATTCCGAATTCAG-3' |
| | | R-5'-GAGTTAAACGTGCTGCCCGCC-3' |
| SDR3 | *P. lavamentivorans* DS-1 | F-5'-TGGGTAAGGATCTGGATTTCTCGGGC-3' |
| | | R-5'-GAGTCAGGAAAGGATGAGGCCGCC-3' |
| SDR4 | *P. lavamentivorans* DS-1 | F-5'-TGGGTAAAAAGTATGCGTTAGTAACTG-3' |
| | | R-5'-GAGTTAGCAGTGCCAACCGCCATTCAC-3' |
| SDR5 | *P. lavamentivorans* DS-1 | F-5'-TGGCAGAATTATTACAAGGC-3' |
| | | R-5'-GAGCTATGGATTAGTAGACCAAAG-3' |
| SDR6 | *P. lavamentivorans* DS-1 | F-5'-CATGGCGCGCAGCATTCTC-3' |
| | | R-5'-CTCGAGCTAGCGCGCGGTGGCCC-3' |

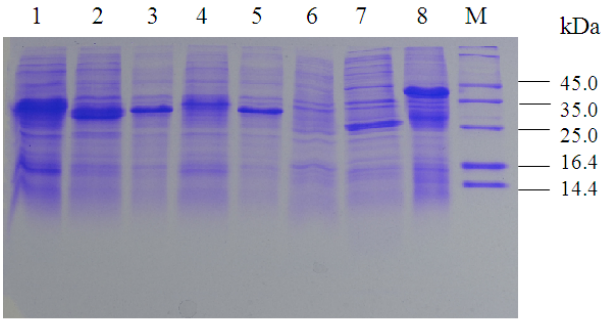


**Figure S1.** SDS-PAGE assay for the expression of SDRs. Lane M: standard proteins marker of different molecular weights; Lane 1: SDR1; Lane 2: SDR2; Lane 3: SDR3; Lane 4:SDR4; Lane 5: SDR5; Lane 6: SDR6-1 expressed with plasmid pET28a (+); Lane 7: SDR6-2 expressed with plasmid pET20b (+); Lane 8: SDR6-3 expressed with plasmid pET32a (+).

## 2. Expression of HHDHs ($HheA_{AD2}$, $HheB_{GP1}$ and HheC)

Three HHDH genes, $HheA_{AD2}$ from group A, $HheB_{GP1}$ from group B and HheC from group C, were synthesized using PCR assembly method[1] after optimization of the codons by a gene designer software against *E. coli* as host[2]. The primers for cloning HHDHs were listed in Table S2. The corresponding fragments were amplified and inserted into plasmid pET28a (+). Then the plasmids were transformed into *E. coli* BL21 (DE3) to construct the recombinant strains for HHDHs. Recombinant HHDHs were grown at 37 °C in LB medium

containing Kan (50 μg·mL$^{-1}$) until the value of $OD_{600}$ reached 0.6-0.8. IPTG (0.1 mM) was added and induced overnight at 28 °C. Finally, the successfully constructed HHDHs were determined by sequencing and SDS-PAGE assay (Figure S2).

**Table S2.** Oligonucleotide primers for HHDHs cloning

| HHDH | Genebank accession | Primers |
|---|---|---|
| HheA-AD2 | AAK92100.1 | F-5'-CATATGGCCGCAGCGTTCGACGG-3'<br>R-5'-CTCGAGTTATTGGGCGACATAGCC-3' |
| HheB-GP1 | AAK73175.1 | F-5'-CATATGACACAAATTCCGAATTCAG-3'<br>R-5'-CTCGAGTTAAACGTGCTGCCCGCC-3' |
| HheC | AAK92099.1 | F-5'-CCATGGGTAAGGATCTGGATTTCTCGGGC-3'<br>R-5'-CTCGAGTCAGGAAAGGATGAGGCCGCC-3' |

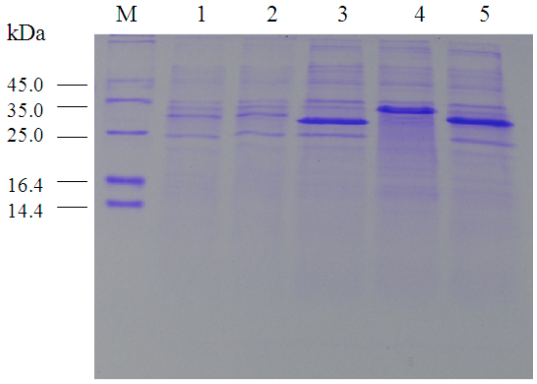


**Figure S2:** SDS-PAGE assay for the expression of HHDHs. Lane M: standard proteins marker of different molecular weights; Lane 1: *E. coli* BL21(DE3) without pET28a (+); Lane 2: *E. coli* BL21(DE3) with pET28a(+); Lane 3: expressed HheA-AD2; Lane 4: expressed HheB-GP1; Lane 5: expressed HheC.

## 3. Purification of HHDH-PL

Recombinant HHDH-PL was cultured and harvested as described above. 2 g (wet cell weight) HHDH-PL cells were resuspended in phosphate buffer (50 mM pH 7.5) and sonicated. After centrifugation at 15,000×g at 4 °C for 20 min, the transparent supernatant was collected and applied to a Nickel-NTA column (MC/20, 16×100, A&B applied biosystem). The column was preequilibrated with buffer A (20 mM imidazole, 50 mM Tris sulfate, 500 mM NaCl, pH 8.0) at the flow rate of 2.0 mL·min$^{-1}$. Nonabsorbed protein was washed off with 10 column volumes of buffer A and 10 column volumes of buffer B (50 mM imidazole, 50 mM Tris

sulfate, 500 mM NaCl, pH 8.0). Bound protein was eluted with buffer C (200 mM imidazole, 50 mM Tris sulfate, 500 mM NaCl, pH 8.0). The fractions eluted showing HHDH activity were pooled, concentrated, and buffer exchanged in 50 mM Tris sulfate (pH 8.0) using a 10-kDa-molecular-mass-cutoff ultrafiltration membrane. A 12% gel of SDS-PAGE assay was carried out to determine the molecular weight and purity of the purified enzyme (Figure S3). After denaturation, the purified enzyme was identified by MALDI-TOF MS (Figure S4). The result revealed the mass of HHDH-PL was about 26 kDa with the16 kDa of fusion protein (Trix-tag, His-tag and S-tag), which was similar to the predicted value.

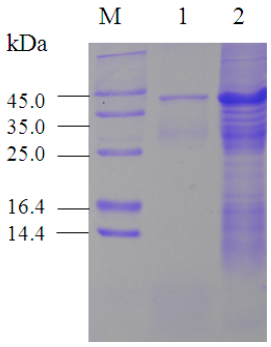


**Figure S3:** SDS-PAGE analysis of the purified HHDH-PL. Lane M: standard proteins marker of different molecular weights; Lane1: Purified HHDH-PL; Lane 2: The crude enzyme of HHDH-PL.

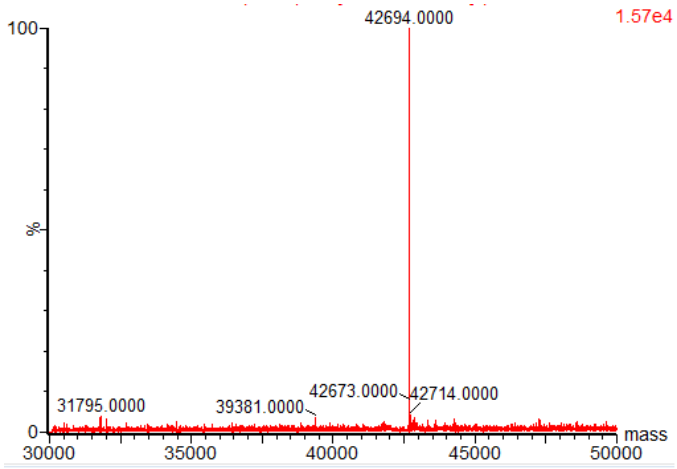


**Figure S4.** MALDI-TOF MS assay of the purified HHDH-PL. The result showed that the mass of expressed HHDH-PL was 42.694 kDa.

## 4. Substrate specificity of HHDH-PL

50 μL of the purified HHDH-PL was added to 650 μL 50 mM PBS buffer containing each of the eleven substrates (listed in Table 3) in 20 mM final concentration. Reactions were performed at 40 $^{o}$C and 700 μL of ethyl acetate containing 1-chlorohexane (1 mM) as an internal standard were added to the each reaction for extracting the samples for activity assay. The organic extracts were determine on a GC-7890A system equipped with a HP-5 capillary column (30 m × 0.32 mm, 0.25 μm film thickness) fitted with a flame ionization (FID) detector. Nitrogen was used as carrier gas at a flow rate of 1.0 mL·min$^{-1}$. The inlet and detector temperatures were 230 $^{o}$C and 250 $^{o}$C**,** respectively. The reaction rates were calculated by using the consuming of the halohydrin substrates. The GC programs for analysis of the substrates were listed below:

Program 1: 90 $^{o}$C for 10 min. The retention times of 1-chlorohexane, **1**, **3**, **7**, **8**, **9**, **10** and **11** were 3.8, 4.6, 4.8, 7.9, 3.4, 3.1, 9.1 and 4.8 min, respectively.

Program 2: 60 $^{o}$C for 10 min. The retention times of 1-chlorohexane and **2** were 6.9 and 3.8 min, respectively.

Program 3: 60 $^{o}$C for 4 min and heating with 20 $^{o}$C min$^{-1}$ to 160 $^{o}$C for 2 min. The retention times of 1-chlorohexane, **4** and **5** were 5.8, 8.4 and 9.2 min, respectively.

Program 4: 120 $^{o}$C for 4 min and heating with 20 $^{o}$C min$^{-1}$ to 180 $^{o}$C. The retention times of 1-chlorohexane and **6** were 2.8 and 4.8 min, respectively.

Halohydrins **1**, **4**, **5**, **6**, **7**, **8**, **9** and **10** were selected to measure kinetic parameters at the concentrations ranging from 10 to 100 mM using the purified HHDH-PL. The reactions were carried out at 650 μL of PBS buffer (50 mM, pH 7.5) containing 10 μL purified HHDH-PL and the gradient concentrations of each substrate. The mixtures were incubated at 40 $^{o}$C for 10 min and 700 μL of ethyl acetate was added to extract the sample for GC assay. The initial rates were calculated using the consuming of substrates and then fitted to the equation: $V = V_{max}\cdot[S]/K_m+[S]$, where $V_{max}$ is the maximum rate, [S] is substrate concentration, and $K_m$ is the Michaelis constant. The concentration of the enzyme was determined by measuring the absorbance at 590 nm with BCA Protein Assay Kit (KeyGEN Biotech, China).

## 5. Determination of the activities of HHDHs for HN

$HheA_{AD2}$, $HheB_{GP1}$, HheC and HHDH-PL were used to prepare HN using the crude enzyme. Each 0.1 g (wet weight) of the recombinant HHDHs was resuspended in 20 mL PBS (50 mM, pH 7.0). After sonication for 15 min at 0 °C, the mixtures were centrifuged 15,000×g for 20 min at 4 °C. The supernatants were used as crude enzyme for biotransformation of (*S*)-CHBE. The 18 mL PBS buffer (50 mM, pH 7.0) mixed with 0.6 g (*S*)-CHBE and 200 uL of 30% NaCN solution. The mixture was heated to 40 °C and 2 mL crude enzyme was added. 800 μL of the mixture was taken after 15 min and extracted with 800 μL of ethyl acetate. The organic extracts were analyzed by GC-7890A using the program 4 as described above. The retention times of (*S*)-CHBE and HN was 4.8 and 6.0 min, respectively. The reaction rates were calculated using the yield of HN.

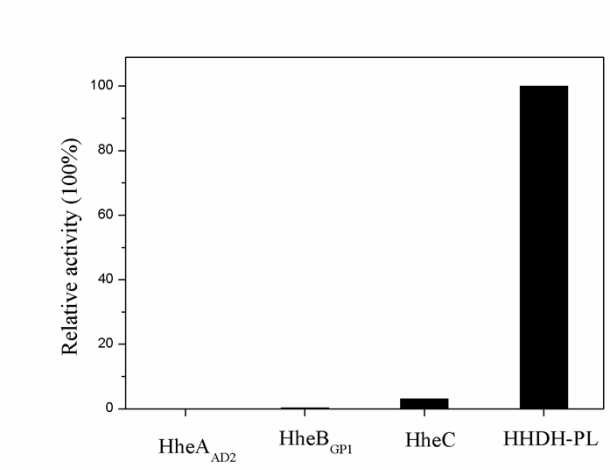


**Figure S5.** Relative activities of HHDHs for preparation of HN.

## 6. Docking Study

The modeling structure of HHDH-PL was constructed by Modeller[3] (version 9.12) software using crystal structures $HheA_{AD2}$ (PDB code: 1ZMO, 2.00 Å) and HheC (PDB code: 1PWX, 1.80 Å) as templates. The obtained models were sent to Procheck[4] and Profile-3D for analysis and validation. The optimal model was selected for docking study against (*S*)-CHBE using AutoDocK (version 4.20)[5]. The docking study of HheC was also performed to compare with HHDH-PL. (*S*)-CHBE was treated using the program LigPrep of Maestro 9.0

(Schrodinger LLC) to generate lowest energy conformations before docking studies. The docking results were analyzed and exhibited using PyMoL[6] software (academic version).

## 7. Optimization of the conditions for preparation of HN

All the reactions for synthesis of HN were performed on 902 Titrando system (Metrohm, Switzerland) equipped with a magnetic stirrer (IkA, Japan) and pH stat. The commercial substrate (*S*)-CHBE (99% ee, 98% purity) was provided by Zhejiang Neo-Dankong Pharmaceutical Co.,Ltd.(Taizhou, China). The temperature optimizations were carried out at 40 $^{o}$C under the following conditions: 20 mL PBS buffer (50 mM, pH 7.5, 500 rpm) containing 2 g (*S*)-CHBE and 0.4 g lyophilized whole cell of HHDH-PL. The dehalogenation reaction released proton and lowered the mixture pH, for which 30% of sodium cyanide solution was added to set pH 7.8 by a pH stat. Each 800 μL of samples were taken from mixture and extracted with 800 μL ethyl acetate for GC analysis after 18 h. The pH optimizations were carried out at 40 $^{o}$C under the following conditions: 20 mL PBS buffer (50 mM, pH 7.5, 500 rpm) containing 2 g (*S*)-CHBE and 0.4 g lyophilized whole cell of HHDH-PL. The different pHs were set and controlled by adding 30% of sodium cyanide solution with a pH stat. The reactions were monitored by taking samples for GC assay under the program 4. The retention times of (*S*)-CHBE and HN were 4.8 and 6.0 min, respectively (Figure S6).

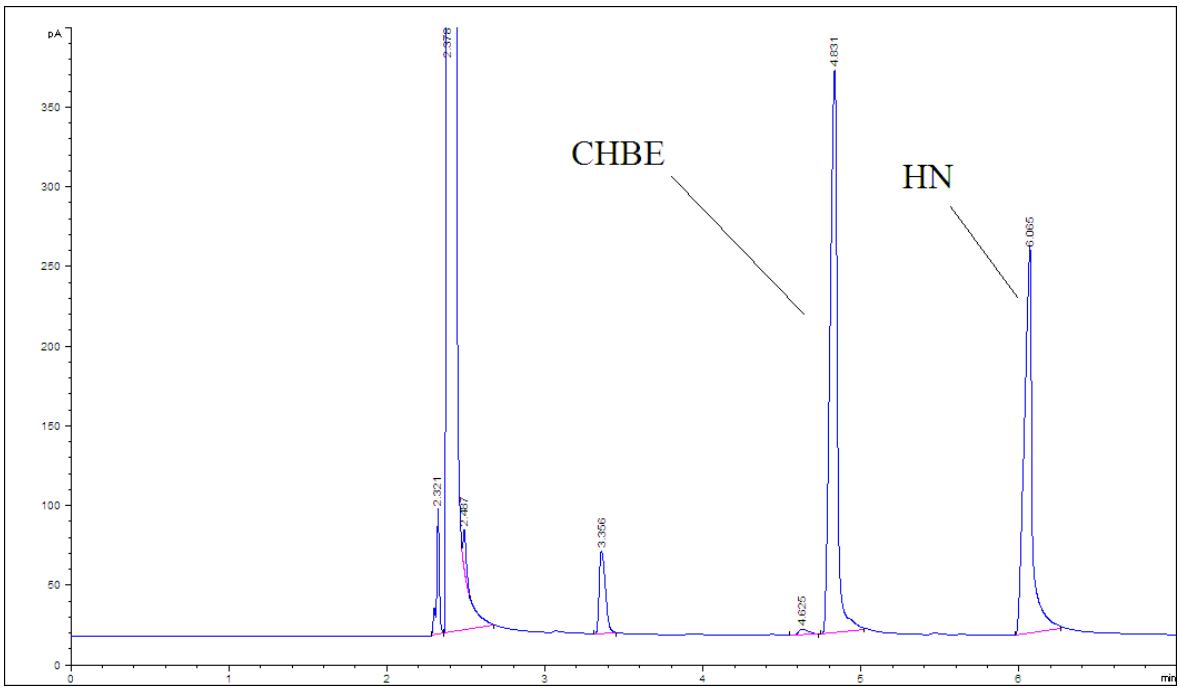


**Figure S6.** GC chart for (*S*)-CHBE and HN

## 8. Characterization of the product (HN) by GC and NMR

The enantiomeric purity of HN was analyzed by using GC-14C (Shimadzu, Japan) equipped with a FID detector and column BGB-174 (30 m×0.25 mm, 0.25 μm film thickness, BGB Analytik). Both inlet and detector temperatures were set at 220 °C. Helium was used as carrier at the flow rate of 1.5 mL·min$^{-1}$. The program was 160 °C for 30 min and the retentions of (*S*)-HN and (*R*)-HN were 23.7 and 24.2 min, respectively (Figure S7). The standards of (*R*)-HN and (*S*)-HN were purchased from J&K Scientific (Shanghai, China). The enantiopurity of the product was determined and calculated by GC assay ((*R*)-HN, ee > 99%) (Figure S8). GC-MS (6890-5975C, Aglient) characterization of the product was performed under program 4 (Figure S9). $^{1}$H and $^{13}$C NMR spectra were recorded on a Agilent 600 MHz DD2 spectrometer in $CDCl_3$. Chemical shift (δ) are given in ppm downfield from TMS as the internal standard. $^{1}$H NMR (600 MHz, $CDCl_3$) δ 4.55-4.52 (m, 1H), 4.19-4.16 (q, $J$ = 7.1 Hz, 2H), 2.65-2.60 (m, 4H), 1.28-1.25 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 174.35, 119.78, 66.97, 64.02, 42.90, 27.83, 16.83.

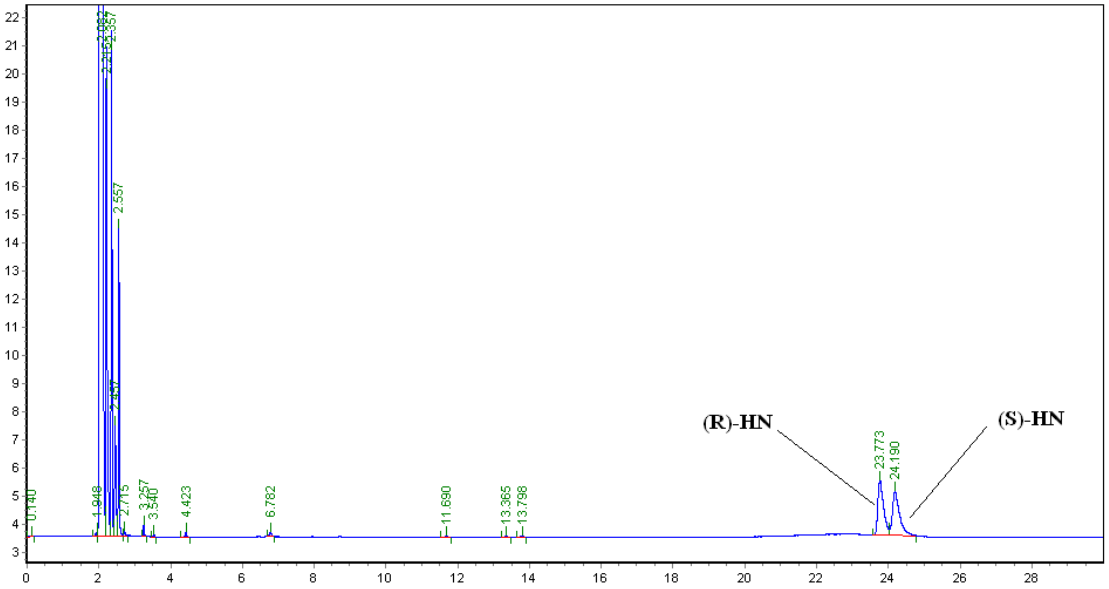


**Figure S7.** GC assay for racemic HN

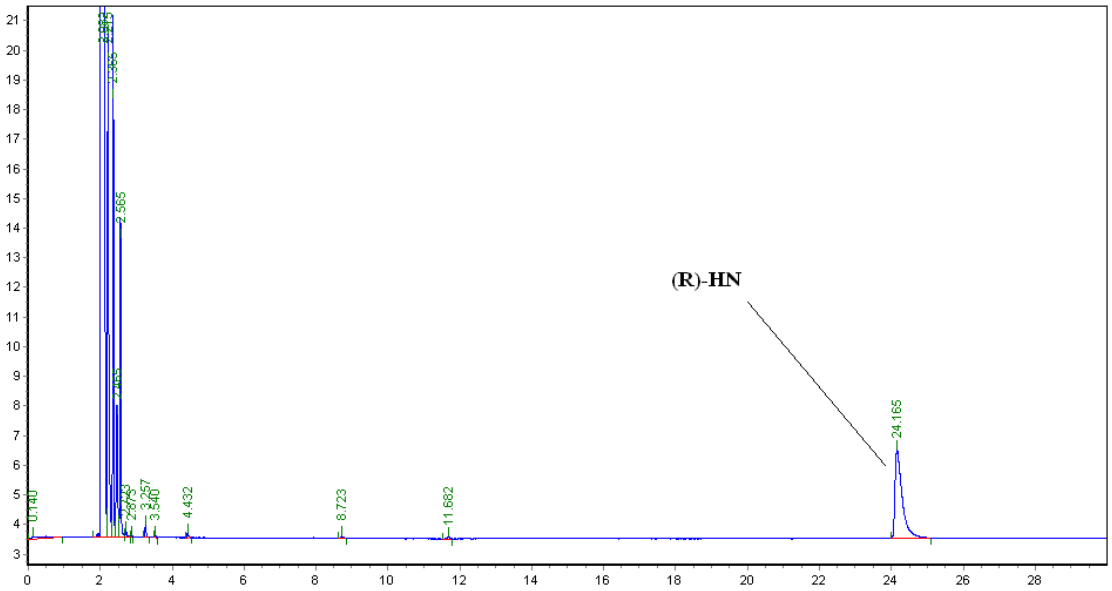


**Figure S8.** GC assay for (*R*)-HN

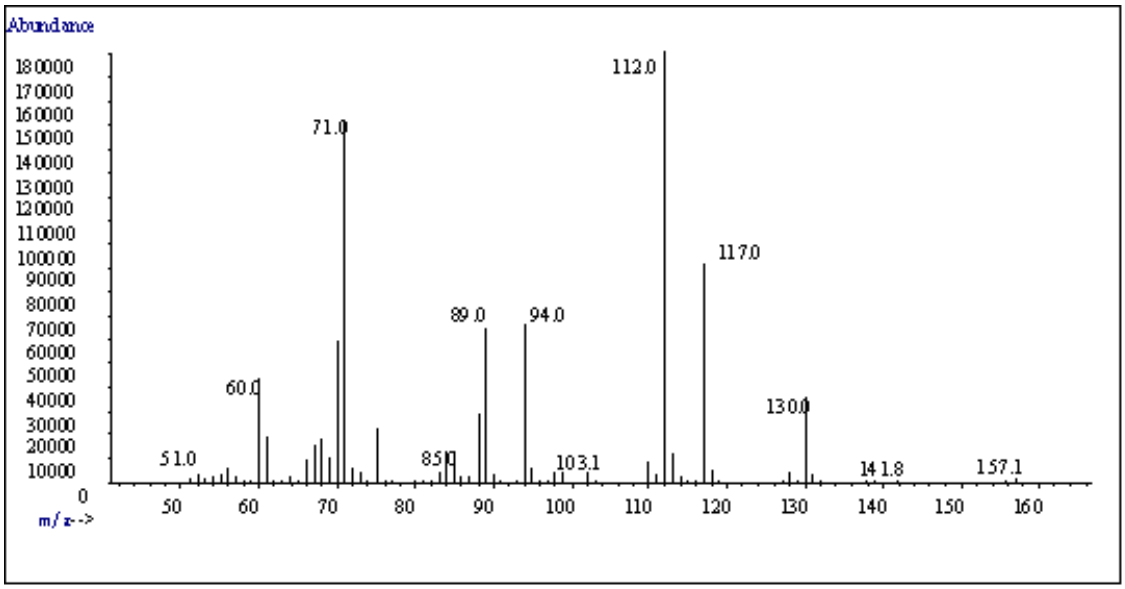


**Figure S9.** GC-MS assay for (*R*)-HN.

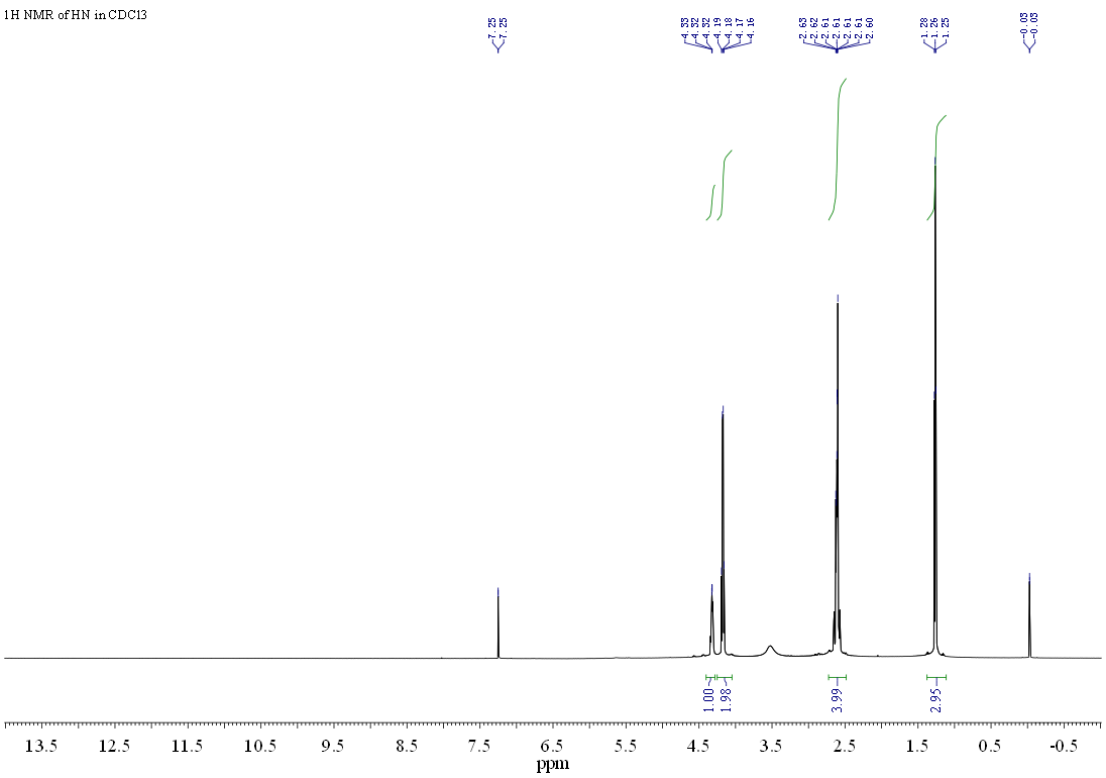


**Figure S10.** $^{1}$H NMR spectra for (*R*)-HN.

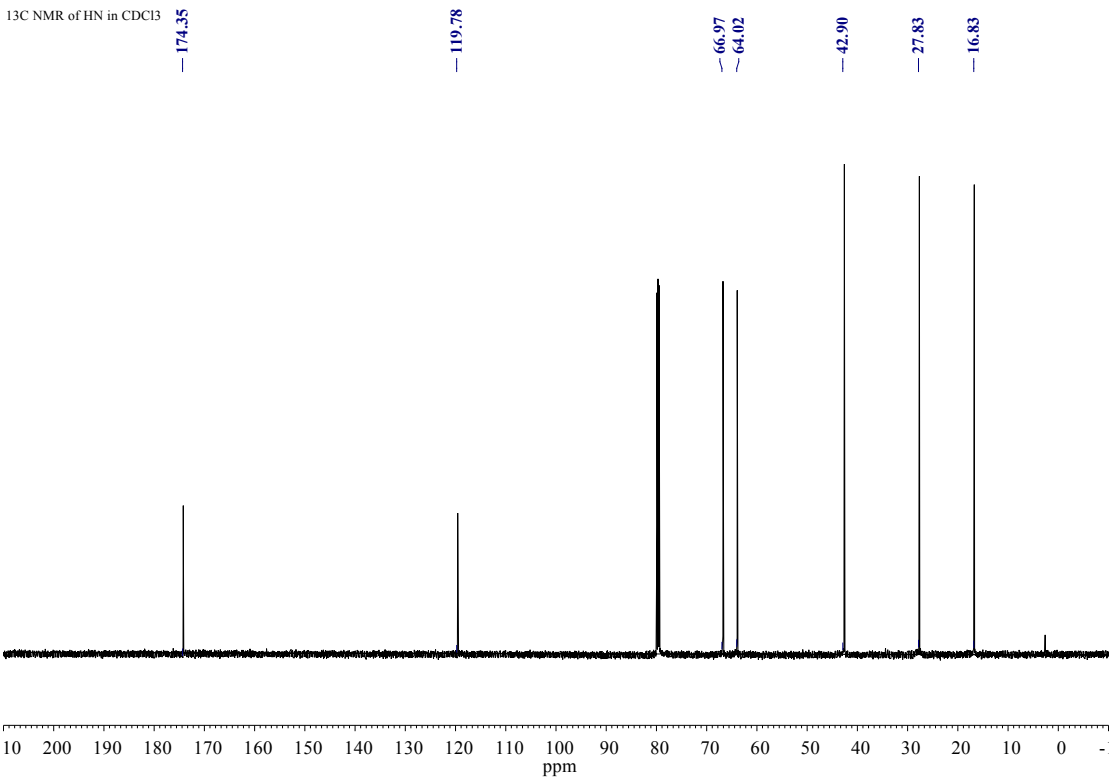


**Figure S11.** $^{13}C$ NMR spectra for (*R*)-HN.

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