

Supporting Information

Structure-guided Tuning of a Hydroxynitrile Lyase to Accept Rigid Pharmacological Aldehydes

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1. Experimental Procedures

1.1 General methods

Strains of *E. coli* BL21(DE3) and *E. coli* DH5 α were purchased from TransGen Biotech Co., Ltd. Beijing, China, competent cells preparation and transformation were carried out according to standard calcium chloride method.^[S1] *Pichia pastoris* X33 was kindly provided from Prof. Liming Liu (State Key Laboratory of Food Science and Technology, Jiangnan University); competent cells preparation and transformation were conducted according to Letchworth's work.^[S2] Kanamycin, ampicillin and isopropyl- β -D-1-thiogalactoside (IPTG) were purchased from Sangon Biotech (Shanghai) Co., Ltd., China. Zeocin was purchased from Thermo Fisher Scientific Inc., Shanghai, China. The gene of endo- β -*N*-acetylglucosaminidase H originated from *Streptomyces plicatus* (*Sp*Endo H,) was codon optimized, synthesized and subsequently cloned into pUC-57 by Genscript Biotech (Nanjing) Co., Ltd (Nanjing, China). The plasmid pPICZ α A containing the codon optimized gene of hydroxynitrile lyase from *Prunus communis* (PcHNL5) for expression was constructed according to our previous work by Zheng *et al.*^[S3]

Unless specified otherwise, chemicals and solvents were purchased from suppliers and used without further purification. ¹H NMR, and ¹³C NMR spectra were recorded on a Bruker AM-400 or a Bruker avance-600 spectrometer in CDCl₃, acetone-*d*₆, MeOH-*d*₄ or DMSO-*d*₆. The chemical shift (δ) of ¹H NMR and ¹³C NMR was given in ppm relative to solvent residual peak according to the work of Fulmer *et al.*,^[S4] Fast column chromatography was carried out using 200–300 mesh silica gel. Thin layer chromatography (TLC) analysis was carried out on glass sheets coated with 0.2 mm thickness silica gel, and visualized using potassium permanganate solution, alkaline potassium manganate (VII) solution, and/or UV light at 254 nm. Analyses of biotransformation and enantiomeric excess were performed on a Shimadzu GC 2014 system equipped with an Agilent CP-Chirasil-DEX CB column (25 m \times 0.25 mm \times 0.25 μ m), using nitrogen as carrier gas. Analyses of enantiomeric excess of intermediates in (R)-salmeterol synthesis were performed on a HPLC SHIMADAZU LC-20A system, equipped with a chiral column (Shanghai Chiralway Biotech Co., Ltd, China, 15 cm \times 0.46 cm \times 5 μ m), and a UV-detector. The optical rotation was recorded on a JASCO P-1030 polarimeter.

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1.2 Stock solutions and medium

Lysogeny broth (LB) medium: tryptone (10 g·L⁻¹), yeast extract (5 g·L⁻¹) and sodium chloride (10 g·L⁻¹) were dissolved in deionized water and subsequently autoclaved (121 °C for 20 min); before use, kanamycin was added with a final concentration 50 µg mL⁻¹.

LB-agar plates: tryptone (10 g·L⁻¹), yeast extract (5 g·L⁻¹), sodium chloride (10 g·L⁻¹) and agar (20 g·L⁻¹) were dissolved in deionized water and subsequently autoclaved (121 °C for 20 min); after cooling to approximately 60 °C, kanamycin was added resulting a final concentration 50 µg mL⁻¹.

Low salt lysogeny broth (LLB) medium: tryptone (10 g·L⁻¹), yeast extract (5 g·L⁻¹) and sodium chloride (5 g·L⁻¹) were dissolved in deionized water and subsequently autoclaved (121 °C for 20 min); before use, zeocin was added resulting a final concentration of 40 µg mL⁻¹.

LLB-agar plates: tryptone (10 g·L⁻¹), yeast extract (5 g·L⁻¹), sodium chloride (5 g·L⁻¹) and agar (20 g·L⁻¹) were dissolved in deionized water and subsequently autoclaved (121 °C for 20 min); after cooling to approximately 60 °C zeocin was added resulting a final concentration of 40 µg mL⁻¹.

10 × Glycerol stock: Glycerol was dissolved in deionized water resulting to a final concentration of 100 g·L⁻¹, and then autoclaved (121 °C for 20 min).

10 × Glucose stock: Glucose was dissolved in deionized water resulting to a final concentration of 200 g·L⁻¹, and then autoclaved (110 °C for 20 min).

10 × Yeast Nitrogen Base (YNB) stock: Yeast nitrogen base without amino acids and ammonium sulfate (34 g·L⁻¹) and ammonium sulfate (100 g·L⁻¹) were dissolved in deionized water; the resulting solution was subsequently autoclaved (110 °C for 20 min) and stored in dark at 4°C until use.

5 × Potassium phosphate buffer: Potassium phosphate Monobasic was dissolved in deionized water, the pH of resulting solution was adjusted to 6.0 by potassium hydroxide pellets, and the final concentration of phosphate was adjusted to 1 M, then autoclaved (121 °C for 20 min).

Yeast extract peptone dextrose (YPD) medium: peptone (22 g·L⁻¹), yeast extract (11 g·L⁻¹) were firstly dissolved in deionized water, and the resulting solution was autoclaved (121 °C for 20 min). Afterward, 10 × glucose stock and zeocin were added to a final concentration of 20 g·L⁻¹ and 200 µg mL⁻¹, respectively.

YPD-agar plates: peptone (22 g·L⁻¹), yeast extract (11 g·L⁻¹) and agar (22 g·L⁻¹) were firstly dissolved in deionized water (90 mL), and the resulting mixture was autoclaved (121 °C for 20 min). Afterward, 10 × glucose stock and zeocin were added to a final concentration of 20 g·L⁻¹ and 1 mg mL⁻¹, respectively.

Modified glycerol-complex (MMGY) medium: tryptone (12.5 g·L⁻¹) and yeast extract (12.5 g·L⁻¹) were firstly dissolved in deionized water (80 mL), and the resulting solution was autoclaved (121 °C for 20 min). Afterward, 10 × glycerol stock (10 mL, final conc. 10 g·L⁻¹, w/v) and 10 × YNB stock (10 mL, final conc. 1.34%, w/v) was added, respectively.

Modified buffered methanol-complex (MBMMY) medium: tryptone (15 g·L⁻¹) and yeast extract (15 g·L⁻¹) were firstly dissolved in deionized water (70 mL), and the resulting solution was autoclaved (121 °C for 20 min). Afterward, 10 × YNB stock (10 mL, final conc. 1.34%, w/v), 5 × potassium phosphate buffer (20 mL, final conc. 200 mM) and neat methanol (2 mL, final conc. 2%, v/v) were added, respectively.

PTM₁ trace salts solution: biotin (0.2 g·L⁻¹), CuSO₄·5H₂O (6.0 g·L⁻¹), KI (0.09 g·L⁻¹), MnSO₄·H₂O (3 g·L⁻¹), Na₂MoO₄·2H₂O (0.2 g·L⁻¹), H₃BO₃ (0.02 g·L⁻¹), CoCl₂·6H₂O (0.9 g·L⁻¹), ZnSO₄·7H₂O (42.2 g·L⁻¹), concentrated H₂SO₄ (5 mL·L⁻¹), FeSO₄·7H₂O (65 g·L⁻¹) were dissolved in preheated (~45 °C) deionized water (1 L) in order, and the resulting solution was sterilized by filtration.

Basal salts medium (BSM): phosphoric acid (85%, 21 mL·L⁻¹), CaSO₄ (0.9 g·L⁻¹), K₂SO₄ (14.3 g·L⁻¹), MgSO₄ (6.0 g·L⁻¹), potassium hydroxide (3.3 g·L⁻¹) and glycerol (40 g·L⁻¹) were dissolved in water (1.8 L), and the resulting solution was autoclaved (121 °C for 30 min) and then cooled to room temperature. The pH of the medium was adjusted by ammonium hydroxide to 5.0 and supplemented by PTM₁ trace salts solution (4.4 mL·L⁻¹).

Glycerol feeding medium: glycerol (600 g·L⁻¹) was dissolved in 500 mL deionized water, autoclaved (121 °C for 30 min), cooled to room temperature, and then supplemented with PTM₁ trace salts solution (13 mL·L⁻¹).

Methanol feeding medium: neat methanol (1 L) was supplemented by PTM₁ trace salts solution (13 mL·L⁻¹).

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1.3 Primers employed in this work

Primers were synthesized by Shanghai Generay Biotech Co., Ltd. Shanghai, China; for details of each primer, see **Table S1**.

Table S1. List of Primers used in this study.

Name	Sequence
F72V-FP	ACACCTTAAGTGCAGACGGAG GTC GCCTACAACCTGCAACAACA
F72V-RP	TGTTGTTGCAAGTTGTAGGC GACT CCGTCTGCAGTTAAGGTGT
L331V-FP	TACTACCAAGTCTCT GTC TCTTCCTTGCCATTCTCT
L331V-RP	AGAGAATGGCAAGGAAGA GAC AGAGACTTGGTAGTA
L343V-FP	TCTCTACTCCACCTTTCTCT GTT TTCCCAACCACTTCTTACCCA
L343V-RP	AGAAGTGGTTGGGAA AAC AGAGAAAGGTGGAGTAGAGAA
H358V-FP	CTTCCTAACTCCACTTTTCGCT GTC ATTGTGTCTCAAGTTCCTGG
H358V-RP	CCAGGAACCTGAGACACAAT GAC AGCGAAAGTGGAGTTAGGAAG
L331W-FP	TACTACCAAGTCTCT TGG TCTTCCTTGCCATTCTCT
L331W-RP	AGAGAATGGCAAGGAAGA CCA AGAGACTTGGTAGTA
L331S-FP	CCGATTACTACCAAGTCTCT TCC TCTTCCTTGCCATTCTCTAC
L331S-RP	GTAGAGAATGGCAAGGAAGA GGA AGAGACTTGGTAGTAATCGG
L331T-FP	CCGATTACTACCAAGTCTCT ACT TCTTCCTTGCCATTCTCTAC
L331T-RP	GTAGAGAATGGCAAGGAAGA AGT AGAGACTTGGTAGTAATCGG
L331F-FP	TACTACCAAGTCTCT TTC TCTTCCTTGCCATTCTCT
L331F-RP	AGAATGGCAAGGAAGA GAA AGAGACTTGGTAGTA
L331A-FP	TACTACCAAGTCTCT GCA TCTTCCTTGCCATTCTCT
L331A-RP	AGAGAATGGCAAGGAAGA TGC AGAGACTTGGTAGTA
L331G-FP	TACTACCAAGTCTCT GGT TCTTCCTTGCCATTCTCT
L331G-RP	AGAGAATGGCAAGGAAGA ACC AGAGACTTGGTAGTA
<i>Sp</i> Endo H-(<i>Nde</i> I)-FP	GGGTTT CATATG ATGTTACCCCGGTTTCGTCG
<i>Sp</i> Endo H-(<i>Xho</i> I)-RP	CCG CTCGAG TTATTTTTCGAACTGAGGGTGAGACCACTGATGATGAT GATGATGCGGGGTACGAACAGCCTC

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1.4 Site-directed mutagenesis

Based on the protocol of QuikChange® Site-Directed Mutagenesis Kit, the site-directed mutagenesis was performed in 200 μL Eppendorf PCR tubes. Previously constructed plasmid pPICZ α A-*PcHNL5* was used as template.^[53] The following components were mixed on ice (total volume 20 μL).

Table S2. PCR conditions.

Component	Volume (μL)	Final concentration
Template	0.5	0.5 – 2 ng μL^{-1}
Primer FP	0.5	0.25 μM
Primer RP	0.5	0.25 μM
DMSO	0.5	2.5 %
ddH ₂ O	6	-
2 \times PrimeStar® HS Premix solution	10	1 \times

Samples were transferred into a Bio-Rad C1000 Touch™ Thermal Cycler QX100 Droplet Digital PCR, and the following temperature program was applied.

Table S3 PCR program.

Temperature ($^{\circ}\text{C}$)	Time (s)	Notes
95	180	
98	10	15 cycles
54	15	
72	315	
72	360	
4	hold	

Afterward, 2 μL of each sample was tested by 1% agarose gel electrophoresis. Successfully amplified samples were incubated after adding 2 μL 10 \times CutSmart buffer and 1 μL *Dpn* I (New England Biolabs (Beijing) Ltd. Beijing, China) at 37 $^{\circ}\text{C}$ for 3 h. 5 μL of the reaction mixture was directly used for *E. coli* DH5 α transformation. Cells were spread on LLB-agar plates which contained 40 $\mu\text{g mL}^{-1}$ of zeocin and incubated at 37 $^{\circ}\text{C}$ for 20 h. Two positive transformants were chosen for plasmid extraction, and further sent to Sangon Biotech (Shanghai) Co., Ltd., China for sequencing. About 5 μg of the plasmid was digested by *Sac* I (New England Biolabs (Beijing) Ltd., China) for 6 h, and recovered with PCR purification Kit (Aidlab Biotechnologies Co., Ltd, Beijing, China). Competent cells preparation and transformation of *P. pastoris* X33 were conducted according to as described in Letchworth's work.^[52] Cells were directly spread on YPD-agar plates which contained 1 mg mL^{-1} zeocin and incubated at 30 $^{\circ}\text{C}$ for 48 h. Five transformants were verified by PCR experiments and one of them which contained mutated HNL gene was selected for further expression.

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1.5 Biocatalyst preparation and purification

1.5.1 PchNL5

One yeast colony was picked into 4 mL YPD media which contained 200 $\mu\text{g}\cdot\text{mL}^{-1}$ zeocin and incubated at 30 °C, 250 rpm for 24 h. 1 mL of the culture was inoculated into a 500 mL baffled flask which contained 100 mL MMGY medium and was grown at 28 °C, 200 rpm until OD₆₀₀ arrived at 2.0 to 6.0. Clear broth was carefully removed after spontaneous subsidence of yeast cells for 4 h, and cell pellets were resuspended with 100 mL MBMMY medium and induced at 28 °C, 200 rpm. 1.5 mL of neat methanol was added after further 24, 48, 72, and 96 h.

Purification of PchNL5 was modified from our previous work.^[S3] All of purification procedures were carried out at 4 °C. Briefly, after about 110 to 120 h of methanol induction, fermentation culture was collected, and biomass as well as cell debris were removed by centrifugation at 8000 g for 1 h. About 500 mL of clear culture broth was subjected to 0.45 μm micro-filtration and subsequently concentrated to 50 mL by ultrafiltration on a LabScale™ Tangential Flow Filtration System (Merck Millipore, German) equipped with a 30 kDa cut off module (Pellicon® XL, 50 cm², Millipore, Germany), prior to the further purification procedure. The concentrated broth was diluted and concentrated twice with citrate buffer (20 mM, pH 5.5) to remove pigment and salts. Concentrated HNL solution was subsequently processed by 30–60% fractional precipitation with 1,2-dimethoxyethane, HNL precipitant was dissolved in ice cold Ni-NTA buffer A (sodium phosphate, 20 mM, pH 7.4; NaCl, 500 mM; 2-mercaptoethanol, 5 mM) and then purified by Ni-affinity chromatography applying a 0 to 500 mM imidazole by gradient elution of Ni-NTA buffer B (sodium phosphate, 20 mM, pH 8.0; NaCl, 500 mM; imidazole, 500 mM; 2-mercaptoethanol, 5 mM). Fractions were analyzed by SDS-PAGE, and highly pure PchNL5 samples were pooled, concentrated and desalted with Ni-NTA buffer C (sodium phosphate, 20 mM, pH 7.4; glycerol, 20%, v/v) by ultrafiltration (15 mL, 30 kDa cut-off, Millipore, Germany). Samples were fast frozen in liquid nitrogen and stored at -80 °C.

The molecular extension of PchNL5 was predicted according to the online server (<http://expasy.org/tools/protparam.html>), and the protein concentration was determined by a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA). The calculation of molarity of over-glycosylated PchNL5 was according to the procedure from Zheng et al.^[S3]

1.5.2 High-density fermentation of PchNL5 L331X mutants in 5-L bioreactor

Production of PchNL5 L331X was performed in a 5-L bioreactor. Strains obtained in 2.5.1 was maintained at -80 °C, a single yeast colony was isolated on YPD agar plate (incubated at 30 °C for 48 h) contained 200 $\mu\text{g}\cdot\text{mL}^{-1}$ zeocin and picked into a 50 mL shake flask containing 10 mL YPD medium which is supplemented with Zeocin (200 $\mu\text{g}\cdot\text{mL}^{-1}$), and cultured at 30 °C, 250 rpm for 12 to 16 h. 1 mL of the culture was inoculated into a 500 mL baffled flask which contained 100 mL YPD medium and grown at 30 °C, 250 rpm until the OD₆₀₀ was about 2.0. Two hundred milliliter culture of precultured recombinant yeast containing L331X mutants was inoculated into 1.8 L initial medium (BSM). The fermentation was carried out at 28 °C, DO > 20%, and pH 5.0. After about 18 h of the fermentation, the DO value was suddenly rebounded to 100%, indicating the consumption of glycerol in the initial medium, and then glycerol feeding was started until an OD₆₀₀ of about 200. A starvation was kept for 1 h to make sure the metabolism intermediates of glycerol were consumed. Then methanol induction was started with an initial rate of 4 mL/h during the first 6 h to make the yeast adapt to methanol before being stepped to a higher rate of about 15 mL/h. The expression of L331X mutants was monitored by determining the cleavage activity toward racemic mandelonitrile (*rac-2a*, see 2.12 for experimental details). The induction was continued for about 72 – 96 h until the HNL titer did not grow further. The supernatant of culture broth was collected by centrifugation at 8000 × g, 4 °C for 15 min, and kept on ice for further treatment.

1.5.3 SpEndo H

The gene of SpEndo H (GenBank: K02182.1) was further amplified by PCR (See primers at **Table S1**), ligated on pET-28(a)+ and transferred into *E. coli* BL21(DE3). One positive transformant was inoculated into 4 mL LB medium which contained 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin, and cultured at 37 °C, 200 rpm overnight. 1 mL of the culture was inoculated into 100 mL LB medium which contained 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin until OD₆₀₀ arrived 0.6 to 0.8 at 37 °C, 180 rpm, 1 M IPTG stock was added to a final concentration of 0.3 mM. Cells were induced at 28 °C, 180 rpm for 12 h, and then harvested by centrifugation. Cell pellets were resuspended in Ni-NTA buffer A2 (sodium phosphate, 20 mM, pH 7.4; NaCl, 500 mM; imidazole, 10 mM; 2-mercaptoethanol, 5 mM). A clear lysate was obtained by sonication and subsequent centrifugation at 10000 g, 4 °C for 30 min. His-tagged SpEndo H was purified by Ni-affinity chromatography applying a 10 to 500 mM imidazole by gradient elution of Ni-NTA buffer B (sodium phosphate, 20 mM, pH 8.0; NaCl, 500 mM; imidazole, 500 mM; 2-mercaptoethanol, 5 mM). Fractions were analyzed by SDS-PAGE, highly pure SpEndo H were pooled, concentrated and desalted with deglyco-buffer (sodium phosphate, 50 mM, pH 6.0) by ultrafiltration (15 mL, 10 kDa cut-off, Millipore, Germany). Samples were fast frozen in liquid nitrogen and stored at -80 °C until for further experiments.

The molecular extension of SpEndo H was predicted according to the online server (<http://expasy.org/tools/protparam.html>), and the protein concentration determined by NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA).

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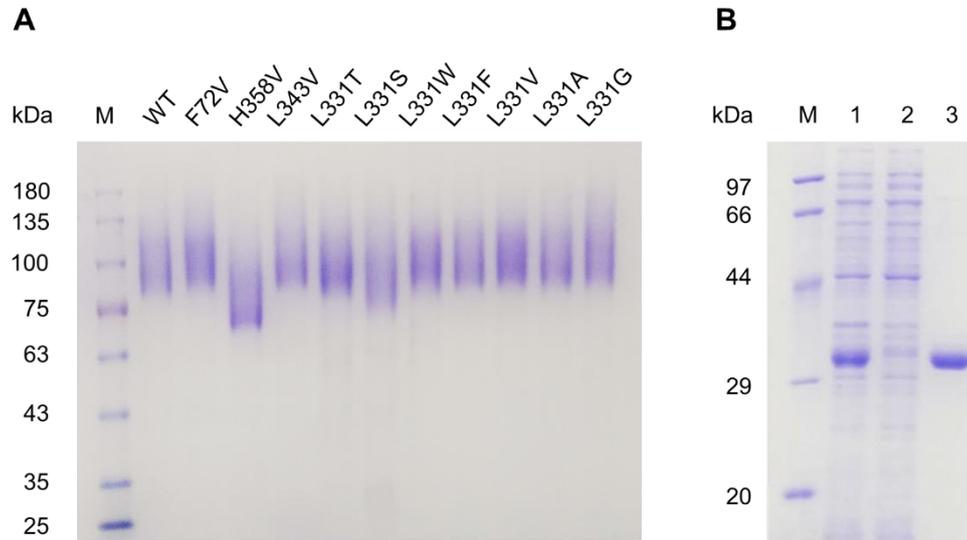


Figure S1. A. SDS-PAGE analysis of Ni-NTA purified PcHNL5 variants. Lane M: standard protein marker. B. SDS-PAGE analysis of Ni-NTA purification *SpEndo H*. Lane M: standard protein marker; Lane 1: cell free extract; Lane 2: flow through; Lane 3: 5% Ni-NTA buffer B washed fraction.

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1.6 Protein crystallization

For protein crystallization, Ni-NTA purified PcHNL5 was desalted with deglyco-buffer (sodium phosphate, 50 mM, pH 6.0) and concentrated to about 10 mg mL⁻¹. 60 mg of PcHNL5 was mixed with 6 mg SpEndo H and incubated at 37 °C for more than 6 h. The deglycosylation was monitored by SDS-PAGE, and deglycosylated PcHNL5 was purified by a ÄKTA pure (GE Healthcare, Piscataway, USA) equipped with a Superdex 75 column (120 mL, GE Healthcare, Piscataway, USA) using Superdex buffer (sodium phosphate, 20 mM, pH 7.4; NaCl, 150 mM; dithiothreitol, 1 mM). Fractions containing highly pure deglycosylated HNLs were pooled, concentrated by ultrafiltration (15 mL, 30 kDa cut-off, Millipore, Germany). Deglycosylated HNLs were fast frozen in liquid nitrogen and stored at -80 °C.

Before crystallization, samples were thawed at 0 °C and subsequently centrifuged at 12,000 × *g*, 4 °C for 10 min to remove inactivated proteins. Initial screening of crystallization conditions was performed using commercially available Morpheus[®] Screen MD 1–46, JCSG + MD1–37 (Molecular Dimensions), and Index HT HR2–107, 109, 110, 112 (Hampton Research), The PEGs II Suite and the Protein Complex Suite (QIAGEN), using the sitting drop vapor-diffusion method in 96-well plates at 18 °C. Further optimization of crystallization conditions was performed in 24-well sitting drop plates to obtain optimal crystals for X-ray diffraction.

Crystals of deglycosylated PcHNL5 was obtained by mixing 2 μL protein sample (19 mg/mL) with 2 μL reservoir solution (tris-bicine, 100 mM, pH 8.5; CaCl₂, 60 mM; MgCl₂, 60 mM; PEG 500MME, 20%, v/v; PEG 20000, 10%, w/v). Crystals of PcHNL5_{L331A} was obtained by mixing 2 μL protein sample (19 mg/mL) with 2 μL reservoir solution (tris-bicine, 100 mM, pH 8.25; CaCl₂, 60 mM; MgCl₂, 60 mM; PEG 500MME, 24%, v/v; PEG 20000, 12%, w/v) after a further optimization based on the crystallization conditions of the wild type enzyme. The crystals were transferred into a pool which containing their corresponding reservoir solution, then flash-cooled in liquid nitrogen by using nylon CryoLoops[™] (Hampton Research) without any cryoprotectant.

Soaking experiments were carried out in a freshly prepared mother liquor which derived from reservoir solution (tris-bicine, 100 mM, pH 8.5; CaCl₂, 60 mM; MgCl₂, 60 mM; PEG 500MME, 20%, v/v; PEG 20000, 10%, w/v, 25 mM (*R*)-mandelonitrile (**2a**), 10% DMSO, v/v). Deglycosylated PcHNL5 crystals were transferred into the mother liquor by CryoLoops, and after about 15 s, 30 s, 1 min of the soaking time, the crystal was flash cooled in liquid nitrogen.

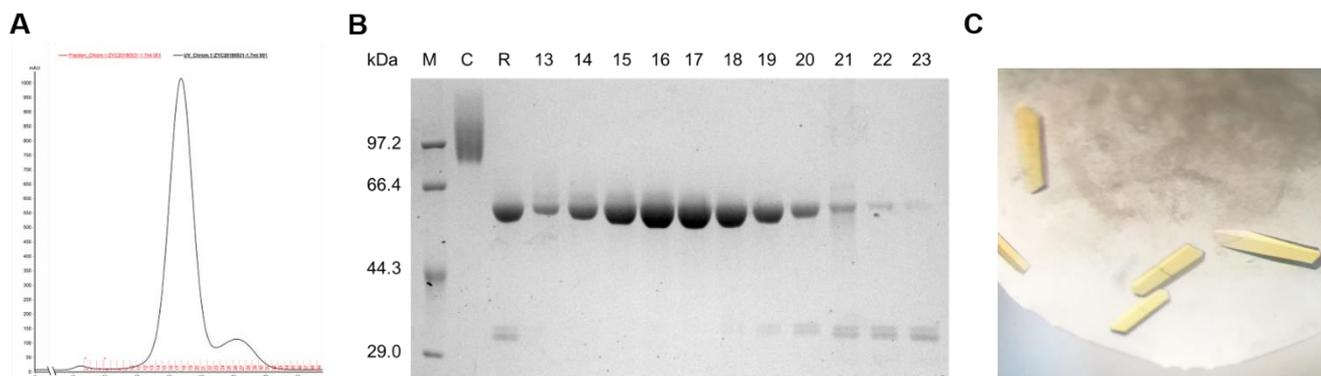


Figure S2. Deglycosylation and crystallization of PcHNL5. A. Chromatogram of purification of deglycosylated PcHNL5; B. SDS-PAGE analysis of fractions collected during the size exclusion chromatography by Superdex 75 column. Lane M: standard protein marker; Lane C: Ni-NTA purified PcHNL5; Lane R: deglycosylation of PcHNL5 by incubation with SpEndo H at 37 °C overnight; Lanes 13–23: fractions of separation of deglycosylated PcHNL5 with SpEndo H by Superdex 75 column. C. Crystals of deglycosylated PcHNL5 used for data collection.

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1.7 Data collection, processing and refinement

All data were collected at 100 K, with the wavelength of 0.9789 Å by using an ADSC Quantum 315r detector at beamline BL17U1 or a DECTRIS PILATUS3 6M detector at beamline BL19U1 of the Shanghai Synchrotron Radiation Facility (SSRF), data were processed with HKL3000.^[55] The crystal structure was solved by molecular replacement by employing CCP4 Suite,^[56] and the peptide chain A was extracted from PDB 5EB4 as input template. Structure refinement was processed with software COOT^[57] and Phenix 1.12.^[58] Ligand FAD, benzaldehyde (HBX) and NAG were added according to observed electron density. Linkages between NAG and corresponding Asn residue were constructed during Phenix refinement. Figures were generated with PyMOL 2.2. Crystallographic details, processing and refinement statistics are collected in **Table S4**.

Table S4. Crystal structure refinement statistics.

	<i>PcHNL5_{WT}</i>	<i>PcHNL5_{WT} + 1a</i>	<i>PcHNL5_{WT} + (R)-2a</i>	<i>PcHNL5_{L331A}</i>
PDB code	6JBY	6LQY	7BWP	6LR8
Space group	P2 ₁ 2 ₁ 2 ₁			
Cell dimens				
a, b, c (Å)	49.5, 91.3, 132.0	49.6, 91.5, 130.8	49.5, 91.1, 130.2	49.6, 91.2, 130.9
α, β, γ (deg)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution range (Å)	46.37–1.60	46.35–1.60	46.30–1.80	41.31–1.60
Highest resolution shell (Å)	1.63–1.60	1.63–1.60	1.83–1.80	1.63–1.60
<i>R</i> _{merge}	0.109	0.117	0.247	0.089
<i>I</i> / σ (<i>I</i>)	20.74	46.46	34.33	22.2
No. of unique reflections	79762 (3877/4108)	79552 (3921/4094)	55236 (2751/2995)	78549 (3811/4088)
Completeness (%)	100	100	99.8	98.8
Redundancy	13.3 (13.6)	12.9 (12.9)	12.6	12.1 (12.4)
Refinement				
Resolution (Å)	1.60	1.60	1.80	1.60
<i>R</i> _{work} / <i>R</i> _{free} (%)	15.8/18.3	16.3/18.8	16.0/19.7	15.2/17.4
No. of water molecules	671	548	448	687
B factors				
protein (Å ²)	12.8	22.5	18.2	14.1
FAD (Å ²)	7.8	17.5	12.0	9.0
NAG (Å ²)	27.4	35.8	30.8	30.3
ligand (Å ²)	-	20.0	26.8	-
water (Å ²)	25.4	33.7	29.9	28.7
RMS deviations				
bond lengths (Å)	0.060	0.006	0.006	0.006
bond angles (deg)	0.908	0.891	0.863	0.920
Ramachandran plot				
favored (%)	96.15	96.68	96.67	96.68
allowed (%)	3.66	3.52	3.53	3.52
outliers (%)	0.19	0.20	0.20	0.20

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1.8 Computational details

1.8.1 Docking simulation

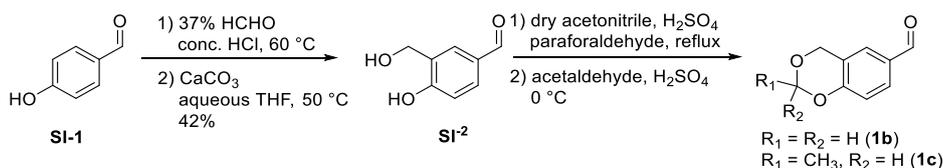
Docking simulation was performed by using AutoDock suite.^[S9] Crystal structure of apo *PcHNL5* (PDB: 6JBY) and *PcHNL5*_{L331A} (PDB: 6LR8) without water molecules was set as input receptor, respectively. The size of grid box was set as 40 × 40 × 40 Å³ and the center of box was surrounded by side chain of Tyr 458, His460, His498 and isoalloxazine ring of FAD. Substrate **1d** was docked into the active pocket and a total number of 200 binding poses for protein receptor were searched. A clustering analysis of the output results was conducted by AutoDock Tools 4.2. A typical predicted binding pose from the cluster was selected for further analysis.

1.8.2 Modelling of substrate access

Prediction of the substrate tunnel was performed by using CAVER PyMOL 3.0 Plugin.^[S10] Water molecules were removed from the crystal structure of *PcHNL5* (PDB: 6LQY) or *PcHNL5*_{L331A} (PDB: 6LR8), and the protein structure was chosen as input file. The minimum probe radius was 0.9 Å and the coordinate of starting point was set as the geometric center of catalytic residue Tyr 458, His460, His498, calculated by VMD 1.9.3 program.^[S11]

SUPPORTING INFORMATION

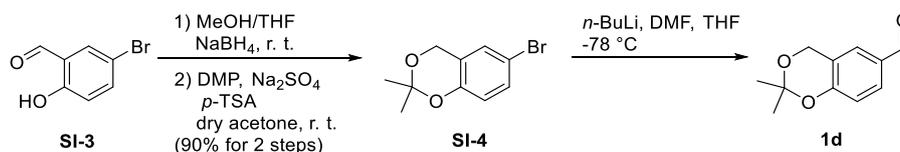
1.9 Chemical preparation of aldehydes



Based on Gisch's work,^[S12] to a suspension of compound **SI-1** (24.4 g, 200 mmol) in concentrated HCl (180 mL) was added formaldehyde (37% w/w, 20 mL). The reaction mixture was stirred at 60 °C for 5 h, then cooled on ice. All the opaque solid was filtrated and washed with appropriate ice-cold water. The resultant solid was re-dissolved in tetrahydrofuran (180 mL) and vigorously mixed with CaCO₃ (30 g, 0.3 mol) and water (75 mL). The mixture was stirred at 50 °C for 12 h. The pH value of resultant mixture was then adjusted to < 2 by adding 1N HCl (aq.) at 0°C. The resulting mixture was evaporated under reduced pressure to remove most of tetrahydrofuran, and then was extracted by ethyl acetate (4 × 80 mL). The combined organic layers were dried by anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was subjected to recrystallization in ethyl acetate and methanol to give **SI-2** as white solid (13 g, 42% yield). **4-hydroxy-3-(hydroxymethyl)benzaldehyde (SI-2)**:^[S12] ¹H NMR (600 MHz, DMSO-*d*₆) δ ppm 10.60 (s, 1H), 9.80 (s, 1H), 7.90 – 7.86 (m, 1H), 7.65 (dd, *J* = 8.2, 2.2 Hz, 1H), 6.94 (d, *J* = 8.2 Hz, 1H), 5.17 (s, 1H), 4.51 (d, *J* = 3.8 Hz, 2H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ ppm 191.3, 160.0, 130.5, 129.8, 128.7, 128.3, 114.8, 57.7.

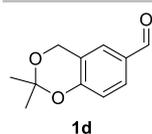
To a solution of compound **SI-2** (3.6 g, 23.6 mmol) in dry acetonitrile (80 mL) was added paraformaldehyde (9 g, 300 mmol). The mixture was refluxed under nitrogen for 5 h, then cooled to room temperature, diluted by dichloromethane (100 mL), and washed with NaOH (aq. 3 M, 3 × 30 mL). The aqueous phase was extracted by dichloromethane (4 × 30 mL). The combined organic layers were dried over anhydrous Na₂SO₄. After concentration under reduced pressure, the resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate /petroleum ether: 1/6) to afford **1b** as white solid (1.3 g, 33% yield). **4H-benzo[d][1,3]dioxine-6-carbaldehyde (1b)**:^[S13] ¹H NMR (600 MHz, CDCl₃) δ ppm 9.82 (s, 1H), 7.67 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.49 (dt, *J* = 2.2, 1.0 Hz, 1H), 6.95 (d, *J* = 8.4 Hz, 1H), 5.28 (s, 2H), 4.91 (s, 2H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 190.8, 157.8, 130.5, 130.2, 127.3, 121.7, 117.5, 91.7, 66.2.

Based on the procedure by Manetsch *et al.*,^[S14] to a suspension of compound **SI-2** (3.5 g, 23.0 mmol) in anhydrous acetaldehyde (60 mL) at 0 °C, three drops of concentrated sulfuric acid were added. The reaction mixture was further stirred at 0 °C for 5 h, then diluted by 100 mL methyl *tert*-butyl ether and washed with NaOH (aq., 3 M, 30 mL). The aqueous phase was extracted by methyl *tert*-butyl ether (3 × 30 mL). The combined organic layers were washed with brine and dried over anhydrous Na₂SO₄. After concentration under reduced pressure, the resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/8) to afford **1c** as white solid (3.4 g, 84% yield). **2-methyl-4H-benzo[d][1,3]dioxine-6-carbaldehyde (1c)**:^[S15] ¹H NMR (600 MHz, CDCl₃) δ ppm 9.83 (s, 1H), 7.68 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.51 (dt, *J* = 2.0, 0.9 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 1H), 5.24 (q, *J* = 5.1 Hz, 1H), 5.00 (d, *J* = 14.6 Hz, 1H), 4.87 (d, *J* = 14.6 Hz, 1H), 1.56 (d, *J* = 5.1 Hz, 3H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 190.9, 158.3, 130.6, 130.0, 127.1, 121.1, 117.4, 98.0, 66.3, 20.6. HRMS(EI-TOF): *m/z* calcd for C₁₀H₁₀O₃ [M⁺]: 178.0630, found: 178.0629.

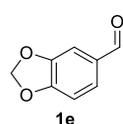
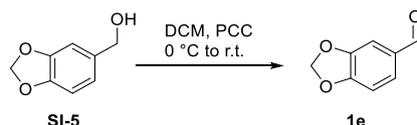


Modified from the work by Gisch *et al.*,^[S12] to a solution of compound **1-3** (25 g, 124 mmol) in methanol/tetrahydrofuran (1:1, v/v, 400 mL) was added sodium borohydride (10 g, 265 mmol) by small portions at 0 °C. The mixture was allowed to warm up to room temperature and further stirred for 0.5 h. The reaction was quenched by dropping 4N HCl (aq.) until the pH < 2, then evaporated under reduced pressure to remove most of the tetrahydrofuran and MeOH. The resultant mixture was extracted by ethyl acetate, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant white solid was re-dissolved in dry acetone (300 mL) and added by 2,2-dimethoxypropane (25 mL, 187 mmol), anhydrous Na₂SO₄ (30 g), and *p*-toluenesulfonic acid (2.2 g, 12.4 mmol). The reaction mixture was stirred at room temperature for 24 h and poured into an aqueous solution of ice-cold saturated sodium bicarbonate and evaporated under reduced pressure. The resultant mixture was extracted by dichloromethane (3 × 50 mL), dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/30) to afford compound **SI-4** as colorless oil (27 g, 90% yield for 2 steps). **6-bromo-2,2-dimethyl-4H-benzo[d][1,3]dioxine (SI-4)**:^[16] ¹H NMR (400 MHz, CDCl₃) δ ppm 7.24 (d, *J* = 2.4 Hz, 1H), 7.10 (d, *J* = 2.6 Hz, 1H), 6.70 (d, *J* = 8.7 Hz, 1H), 4.81 (s, 2H), 1.53 (s, 6H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 150.4, 131.1, 127.5, 121.5, 119.0, 112.5, 99.9, 60.4, 24.7.

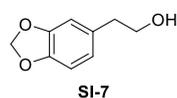
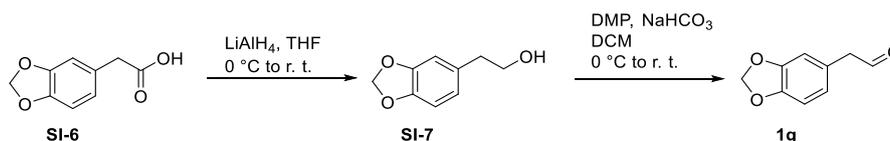
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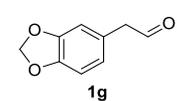
Following the procedure by Gisch et al.,^[S12] a solution of compound **SI-4** (13 g, 54 mmol) in dry tetrahydrofuran (500 mL) was cooled at -78 °C under argon for 15 min, then *n*-butyllithium (2.5 M in *n*-hexane, 30 mL) was added dropwise and further stirred at -78 °C for 2 h. Dry *N,N*-dimethylformamide (11.2 mL) was added over 5 min and the resultant was further stirred at -78 °C for 0.5 h, then the reaction mixture was allowed to stirred at 0 °C for 1 h. The reaction was quenched by slowly addition of saturated NH₄Cl aqueous solution (50 mL) at 0 °C. The opaque solid was formed and re-dissolved by adding 50 mL water and separated. The aqueous phase was extracted by dichloromethane (3 × 100 mL), the combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/30) and subsequent recrystallization in *n*-hexane to afford **1d** as white solid (8.9 g, 86% yield). **2,2-Dimethyl-4H-benzo[d][1,3]dioxine-6-carbaldehyde (1d)**:^[S16] ¹H NMR (400 MHz, CDCl₃) δ ppm 9.84 (s, 1H), 7.69 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.54 (d, *J* = 2.2 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 4.90 (s, 2H), 1.56 (s, 6H). ¹³C NMR (100 MHz, CDCl₃) δ ppm 190.9, 156.9, 130.6, 129.6, 127.0, 119.9, 117.9, 100.9, 60.7, 24.9. HRMS(EI-TOF): *m/z* calcd for C₁₁H₁₂O₃ [M⁺]: 192.0786, found: 192.0787.



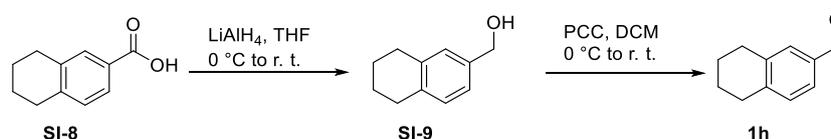
To a suspension of 4Å molecular sieve (10 g), Celite (10 g) and pyridinium chlorochromate (21 g, 97 mmol) in dichloromethane (200 mL) was added a solution of benzo[d][1,3]dioxol-5-ylmethanol **SI-5** (10 g, 65.7 mmol) in dichloromethane (50 mL) at 0 °C. The mixture was allowed to warm up to room temperature spontaneously and further stirred for 24 h. The reaction mixture was filtrated and the filtrate was concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/10) to afford **1e** as white solid (9.3 g, 94% yield). **benzo[d][1,3]dioxole-5-carbaldehyde (1e)**:^[S16] ¹H NMR (600 MHz, CDCl₃) δ ppm 9.73 (s, 1H), 7.34 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.25 (d, *J* = 1.6 Hz, 1H), 6.86 (d, *J* = 7.9 Hz, 1H), 6.01 (s, 2H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 190.3, 153.0, 148.7, 131.8, 128.7, 108.3, 106.7, 102.1. HRMS(EI-TOF): *m/z* calcd for C₁₁H₁₂O₃ [M⁺]: 150.0317, found: 150.0316.



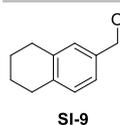
To a suspension of lithium aluminium hydride (4.2 g, 110 mmol) in dry tetrahydrofuran (180 mL) was added a solution of compound **SI-6** (10 g, 56 mmol) in dry tetrahydrofuran (50 mL) under argon at 0 °C. The mixture was allowed to spontaneously warm up to room temperature and further stirred for 4 h. The reaction was quenched by slow addition of NaOH aqueous solution (10%, w/v, 8 mL) and distilled water (13 mL) at 0 °C, respectively. The mixture was further vigorously stirred over night at room temperature until the mixture was layered easily. After filtration, the filtrate was dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The resultant residue was purified by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/4) to afford compound **SI-7** as yellow oil (8.8 g, 95% yield). **2-(benzo[d][1,3]dioxol-5-yl)ethan-1-ol (SI-7)**:^[S17] ¹H NMR (600 MHz, CDCl₃) δ ppm 6.76–6.68 (m, 2H), 6.65 (dd, *J* = 7.8, 1.7 Hz, 1H), 5.90 (s, 2H), 3.76 (t, *J* = 6.6 Hz, 2H), 2.75 (t, *J* = 6.6 Hz, 2H), 2.13 (s, 1H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 147.8, 146.1, 132.3, 121.9, 109.4, 108.3, 100.9, 63.7, 38.9.



To a solution of 2-(benzo[d][1,3]dioxol-5-yl)ethan-1-ol **SI-7** (3.24 g, 20 mmol) and NaHCO₃ (8.4 g, 100 mmol) in dichloromethane (100 mL) at 0 °C, Dess-Martin periodinane (DMP, 12.8 g, 30 mmol) was added in portions. The mixture was allowed to spontaneously warm up to room temperature and further stirred for 1 h. The reaction was neutralized by aqueous solution of saturated NaHCO₃ and saturated sodium thiosulfate at 0 °C. The resultant mixture was extracted by dichloromethane (3 × 50 mL), washed with brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant residue was purified by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/6) to afford **1g** as yellow oil (2.9 g, 90% yield). **2-(benzo[d][1,3]dioxol-5-yl)acetaldehyde (1g)**:^[S18] ¹H NMR (400 MHz, CDCl₃) δ ppm 9.69 (t, *J* = 2.3 Hz, 1H), 6.79 (d, *J* = 7.9 Hz, 1H), 6.70–6.63 (m, 2H), 5.95 (s, 2H), 3.59 (d, *J* = 2.3 Hz, 2H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 198.5, 147.2, 146.1, 124.4, 121.9, 109.0, 107.8, 100.2, 49.2. HRMS (EI-TOF): *m/z* calcd for C₉H₈O₃ [M⁺]: 164.0473, found: 164.0474.

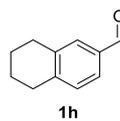


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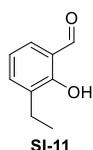
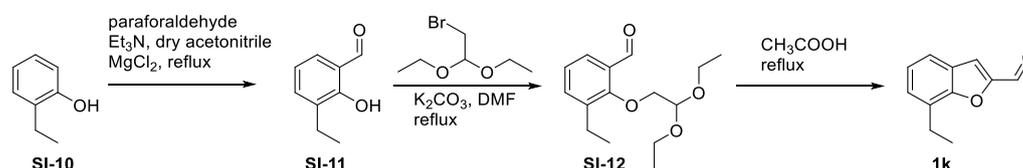


To a suspension of lithium aluminium hydride (4.2 g, 110 mmol) in dry tetrahydrofuran (180 mL) was added a solution of compound **SI-8** (10 g, 57 mmol) in dry tetrahydrofuran (50 mL) under argon at 0 °C. The mixture was allowed to spontaneously warm up to room temperature and further stirred for 3 h. The reaction was quenched by slow addition of distilled water (4.2 mL), NaOH aqueous solution (10%, w/v, 8 mL), and distilled water (13 mL) at 0 °C, respectively.

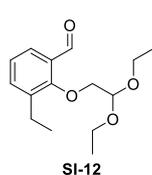
The mixture was further vigorously stirred over night at room temperature until the mixture was layered easily. After filtration, the filtrate was dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/4) to afford compound **SI-9** as colorless oil (9.0 g, 99% yield). **(5,6,7,8-tetrahydronaphthalen-2-yl)methanol (1-9)**:^[S19] ¹H NMR (600 MHz, CDCl₃) δ ppm 7.10 – 7.03 (m, 3H), 4.60 (s, 2H), 2.79 – 2.73 (m, 4H), 1.79 (ddd, *J* = 6.6, 4.0, 2.7 Hz, 4H), 1.70 (s, 1H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 138.1, 137.5, 136.8, 129.5, 128.0, 124.5, 65.5, 29.5, 29.3, 23.3.



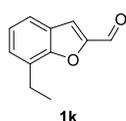
To a suspension of 100-200 mesh silica gel (10 g), Celite (10 g) and pyridinium chlorochromate (PCC, 12 g, 55 mmol) in dichloromethane (180 mL) was added a solution of compound **SI-9** (8.4 g, 51.6 mmol) in dichloromethane (30 mL) at 0 °C. The mixture was allowed to warm to room temperature spontaneously and further stirred for 7 h. The reaction mixture was filtrated by Celite and the filtrate was concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/20) to afford **1** as colorless oil (8.2 g, 99% yield). **5,6,7,8-tetrahydronaphthalene-2-carbaldehyde (1h)**:^[S20] ¹H NMR (600 MHz, CDCl₃) δ ppm 9.92 (s, 1H), 7.61 – 7.56 (m, 2H), 7.21 (d, *J* = 7.7 Hz, 1H), 2.84 (dq, *J* = 6.5, 3.1 Hz, 4H), 1.84 – 1.81 (m, 4H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 192.5, 145.1, 138.2, 134.3, 131.0, 130.0, 126.8, 30.0, 29.4, 24.0, 22.9. HRMS (EI-TOF): *m/z* calcd for C₁₁H₁₂O [M⁺]: 160.0888, found: 160.0891.



Following the procedure by Chen et al.,^[S21] to a solution of 2-ethylphenol **SI-10** (6.1 g, 50 mmol) and anhydrous MgCl₂ (7.2 g, 75 mmol) in dry acetonitrile (130 mL) was added triethylamine (27 mL, 113 mmol). The mixture was stirred at room temperature for 30 min. Then paraformaldehyde (7.5 g, 250 mmol) was added in one portion. The mixture was refluxed for 2 h under nitrogen, then cooled to room temperature and the pH was adjusted to < 2 by adding 3*N* HCl (aq.) at 0 °C. The resultant solution was extracted by ethyl acetate (3 × 50 mL), washed with brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The corresponding residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/200) to afford compound **SI-11** as slight yellow oil (6.2 g, 83% yield). **3-ethyl-2-hydroxybenzaldehyde (SI-11)**:^[S21] ¹H NMR (600 MHz, CDCl₃) δ ppm 11.28 (s, 1H), 9.88 (s, 1H), 7.43 – 7.38 (m, 2H), 6.96 (t, *J* = 7.5 Hz, 1H), 2.70 (q, *J* = 7.6 Hz, 2H), 1.23 (t, *J* = 7.6 Hz, 3H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 197.0, 159.9, 136.4, 132.9, 131.5, 120.3, 119.7, 22.4, 13.8.



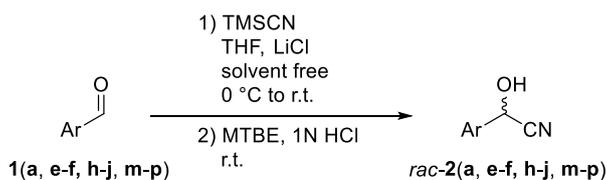
To a mixture of anhydrous K₂CO₃ and 3-ethyl-2-hydroxybenzaldehyde **SI-11** (6.2 g, 41.3 mmol) in *N,N*-dimethylformamide (30 mL) was added 2-bromo-1,1-diethoxyethane (9.2 g, 47 mmol). The reaction mixture was stirred at 170 °C under nitrogen for 4 h, then cooled to room temperature, diluted by ether (150 mL) and filtrated. The filtrate was washed with water (3 × 20 mL), the organic layer was washed with brine. The combined aqueous phase was extracted by ethyl ether (3 × 30 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/30) to afford **1-6** as yellow oil (8.4 g, 77% yield). **2-(2,2-diethoxyethoxy)-3-ethylbenzaldehyde (SI-12)**:^[S22] ¹H NMR (400 MHz, CDCl₃): δ ppm 10.44 (s, 1H), 7.73–7.63 (m, 1H), 7.47 (d, *J* = 7.5 Hz, 1H), 7.19 (t, *J* = 7.6 Hz, 1H), 4.87 (t, *J* = 5.1 Hz, 1H), 3.98 (d, *J* = 5.1 Hz, 2H), 3.76 (tt, *J* = 14.2, 7.1 Hz, 2H), 3.63 (dq, *J* = 9.2, 7.1 Hz, 2H), 2.76 (q, *J* = 7.6 Hz, 2H), 1.25 (qd, *J* = 7.3, 1.6 Hz, 9H). ¹³C NMR (100 MHz, CDCl₃) δ ppm 190.7, 159.9, 138.2, 135.8, 129.4, 126.5, 124.6, 100.7, 76.3, 62.8, 22.3, 15.3, 14.6.



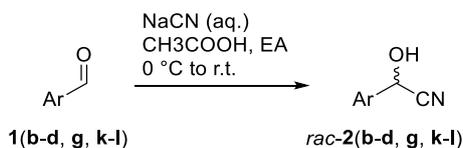
A solution of 2-(2,2-diethoxyethoxy)-3-ethylbenzaldehyde **SI-12** (8.4 g, 31.8 mmol) in acetic acid (30 mL) was refluxed under nitrogen for 24 h and cooled to room temperature. The solvent was removed under reduced pressure and the resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/30) to afford **1k** as orange solid (3.3 g, 60% yield). **7-ethylbenzofuran-2-carbaldehyde (1k)**:^[S22] ¹H NMR (600 MHz, CDCl₃) δ ppm 9.86 (s, 1H), 7.59 – 7.53 (m, 2H), 7.33 (dd, *J* = 7.3, 1.1 Hz, 1H), 7.26 (d, *J* = 15.1 Hz, 1H), 2.98 (q, *J* = 7.6 Hz, 2H), 1.35 (t, *J* = 7.6 Hz, 3H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 179.9, 155.1, 152.6, 129.4, 128.2, 126.4, 124.5, 121.1, 22.7, 14.1. HRMS (EI-TOF): *m/z* calcd for C₁₁H₁₀O₂ [M⁺]: 174.0681, found: 174.0682.

SUPPORTING INFORMATION

1.10 Preparation of racemic cyanohydrins for use as GC standards



Procedure 1: Modifying the procedure by our previous work,^[53] to a mixture of 0.5–5 mmol aldehyde (**1a**, **1e** to **1f**, **1h** to **1j**, **1m** to **1p**) and 1.1 equiv. trimethylsilyl cyanide was added one drop of anhydrous LiCl (100 mM) in tetrahydrofuran at 0 °C and keep stirring for 15 min. The reaction mixture was spontaneously warmed to room temperature and further stirred for 45 min. The reaction mixture was diluted by adding methyl *tert*-butyl ether (10 mL) and subsequently added HCl (aq. 1N, 10 mL). The mixture was vigorously stirred at room temperature (traced by TLC, usually 2 to 4 h) and the organic phase was separated. The aqueous phase was extracted by methyl *tert*-butyl ether (3 × 10 mL). The combined organic phase was washed with brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether) to afford the desired products (70–90% yield).



Procedure 2: To a mixture of 0.5–5 mmol aldehyde (**1b** to **1d**, **1g**, **1k**, **1l**) in acetic acid (2 mL) was slowly added aqueous solution of NaCN (8 M, 2 mL) at 0 °C and kept stirring for 15 min, then ethyl acetate (8 mL) was added and the mixture was spontaneously warmed to room temperature and further stirred until aldehydes were consumed (traced by TLC). The reaction mixture was extracted by ethyl acetate (3 × 10 mL). The combined organic phase was washed with saturated FeCl₃/FeSO₄ aqueous solution until the color of aqueous phase unchanged. The organic layer was subsequently washed with brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether) to afford the desired products (60 – 85% yield).

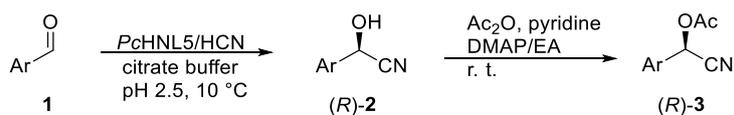
SUPPORTING INFORMATION

1.11 Preparation of racemic compounds for use in HPLC analysis

For the synthesis of *rac*-**4**, *rac*-**6**, *rac*-salmeterol, the reaction was performed in a mmol-scale (usually 0.2 to 1 mmol) while a gram scale synthesis was designed for the optically pure form of salmeterol. For detailed procedure, see part **1.20**.

SUPPORTING INFORMATION

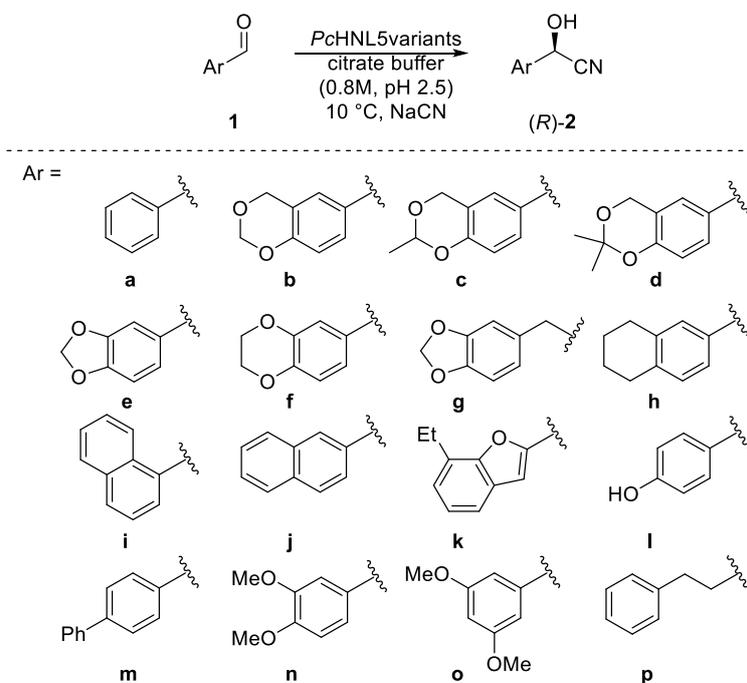
1.12 Activity assays and kinetic parameters of biohydrocyanation



General activity assays: Hydrocyanation was monitored by chiral GC in 0.5 mL scale aqueous reaction which employed citrate buffer (0.8 M, pH 2.5), sodium cyanide (100 mM) and appropriately diluted HNLs, the reaction was started by adding aldehyde dissolved in dimethyl sulfoxide (10%, v/v) to a final concentration of 10 mM (for substrate **1g**, **1h**, **1i**, **1j**, **1k**, **1l**, and **1m**, the final concentration was decreased to 1 mM due to solubility issues). The reaction was agitated at 10 °C, 1000 rpm for 5 – 15 min. Then extracted by EA (1 mL). The organic phase was mixed with acetic anhydride (20 μ L), pyridine (20 μ L) and 4-dimethylaminopyridine (20 μ L, 10 mM stock in ethyl acetate) stock. (For determining substrate **1l**, the 0.5 mL aqueous reaction system was extracted by ethyl acetate (0.5 mL), and mixed with dichloromethane (0.5 mL), acetic anhydride (80 μ L), pyridine (80 μ L) and 4-dimethylaminopyridine (80 μ L, 10 mM stock in ethyl acetate stock). The mixture was incubated at 30 °C for more than 2 h, then washed with saturated CuSO_4 aqueous solution (900 μ L), saturated NaHCO_3 aqueous solution (900 μ L), dried over anhydrous Na_2SO_4 and analyzed by Chiral GC. The conversion of the reaction was maintained within 10%.

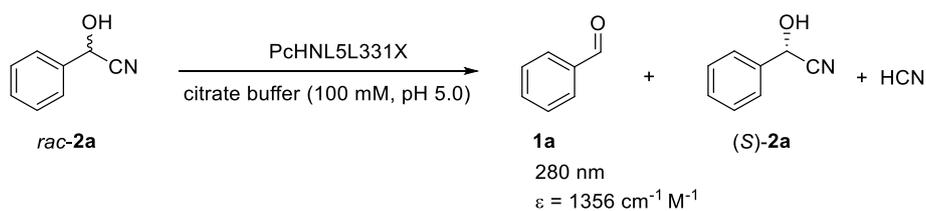
Kinetic assays: For determination of saturation kinetic behavior of PcHNL5 variants. Reaction conditions and the following analytical procedure were the same with that of general activity assays except for the concentration of the final aldehyde (**1a**, **1b**, **1c** or **1d**) was varied from 0.5 to 30 mM (for detailed data point see **Figures S116-S119**). The kinetic parameters were fitted by using Origin 9.0 suite.

Table S5. Hydrocyanation activity of PcHNL5 mutants toward aldehydes 1a – 1p.



Enzyme	Specific activity (U/mg HNL)										
	WT	F72V	L343V	H358V	L331G	L331A	L331V	L331F	L331W	L331T	L331S
1a	84	3.1	17	5.3	6.4	50	66	28	3.4	21	7.2
1b	1.5	0.15	1.2	0.87	0.71	11	11	0.67	2.6	3.1	3.2
1c	8.7×10^{-2}	3.7×10^{-2}	0.57	0.17	3.3	9.1	2.8	2.4×10^{-2}	0.16	0.47	2.6
1d	4.4×10^{-3}	3.6×10^{-2}	1.9×10^{-2}	5.2×10^{-2}	0.77	2.4	0.31	1.6×10^{-2}	N. D.	3.0×10^{-2}	0.37
1e	12	5.2	5.8	6.7	0.57	4.2	15	5.5	1.5	6.9	3.0
1f	0.43	0.42	0.43	2.1	0.22	3.2	1.0	0.75	1.9	0.28	0.62
1g	1.5×10^{-2}	0.15	1.4×10^{-2}	4.9×10^{-2}	0.45	0.29	8.4×10^{-2}	1.9×10^{-2}	2.7×10^{-2}	9.2×10^{-2}	6.0×10^{-3}
1h	0.83	0.59	1.3	1.0	1.1	12	14	1.2	4.9	1.1	2.9
1i	0.12	3.2×10^{-2}	9.6×10^{-3}	3.3×10^{-2}	5.3×10^{-3}	0.26	0.46	0.14	1.6	4.2×10^{-2}	3.3×10^{-2}
1j	8.4	18	2.7	18	1.2	27	25	3.7	2.8	6.9	3.8
1k	0.43	0.29	7.1×10^{-2}	0.30	N.D.	1.3	2.4	0.29	0.60	0.27	7.1×10^{-2}
1l	0.44	0.15	0.13	5.4×10^{-2}	2.6×10^{-2}	0.41	0.58	9.3×10^{-2}	0.63	0.54	0.48
1m	0.43	2.6×10^{-2}	2.6×10^{-2}	1.0	0.97	1.4	3.6	0.12	0.02	0.28	0.28
1n	3.0×10^{-2}	9.9×10^{-2}	2.0×10^{-2}	N.D.	N.D.	0.14	0.17	3.5×10^{-2}	7.0×10^{-2}	1.6×10^{-2}	1.6×10^{-2}
1o	1.5	0.44	0.77	8.5	0.39	4.9	4.2	7.9	11	0.78	0.79
1p	9.6	1.0	22	26	7.4	30	50	3.9	1.5	6.2	3.3

1.13 Activity assays of cyanohydrin cleavage



To monitor the expression of HNL during high density fermentation of recombinant *P. pastoris* X33 strains which harboring PcHNL5 L331X mutants. At the schedule time, samples were centrifugated at 10000 g, 4 °C for 2 min, the supernatant portion of the sample was appropriately diluted by citrate buffer (100 mM, pH 5.0). The dehydrocyanation activity was determined by UV-Vis absorbance at 280 nm of benzaldehyde that is cleaved from racemic mandelonitrile (*rac-2a*). The cleavage of *rac-2a* was then performed at 25 °C, in citrate buffer (100 mM, pH 5.0) with 2 mM *rac-2a*, 1% dimethyl sulfoxide (v/v) and appropriate diluted HNL to maintain the the variation within 0 – 0.1 units.

SUPPORTING INFORMATION

1.14 HCN-MTBE formation

Caution: all of experiments related to cyanide formation must be carried out in a well-ventilated hood for self-protection. All cyanide waste should be decontaminated by saturated $\text{FeCl}_3/\text{FeSO}_4$. It is suggested that all the apparatus should be soaked in 10% hydrogen peroxide for 12 h before reuse.

HCN-MTBE should be used as freshly prepared, modified from the procedure by Pohl *et al.*,^[S23] sodium cyanide (1.96 g) was mixed homogeneously with water (4 mL) and methyl *tert*-butyl ether (10 mL) at 0 °C, the mixture was stirred for at least 15 min. Then sulfuric acid (4 M, 5 mL) was slowly added dropwise. The mixture was further stirred for 15 min. The organic phase was separated and transferred to another small dark bottle at 0 °C. The residual aqueous phase was extracted by methyl *tert*-butyl ether (2.8 mL) twice. The combined organic phase could be directly used for enzymatic transformation. For preparative scale hydrocyanation reaction, preparation of HCN-MTBE was scaled-up.

SUPPORTING INFORMATION

1.15 Determination of biocatalytic turnover frequency, conversion and enantiomeric excess of the cyanohydrins

At the schedule time, 10 μL of the organic layer from the reaction system was taken and mixed with dichloromethane (300 μL), acetic anhydride (20 μL), pyridine (20 μL) and 4-dimethylaminopyridine (10 mM stock in ethyl acetate, 20 μL) stock. Samples were incubated at 30 $^{\circ}\text{C}$ for more than 2 h, then diluted by 400 μL ethyl acetate. The organic layer was washed with saturated CuSO_4 aqueous solution (900 μL), saturated NaHCO_3 aqueous solution (900 μL), dried over anhydrous Na_2SO_4 and analyzed by Chiral GC.

SUPPORTING INFORMATION

1.16 Acidic tolerance of the PcHNL5 mutants

Purified PcHNL5 mutants were diluted to 1 mg/mL with citrate buffer (100 mM, pH 2.5) and vortexed vigorously (1000 rpm) at 30 °C. Relative activity was shown as a percentage of the activity for cleaving *rac-2a* before incubation at pH 2.5. At the schedule time, the sample was appropriately diluted by citrate buffer (100 mM, pH 5.0). The dehydrocyanation activity was determined by UV-Vis absorbance at 280 nm of benzaldehyde that is cleaved from *rac-2a*. The cleavage of *rac-2a* was performed at 25 °C, in citrate buffer (100 mM, pH 5.0) with 2 mM *rac-2a*, 1% dimethyl sulfoxide (v/v) and appropriate diluted HNL to maintain the the variation within 0 – 0.1 units.

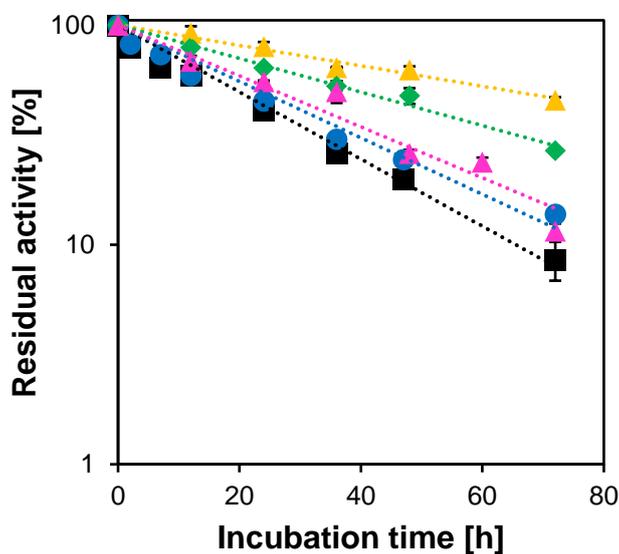


Figure S3. Plot of the relative activities of PcHNL5 mutants after agitated at pH 2.5, 1000 rpm, 30 °C.

PcHNL5 variants: (■) PcHNL5 wild-type; (●) L331V; (▲) L331A; (▲) L331G; (◆) L331W.

SUPPORTING INFORMATION

1.17 Preparative scale hydrocyanation and subsequent O-acetylation

Fermentation broth (the supernatant portion contained L331X mutant) was acidified by phosphoric acid (30%) until pH was adjusted to 3.5, then appropriately diluted by citrate buffer (100 mM, pH 3.5). HNL solution (3 mL) was cooled to 10 °C and mixed with the aldehydes. The reaction was initiated by adding HCN-MTBE (2 M, 7 mL) and further stirred at 10 °C. The reaction was monitored by TLC until no more products were formed. The reaction was separated, the aqueous phase was extracted by methyl *tert*-butyl ether (3 × 10 mL). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The resulting residual was diluted by 30 mL dichloromethane, then acetic anhydride (5 equiv. to aldehydes), pyridine (3 equiv. to aldehydes) and 4-dimethylaminopyridine (5 mg) were added. The reaction mixture was stirred at room temperature for more than 2 h. The reaction mixture was diluted by ethyl acetate (50 mL), and washed with saturated CuSO₄ aqueous solution, water, saturated NaHCO₃ aqueous solution, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel to afford desired O-acetylated cyanohydrins (**3b-3p**).

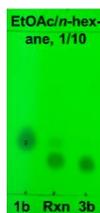
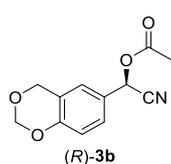
Table S6. Preparative scale bio-hydrocyanation reaction using PcHNL5_{L331X} fermentation supernatant portion.

Substrate	HNL	Specific activity toward <i>rac-2a</i> (U/mg)	Volumetric activity after high-density fermentation (kJ/L)	Substrate loading/HNL dosage (M/(g L ⁻¹))	Reaction time (h)	Conversion ^[a] (%)	Yield ^[b] (%)	<i>E_e</i> ^[c] (%)
1b	L331V	95.4±3.6	104.6±2.9	0.5/0.5	12	98	92	>99
1c	L331A	25.0±1.1	31.7±1.3	0.5/1.0	12	98	96	>99
1d	L331A	25.0±1.1	31.7±1.3	0.2/1.0	12	96	90	>99
1e	L331V	95.4±3.6	104.6±2.9	1.0/0.5	11	96	93	>99
1f	L331A	25.0±1.1	31.7±1.3	0.5/1.0	12	98	96	>99
1g	L331G	8.0±0.2	8.3±0.09	0.5/1.0	10	95	82	97
1h	L331V	95.4±3.6	104.6±2.9	0.5/0.5	12	96	80	>99
1i	L331W	12.1±0.1	18.4±0.4	0.2/1.0	12	52	41	97
1j	L331A	25.0±1.1	31.7±1.3	1.0/1.0	12	99	89	99
1k	L331V	95.4±3.6	104.6±2.9	0.2/1.0	12	94	90	96
1l	L331V	95.4±3.6	104.6±2.9	0.1/1.0	12	92	90	>99
1m	L331V	95.4±3.6	104.6±2.9	0.2/1.0	12	77	70	99
1n	L331V	95.4±3.6	104.6±2.9	0.05/1.0	12	93	81	98
1o	L331F	81.4±0.1	98.2±0.5	1.0/1.0	10	99	97	>99
1p	L331V	95.4±3.6	104.6±2.9	1.0/0.1	10	95	85	99

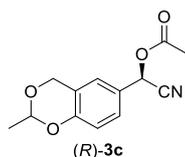
^[a] Determined by chiral GC. ^[b] Calculated based on O-acetylated cyanohydrins obtained after a two-step transformation, after purification of O-acetylated cyanohydrins by fast column chromatography on silica gel. ^[c] Determined by chiral GC for purified O-acetylated cyanohydrin from preparation reactions.

SUPPORTING INFORMATION

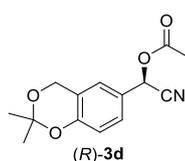
1.18 Characterization of O-acetylated cyanohydrins



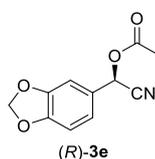
(R)-4-(benzo[d][1,3]dioxin-6-yl)(cyano)methyl acetate ((R)-3b, pale yellow oil, ethyl acetate/*n*-hexane = 1/10, 1.07 g, 92% yield, >99% *ee*). TLC: ethyl acetate/*n*-hexane = 1/10, UV 254 nm, R_f (acetate) = 0.22, R_f (aldehyde) = 0.33. $[\alpha]_D^{25} = -18.55$ ($c = 1.06$ in CHCl_3). $[\alpha]_D^{25} = -6.90$ ($c = 1.08$ in CH_2Cl_2). {lit.^[S15] $[\alpha]_{578}^{20} = -7.5$ ($c = 1.5$ in CH_2Cl_2) for (R)-3b, *ee* = 97%}. ^1H NMR (600 MHz, CHCl_3) δ ppm 7.30 (dd, $J = 8.5, 2.3$ Hz, 1H), 7.15 (d, $J = 2.2$ Hz, 1H), 6.92 (d, $J = 8.5$ Hz, 1H), 5.25 (s, 2H), 4.91 (s, 2H), 2.14 (s, 3H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 169.0, 154.4, 128.1, 125.4, 124.6, 122.1, 117.8, 116.3, 91.5, 66.1, 62.6, 20.6. HRMS (EI-TOF): m/z calcd for $\text{C}_{12}\text{H}_{11}\text{NO}_4$ [M^+]: 233.0688, found: 233.0687.



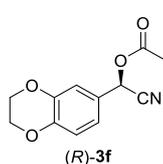
(R)-Cyano(2-methyl-4H-benzo[d][1,3]dioxin-6-yl)methyl acetate ((R)-3c, white solid, ethyl acetate/petroleum ether = 1/15, 1.18 g, 96% yield, >99% *ee*). TLC: ethyl acetate/petroleum ether = 1/15, UV 254 nm, R_f (acetate) = 0.19, R_f (aldehyde) = 0.28. $[\alpha]_D^{25} = -19.68$ ($c = 1.11$ in CH_3Cl). $[\alpha]_D^{20} = -11.82$ ($c = 1.00$ in CH_2Cl_2). {lit.^[S15] $[\alpha]_{578}^{20} = -17.0$ ($c = 1.0$ in CH_2Cl_2) for (S)-3c, *ee* = 96%}. ^1H NMR (600 MHz, CDCl_3) δ ppm 7.31 – 7.29 (m, 1H), 7.15 (dd, $J = 4.9, 2.2$ Hz, 1H), 6.90 (dd, $J = 8.5, 2.8$ Hz, 1H), 6.32 (s, 1H), 5.19 (q, $J = 5.1$ Hz, 1H), 4.99 (dd, $J = 14.7, 5.0$ Hz, 1H), 4.85 (dd, $J = 14.8, 4.4$ Hz, 1H), 2.14 (s, 3H), 1.56 (d, $J = 5.1$ Hz, 3H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 169.1, 154.8, 128.2, 128.1, 125.2, 124.4, 121.7, 117.7, 116.3, 97.6, 66.3, 62.7, 20.7. HRMS (EI-TOF): m/z calcd for $\text{C}_{13}\text{H}_{13}\text{NO}_4$ [M^+]: 247.0845, found: 247.0844.



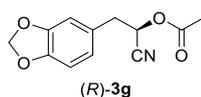
(R)-Cyano(2,2-dimethyl-4H-benzo[d][1,3]dioxin-6-yl)methyl acetate ((R)-3d, colorless oil, ethyl acetate/petroleum ether = 1/30, 468 mg, 90% yield, >99% *ee*). TLC: ethyl acetate/petroleum ether = 1/10, KMnO_4 solution staining, R_f (acetate) = 0.26, R_f (aldehyde) = 0.41. $[\alpha]_D^{25} = -23.75$ ($c = 1.17$ in CHCl_3). ^1H NMR (600 MHz, CDCl_3) δ ppm 7.30 (dd, $J = 8.5, 2.3$ Hz, 1H), 7.15 (d, $J = 2.1$ Hz, 1H), 6.86 (d, $J = 8.5$ Hz, 1H), 6.32 (s, 1H), 4.85 (s, 2H), 2.14 (s, 3H), 1.54 (s, 6H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 169.1, 153.2, 128.2, 125.0, 123.7, 120.3, 118.1, 116.4, 100.3, 62.7, 60.7, 24.8, 24.8, 20.6. HRMS (EI-TOF): m/z calcd for $\text{C}_{14}\text{H}_{15}\text{NO}_4$ [M^+]: 261.1001, found: 261.1002.



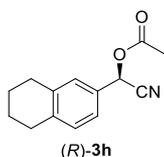
(R)-Benzo[d][1,3]dioxol-5-yl(methyl) acetate ((R)-3e, colorless oil, ethyl acetate/*n*-hexane = 1/15, 2.02 g, 93% yield, >99% *ee*). TLC: ethyl acetate/petroleum ether = 1/10, UV 254 nm, R_f (acetate) = 0.22, R_f (aldehyde) = 0.38. $[\alpha]_D^{25} = -19.62$ ($c = 1.07$ in CHCl_3). {lit.^[S27] $[\alpha]_D^{26} = +14.4$ ($c = 0.14$ in CHCl_3) for (S)-3e, *ee* = 97%}. ^1H NMR (600 MHz, CDCl_3) δ ppm 7.02 – 6.95 (m, 2H), 6.83 (d, $J = 8.0$ Hz, 1H), 6.30 (s, 1H), 6.01 (s, 2H), 2.14 (s, 3H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 169.0, 149.5, 148.5, 125.5, 122.5, 108.7, 108.3, 101.9, 62.8, 20.6. HRMS (EI-TOF): m/z calcd for $\text{C}_{11}\text{H}_9\text{NO}_4$ [M^+]: 219.0532, found: 219.0531.



(R)-Cyano(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)methyl acetate ((R)-3f, colorless oil, ethyl acetate/petroleum ether = 1/10, 1.12 g, 96% yield, >99% *ee*). TLC: ethyl acetate/petroleum ether = 1/10, UV 254 nm, R_f (acetate) = 0.21, R_f (aldehyde) = 0.30. $[\alpha]_D^{25} = -17.58$ ($c = 1.14$ in CHCl_3). ^1H NMR (600 MHz, CDCl_3) δ ppm 7.04 (d, $J = 2.2$ Hz, 1H), 6.99 (dd, $J = 8.4, 2.2$ Hz, 1H), 6.91 (d, $J = 8.4$ Hz, 1H), 6.29 (s, 1H), 4.31 – 4.24 (m, 4H), 2.14 (s, 3H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 169.1, 145.5, 144.1, 124.9, 121.3, 118.1, 117.3, 116.3, 64.5, 64.4, 62.6, 20.6. HRMS (EI-TOF): m/z calcd for $\text{C}_{12}\text{H}_{11}\text{NO}_4$ [M^+]: 233.0688, found: 233.0689.



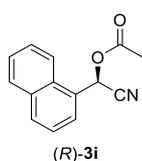
(R)-2-(benzo[d][1,3]dioxol-5-yl)-1-cyanoethyl acetate ((R)-3g, yellow oil, ethyl acetate/petroleum ether = 1/20, 970 mg, 83% yield, 97% *ee*). TLC: ethyl acetate/petroleum ether = 1/10, potassium permanganate solution staining, R_f (acetate) = 0.18, R_f (aldehyde) = 0.34. $[\alpha]_D^{25} = +47.57$ ($c = 1.01$ in CHCl_3). ^1H NMR (600 MHz, CDCl_3) δ ppm 6.77 (d, $J = 7.8$ Hz, 1H), 6.76 – 6.70 (m, 2H), 5.95 (s, 2H), 5.42 (t, $J = 7.0$ Hz, 1H), 3.09 (d, $J = 7.0$ Hz, 2H), 2.12 (s, 3H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 169.1, 148.1, 147.5, 126.9, 116.5, 109.9, 108.7, 101.3, 62.1, 38.4, 20.5. HRMS (EI-TOF): m/z calcd for $\text{C}_{13}\text{H}_{13}\text{NO}_4$ [M^+]: 247.0845, found: 247.0844. HRMS (EI-TOF): m/z calcd for $\text{C}_{12}\text{H}_{11}\text{NO}_4$ [M^+]: 233.0688, found: 233.0687.



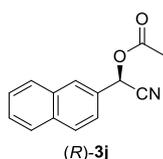
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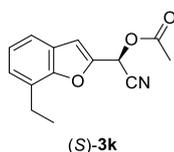
(R)-Byano(5,6,7,8-tetrahydronaphthalen-2-yl)methyl acetate ((R)-3h, colorless oil, EA/*n*-hexane = 1/15, 916 mg, 80% yield, >99% *ee*). TLC: ethyl acetate/petroleum ether = 1/15, UV 254 nm, R_f (acetate) = 0.38, R_f (aldehyde) = 0.73. $[\alpha]_D^{25} = -10.94$ ($c = 1.11$ in CHCl_3). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 7.24 – 7.13 (m, 2H), 7.14 (d, $J = 8.5$ Hz, 1H), 6.34 (s, 1H), 2.82 – 2.77 (m, 4H), 2.16 (s, 3H), 1.81 (p, $J = 3.4$ Hz, 4H). $^{13}\text{C NMR}$ (150 MHz, Chloroform-*d*) δ ppm 169.0, 140.0, 138.4, 130.0, 128.8, 128.7, 125.0, 116.4, 62.9, 29.3, 29.3, 22.9, 20.5. m/z calcd for $\text{C}_{14}\text{H}_{15}\text{NO}_2$ [M^+]: 229.1103,



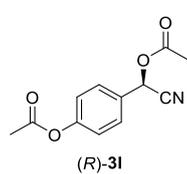
(R)-Cyano(naphthalen-1-yl)methyl acetate ((R)-3i, white solid, ethyl acetate/petroleum ether = 1/30, 186 mg, 41% yield, 97% *ee*). TLC: ethyl acetate/petroleum ether = 1/20, UV 254 nm, R_f (acetate) = 0.33, R_f (aldehyde) = 0.58. $[\alpha]_D^{25} = +47.18$ ($c = 1.20$ in CHCl_3) {lit.^[S28] $[\alpha]_D^{26} = -42.6$ ($c = 0.53$ in CHCl_3) for (S)-3i, *ee* = 87%}. $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 8.04 (d, $J = 8.5$ Hz, 1H), 7.98 (d, $J = 8.3$ Hz, 1H), 7.94 (d, $J = 8.1$ Hz, 1H), 7.82 (dd, $J = 7.0, 1.2$ Hz, 1H), 7.66 – 7.63 (m, 1H), 7.60 – 7.57 (m, 1H), 7.54 – 7.51 (m, 1H), 7.04 (s, 1H), 2.19 (s, 3H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ ppm 169.2, 134.1, 131.7, 130.2, 129.3, 127.8, 127.1, 126.8, 125.2, 122.7, 116.3, 61.4, 20.6. HRMS (EI-TOF): m/z calcd for $\text{C}_{14}\text{H}_{11}\text{NO}_2$ [M^+]: 225.0790, found: 225.0791.



(R)-Cyano(naphthalen-2-yl)methyl acetate ((R)-3j, white solid, ethyl acetate/petroleum ether = 1/35, 2.15 g, 96% yield, 99% *ee*). TLC: ethyl acetate/petroleum ether = 1/20, UV 254 nm, R_f (acetate) = 0.24, R_f (aldehyde) = 0.43. $[\alpha]_D^{25} = -26.08$ ($c = 1.00$ in CHCl_3) {lit.^[S28] $[\alpha]_D^{26} = +18.6$ ($c = 0.6$ in CHCl_3) for (S)-3j, *ee* = 73%}. $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 8.05 – 8.01 (m, 1H), 7.96 – 7.86 (m, 3H), 7.61 – 7.54 (m, 3H), 6.59 (s, 1H), 2.19 (s, 3H). $^{13}\text{C NMR}$ (151 MHz, CDCl_3) δ ppm 169.1, 134.0, 133.0, 129.6, 129.1, 128.5, 128.1, 128.0, 127.7, 127.2, 124.4, 116.3, 63.2, 20.6. HRMS (EI-TOF): m/z calcd for $\text{C}_{14}\text{H}_{11}\text{NO}_2$ [M^+]: 225.0790, found: 225.0788.



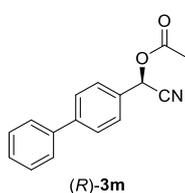
(S)-Cyano(7-ethylbenzofuran-2-yl)methyl acetate ((S)-3k, yellow oil, ethyl acetate/petroleum ether = 1/60, 404 mg, 83% yield, 96% *ee*). TLC: ethyl acetate/petroleum ether = 1/30, UV 254 nm, R_f (acetate) = 0.16, R_f (aldehyde) = 0.26. $[\alpha]_D^{25} = -38.26$ ($c = 1.08$ in CHCl_3). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 7.46 (dd, $J = 6.5, 2.4$ Hz, 1H), 7.25 – 7.21 (m, 2H), 7.06 (s, 1H), 6.63 (s, 1H), 2.96 (q, $J = 7.6$ Hz, 2H), 2.22 (s, 3H), 1.37 (t, $J = 7.6$ Hz, 3H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ ppm 168.8, 154.4, 145.9, 128.4, 126.7, 125.4, 124.0, 119.5, 114.1, 109.4, 56.5, 22.8, 20.4, 14.1. HRMS (EI-TOF): m/z calcd for $\text{C}_{14}\text{H}_{13}\text{NO}_3$ [M^+]: 243.0895, found: 243.0894.



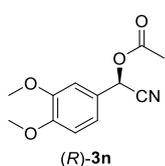
233.0687.



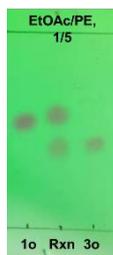
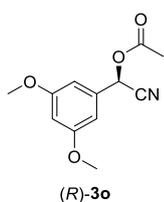
(R)-4-(Acetoxycyano)methyl phenyl acetate ((R)-3l, colorless oil, ethyl acetate/*n*-hexane = 1/10, 420 mg, 90% yield, >99% *ee*). TLC: ethyl acetate/petroleum ether = 1/4, UV 254 nm, R_f (acetate) = 0.36, R_f (aldehyde) = 0.19. $[\alpha]_D^{25} = -8.52$ ($c = 1.05$ in CHCl_3) {lit.^[S29] $[\alpha]_D^{20} = +8.4$ ($c = 1.00$ in CHCl_3) for (S)-3l, *ee* = 91%}. $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 7.57 – 7.52 (m, 2H), 7.21 – 7.16 (m, 2H), 6.41 (s, 1H), 2.31 (s, 3H), 2.16 (s, 3H). $^{13}\text{C NMR}$ (151 MHz, CDCl_3) δ ppm 169.2, 169.0, 152.2, 129.4, 129.4, 122.7, 116.1, 62.3, 21.2, 20.6. HRMS (EI-TOF): m/z calcd for $\text{C}_{12}\text{H}_{11}\text{NO}_4$ [M^+]: 233.0688, found:



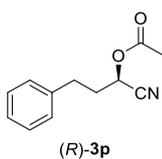
(R)-[1,1'-Biphenyl]-4-yl(cyano)methyl acetate ((R)-3m, white solid, ethyl acetate/petroleum ether = 1/25, 186 mg, 70% yield). TLC: ethyl acetate/petroleum ether = 1/10, UV 254 nm, R_f (acetate) = 0.52, R_f (aldehyde) = 0.70. $[\alpha]_D^{25} = -35.34$ ($c = 0.96$ in CHCl_3). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 7.70 – 7.65 (m, 2H), 7.63 – 7.57 (m, 4H), 7.51 – 7.45 (m, 2H), 7.43 – 7.37 (m, 1H), 6.47 (s, 1H), 2.20 (s, 3H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ ppm 169.1, 143.6, 140.0, 130.7, 129.1, 128.5, 128.1, 127.3, 116.2, 62.8, 20.6. HRMS (EI-TOF): m/z calcd for $\text{C}_{16}\text{H}_{13}\text{NO}_2$ [M^+]: 251.0946, found: 251.0944.



(R)-Cyano(3,4-dimethoxyphenyl)methyl acetate ((R)-3n, white solid, methyl *tert*-butyl ether/petroleum ether = 1/8, 192 mg, 82% yield). TLC: methyl *tert*-butyl ether/petroleum ether = 3/10, UV 254 nm, R_f (acetate) = 0.18, R_f (aldehyde) = 0.24. $[\alpha]_D^{25} = -18.60$ ($c = 1.09$ in CHCl_3). TLC: ethyl acetate/petroleum ether = 1/5, KMnO_4 solution staining, R_f (acetate) = 0.52, R_f (aldehyde) = 0.70. $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 7.09 (dd, $J = 8.2, 2.1$ Hz, 1H), 7.00 (d, $J = 2.2$ Hz, 1H), 6.90 (d, $J = 8.3$ Hz, 1H), 6.36 (s, 1H), 3.92 (s, 3H), 3.91 (s, 3H), 2.16 (s, 3H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ ppm 169.2, 150.9, 149.7, 124.2, 121.4, 116.4, 111.3, 110.9, 63.0, 56.2, 56.2, 20.7. HRMS (EI-TOF): m/z calcd for $\text{C}_{12}\text{H}_{13}\text{NO}_4$ [M^+]: 235.0845, found: 235.0846



(R)-Cyano(3,5-dimethoxyphenyl)methyl acetate ((R)-3o, colorless oil, methyl *tert*-butyl ether/*n*-hexane = 1/7, 2.28 g, 97% yield). TLC: ethyl acetate/petroleum ether = 1/5, UV 254 nm, R_f (acetate) = 0.41, R_f (aldehyde) = 0.57. $[\alpha]_D^{25} = +3.81$ ($c = 1.02$ in CHCl_3). $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 6.62 (d, $J = 2.3$ Hz, 2H), 6.50 (t, $J = 2.3$ Hz, 1H), 6.31 (s, 1H), 3.80 (s, 6H), 2.16 (s, 3H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ ppm 168.9, 161.4, 133.7, 116.1, 105.8, 102.1, 62.8, 55.6, 20.5. HRMS (EI-TOF): m/z calcd for $\text{C}_{12}\text{H}_{13}\text{NO}_4$ [M^+]: 235.0845, found: 235.0844.



(R)-1-Cyano-3-phenylpropyl acetate ((R)-3p, colorless oil, ethyl acetate/*n*-hexane = 1/20, 1.73 g, 85% yield, 99% *ee*). TLC: ethyl acetate/petroleum ether = 1/20, potassium permanganate solution staining, R_f (acetate) = 0.31, R_f (aldehyde) = 0.43. $[\alpha]_D^{25} = +50.79$ ($c = 0.95$ in CHCl_3). {lit.^[S30] $[\alpha]_D^{20} = +26.2$ ($c = 1.0$ in CHCl_3) for (S)-3p, *ee* = 55%}. $^1\text{H NMR}$ (600 MHz, CDCl_3) δ ppm 7.35 – 7.29 (m, 2H), 7.26 – 7.22 (m, 1H), 7.22 – 7.17 (m, 2H), 5.27 (t, $J = 6.8$ Hz, 1H), 2.84 (ddd, $J = 8.1, 6.9, 3.7$ Hz, 2H), 2.26 – 2.24 (m, 2H), 2.13 (s, 3H). $^{13}\text{C NMR}$ (150 MHz, CDCl_3) δ ppm 169.2, 139.1, 128.9, 128.4, 126.8, 116.8, 60.6, 33.9, 30.8, 20.4. HRMS (EI-TOF): m/z calcd for $\text{C}_{12}\text{H}_{13}\text{NO}_2$ [M^+]: 203.0946, found: 203.0947.

SUPPORTING INFORMATION

1.19 GC analytical conditions

GC analysis was carried out with a SHIMADZU GC-2014 system, equipped an Agilent CP-Chirasil-DEX CB column (25 m × 0.25 mm × 0.25 μm) and a flame ionization detector (FID), using nitrogen as carrier gas at a flow rate of 0.55 mL/min. The detailed analytical conditions were described as the follows (**Table S7**)

Table S7. GC analytical conditions.

Entry	Program	Retention time (min)			Coefficient (Compound 1 /Compound 3)
		Aldehyde 1	O-Acetylated- cyanohydrin (<i>R</i>)- 3	O-Acetylated- cyanohydrin (<i>S</i>)- 3	
a	100 °C hold for 3 min, 10 °C/min to 180 °C, 180 °C hold for 2 min	4.391	9.452	10.039	0.787
b	170 °C hold for 2 min, 10 °C/min to 180 °C, 180 °C hold for 13 min	5.218	13.897	14.854	1.134
c	180 °C hold for 15 min	4.538	13.136	13.926	1.065
d	150 °C hold for 3 min, 10 °C/min to 180 °C, 180 °C hold for 13 min	7.543	17.155	18.030	0.816
e	150 °C hold for 3 min, 10 °C/min to 180 °C, 180 °C hold for 7 min	5.200	10.528	11.301	1.480
f	180 °C hold for 18 min	5.037	14.928	16.082	1.149
g	160 °C hold for 3 min, 2 °C/min to 180 °C, 180 °C hold for 4 min	5.137	14.485	15.000	1.347
h	170 °C hold for 1 min, 10 °C/min to 180 °C, 180 °C hold for 13 min	4.611	12.976	13.729	2.777
i	140 °C hold for 5 min, 2 °C/min to 180 °C, 180 °C hold for 2 min	11.982	25.335	25.726	1.699
j	170 °C hold for 3 min, 10 °C/min to 180 °C, 180 °C hold for 13 min	5.161	14.680	15.680	1.427
k	160 °C hold for 4 min, 5 °C/min to 180 °C, 180 °C hold for 6 min	5.892	12.552	12.000	0.847
l	150 °C hold for 2 min, 10 °C/min to 180 °C, 180 °C hold for 8 min	4.458	10.630	11.292	0.830
m	180 °C hold for 38 min	8.287	32.605	34.958	1.165
n	150 °C hold for 4 min, 10 °C/min to 180 °C, 180 °C hold for 7 min	6.710	12.506	13.907	1.625
o	150 °C hold for 4 min, 10 °C/min to 180 °C, 180 °C hold for 7 min	6.172	12.347	12.836	1.088
p	110 °C hold for 3 min, 10 °C/min to 180 °C, 180 °C hold for 5 min	6.495	11.322	11.693	2.336

SUPPORTING INFORMATION

1.20 HPLC analytical conditions

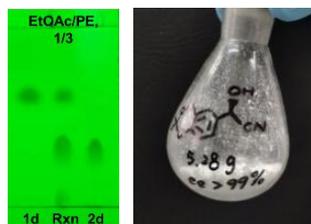
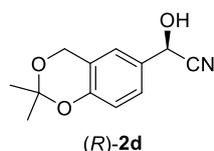
HPLC analysis was carried out with a SHIMADAZU LC-20A system, equipped with a chiral column (Shanghai Chiralway Biotech Co., Ltd, China) and a UV-detector under 220 nm, 40 °C, at a flow rate of 0.9 mL/min. Samples were dissolved in eluent phase. The detailed analytical conditions were described as follows (**Table S8**).

Table S8. HPLC analytical conditions

Compound	Column	Elution phase	Retention time (min)	
			(<i>R</i>)-Isomer	(<i>S</i>)-Isomer
4	Superchiral S-AY (15 cm × 0.46 cm × 5 μm)	<i>n</i> -Hexane/isopropanol/ethanolamine = 80/20/0.1	9.068	12.500
6	Superchiral S-AY (15 cm × 0.46 cm × 5 μm)	<i>n</i> -Hexane/ethanol/ethylenediamine = 85/15/0.05	5.613	6.838
salmeterol	Superchiral S-IG (15 cm × 0.46 cm × 5 μm)	<i>n</i> -Hexane/ethanol/ethylenediamine = 80/20/0.05	5.348	6.852

SUPPORTING INFORMATION

1.21 Chemo-enzymatic and gram-scale synthesis of (*R*)-salmeterol



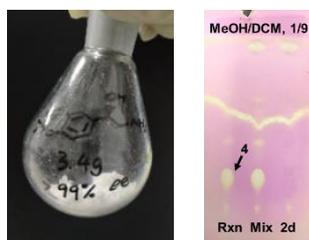
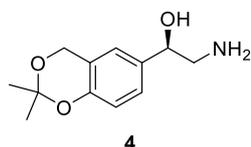
PcHNL5_{L331A} (26 mg) was dissolved in citrate buffer (100 mM, pH 3.5, 50 ml) and pre-cooled to 10 °C, then **1d** (5 g, 26 mmol) was added. After addition of HCN-MTBE (25 mL) and

methyl *tert*-butyl ether (25 mL), the reaction mixture was stirred by a magnetic stirrer for 750 rpm, at 10 °C for 16 h. Then the organic layer was separated, the aqueous phase was extracted by ethyl acetate (3 × 50 mL). The combined organic layers were washed with aqueous solution of FeCl₃ (saturated) until the color of the aqueous phase unchanged, then washed with brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (ethyl acetate/petroleum ether: 1/3) to afford (*R*)-**2d** as white solid (5.3 g, 93% yield, >99% *ee*). (***R***)-**2-(2-(2-Dimethyl-4H-benzo[d][1,3]dioxin-6-yl)-2-hydroxyacetonitrile ((*R*)-2d)**: [α]_D²⁵ = +40.59 (*c* = 1.03 in CH₃Cl). TLC: ethyl acetate/petroleum ether = 1/3, UV 254 nm, *R_f* (aldehyde) = 0.61, *R_f* (cyanohydrin) = 0.32. ¹H NMR (400 MHz, CDCl₃) δ ppm 7.31 (dd, *J* = 8.4, 2.3 Hz, 1H), 7.17 (d, *J* = 1.7 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 1H), 5.46 (s, 1H), 4.85 (s, 2H), 1.55 (s, 6H). ¹³C NMR (100 MHz, CDCl₃) δ ppm 152.6, 127.4, 127.0, 123.6, 120.2, 119.0, 118.0, 100.3, 63.4, 60.8, 24.8, 24.8. MS (ESI-TOF): *m/z* calcd for C₁₂H₁₃NO₃ [*M*⁺]: 219.1, found [*M*+Na⁺]: 242.1.

Table S9. Optimization of enzymatic hydrocyanation of **1d**.^[a]

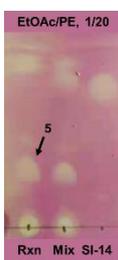
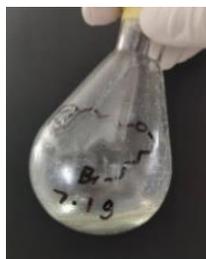
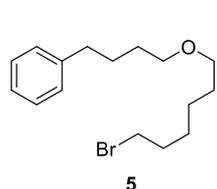
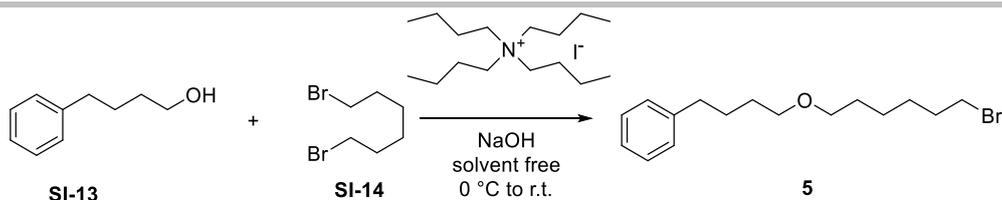
Entry	pH ^[b]	Organic solvent	Aldehyde loading [M]	Ratio ^[c] [v/v]	Conv. [%]	<i>ee</i> [%]	Conv. control ^[d] [%]
1	3.5	DMSO	0.2	-	84	99	<1
2	5	DMSO	0.2	-	82	<i>rac</i>	72
3	6.5	DMSO	0.2	-	22	<i>rac</i>	15
4	3.5	MTBE	0.2	3/7	94	>99	<1
5	5	MTBE	0.2	3/7	86	>99	<1
6	6.5	MTBE	0.2	3/7	54	78	21
7	3.5	MTBE	0.2	1/1	92	>99	-
8	3.5	DIPE	0.2	1/1	88	>99	-
9	3.5	DCM	0.2	1/1	3	94	-
10	3.5	Either	0.2	1/1	90	>99	-
11 ^[e]	3.5	MTBE	0.26 ^[f]	1/1	91	>99	-

^[a] A biphasic reaction mixture (1 mL) was composed of 0.2 mg purified *PcHNL5_{L331A}*, citrate buffer (100 mM), HCN-MTBE (2 M) and aldehydes **1d**. For aqueous reaction mixtures (1 mL), the system was composed of 0.1 mg purified *PcHNL5_{L331A}*, 0.65 mL citrate buffer (2 M), 0.1 mL DMSO and 0.25 mL NaCN aqueous solution (4 M). Reactions were carried out at 10 °C and 1500 rpm for 12 h. ^[b] Initial pH of aqueous phase before NaCN or MTBE adding. ^[c] Ratios for volume aqueous phase/organic phase. ^[d] Nonenzymatic conversion. ^[e] HCN-MTBE was diluted by the same volume of MTBE. ^[f] HNL was adjusted to 0.26 mg.



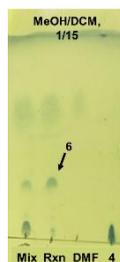
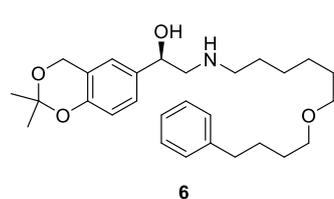
To a suspension of LiAlH₄ (1.3 g, 34 mmol) in dry tetrahydrofuran (120 mL) was added a solution of (*R*)-**2d** (5 g, 23 mmol) in dry tetrahydrofuran (50 mL) under argon, at 0 °C. The mixture was stirred for 2 h at room temperature and additional LiAlH₄ (0.3 g, 8 mmol) was introduced. The mixture was further stirred for 10 h at room temperature. The reaction was quenched by water (distilled, 1.6 mL), NaOH aqueous solution (15%, w/v, 1.6 mL), and distilled water (4.8 mL) at 0 °C, respectively. The resultant mixture was further vigorously stirred over night at room temperature

until the mixture was layered easily. The mixture was filtrated by Celite and the filter cake was washed by dichloromethane. The combined organic layers were evaporated under reduced pressure. The resultant residue was dissolved in dichloromethane, dried over anhydrous Na₂SO₄ and filtrated. The filtration was evaporated under reduced pressure, the resultant residue was washed with appropriate ether and subjected to recrystallization in CHCl₃/*n*-hexane to afford compound **4** as white crystals. (3.4 g, 67% yield, > 99% *ee*). (***R***)-**2-Amino-1-(2-(2-dimethyl-4H-benzo[d][1,3]dioxin-6-yl)ethan-1-ol (4)**: TLC: methanol/dichloromethane = 1/9, KMnO₄ solution staining, *R_f* (**4**) = 0.23. [α]_D²⁵ = +40.59 (*c* = 1.02 in CH₃Cl). ¹H NMR (600 MHz, CDCl₃) δ ppm 7.09 (dd, *J* = 8.4, 2.1 Hz, 1H), 6.95 (d, *J* = 1.9 Hz, 1H), 6.77 (d, *J* = 8.3 Hz, 1H), 4.81 (s, 2H), 4.51 (dd, *J* = 7.9, 4.0 Hz, 1H), 2.90 (dd, *J* = 12.8, 4.0 Hz, 1H), 2.75 (dd, *J* = 12.8, 8.0 Hz, 1H), 2.23 (br, 3H), 1.52 (d, *J* = 2.9 Hz, 6H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 150.7, 134.6, 125.9, 122.3, 119.4, 117.1, 99.7, 74.0, 61.1, 49.3, 24.9, 24.8. HRMS (EI-TOF): *m/z* calcd for C₁₂H₁₇NO₃ [*M*⁺]: 223.1208, found: 223.1207.



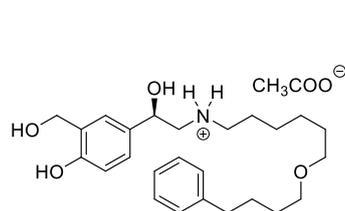
Modify the procedure by Guo et al.,^[S24] to a mixture of powdered sodium hydroxide (1.8 g, 45 mmol), tetrabutylammonium iodide (1.1 g, 3 mmol) and compound **SI-14** (15 g, 60 mmol) was added compound **SI-13** (4.5 g, 30 mmol) at 0 °C. The mixture was stirred at room temperature for 8 h, then diluted by methyl *tert*-butyl ether (100 mL). The organic layer was filtrated and the filtrate was poured into ice-cooled aqueous solution of saturated NH₄Cl for neutralization. The mixture was further agitated for 15 min and separated. The aqueous

phase was extract by dichloromethane (3 × 50 mL), the combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resultant residue was subjected to purification by fast column chromatography on silica gel (methyl *tert*-butyl ether/petroleum ether: 1/50) to afford compound **5** as pale yellow oil (7.1 g, 76% yield). **(4-((6-Bromoethyl)oxy)butyl)benzene (5)**:^[S24] TLC: ethyl acetate/petroleum ether = 1/20, KMnO₄ solution staining, R_f (**5**) = 0.38. ¹H NMR (400 MHz, CDCl₃) δ ppm 7.27 (d, *J* = 1.2 Hz, 2H), 7.20 – 7.15 (m, 3H), 3.40 (tt, *J* = 6.5, 4.5 Hz, 6H), 2.63 (t, *J* = 7.5 Hz, 2H), 1.90 – 1.82 (m, 2H), 1.73 – 1.56 (m, 6H), 1.41 (dddd, *J* = 23.2, 10.7, 9.2, 7.6 Hz, 4H). ¹³C NMR (100 MHz, CDCl₃) δ ppm 142.5, 128.4, 128.2, 125.7, 70.7, 70.7, 35.7, 33.9, 32.7, 29.6, 29.4, 28.1, 28.0, 25.4. HRMS (EI-TOF): *m/z* calcd for C₁₆H₂₅BrO [M⁺]: 312.1089, found: 312.1090.



A solution of compound **4** (3 g, 13 mmol) in dry *N,N*-dimethylformamide (27 mL) was added compound **5** (2.8 g, 9 mmol). The reaction mixture was further stirred at room temperature for 70 h. The mixture was evaporated under reduced pressure, the resulting residue was subjected to purification by fast column chromatography on silica gel (ammonia-MeOH(7M)/dichloromethane: 1/40) to afford compound **6** as white solid (3.5 g, 86% yield).

(6-(4-phenylbutoxy)hexyl)amino)ethan-1-ol (6):^[S25] TLC: methanol/dichloromethane = 1/15, potassium permanganate solution staining, R_f (**6**) = 0.28. [α]_D²⁵ = -14.37 (c = 1.03 in MeOH). ¹H NMR (600 MHz, CDCl₃) δ ppm 7.28 – 7.25 (m, 2H), 7.20 – 7.14 (m, 3H), 7.13 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.01 (d, *J* = 2.0 Hz, 1H), 6.78 (d, *J* = 8.4 Hz, 1H), 4.83 (s, 2H), 4.70 (dd, *J* = 9.4, 3.4 Hz, 1H), 3.39 (dt, *J* = 18.8, 6.5 Hz, 4H), 3.05 (br, 2H), 2.89 (dd, *J* = 12.2, 3.4 Hz, 1H), 2.75 – 2.61 (m, 5H), 1.70 – 1.54 (m, 8H), 1.53 (s, 3H), 1.53 (s, 3H), 1.35 (p, *J* = 3.6 Hz, 4H). ¹³C NMR (150 MHz, CDCl₃) δ ppm 150.8, 142.7, 134.2, 128.6, 128.4, 125.9, 125.8, 122.3, 119.5, 117.1, 99.7, 71.0, 71.0, 70.9, 61.1, 57.0, 49.4, 35.9, 29.8, 29.7, 29.6, 28.2, 27.2, 26.2, 25.0, 24.8. HRMS (ESI-TOF): *m/z* calcd for C₂₈H₄₁NO₄ [M+H⁺]: 456.3069, found: 456.3113.



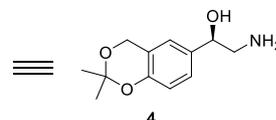
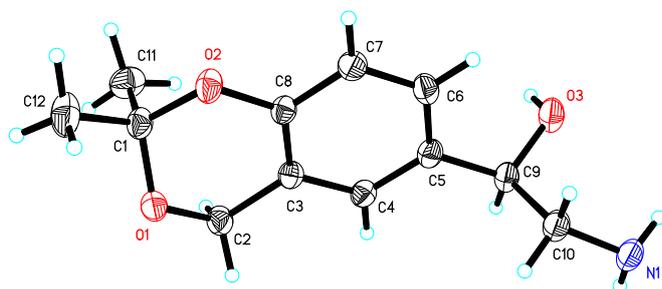
Compound **6** (3 g, 6.6 mmol) was dissolved in a mixed solvent of acetic acid/water (3/1, v/v, 60 mL). The reaction was stirred at 50 °C for 4 h, then diluted by neat methanol, the resulting mixture was evaporated under reduced pressure to afford (*R*)-salmeterol acetate as pale yellow gum (3.1 g, > 99% yield).

(R)-4-(1-Hydroxy-2-((6-(4-phenylbutoxy)hexyl)amino)ethyl)-2-(hydroxymethyl)phenol acetate:^[S26] TLC: methanol/dichloromethane = 1/9, potassium permanganate solution staining, R_f (Salmeterol) = 0.24. [α]_D²⁵ = -20.78 (c = 1.02 in MeOH). ¹H NMR (600 MHz, CD₃OD) δ ppm 7.35 (d, *J* = 2.2 Hz, 1H), 7.27 – 7.21 (m, 2H), 7.19 – 7.10 (m, 4H), 6.79 (d, *J* = 8.2 Hz, 1H), 4.85 (dd, *J* = 9.7, 3.8 Hz, 2H), 4.66 (s, 2H), 3.43 (dt, *J* = 9.7, 6.4 Hz, 4H), 3.11 – 3.04 (m, 2H), 3.03 – 2.97 (m, 2H), 2.62 (t, *J* = 7.6 Hz, 2H), 1.93 (s, 3H), 1.73 – 1.63 (m, 4H), 1.62 – 1.55 (m, 4H), 1.45 – 1.39 (m, 4H). ¹³C NMR (150 MHz, CD₃OD) δ ppm 178.8, 156.3, 143.7, 132.9, 129.4, 129.3, 129.0, 127.0, 126.9, 126.8, 116.0, 71.8, 71.6, 70.1, 60.8, 55.3, 36.7, 30.5, 30.3, 29.3, 27.5, 27.1, 26.9, 23.2. HRMS (ESI-TOF): *m/z* calcd for C₂₅H₃₇NO₄ [M+H⁺]: 416.2756, found: 416.2802.

SUPPORTING INFORMATION

1.22 X-Ray Crystallographic Information

X-Ray crystallographic Information for **4**, CCDC 1938268



Identification code	cu_d8v19594_0m
Empirical formula	C ₁₂ H ₁₇ N O ₃
Formula weight	223.26
Temperature	293(2) K
Wavelength	1.54178 Å
Crystal system	Triclinic
Space group	P 1
Unit cell dimensions	a = 5.49450(10) Å = 89.5530(10)°. b = 5.62720(10) Å = 78.9620(10)°. c = 10.4756(3) Å = 67.6520(10)°.
Volume	293.268(11) Å ³
Z	1
Density (calculated)	1.264 Mg/m ³
Absorption coefficient	0.742 mm ⁻¹
F(000)	120
Crystal size	0.190 x 0.150 x 0.110 mm ³
Theta range for data collection	10.690 to 67.490°.
Index ranges	-6 <= h <= 6, -6 <= k <= 6, -12 <= l <= 12
Reflections collected	6561
Independent reflections	1883 [R(int) = 0.0354]
Completeness to theta = 67.679°	93.6 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7553 and 0.4552
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	1883 / 3 / 157
Goodness-of-fit on F ²	1.075
Final R indices [I > 2sigma(I)]	R1 = 0.0343, wR2 = 0.0964
R indices (all data)	R1 = 0.0346, wR2 = 0.0969
Absolute structure parameter	0.05(8)
Extinction coefficient	0.45(7)
Largest diff. peak and hole	0.152 and -0.133 e.Å ⁻³

1.23 Supporting references

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SUPPORTING INFORMATION

2. Figures and spectra

2.1 NMR spectra

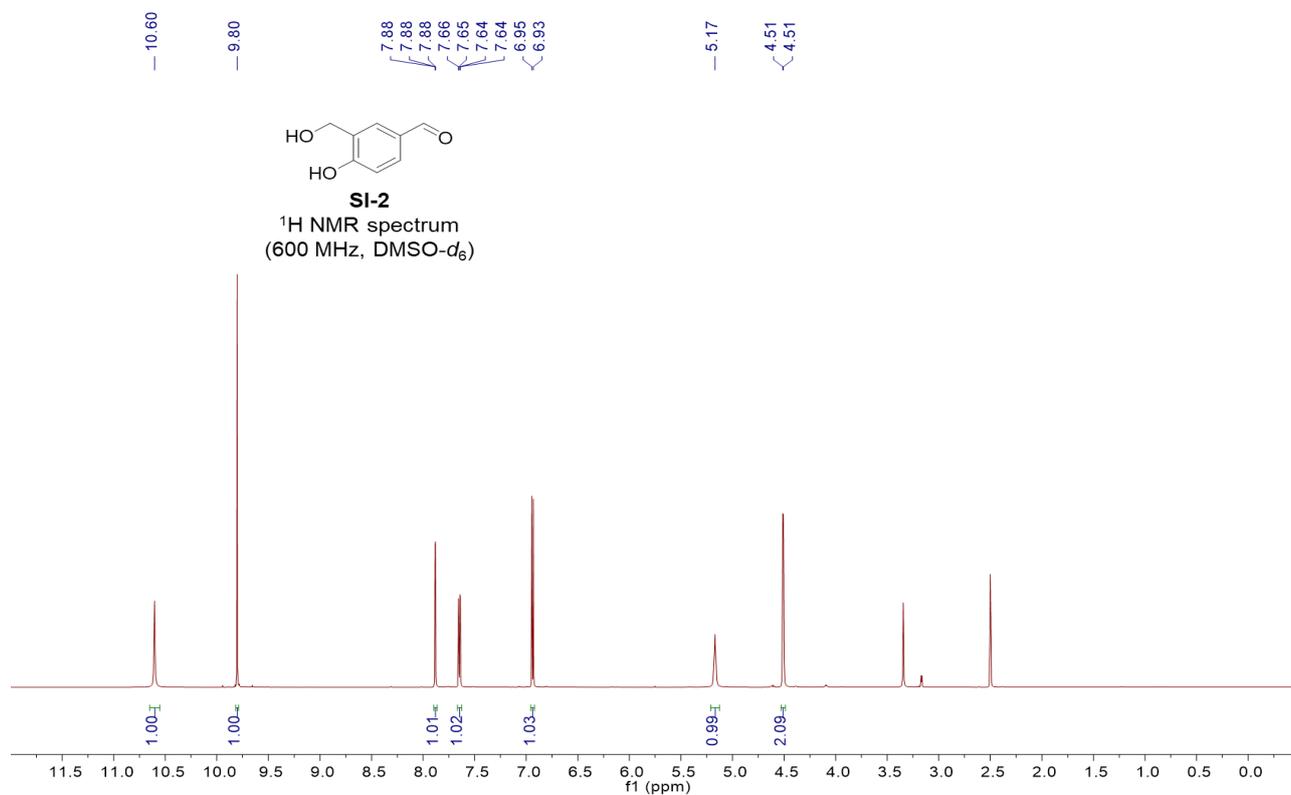


Figure S4. ¹H NMR trace of SI-1-2 synthesized for **1b** and **1c** synthesis.

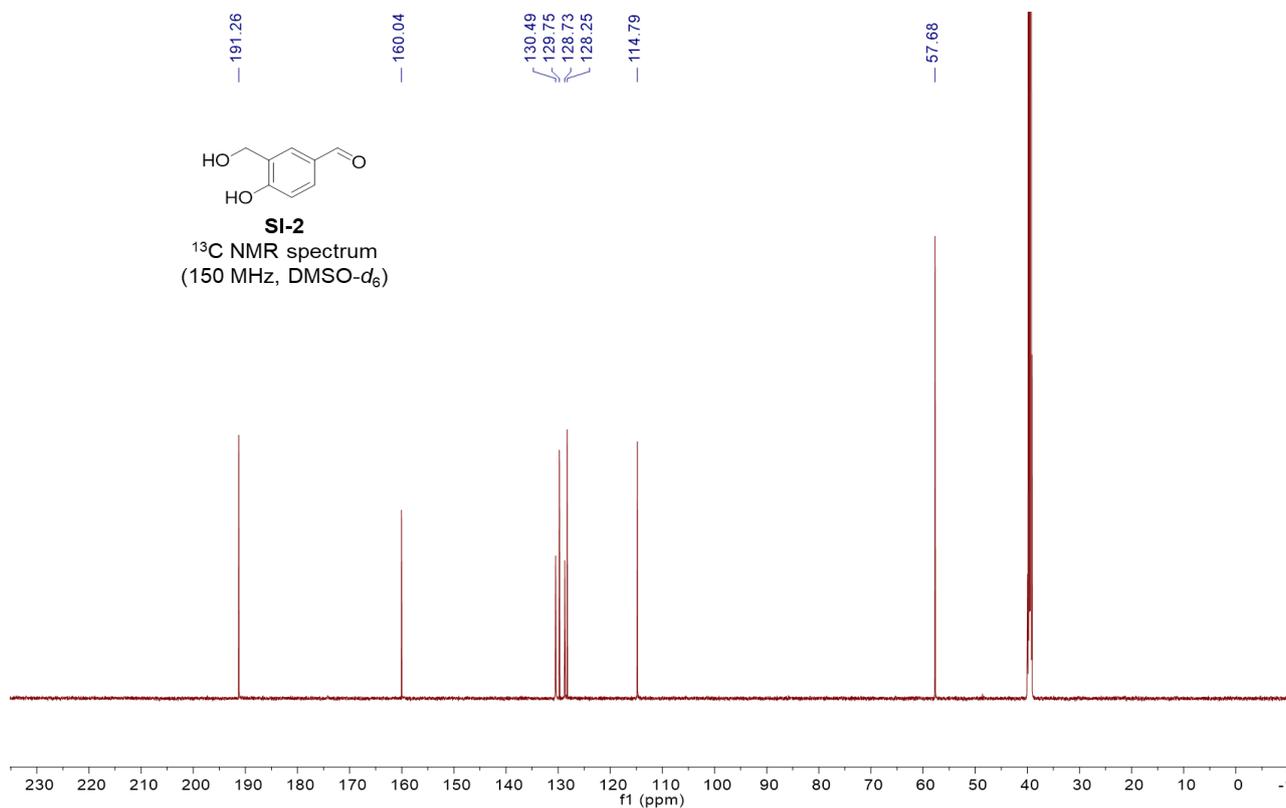


Figure S5. ¹³C NMR trace of SI-1-2 synthesized for **1b** and **1c** synthesis.

SUPPORTING INFORMATION

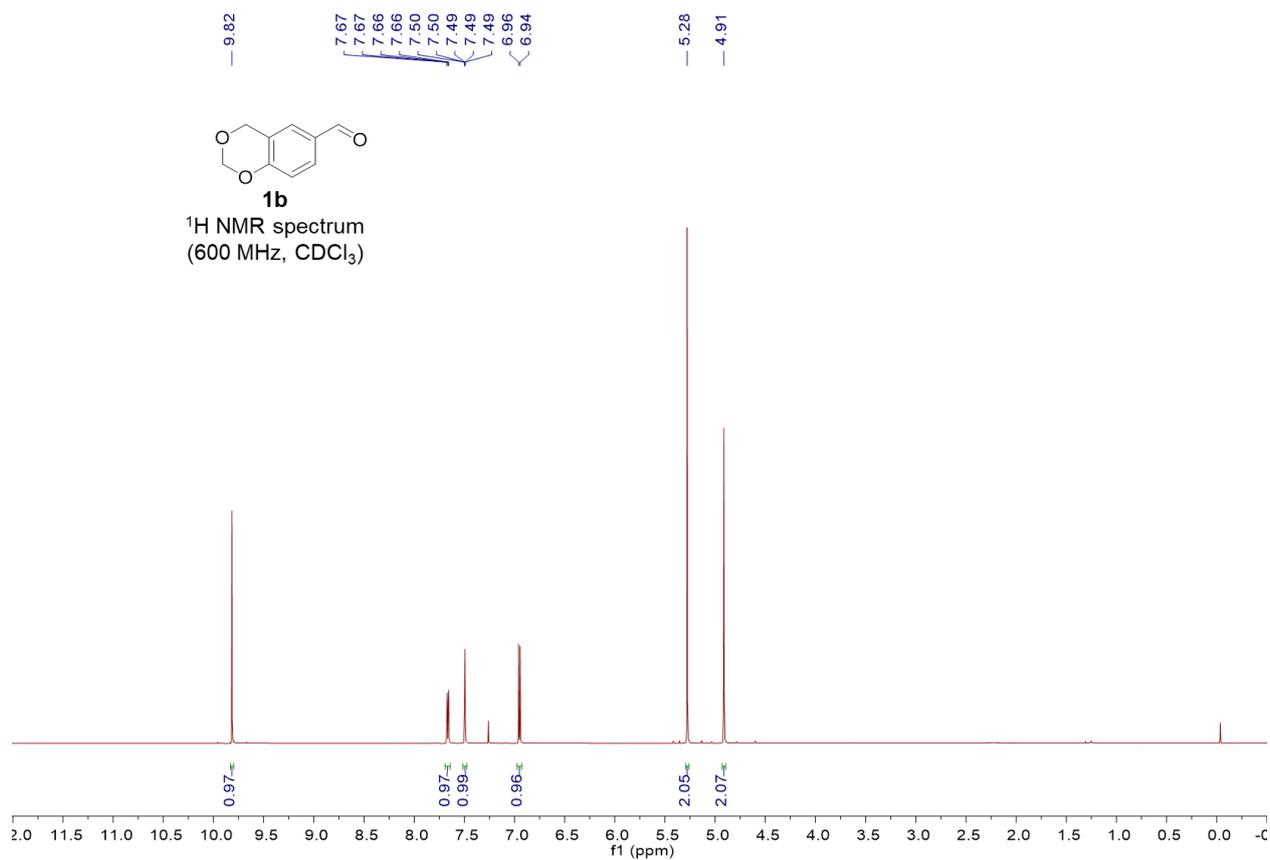


Figure S6. ¹H NMR trace of **1b** synthesized for activity determination.

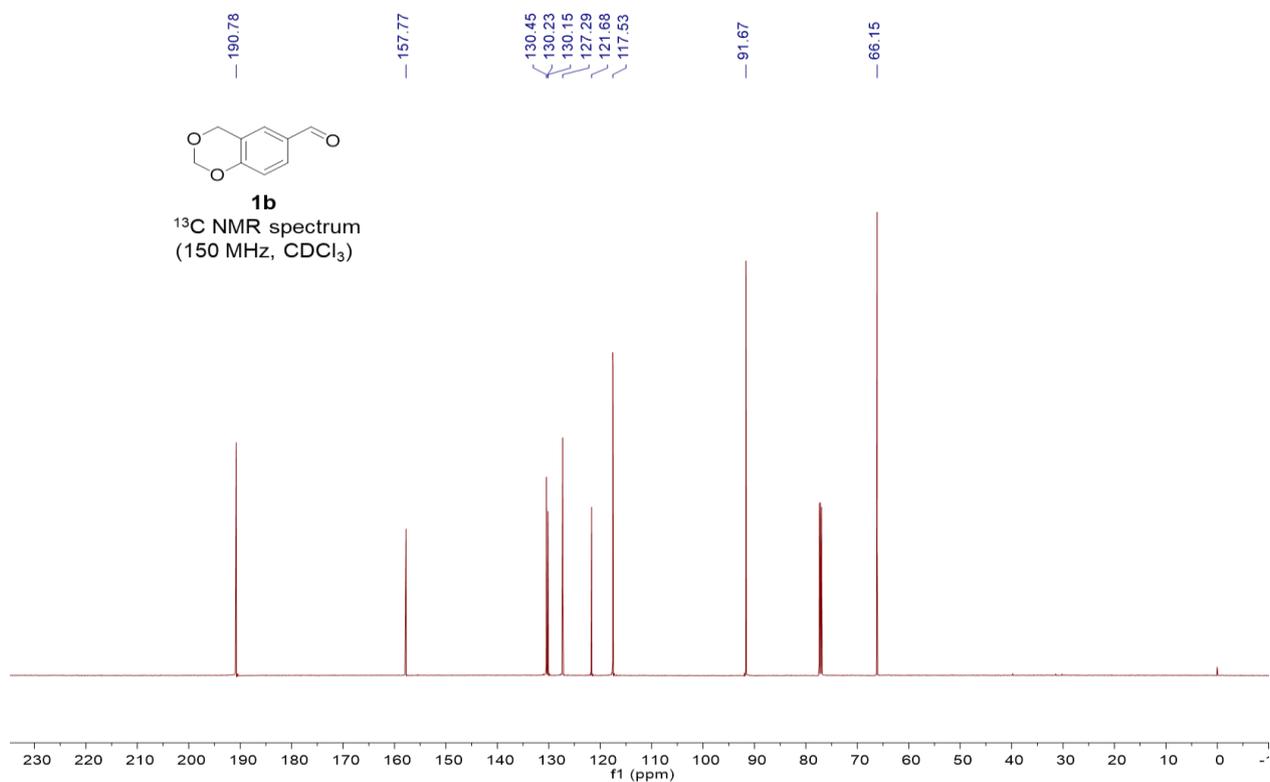


Figure S7. ¹³C NMR trace of **1b** synthesized for activity determination.

SUPPORTING INFORMATION

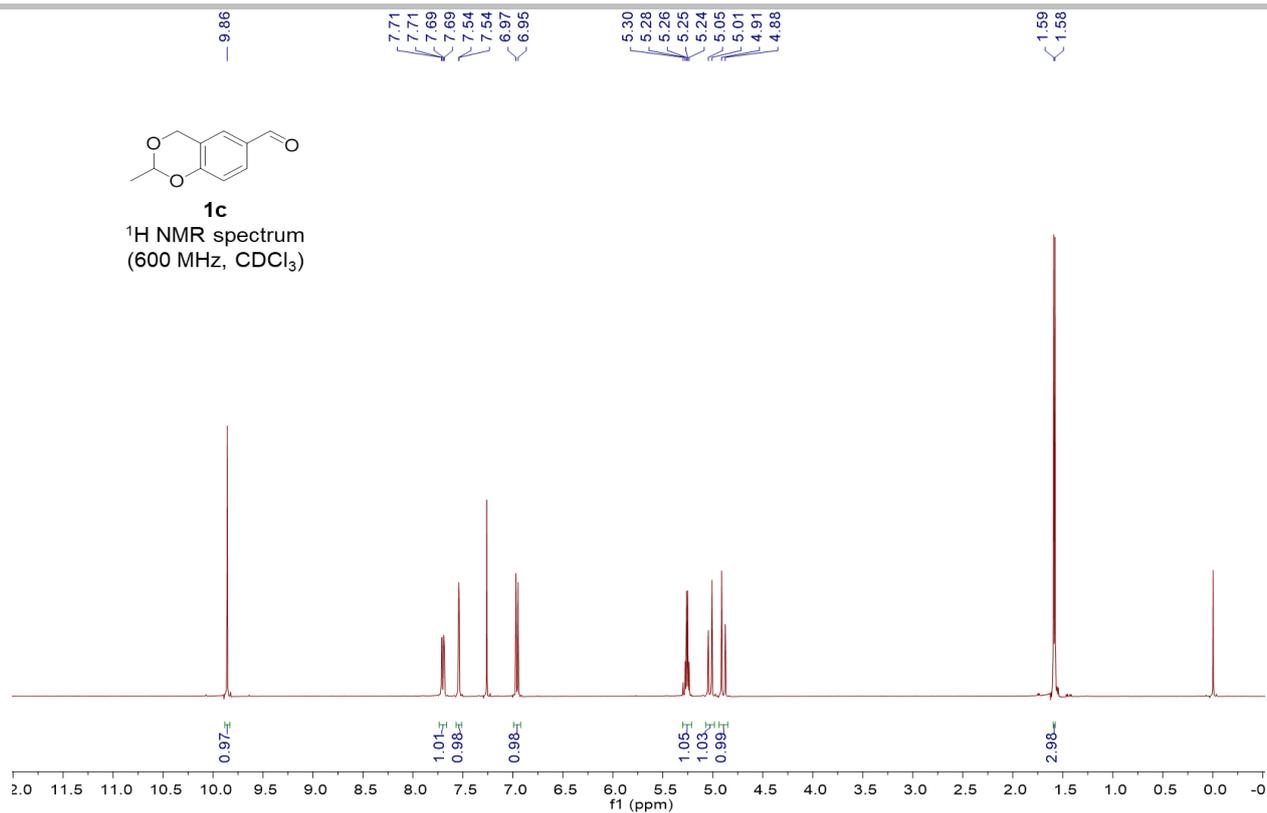


Figure S8. ¹H NMR trace of **1c** synthesized for activity determination.

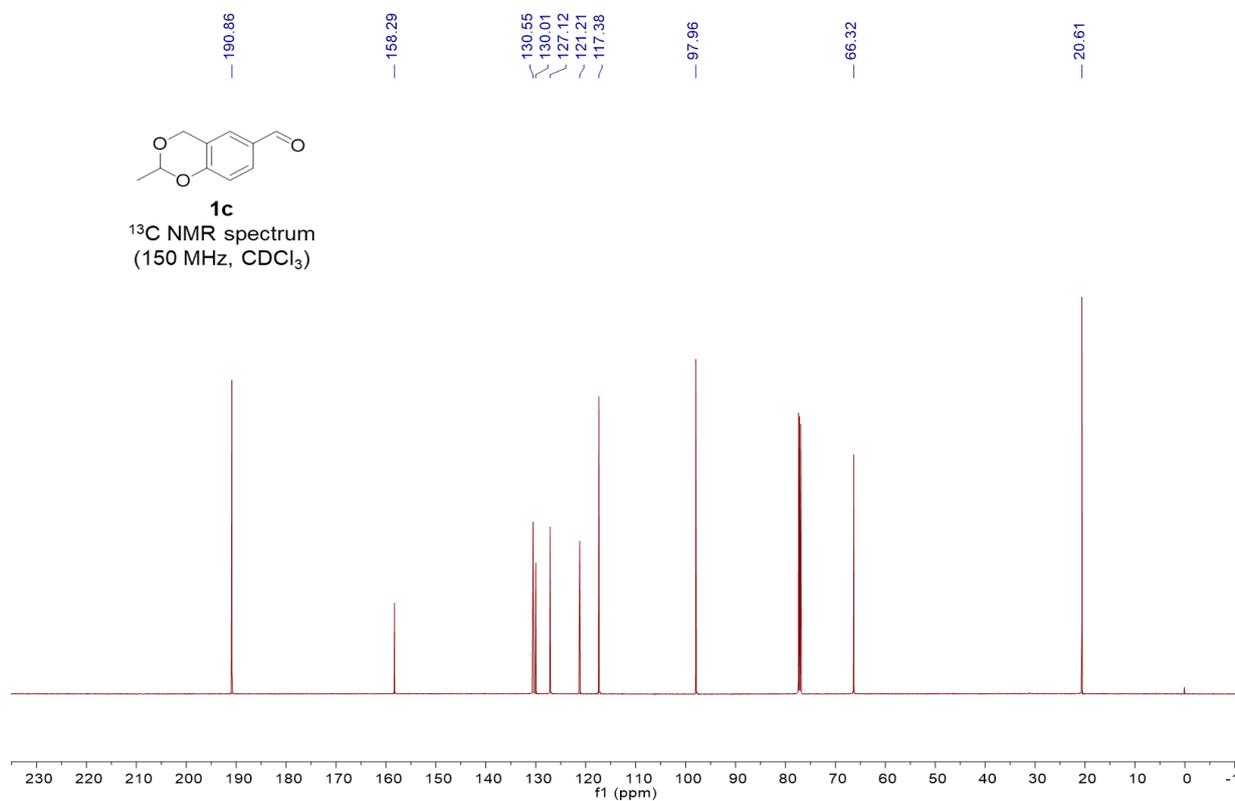


Figure S9. ¹³C NMR trace of **1c** synthesized for activity determination.

SUPPORTING INFORMATION

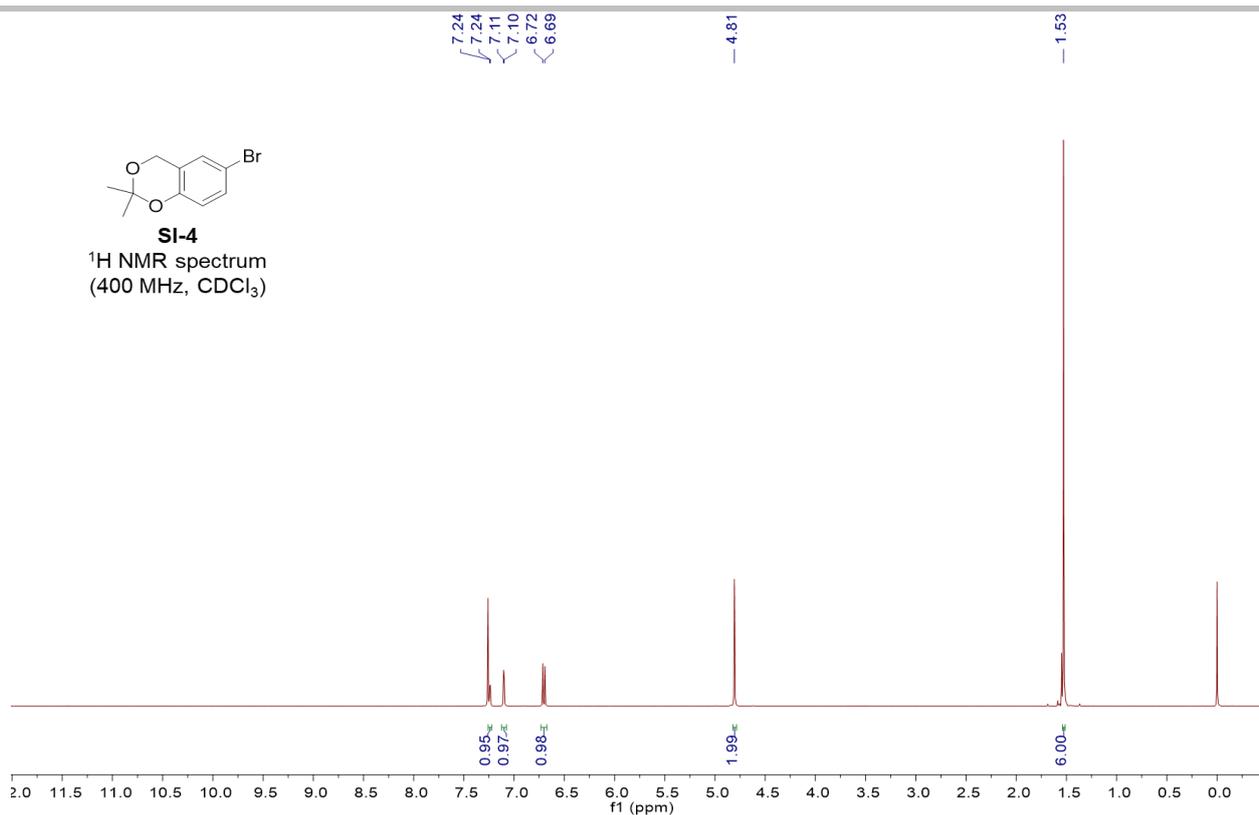


Figure S10. ¹H NMR trace of **SI-4** synthesized for **1d** synthesis.

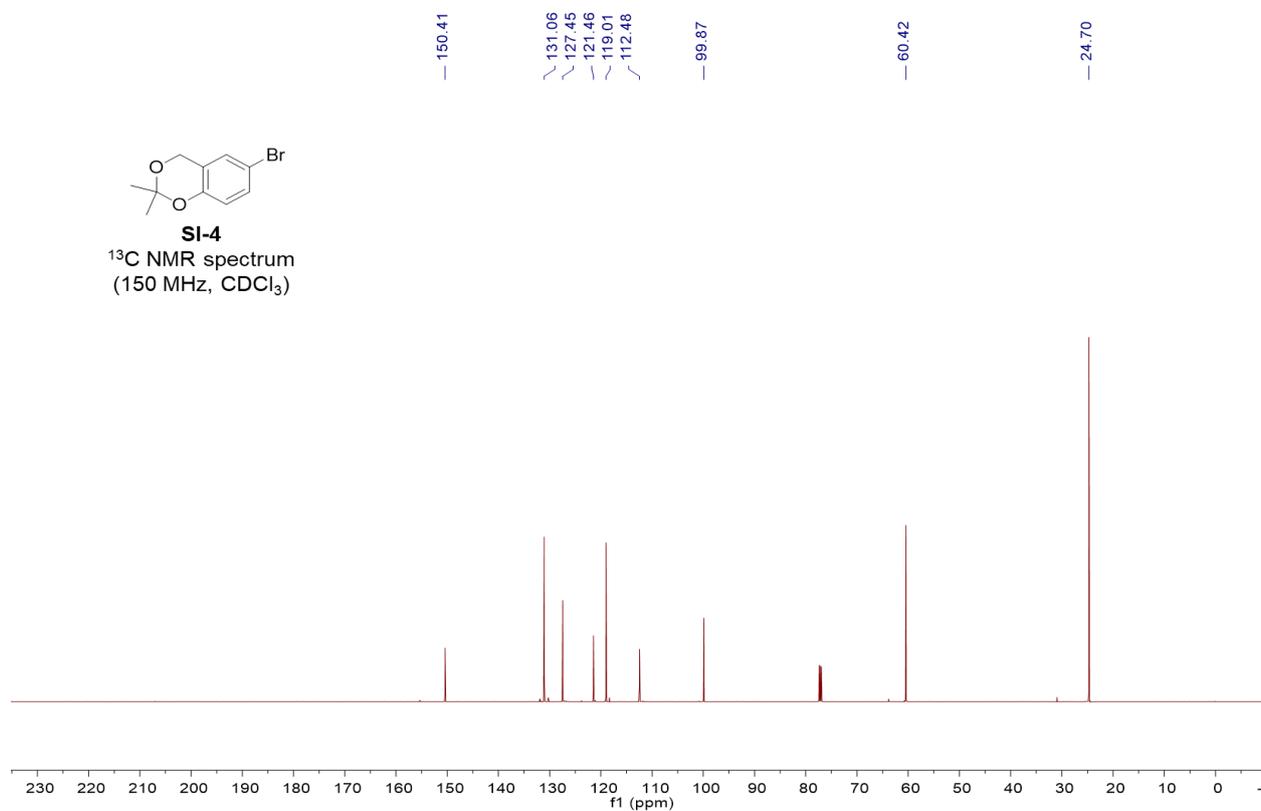


Figure S11. ¹³C NMR trace of **SI-4** synthesized for **1d** synthesis.

SUPPORTING INFORMATION

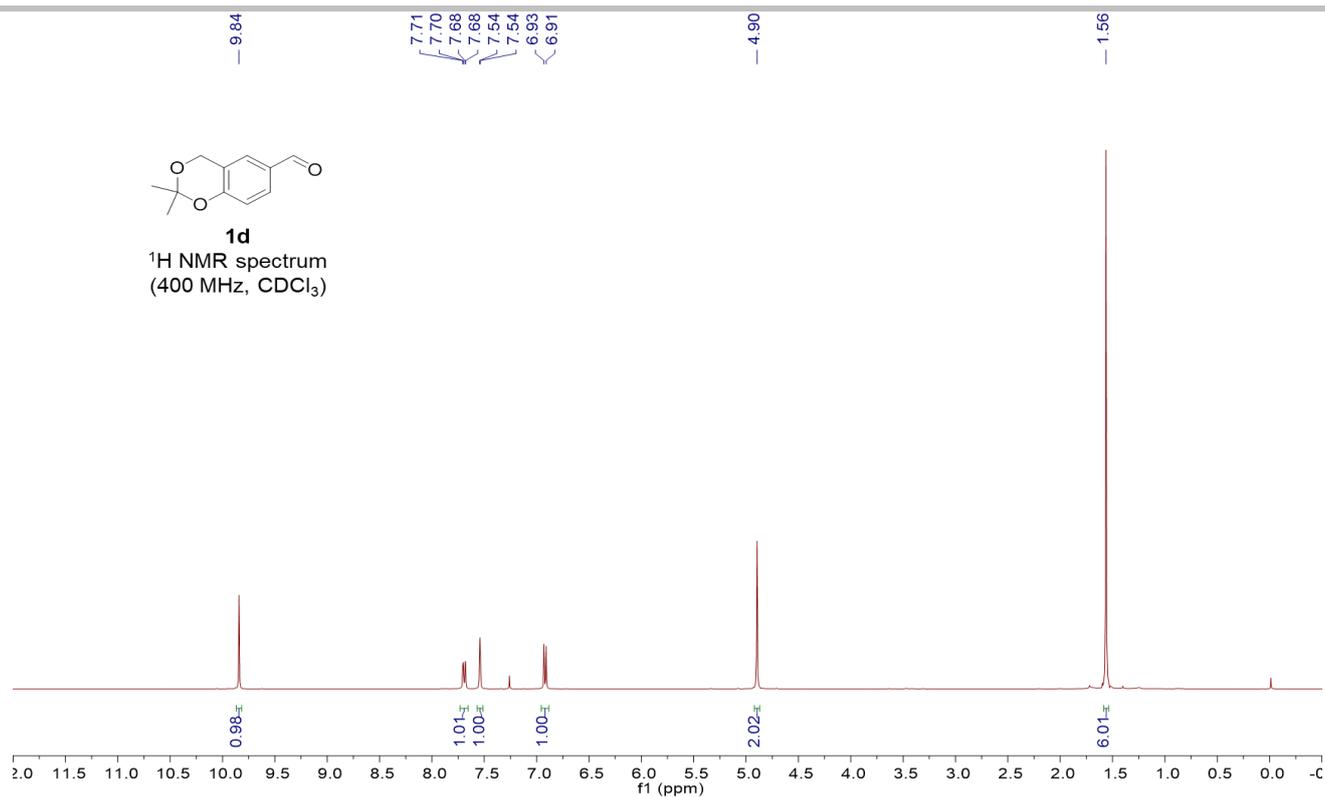


Figure S12. ¹H NMR trace of **SI-1-4** synthesized for **1d** synthesis.

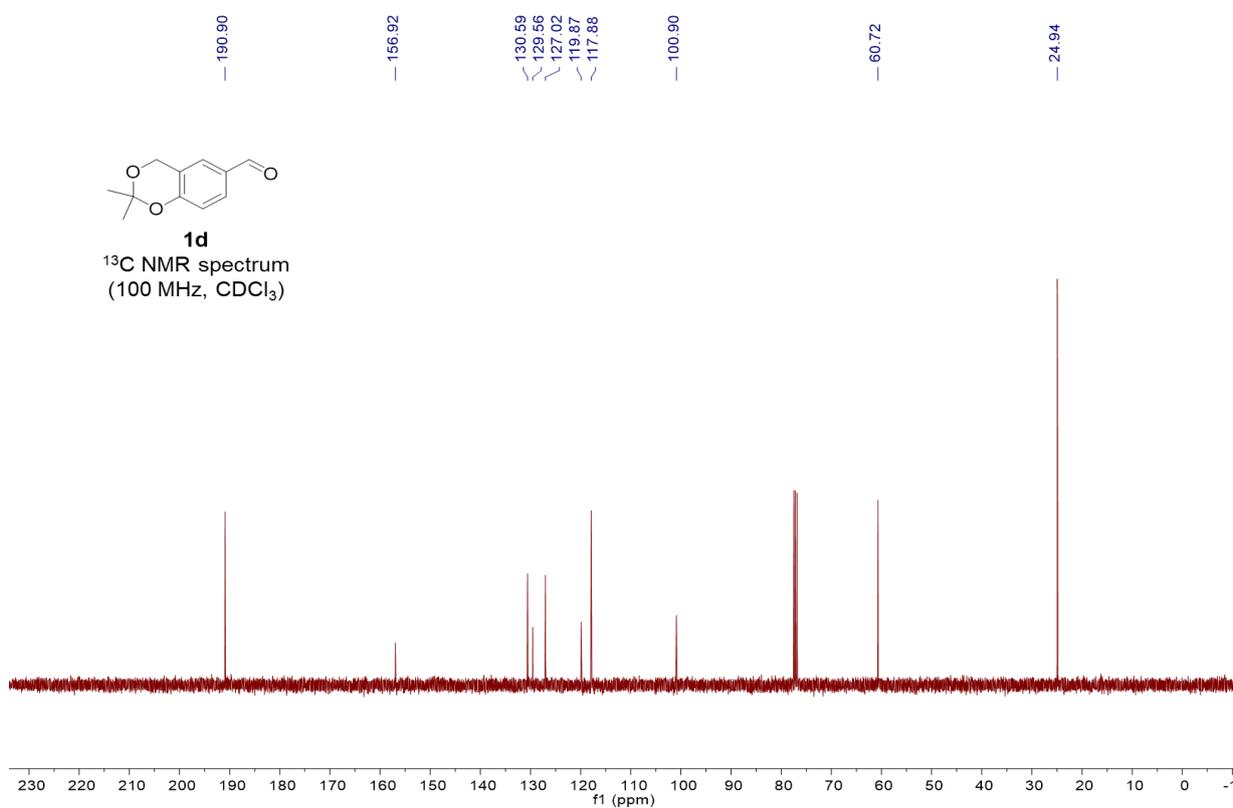


Figure S13. ¹³C NMR trace of **1d** synthesized for activity determination.

SUPPORTING INFORMATION

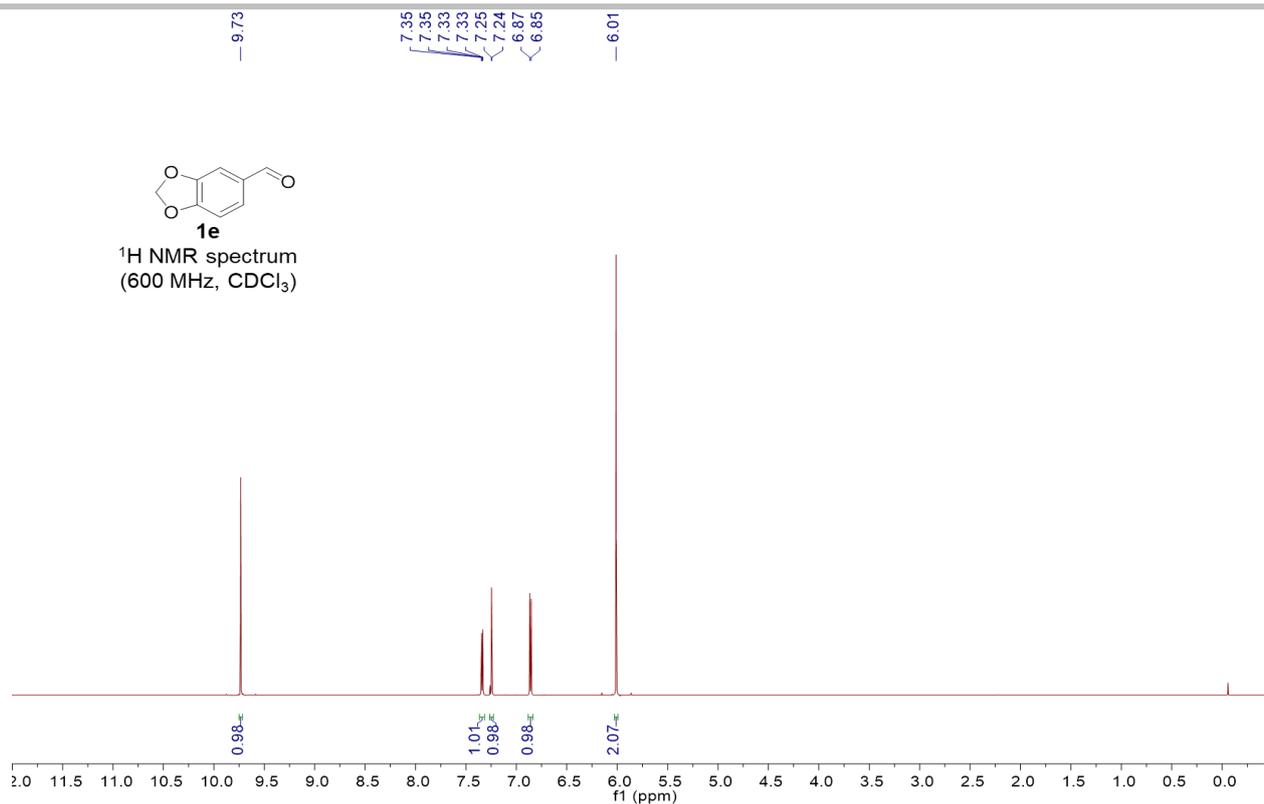


Figure S14. ¹H NMR trace of **1e** synthesized for activity determination.

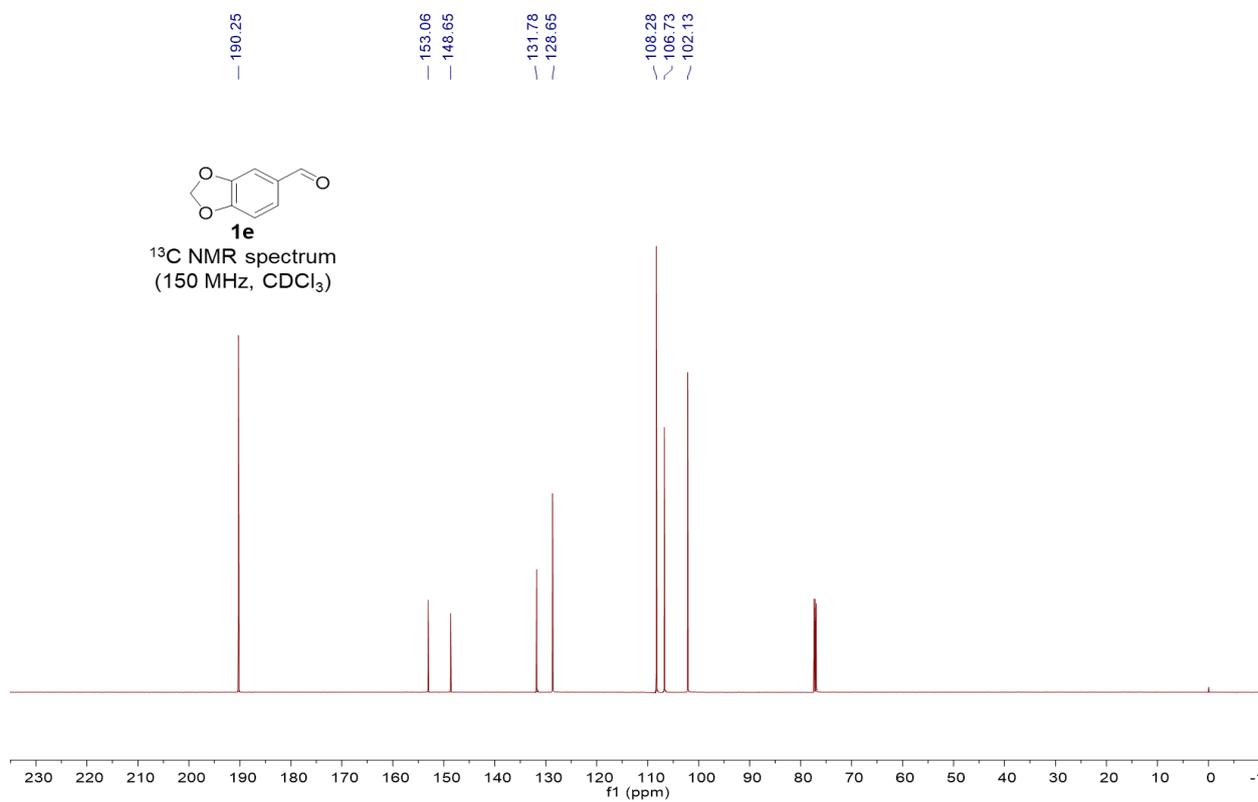


Figure S15. ¹³C NMR trace of **1e** synthesized for activity determination.

SUPPORTING INFORMATION

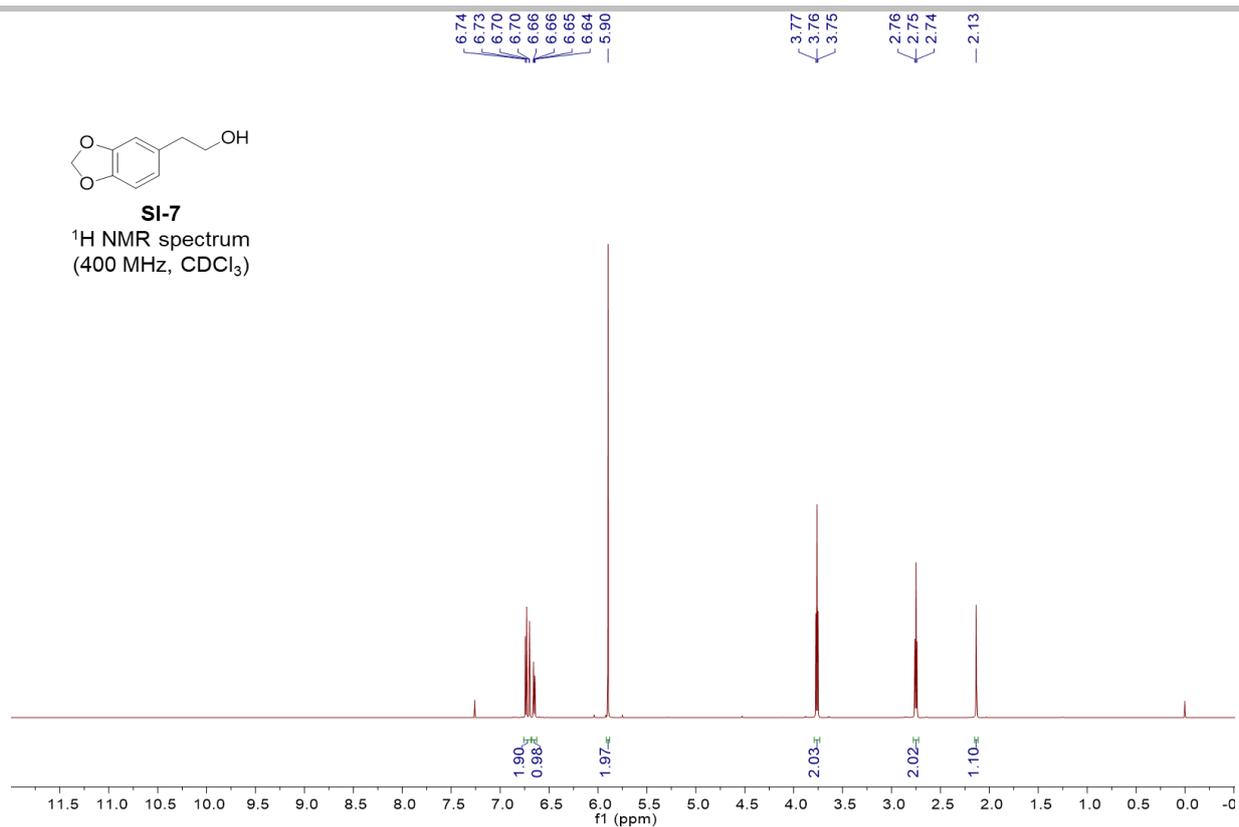


Figure S16. ¹H NMR trace of **SI-7** synthesized for **1g** synthesis.

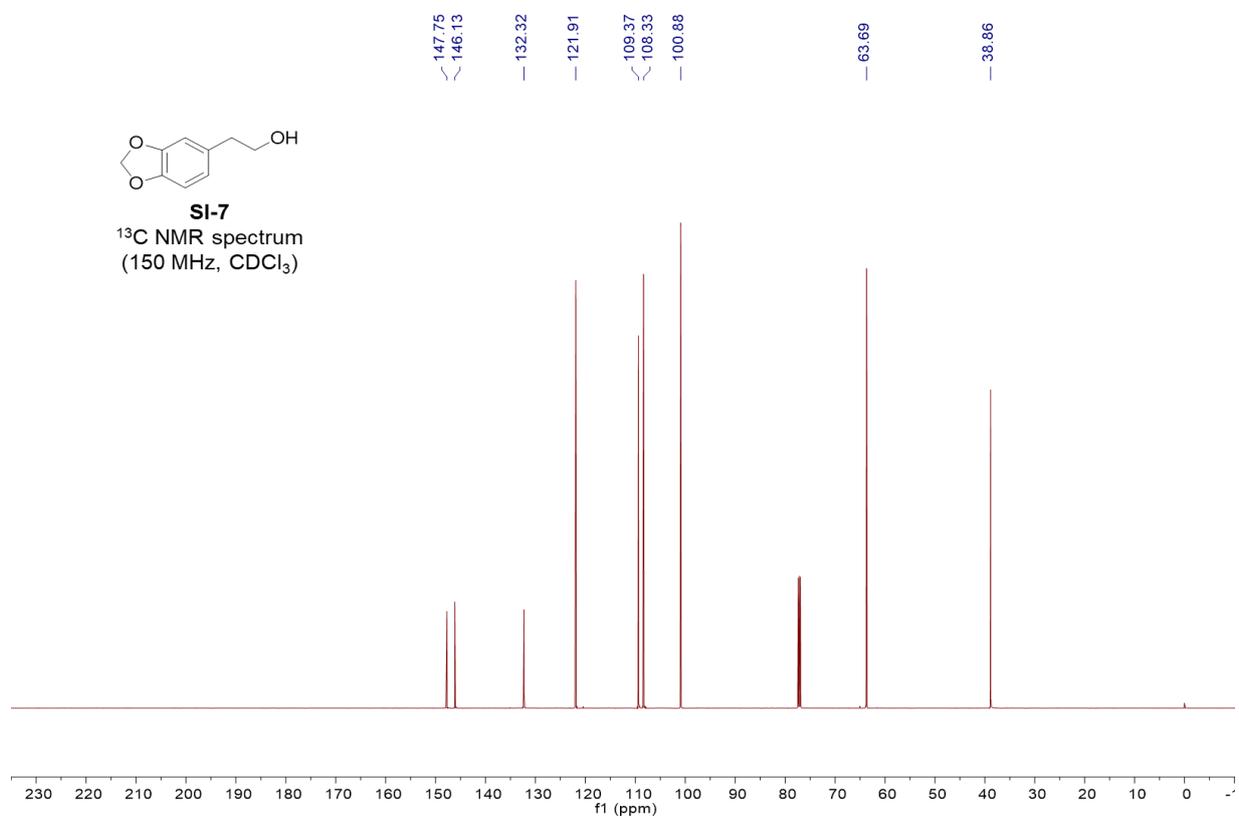


Figure S17. ¹³C NMR trace of **SI-7** synthesized for **1g** synthesis.

SUPPORTING INFORMATION

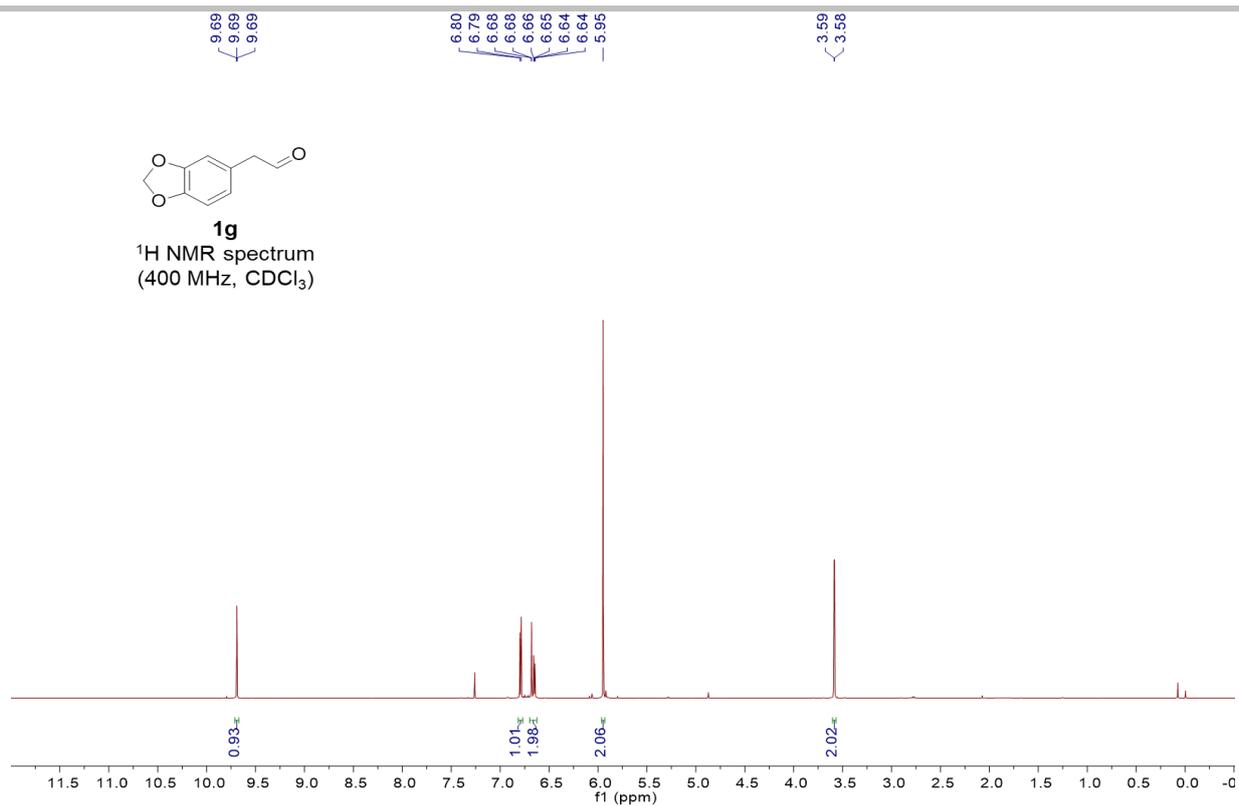


Figure S18. ¹H NMR trace of **1g** synthesized for activity determination.

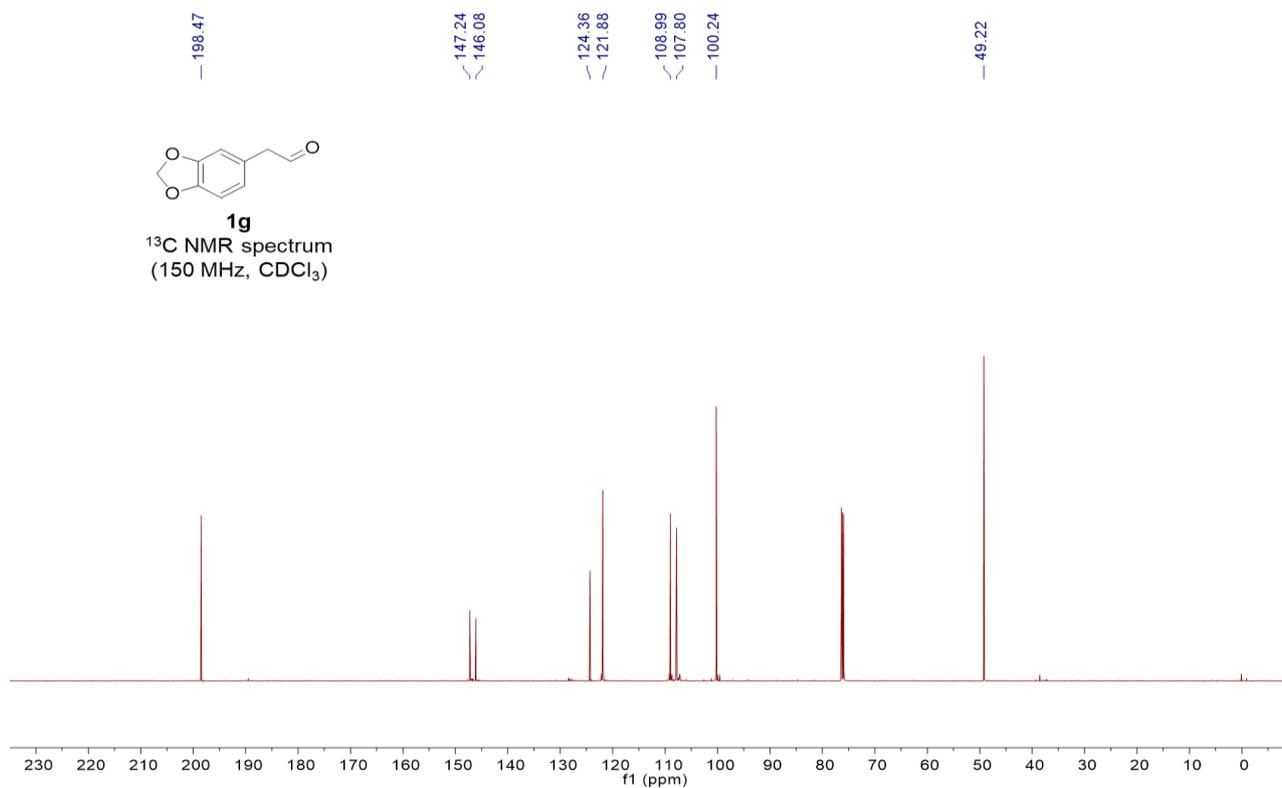


Figure S19. ¹³C NMR trace of **1g** synthesized for activity determination.

SUPPORTING INFORMATION

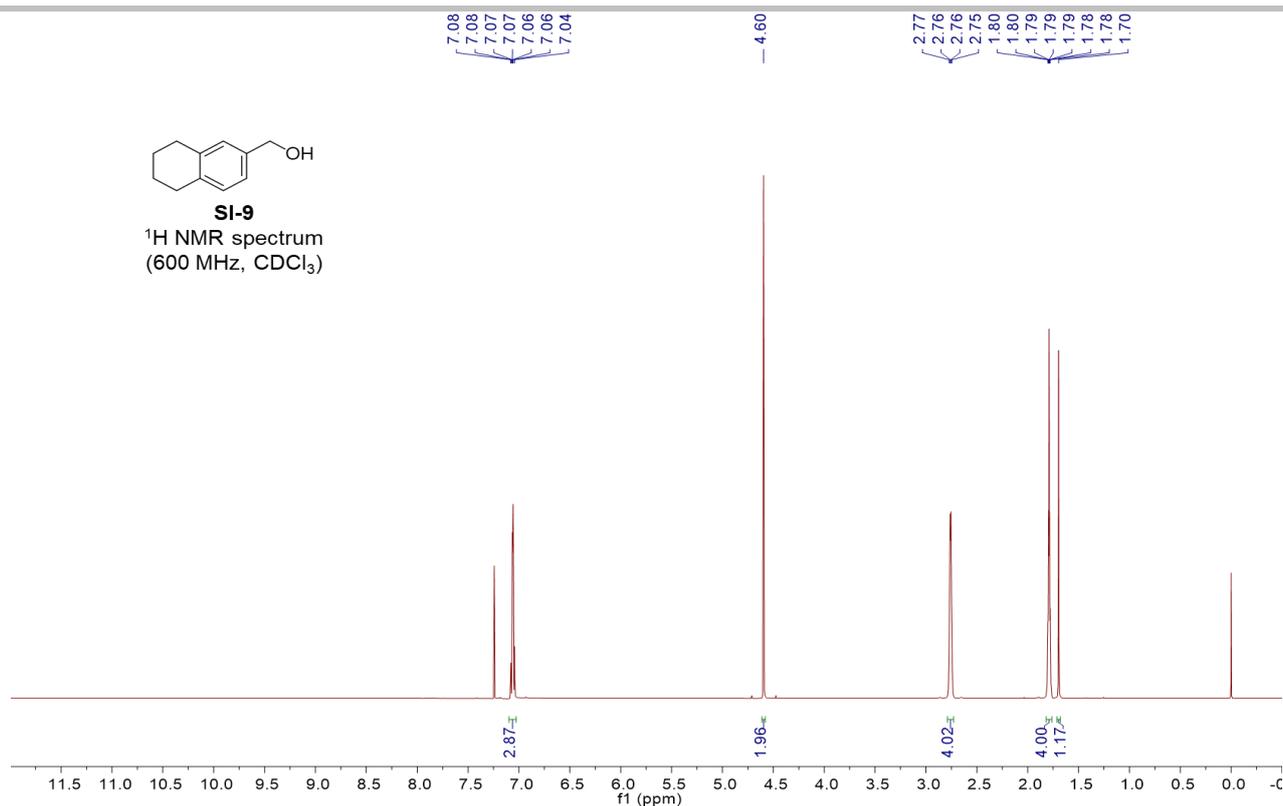


Figure S20. ¹H NMR trace of **SI-9** synthesized for **1h** synthesis.

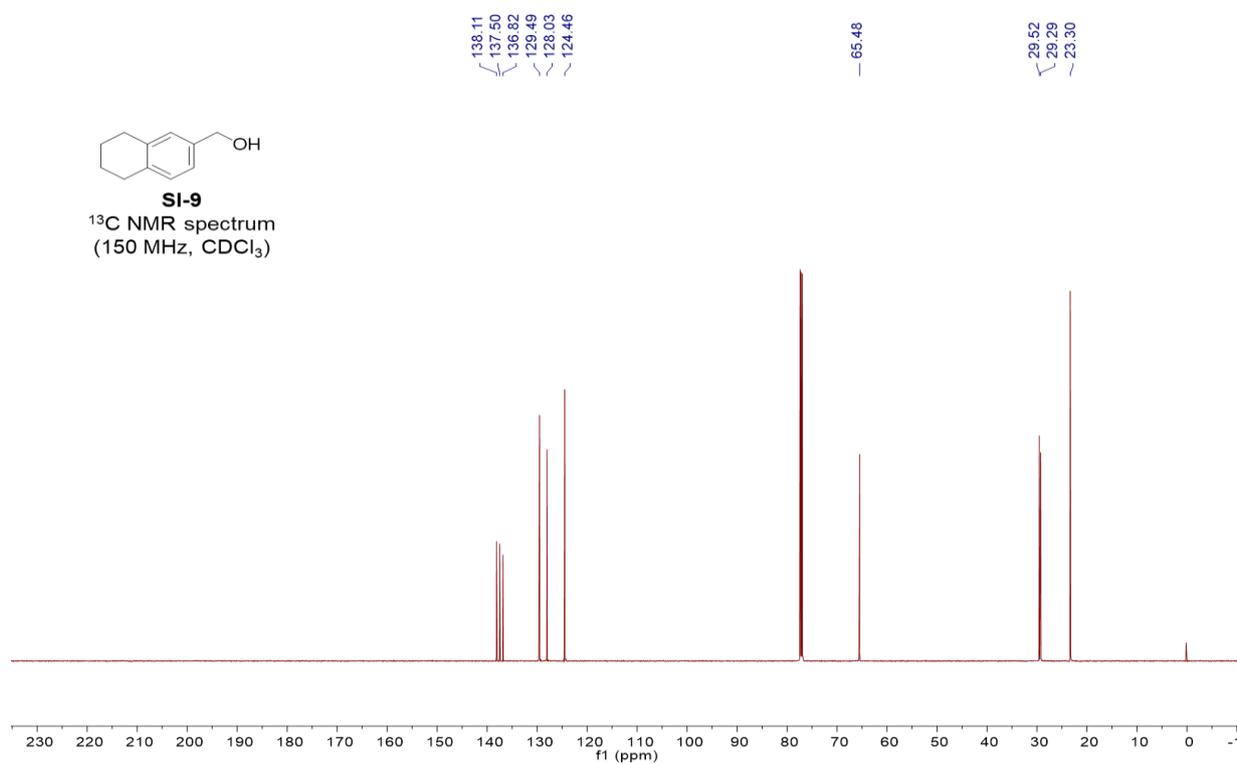


Figure S21. ¹³C NMR trace of **SI-9** synthesized for **1h** synthesis.

SUPPORTING INFORMATION

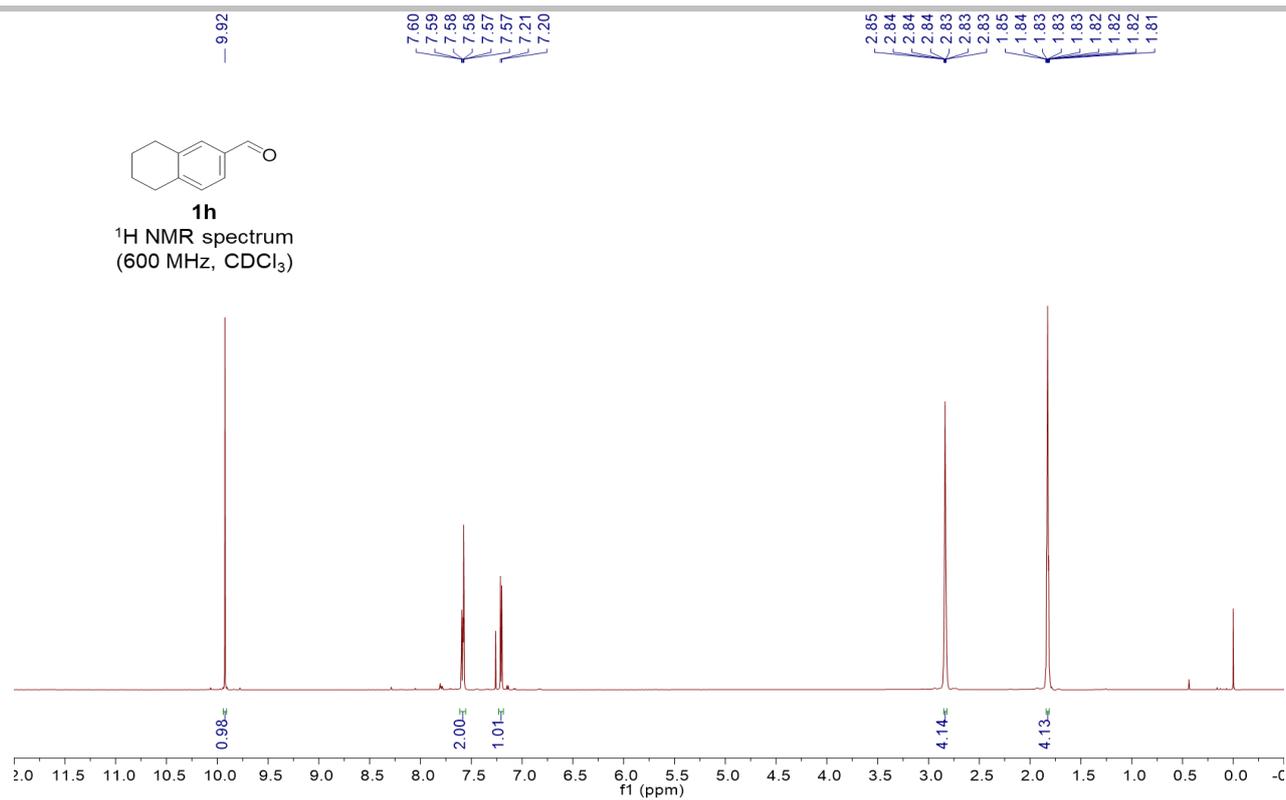


Figure S22. ¹H NMR trace of **1h** synthesized for activity determination.

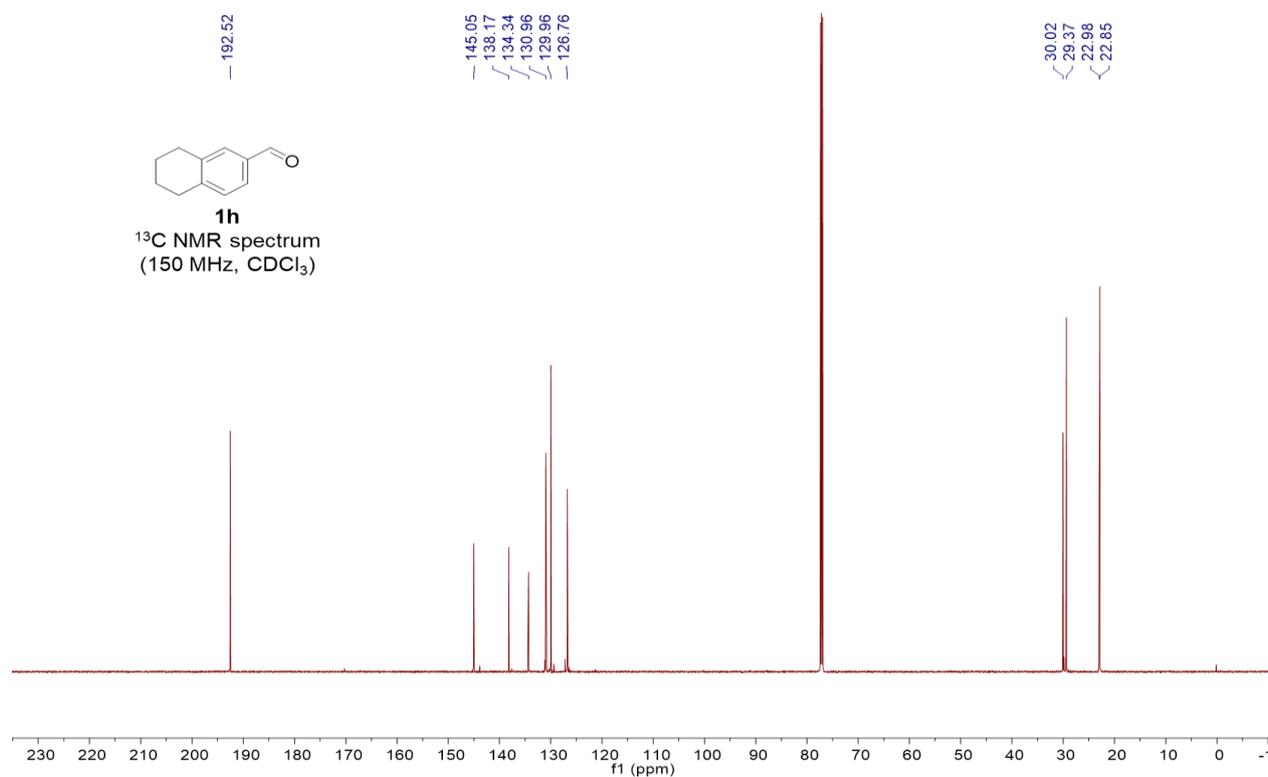


Figure S23. ¹³C NMR trace of **1h** synthesized for activity determination.

SUPPORTING INFORMATION

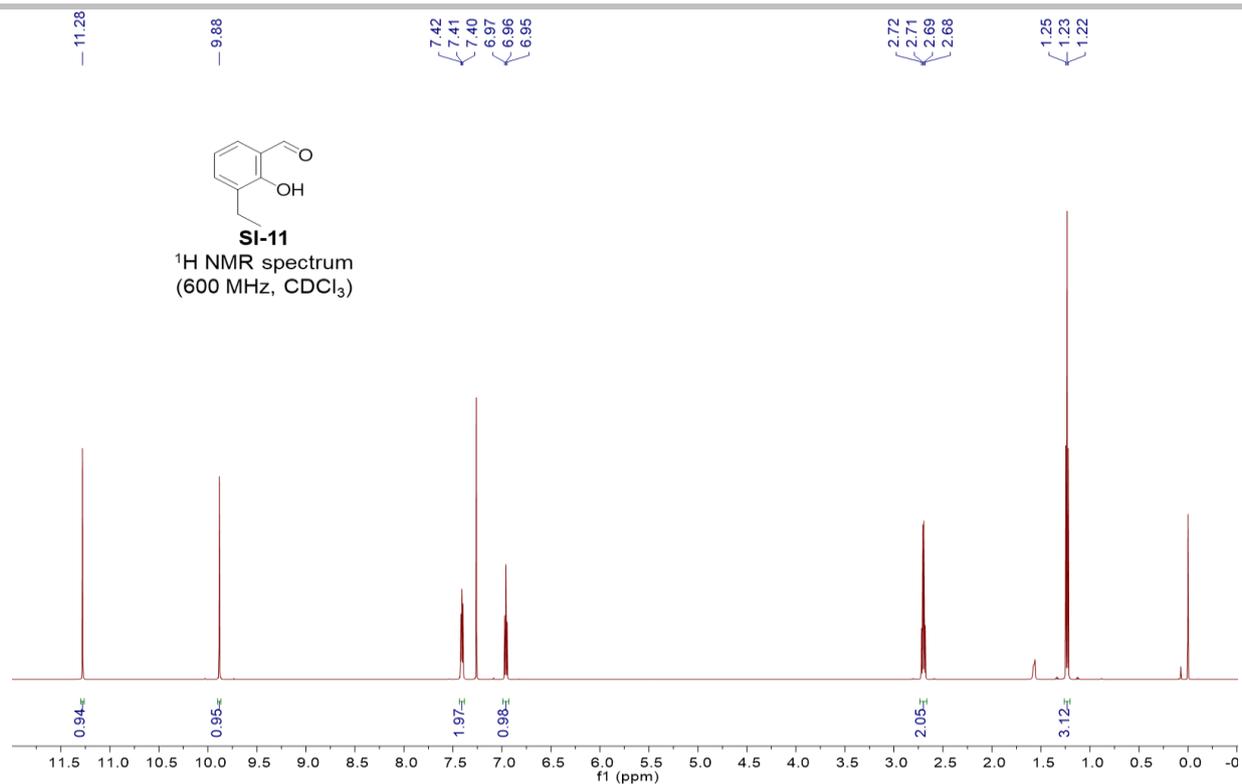


Figure S24. ¹H NMR trace of SI-11 synthesized for 1k synthesis.

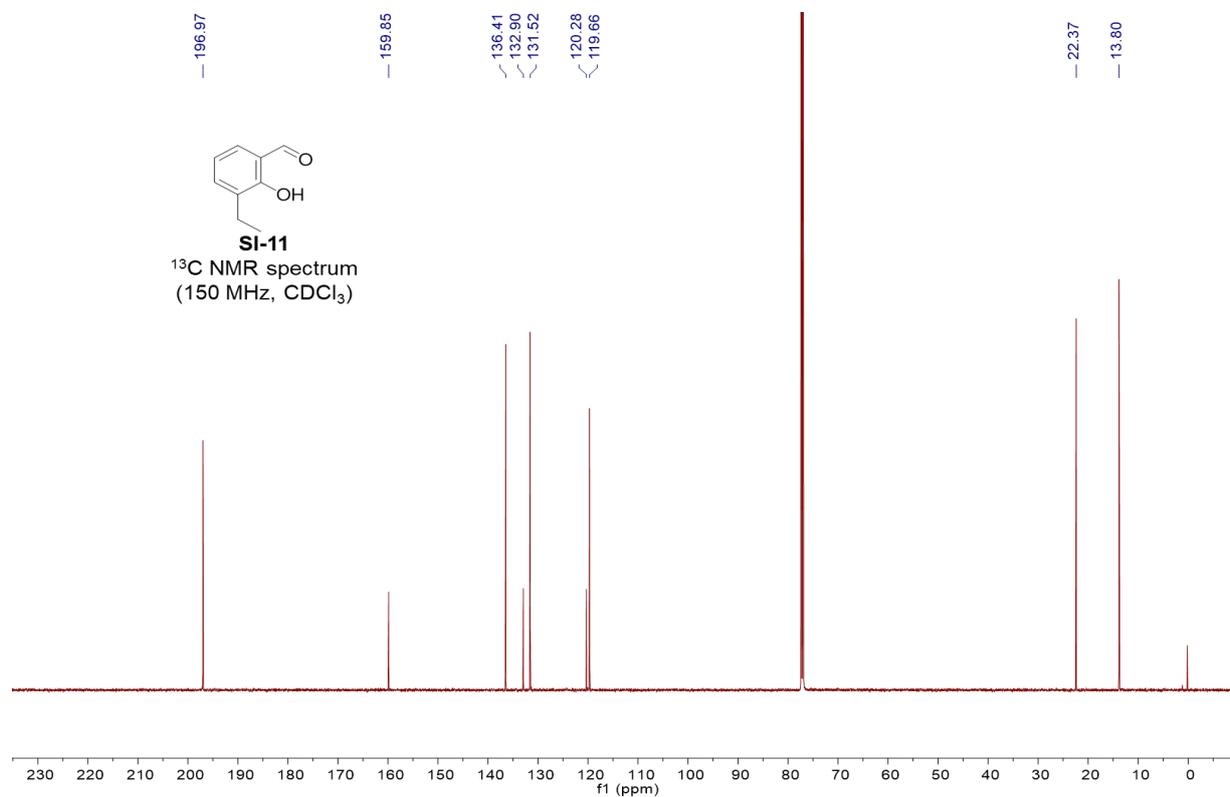


Figure S25. ¹³C NMR trace of SI-11 synthesized for 1k synthesis.

SUPPORTING INFORMATION

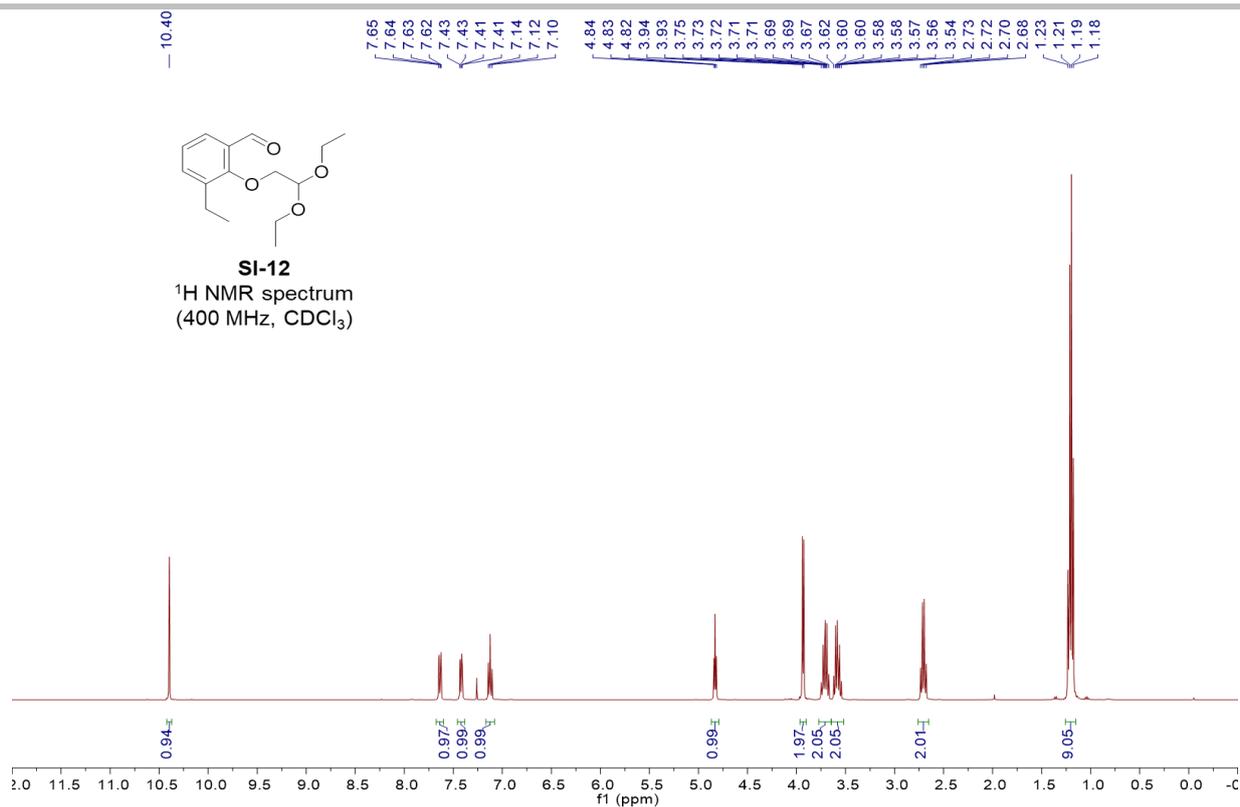


Figure S26. ¹H NMR trace of SI-12 synthesized for 1k synthesis.

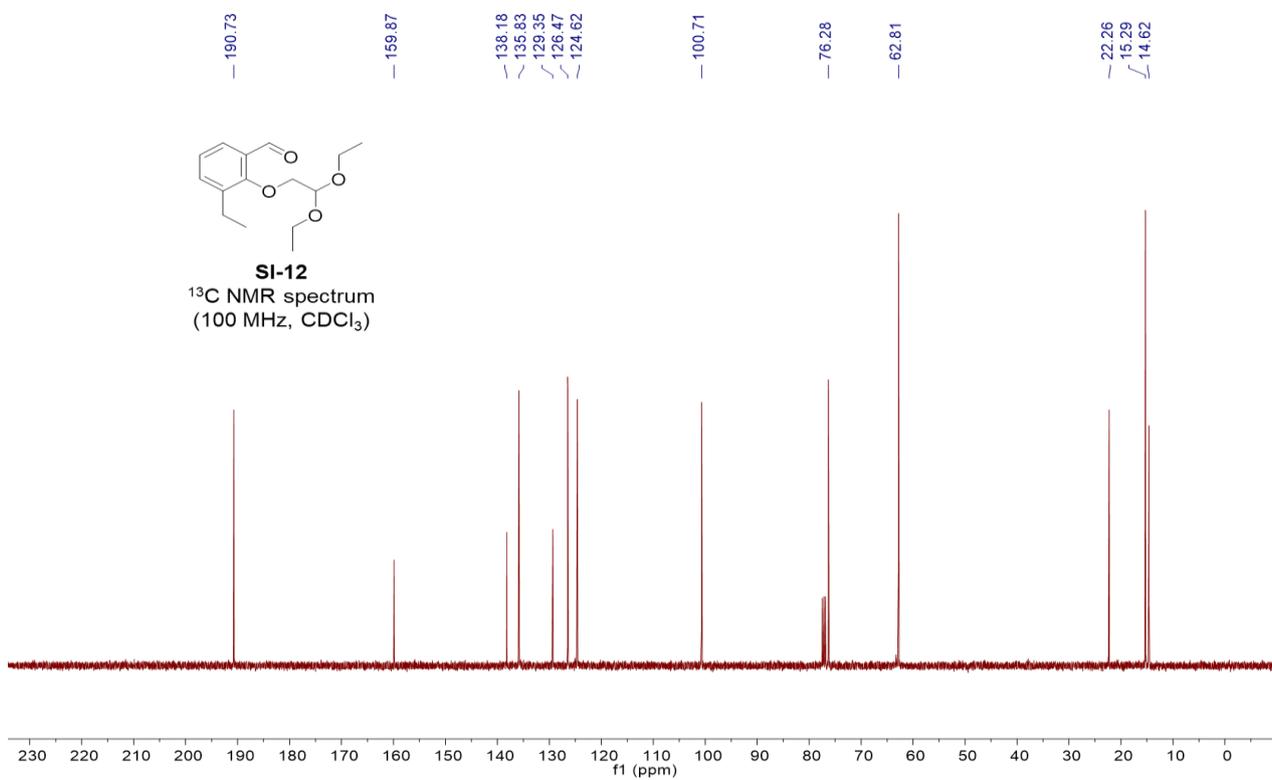


Figure S27. ¹³C NMR trace of SI-12 synthesized for 1k synthesis.

SUPPORTING INFORMATION

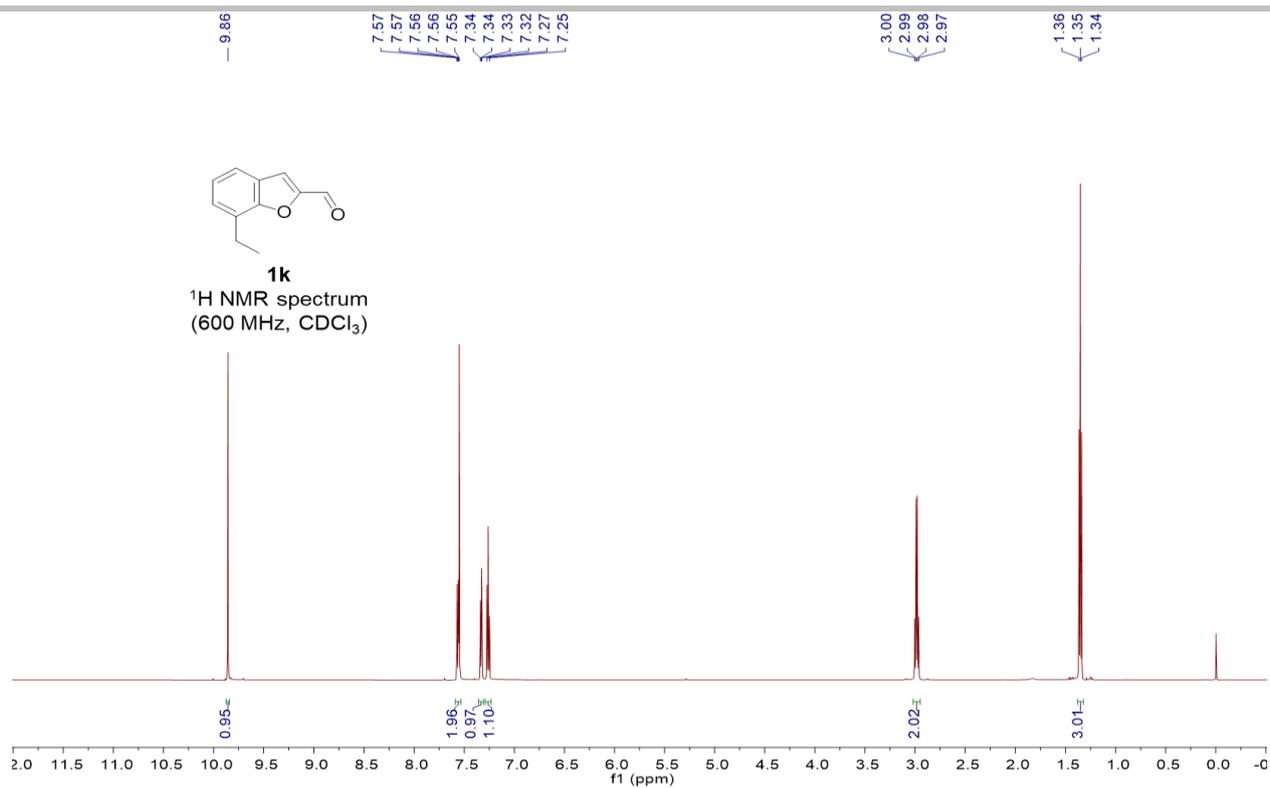


Figure S28. ¹H NMR trace of **1k** synthesized for activity determination.

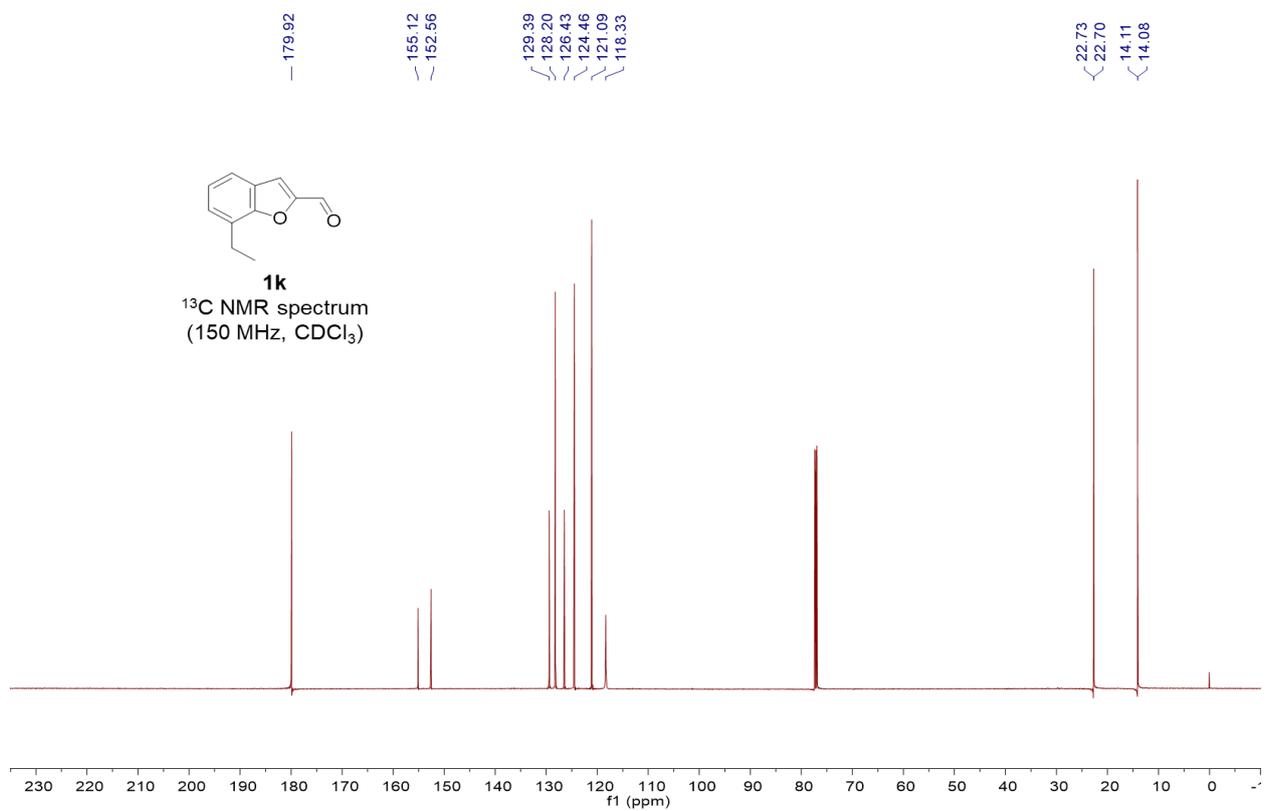


Figure S29. ¹³C NMR trace of **1k** synthesized for activity determination.

SUPPORTING INFORMATION

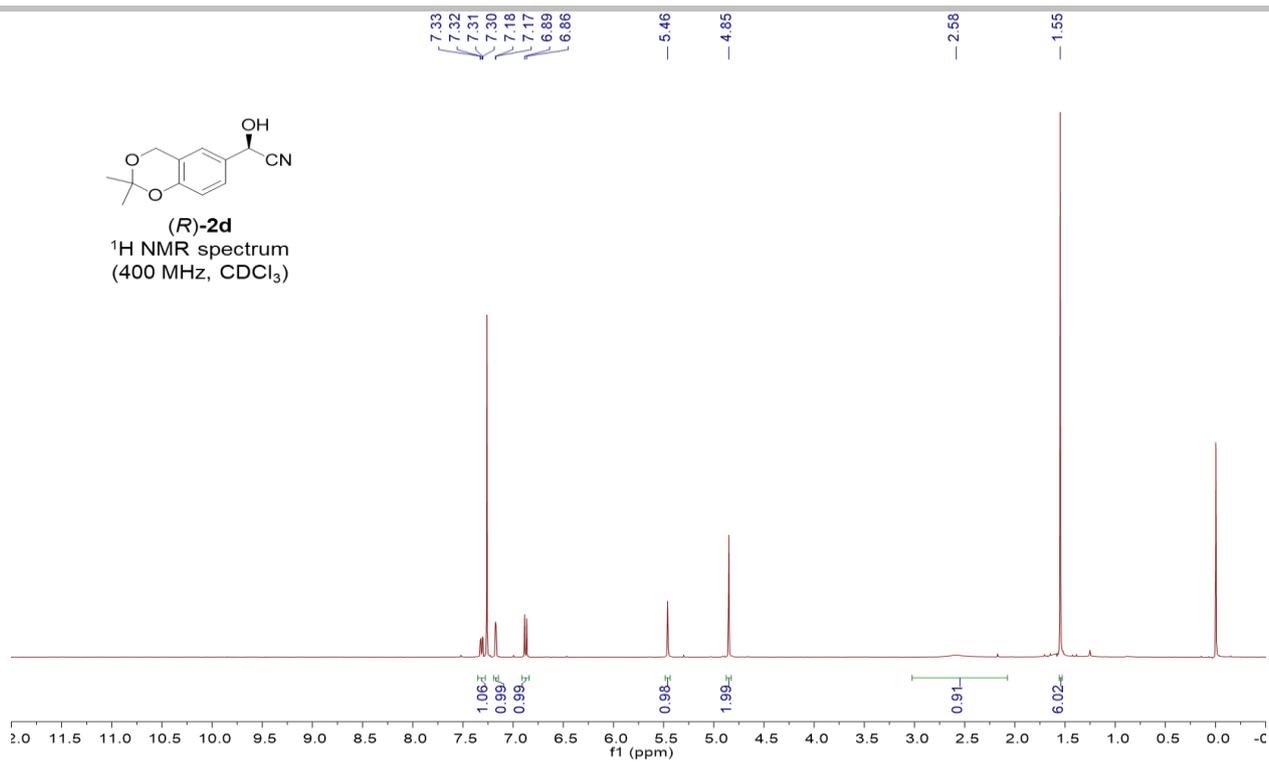


Figure S30. ¹H NMR trace of (*R*)-**2d** prepared for (*R*)-salmeterol synthesis.

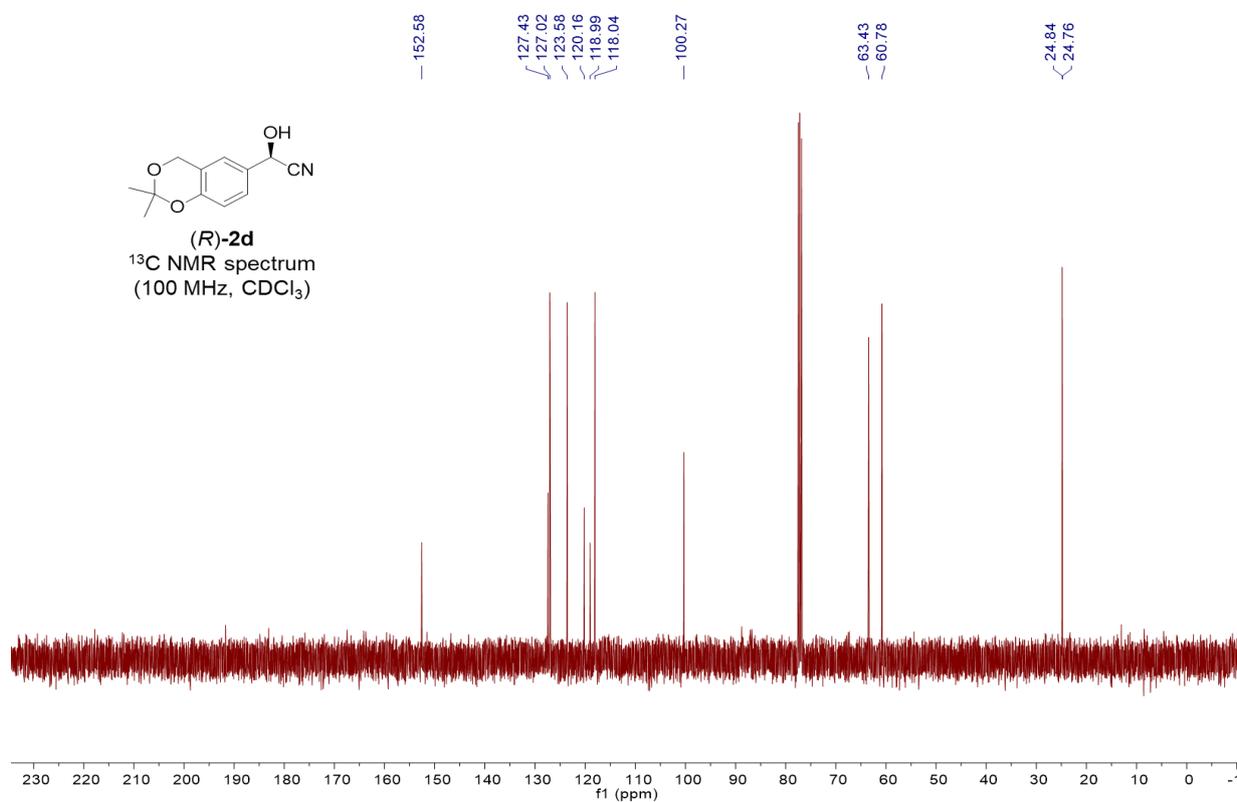


Figure S31. ¹³C NMR trace of (*R*)-**2d** prepared for (*R*)-salmeterol synthesis.

SUPPORTING INFORMATION

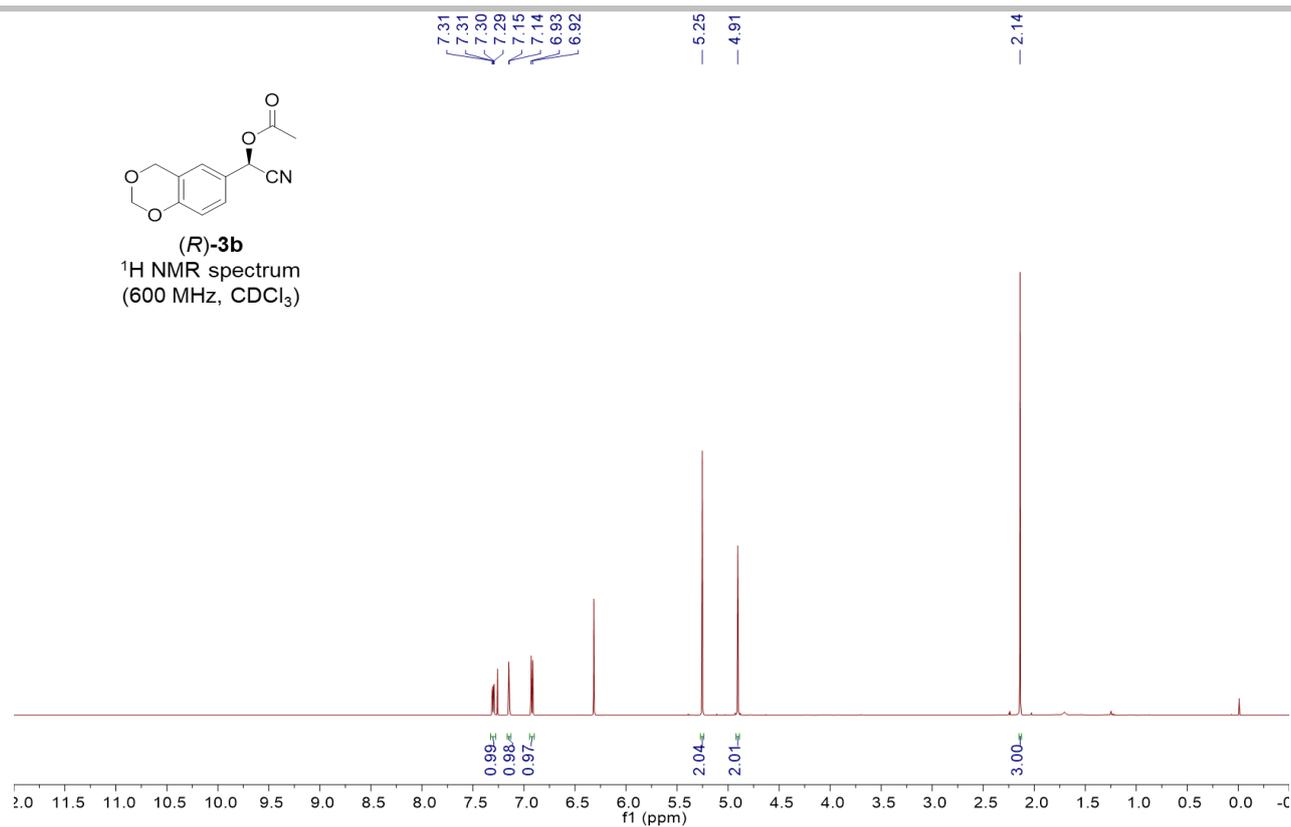


Figure S32. ¹H NMR trace of (*R*)-**3b** prepared in this study.

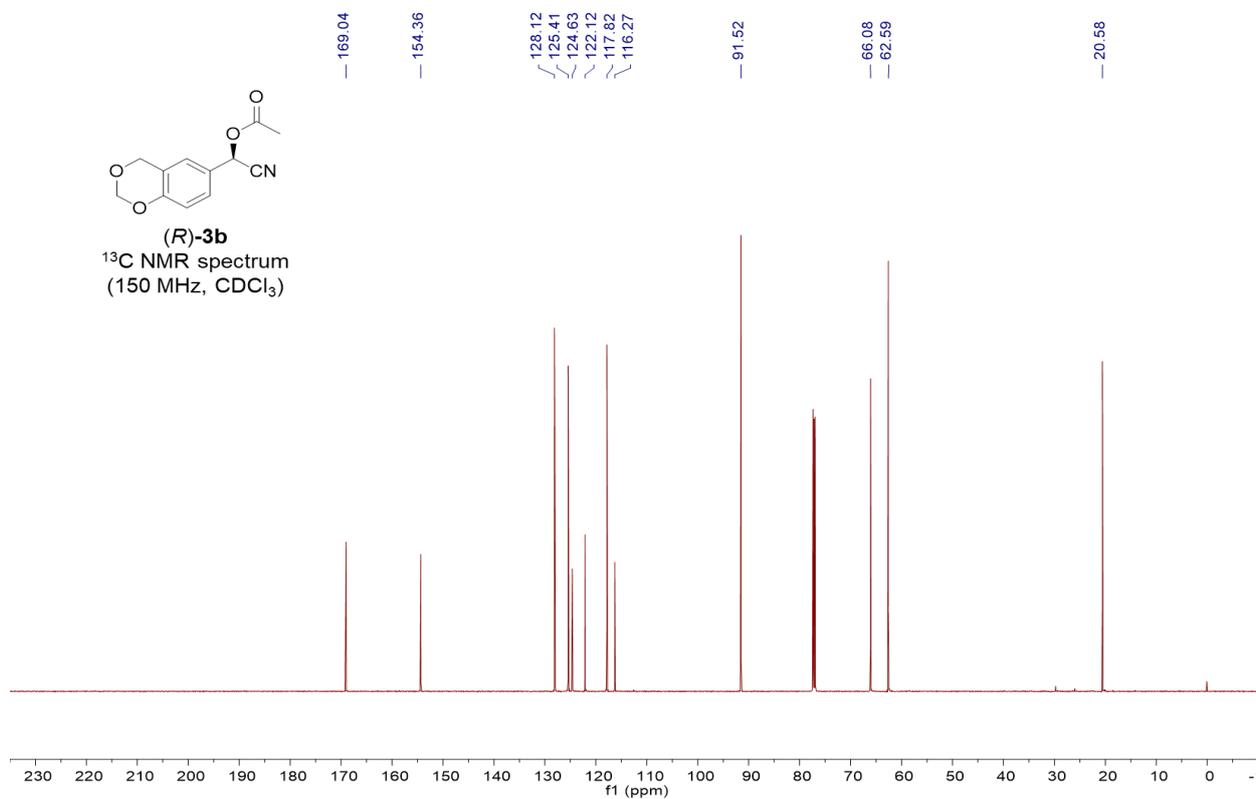


Figure S33. ¹³C NMR trace of (*R*)-**3b** prepared in this study.

SUPPORTING INFORMATION

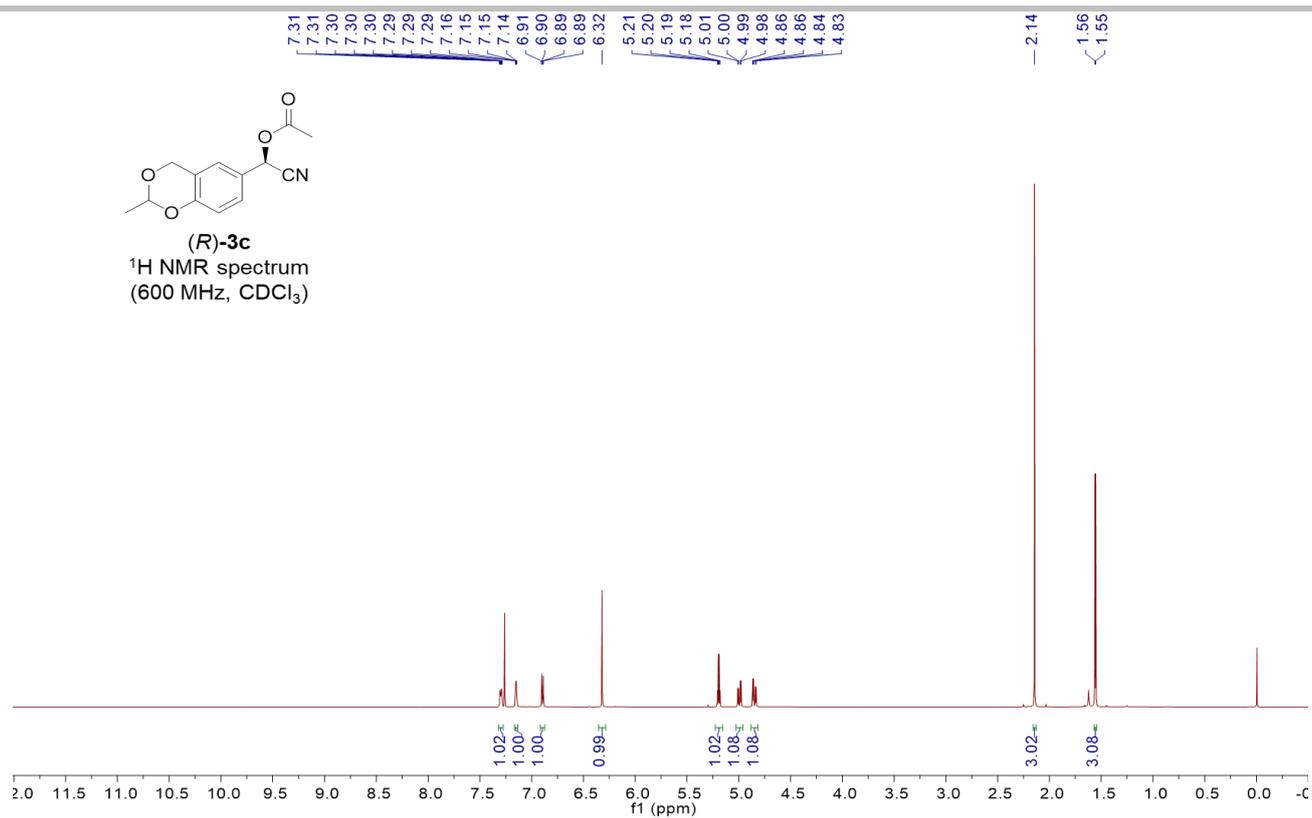


Figure S34. ¹H NMR trace of (R)-3c prepared in this study.

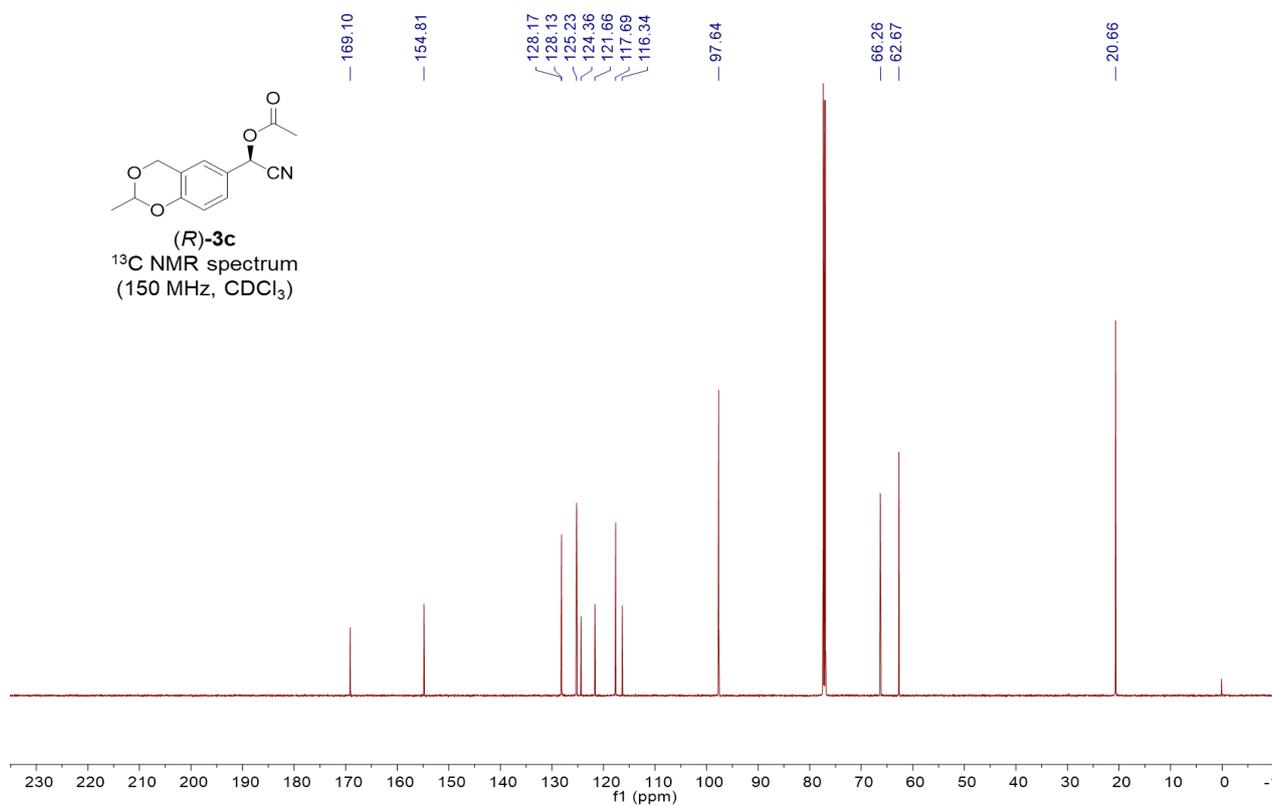


Figure S35. ¹³C NMR trace of (R)-3c prepared in this study.

SUPPORTING INFORMATION

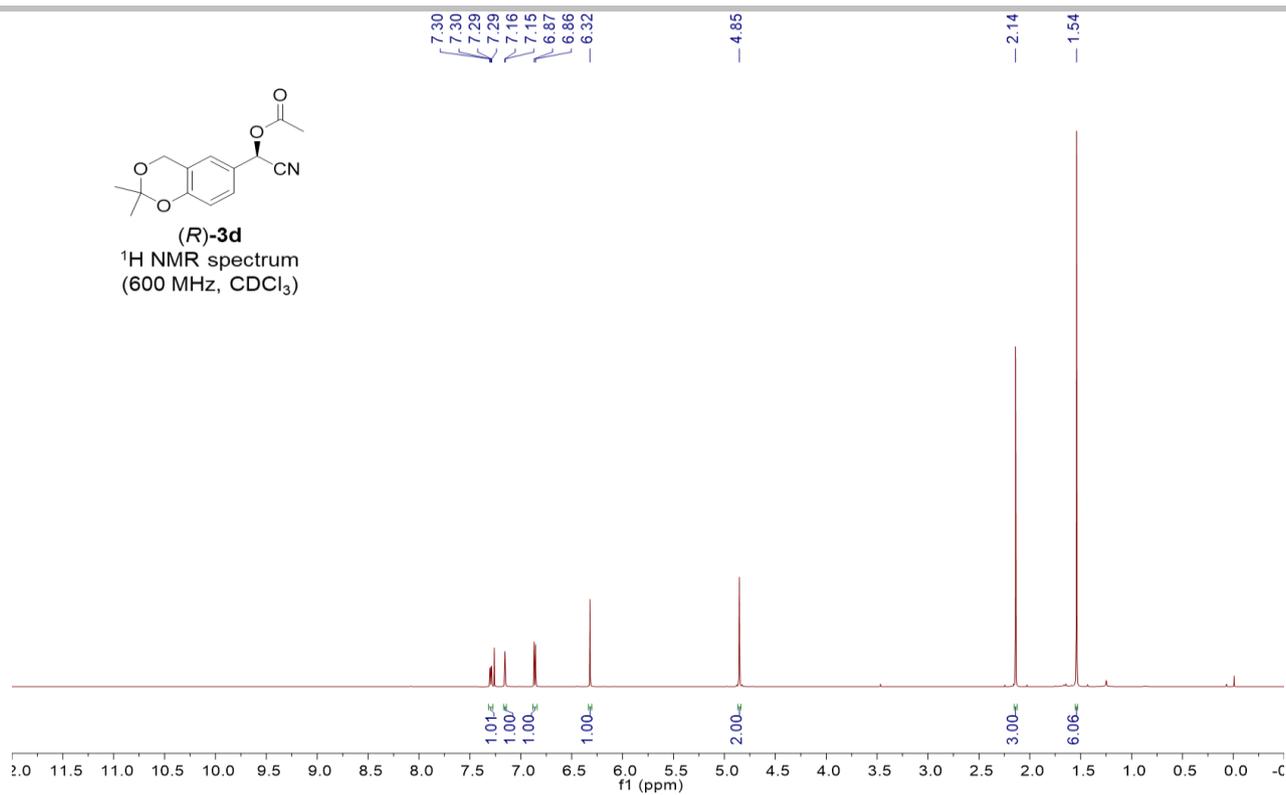


Figure S36. ¹H NMR trace of (*R*)-**3d** prepared in this study.

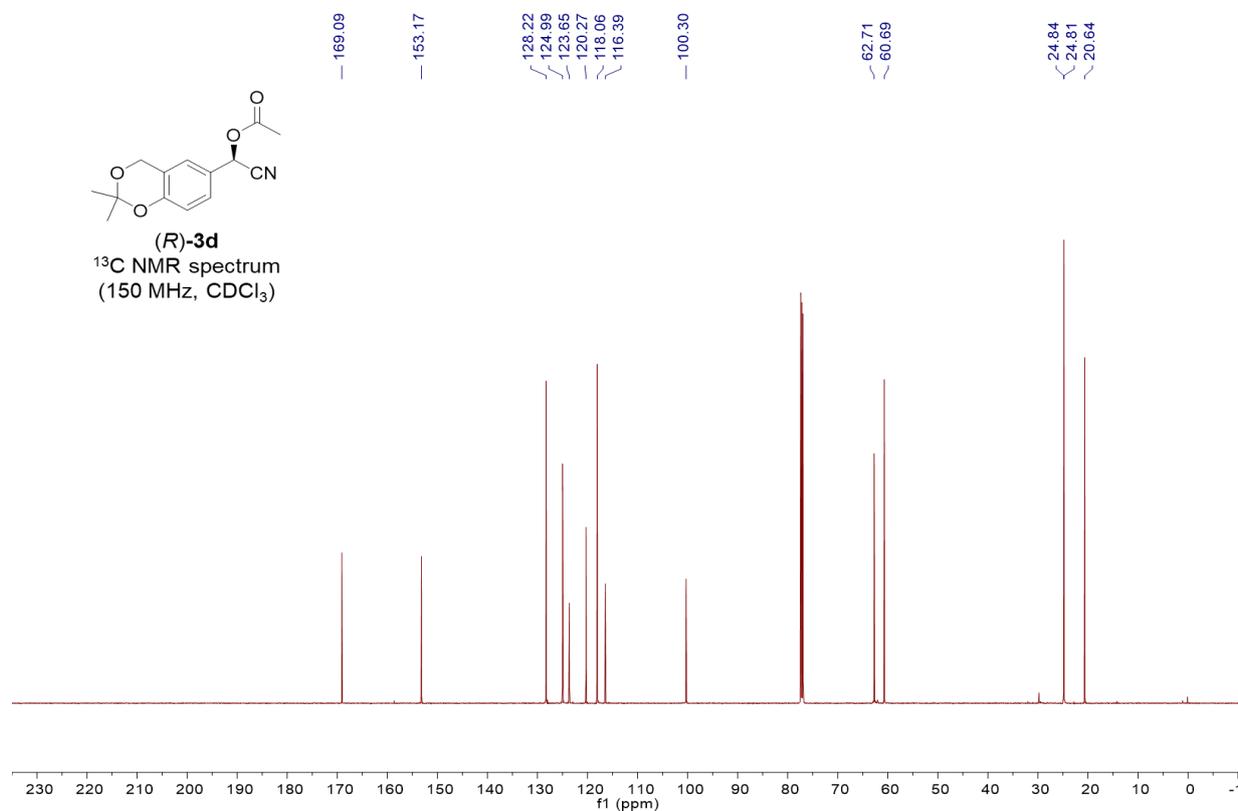


Figure S37. ¹³C NMR trace of (*R*)-**3d** prepared in this study.

SUPPORTING INFORMATION

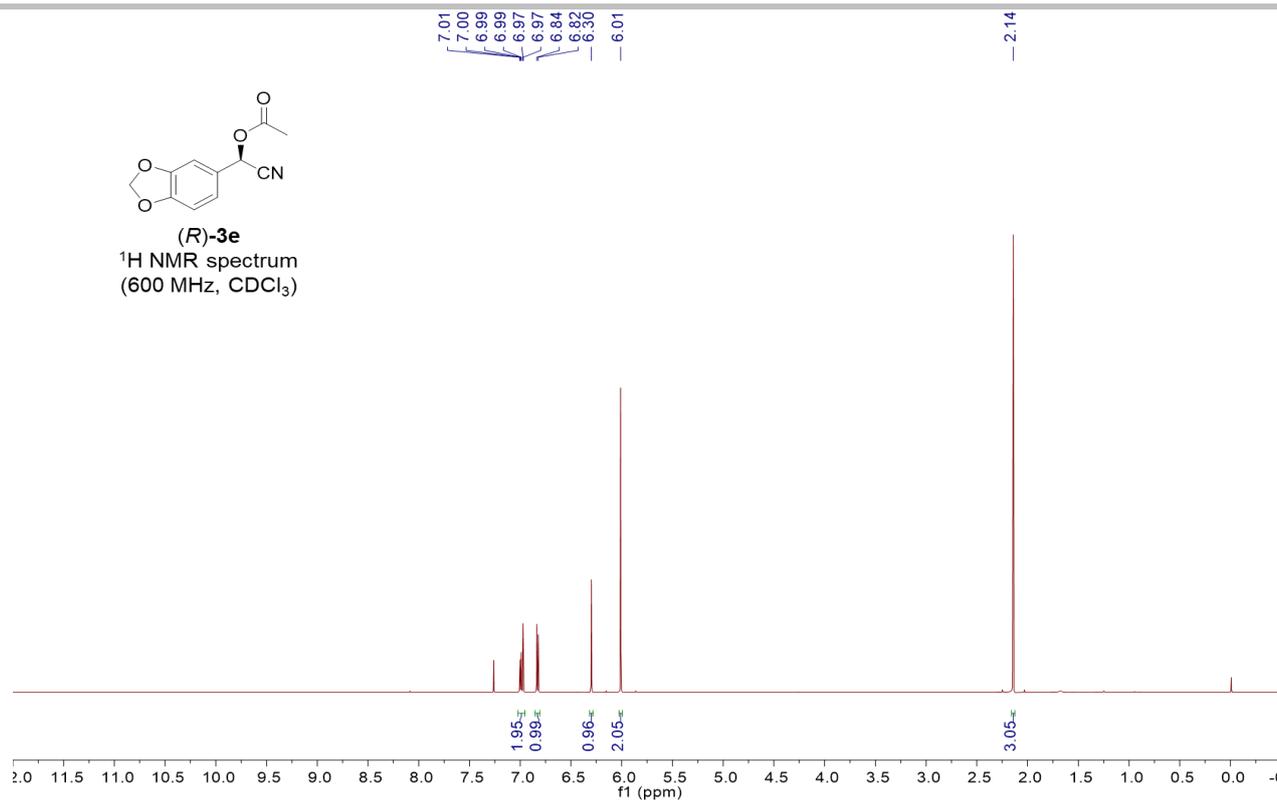


Figure S38. ¹H NMR trace of **(R)-3e** prepared in this study.

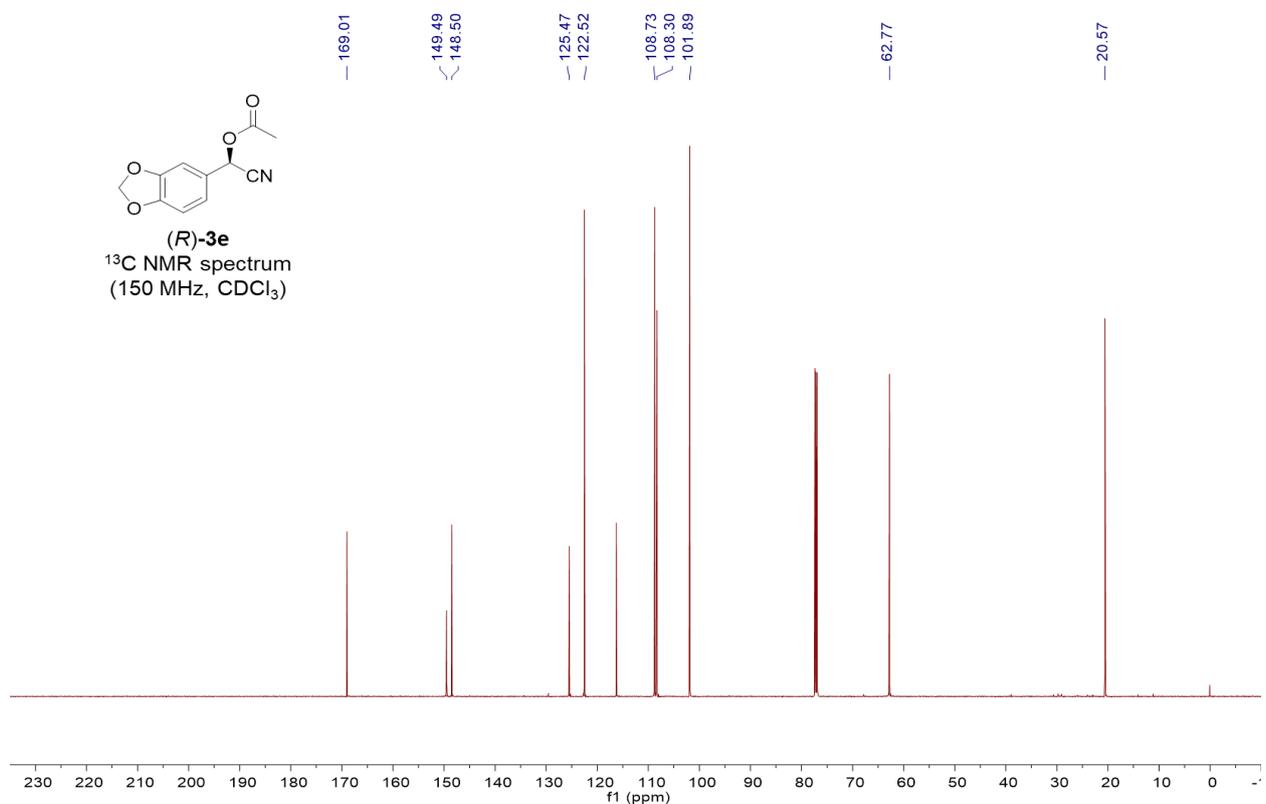


Figure S39. ¹³C NMR trace of **(R)-3e** prepared in this study.

SUPPORTING INFORMATION

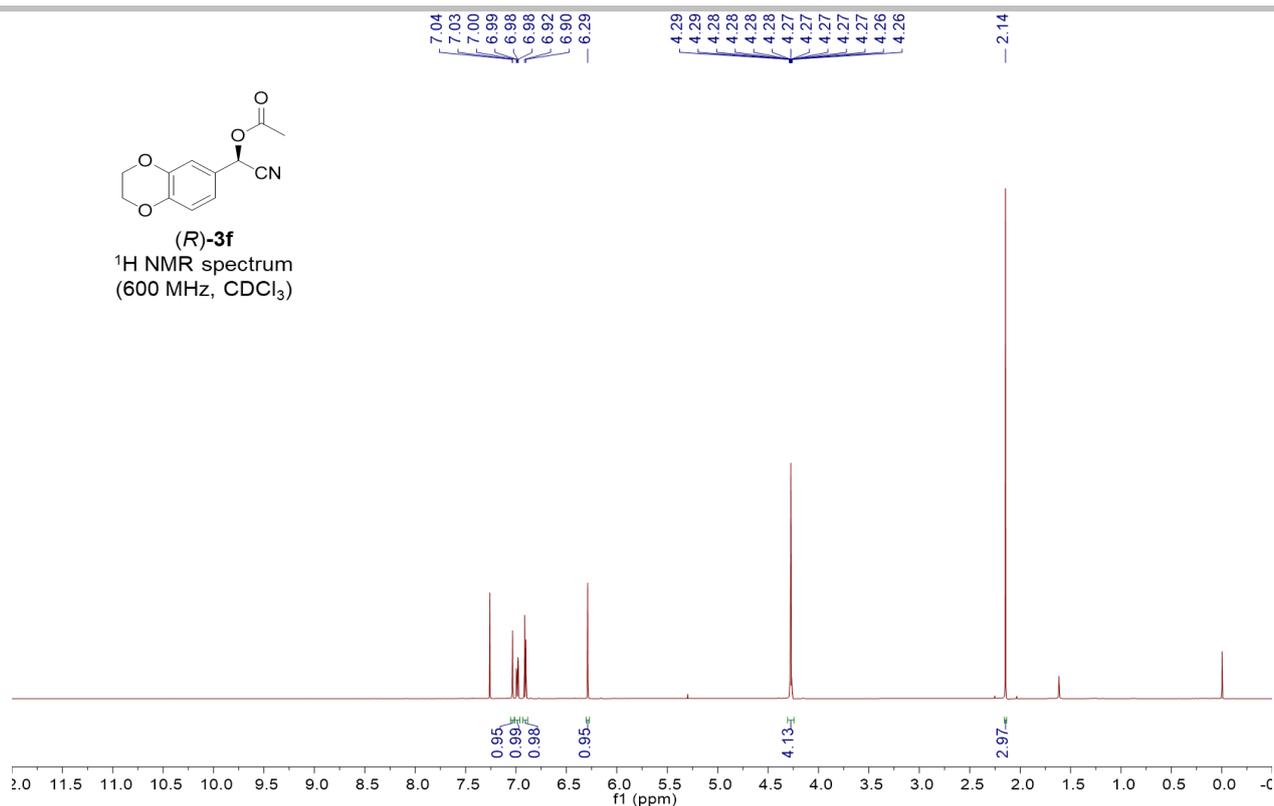


Figure S40. ¹H NMR trace of (*R*)-**3f** prepared in this study.

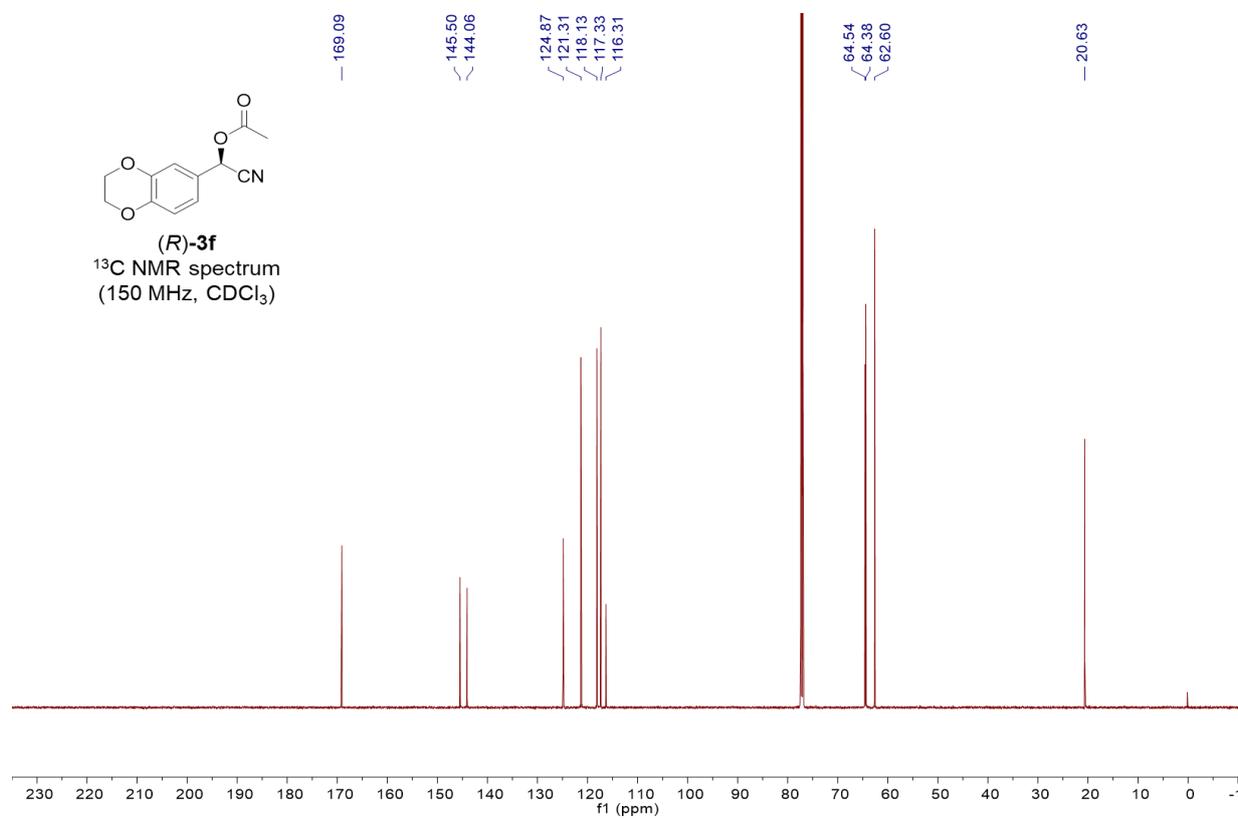


Figure S41. ¹³C NMR trace of (*R*)-**3f** prepared in this study.

SUPPORTING INFORMATION

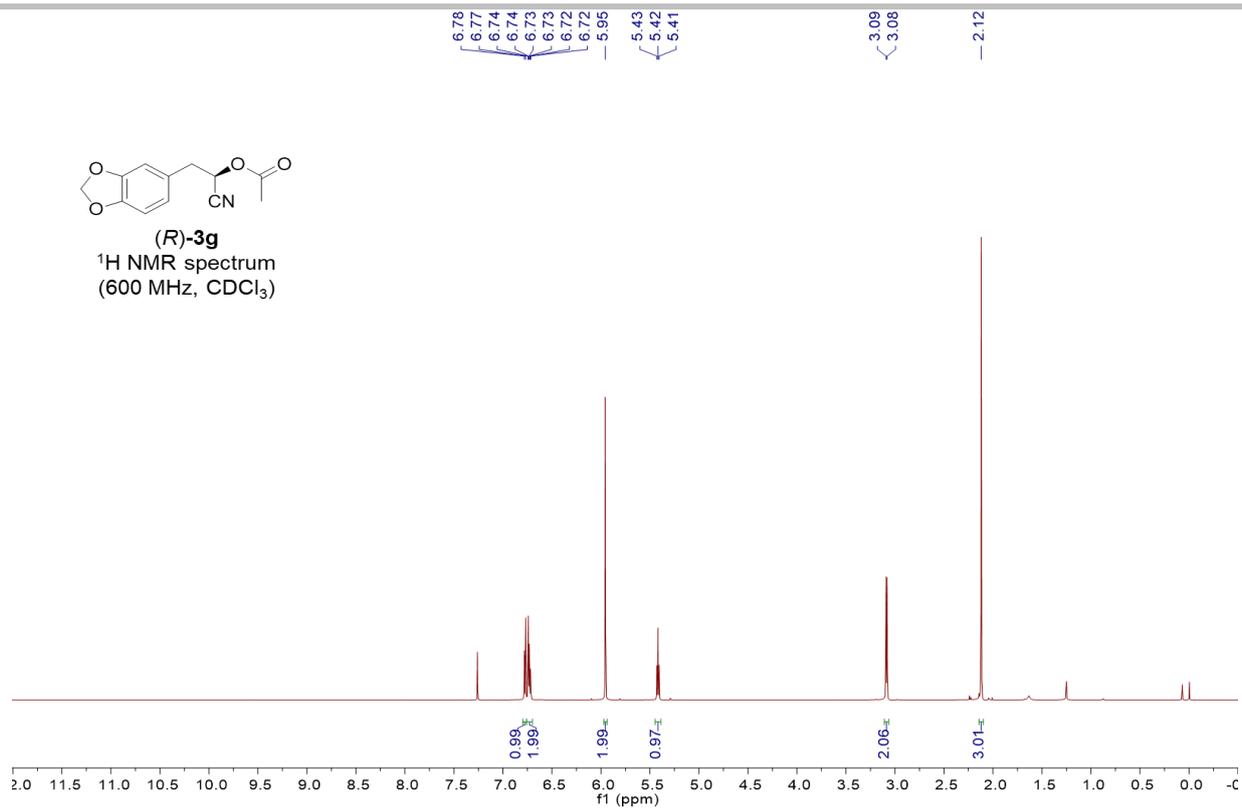


Figure S42. ¹H NMR trace of (R)-3g prepared in this study.

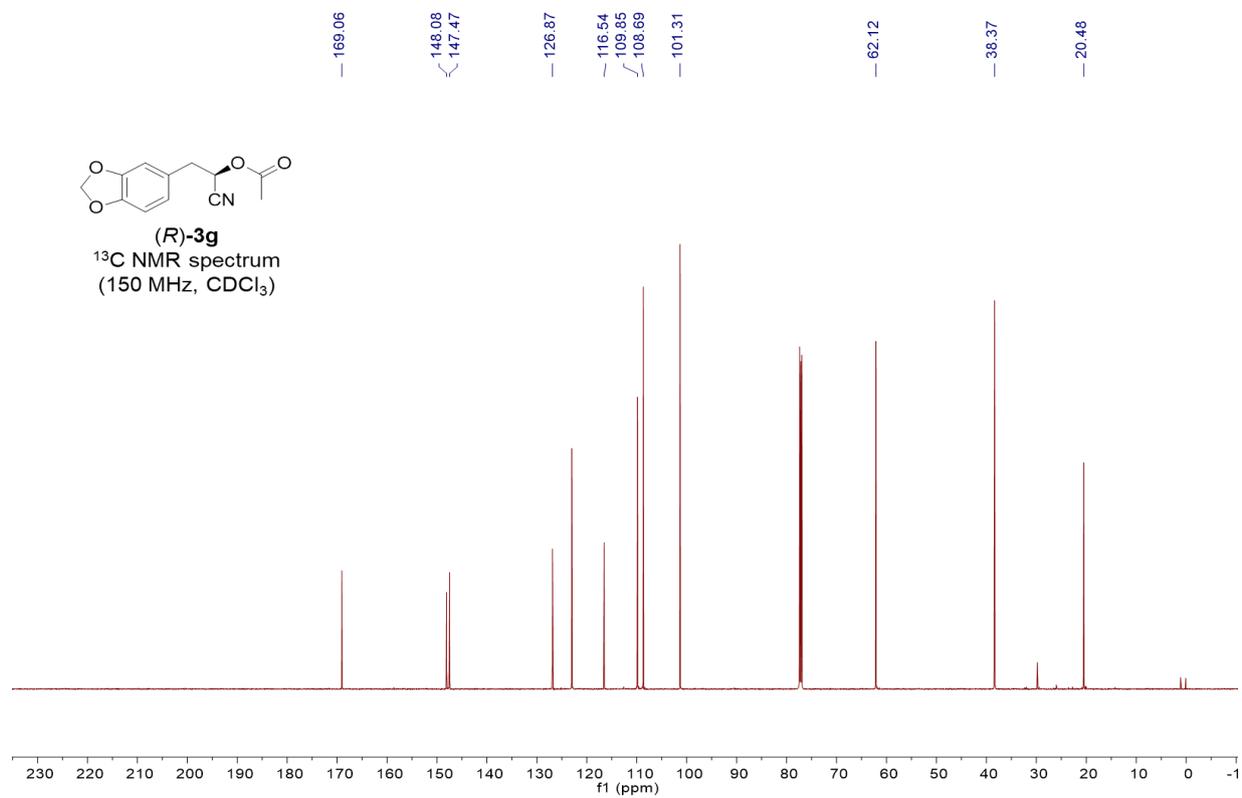


Figure S43. ¹³C NMR trace of (R)-3g prepared in this study.

SUPPORTING INFORMATION

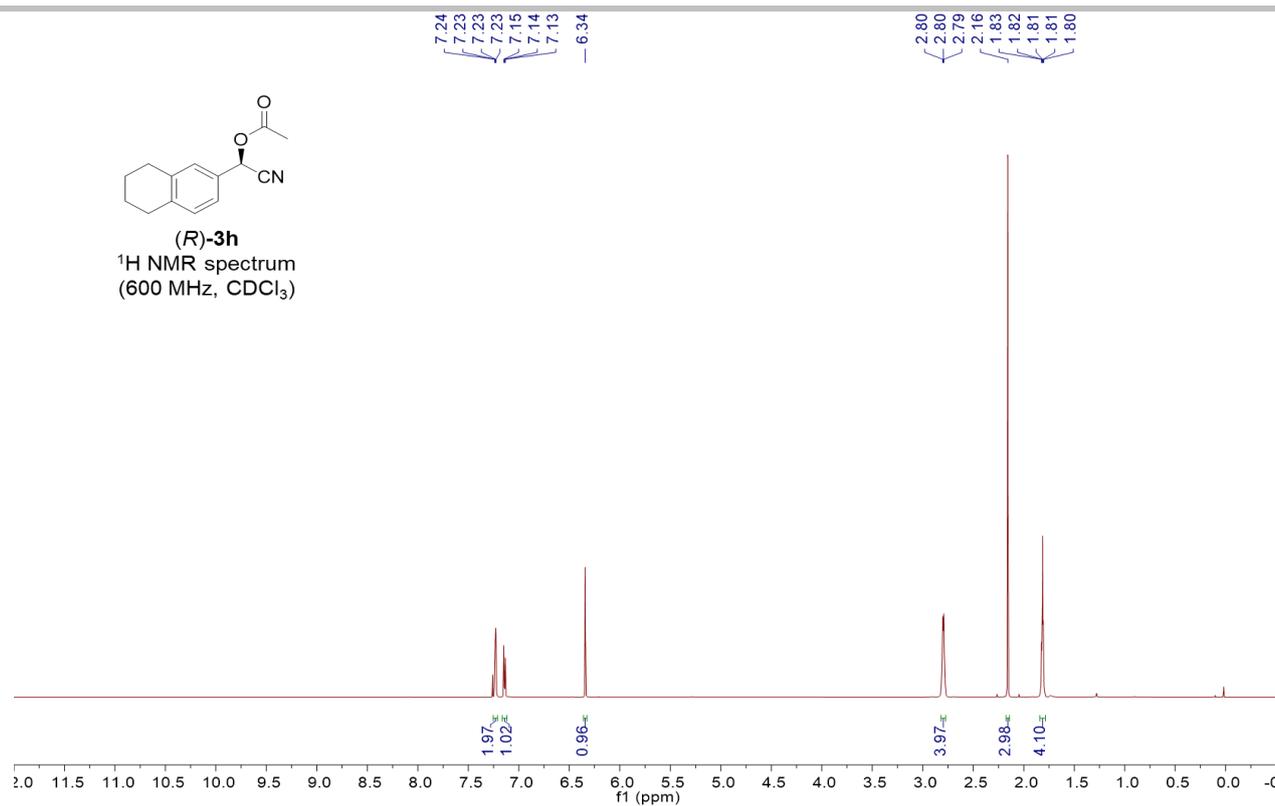


Figure S44. ¹H NMR trace of (*R*)-**3h** prepared in this study.

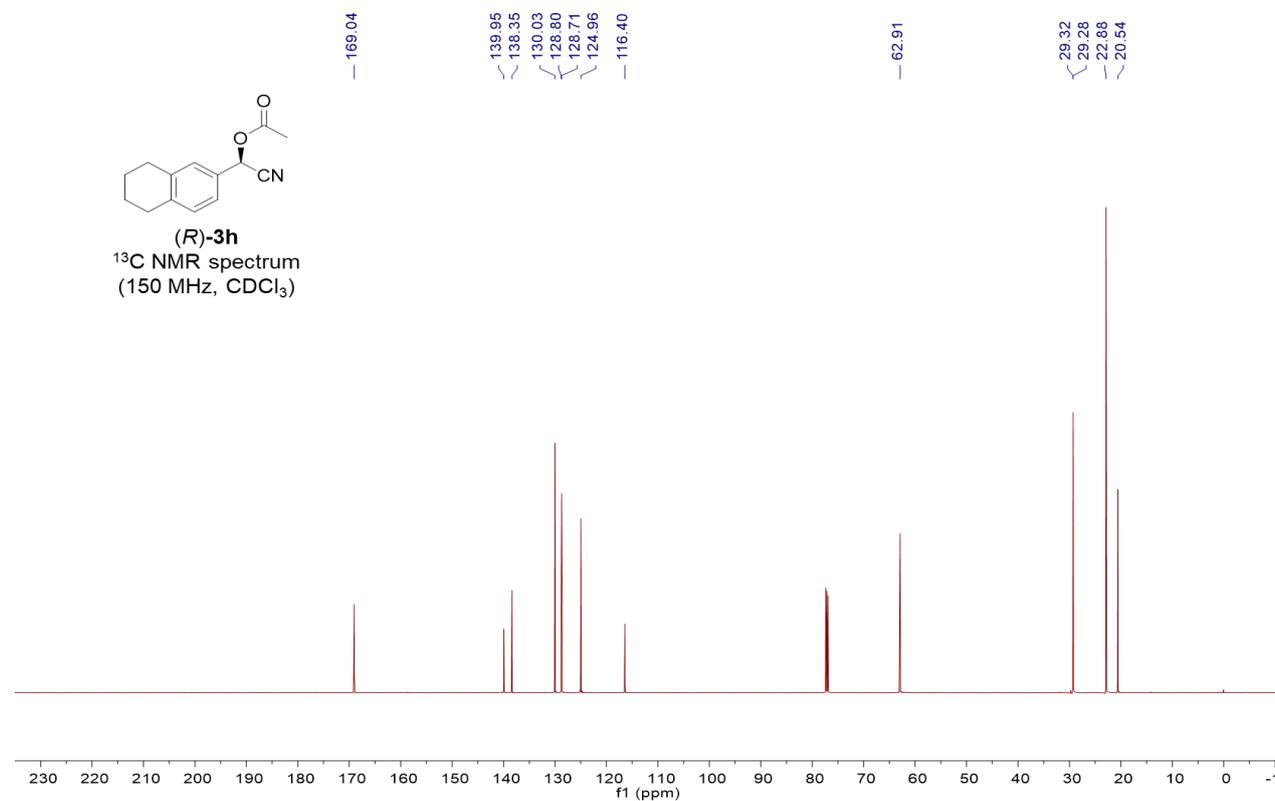


Figure S45. ¹³C NMR trace of (*R*)-**3h** prepared in this study.

SUPPORTING INFORMATION

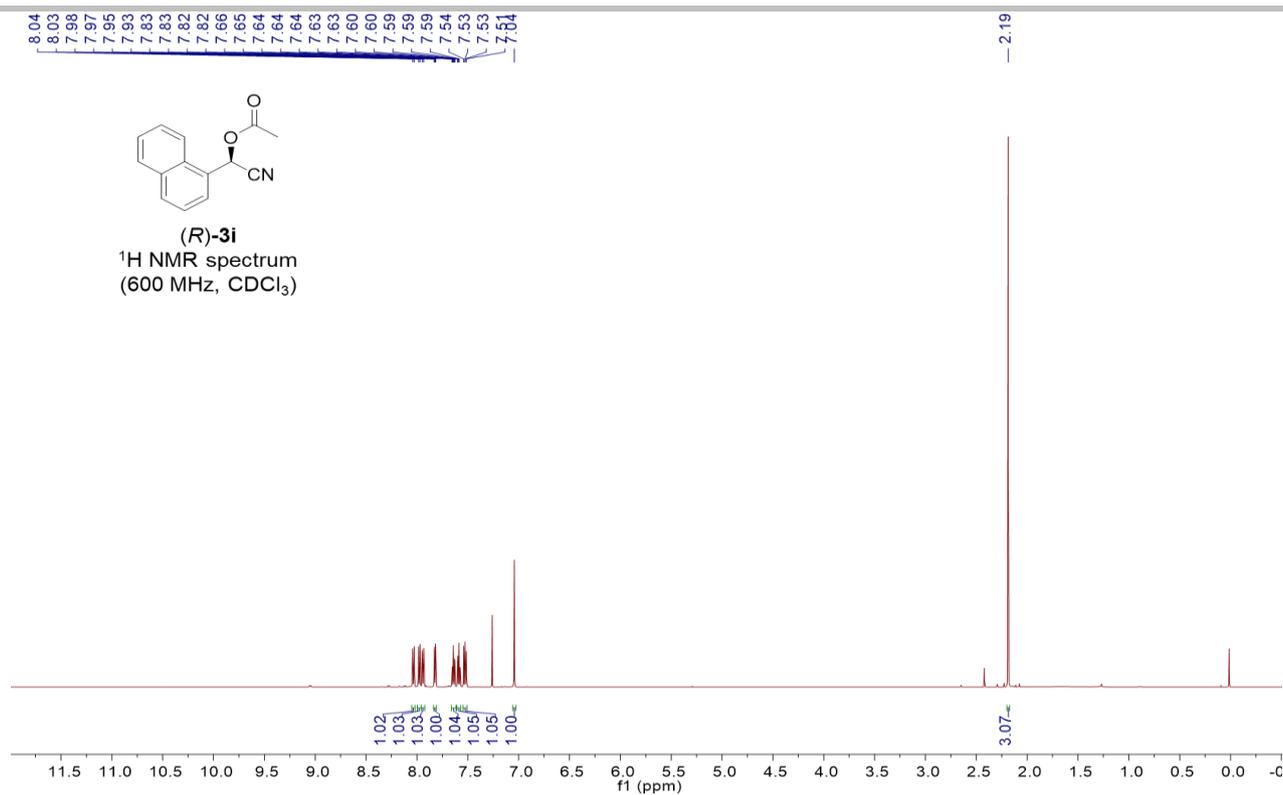


Figure S46. ¹H NMR trace of (R)-3i prepared in this study.

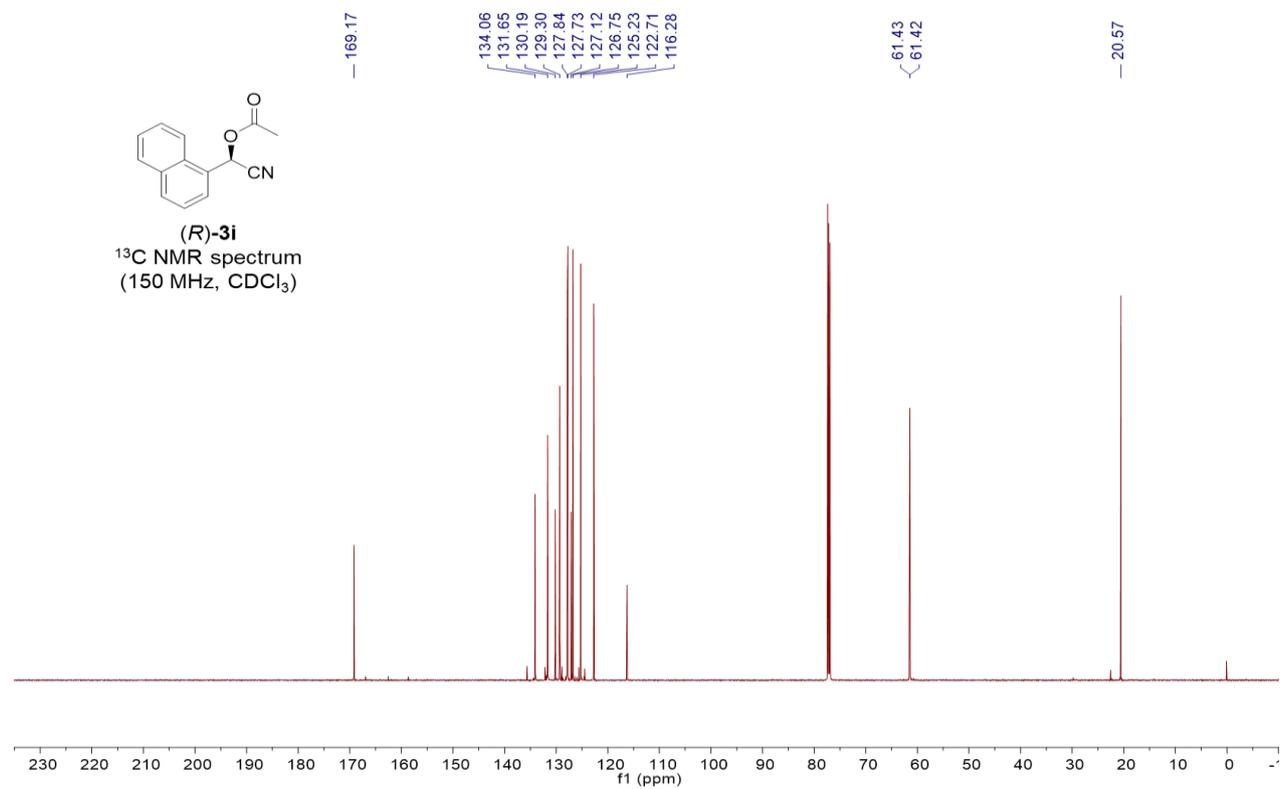


Figure S47. ¹³C NMR trace of (R)-3i prepared in this study.

SUPPORTING INFORMATION

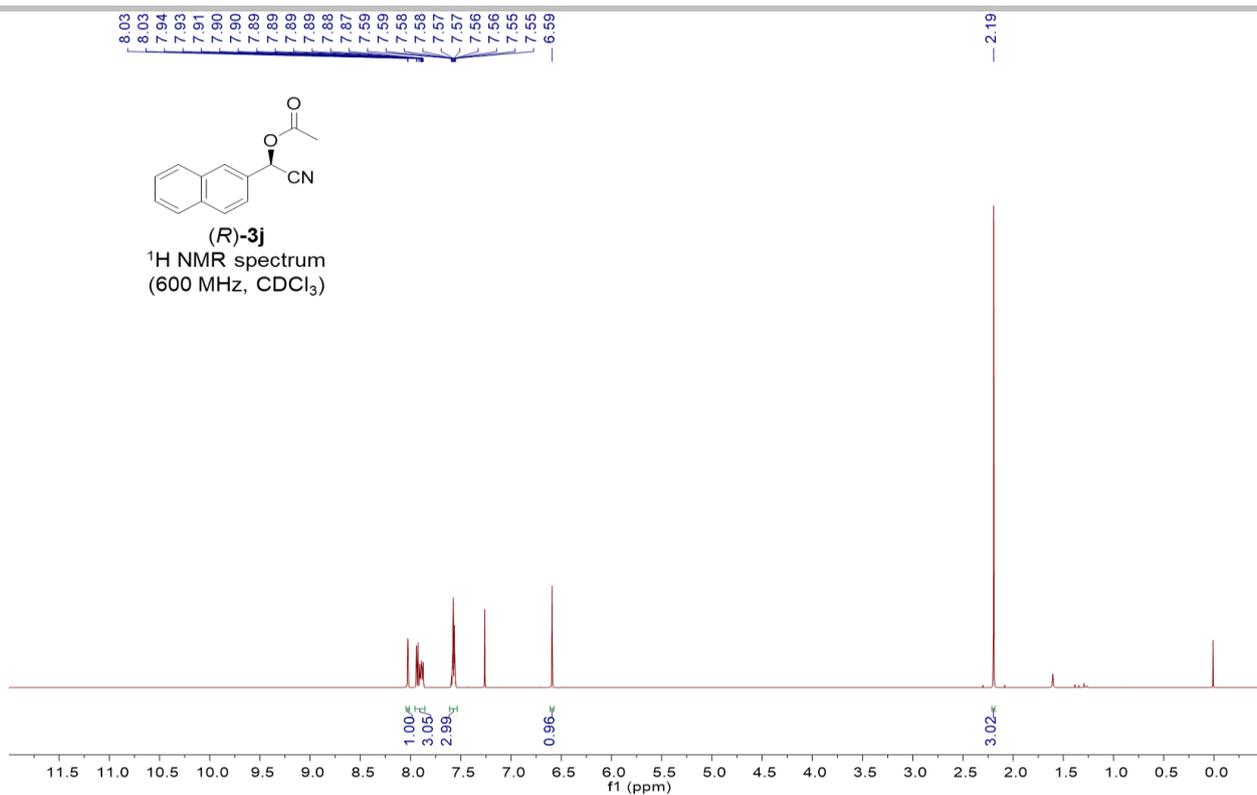


Figure S48. ¹H NMR trace of (R)-3j prepared in this study.

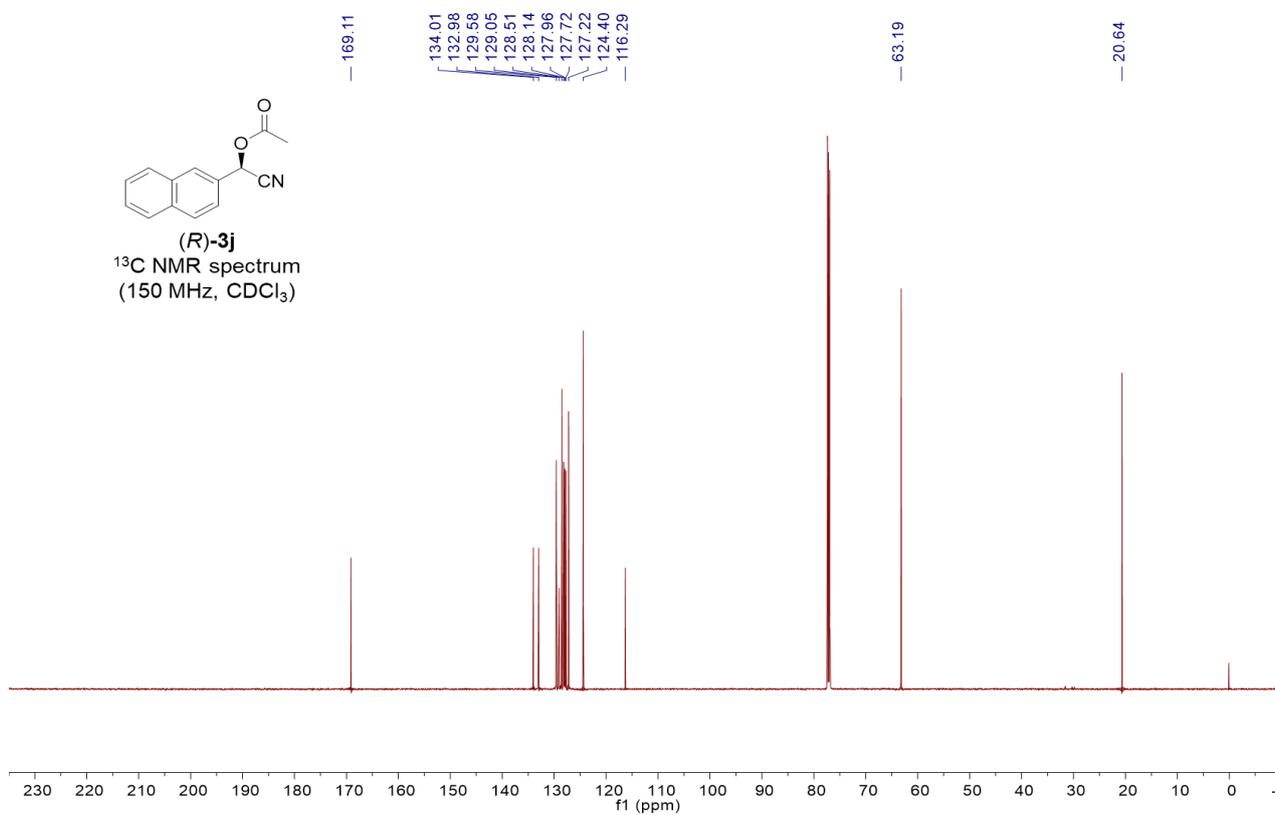
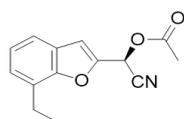


Figure S49. ¹³C NMR trace of (R)-3j prepared in this study.

SUPPORTING INFORMATION



(S)-3k
 ^1H NMR spectrum
 (600 MHz, CDCl_3)

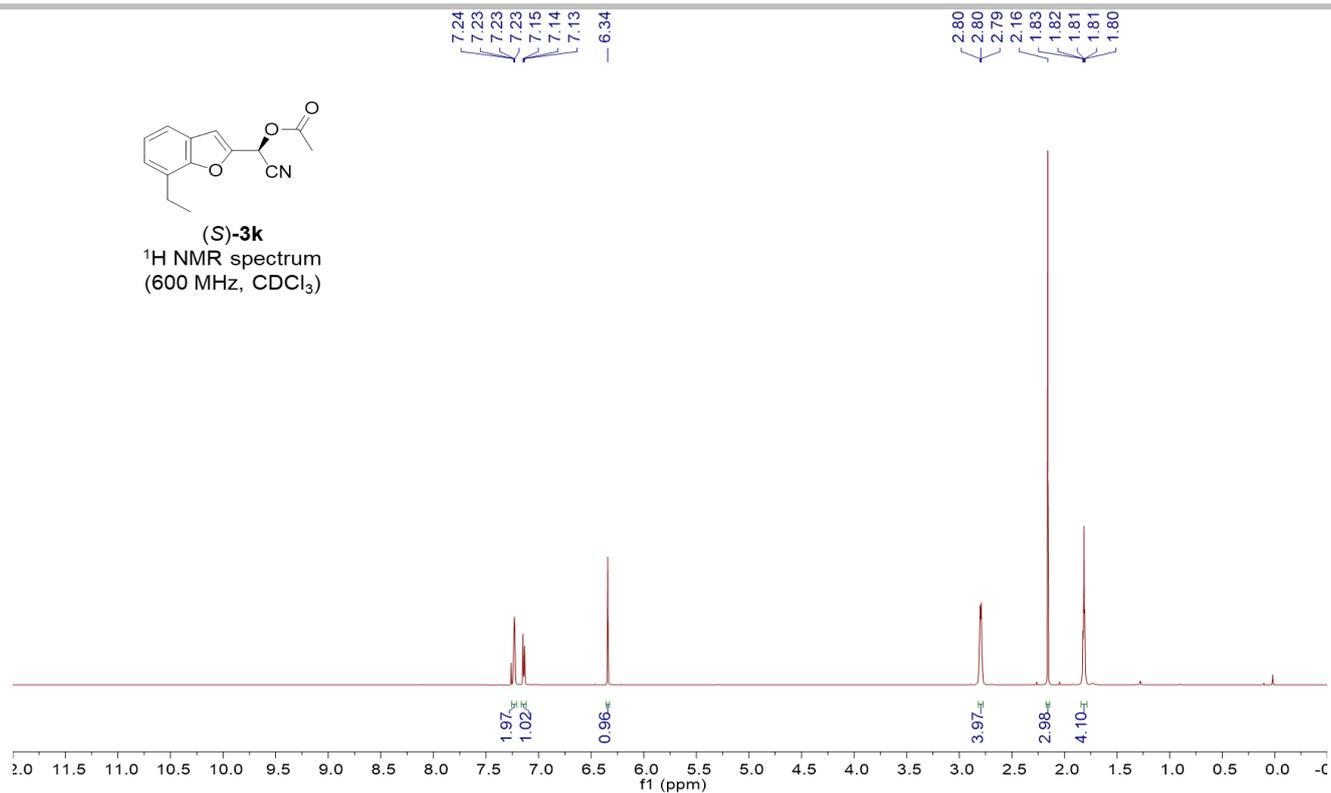
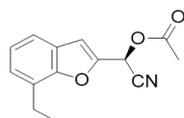


Figure S50. ^1H NMR trace of (S)-3k prepared in this study.



(S)-3k
 ^{13}C NMR spectrum
 (150 MHz, CDCl_3)

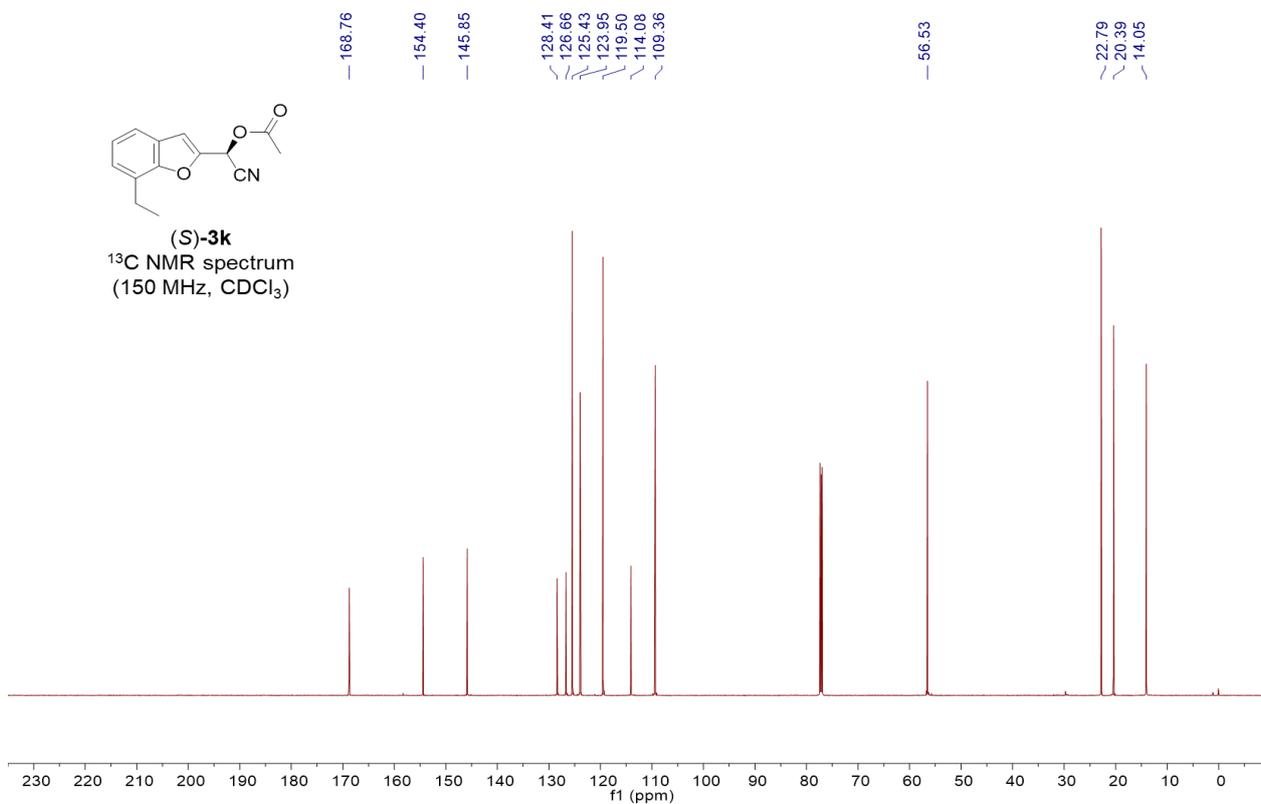


Figure S51. ^{13}C NMR trace of (S)-3k prepared in this study.

SUPPORTING INFORMATION

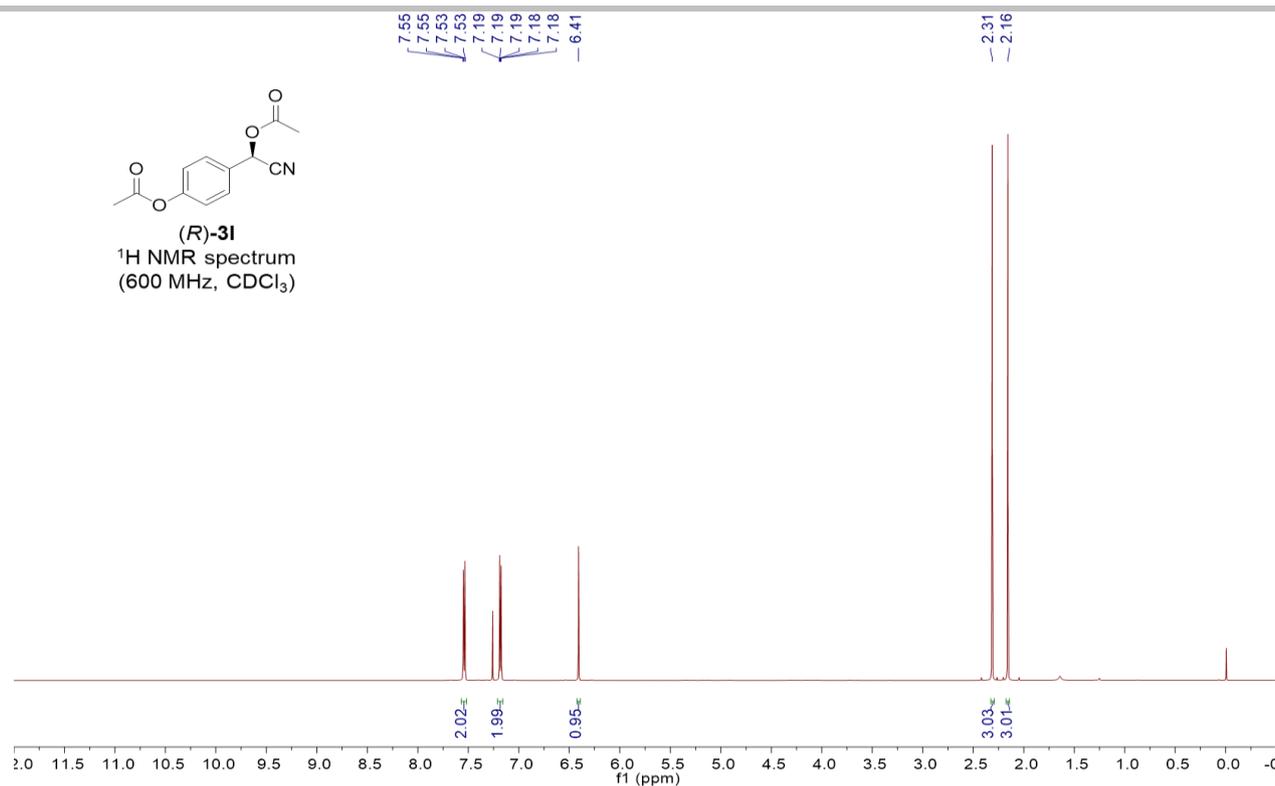


Figure S52. ¹H NMR trace of (R)-3I prepared in this study.

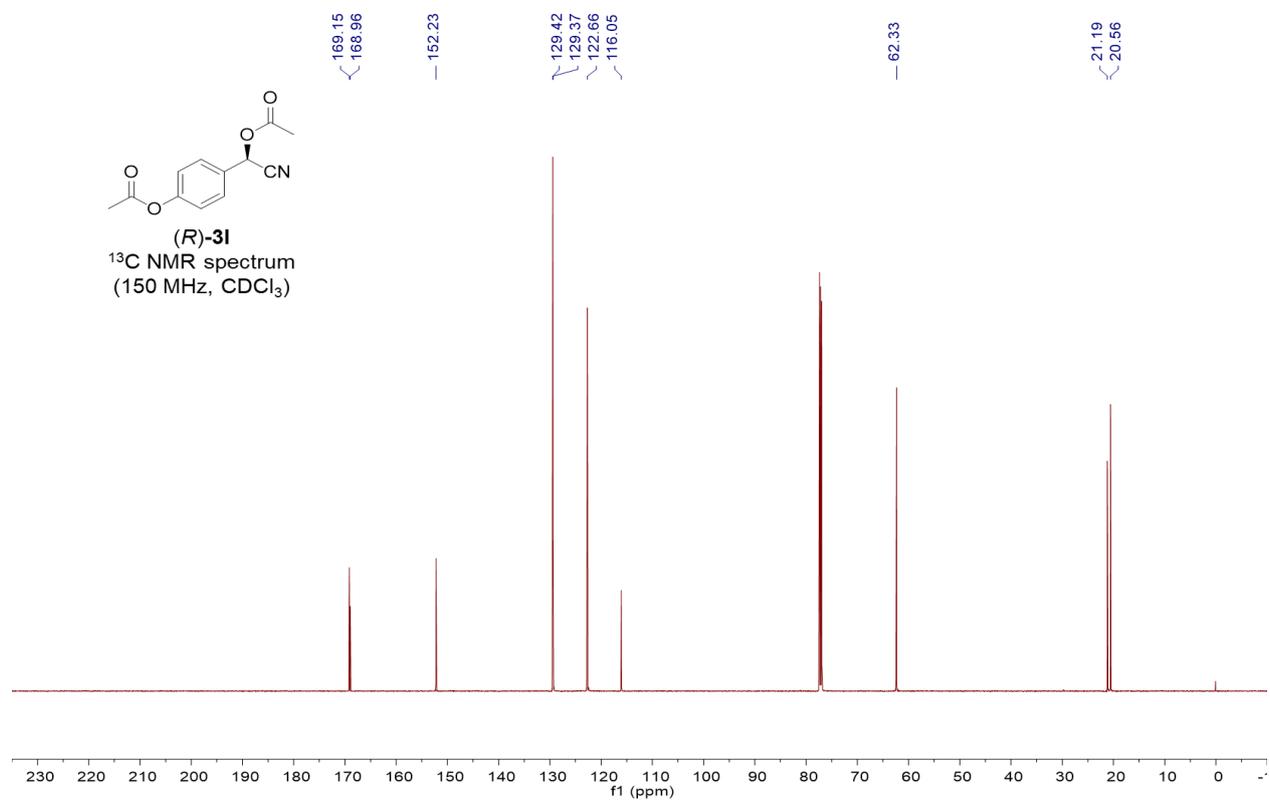


Figure S53. ¹³C NMR trace of (R)-3I prepared in this study.

SUPPORTING INFORMATION

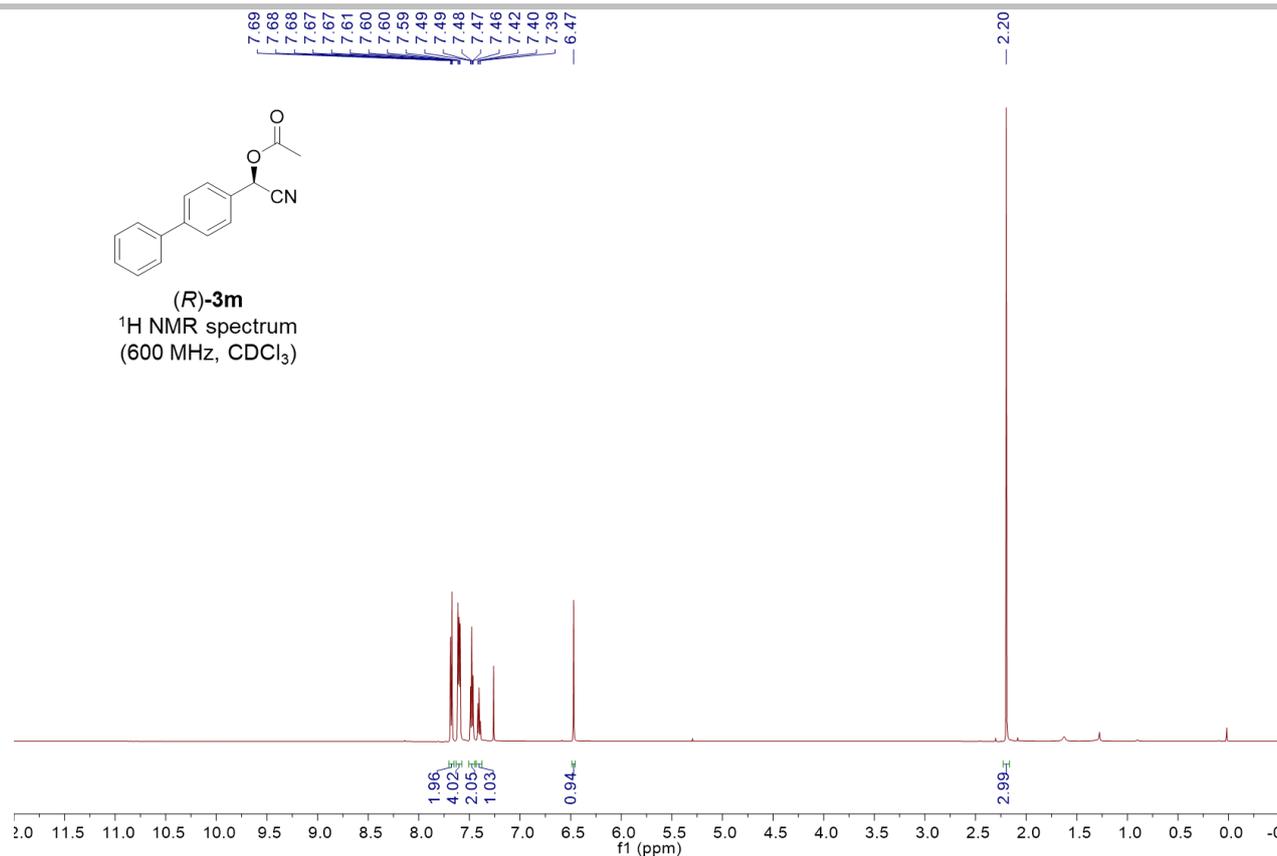


Figure S54. ¹H NMR trace of (R)-3m prepared in this study.

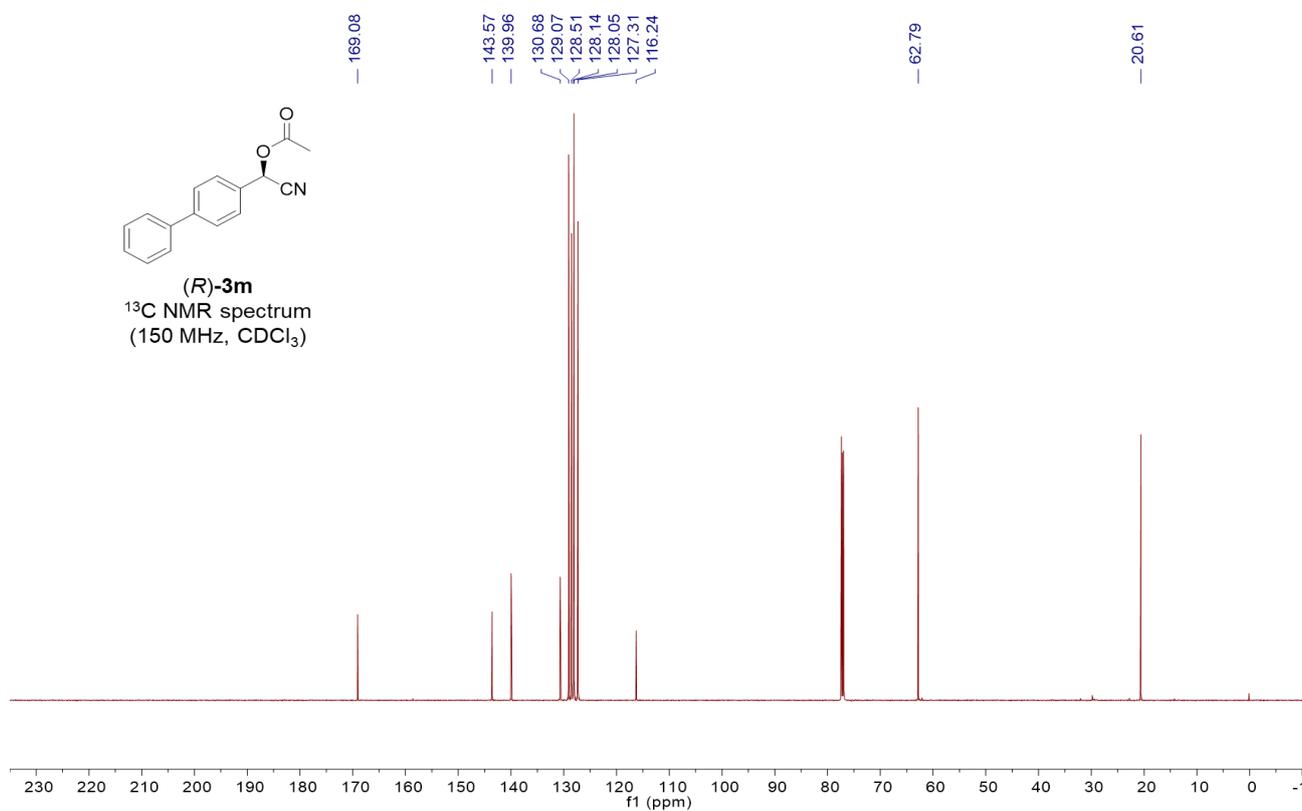


Figure S55. ¹³C NMR trace of (R)-3m prepared in this study.

SUPPORTING INFORMATION

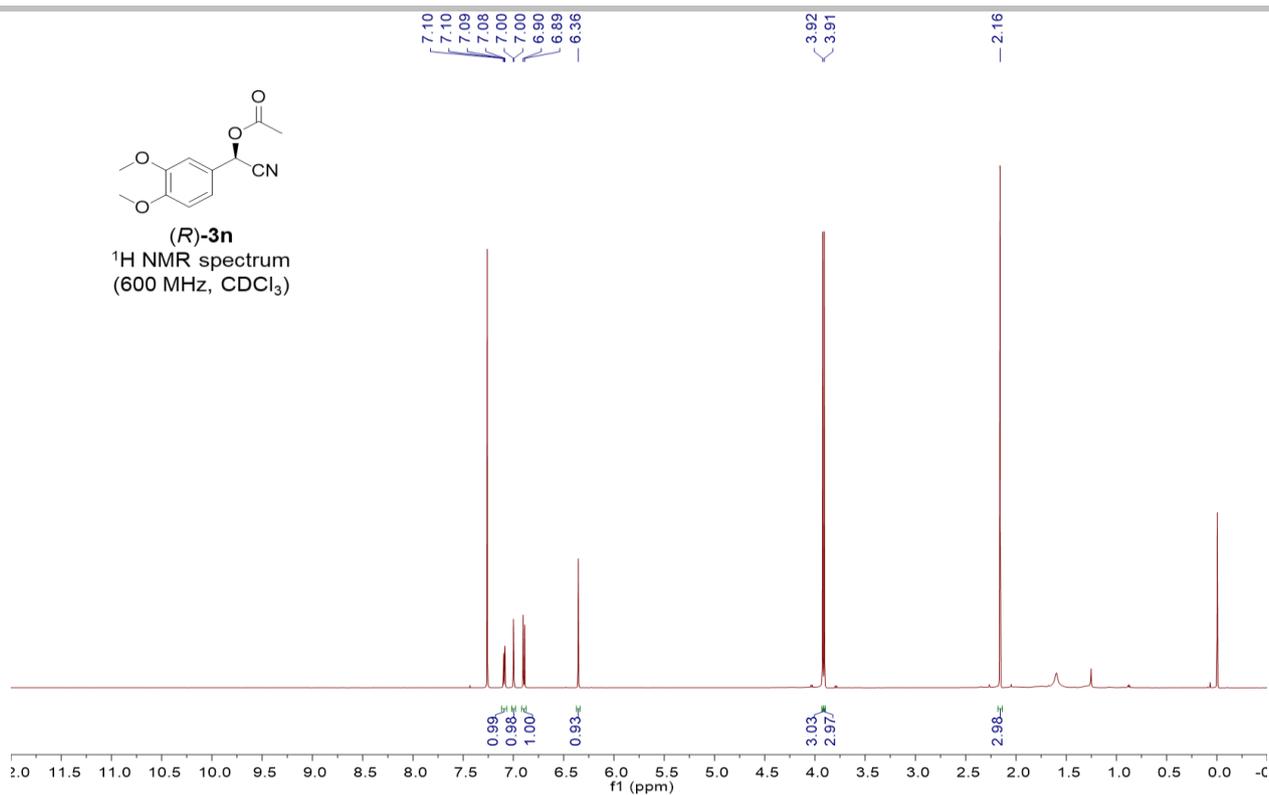


Figure S56. ¹H NMR trace of (R)-3n prepared in this study.

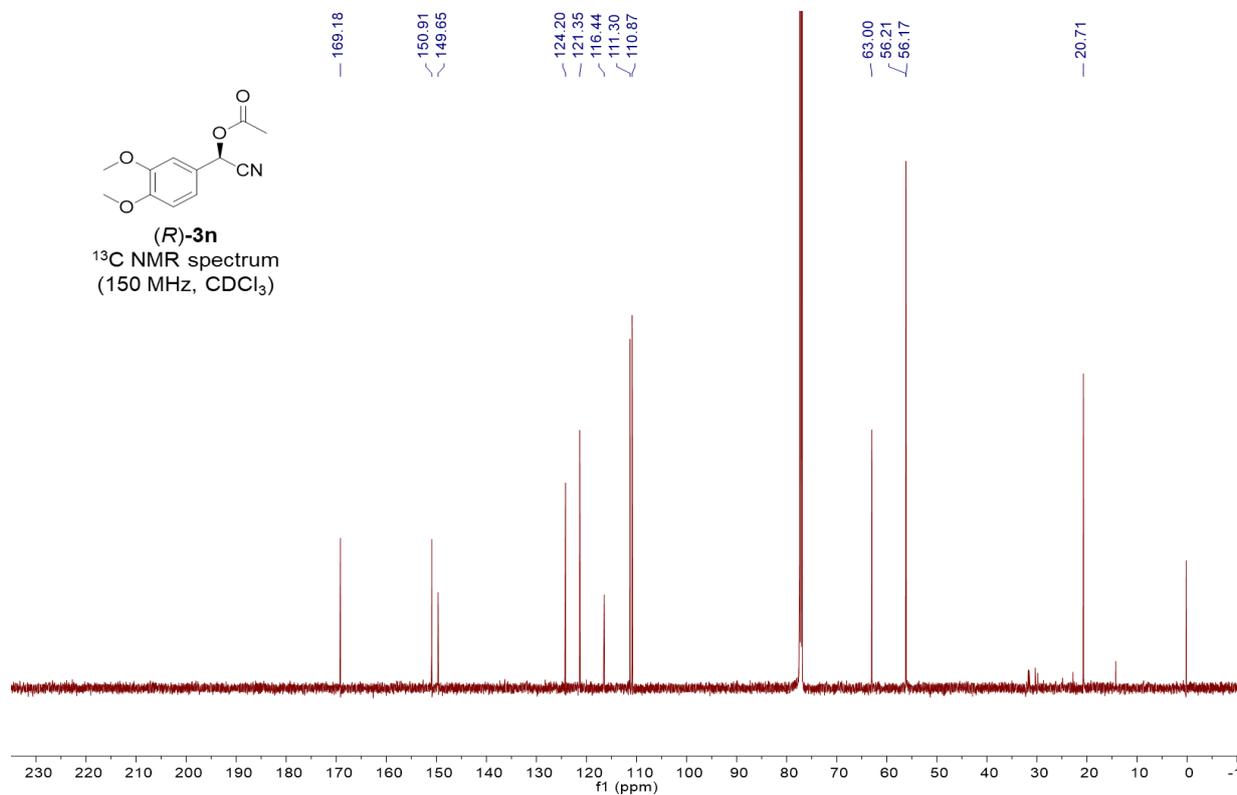


Figure S57. ¹³C NMR trace of (R)-3n prepared in this study.

SUPPORTING INFORMATION

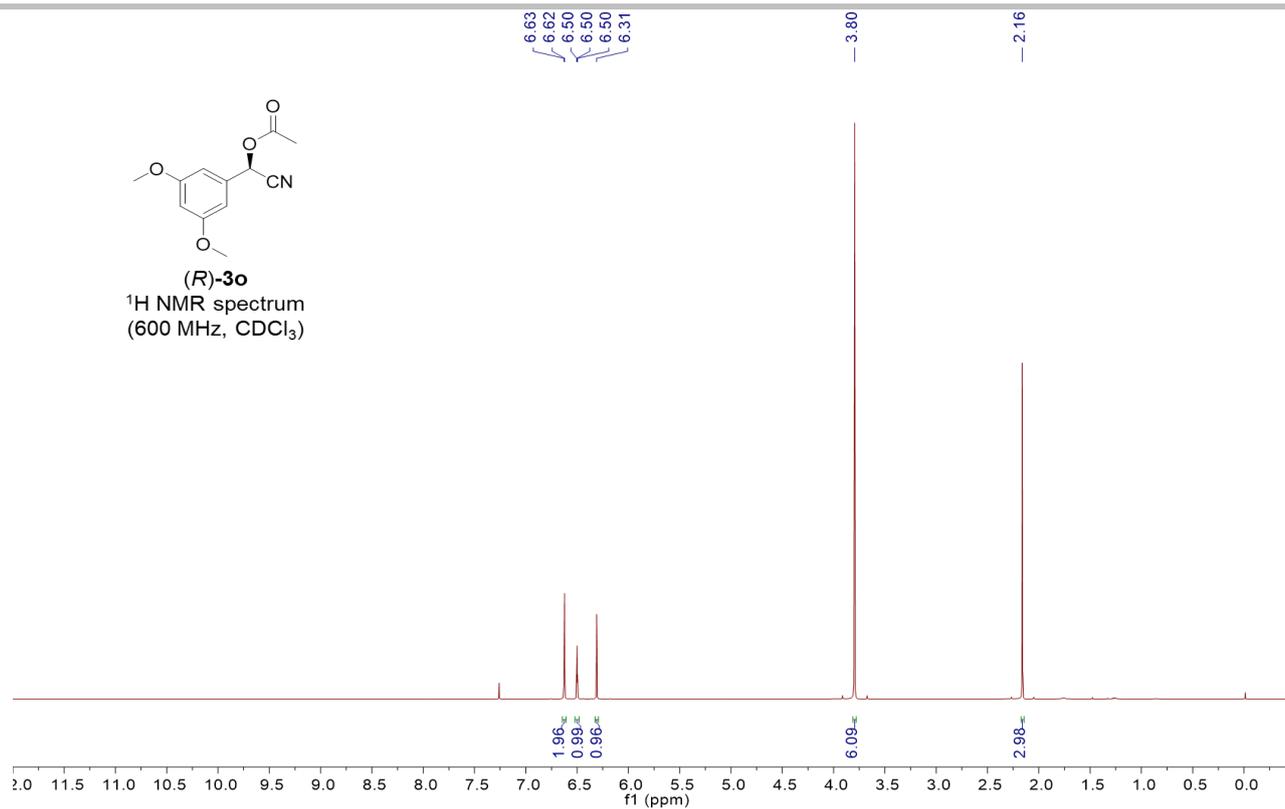


Figure S58. ¹H NMR trace of (R)-3o prepared in this study.

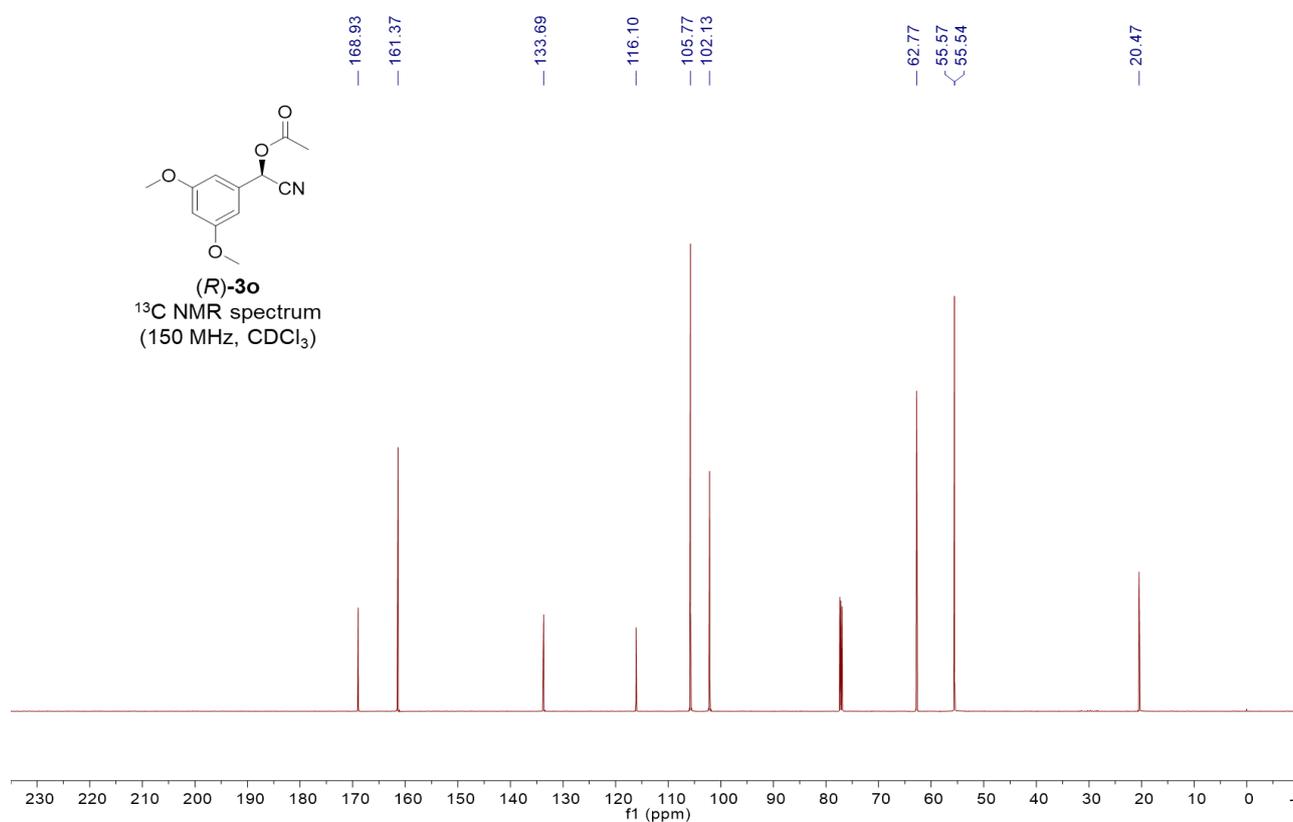


Figure S59. ¹³C NMR trace of (R)-3o prepared in this study.

SUPPORTING INFORMATION

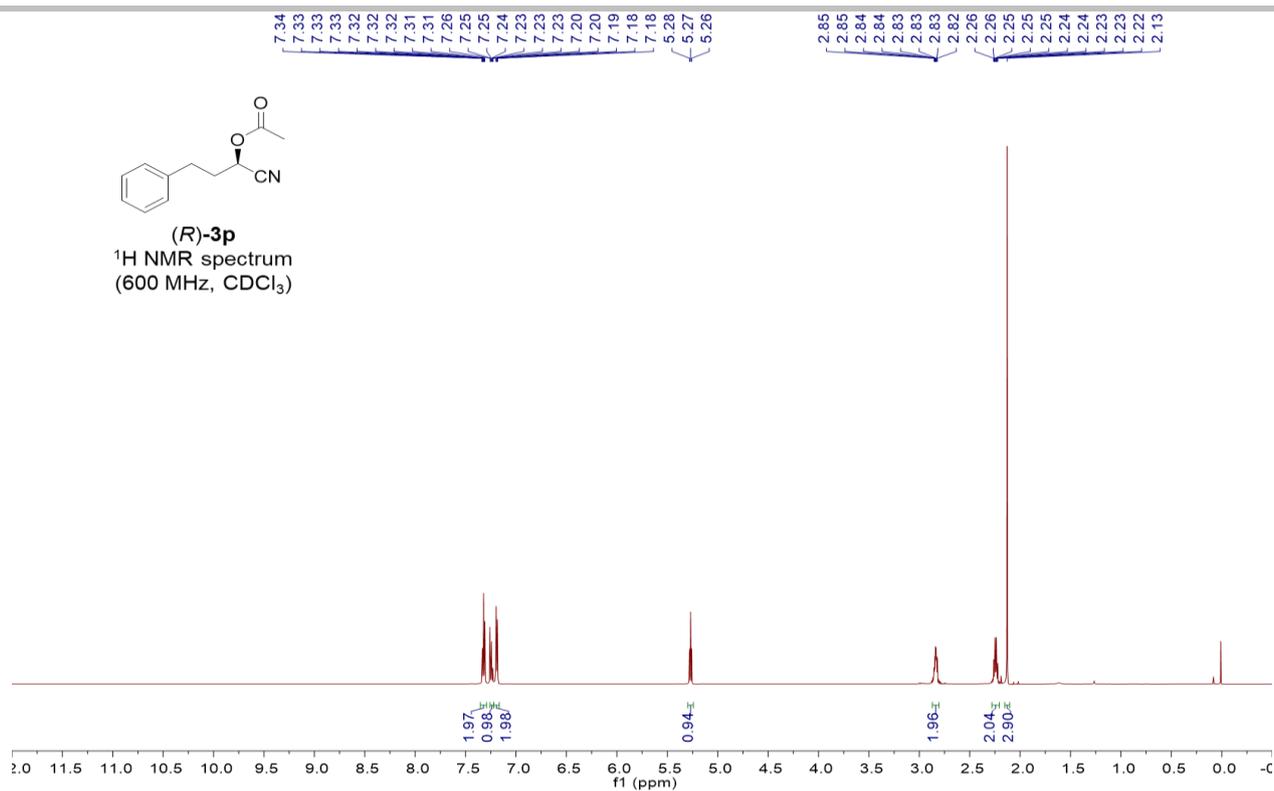


Figure S60. ¹H NMR trace of (R)-3p prepared in this study.

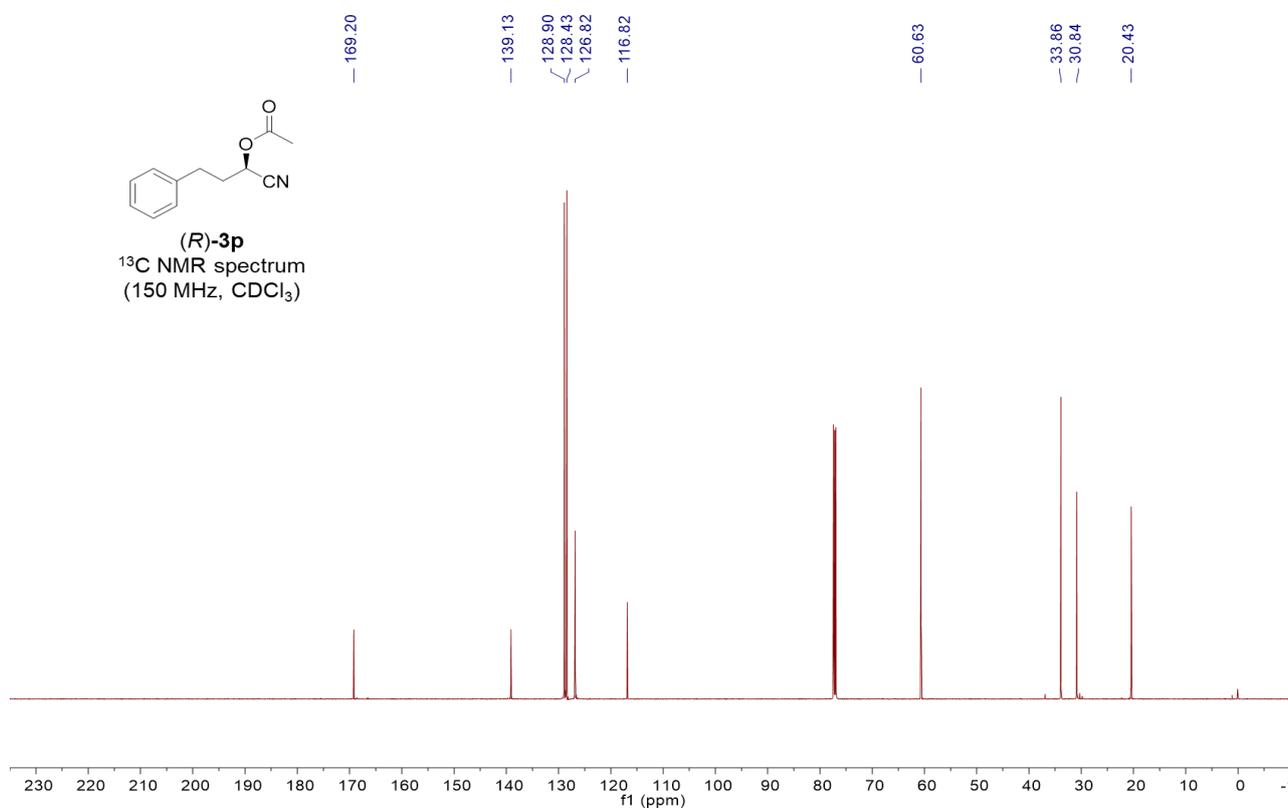


Figure S61. ¹³C NMR trace of (R)-3p prepared in this study.

SUPPORTING INFORMATION

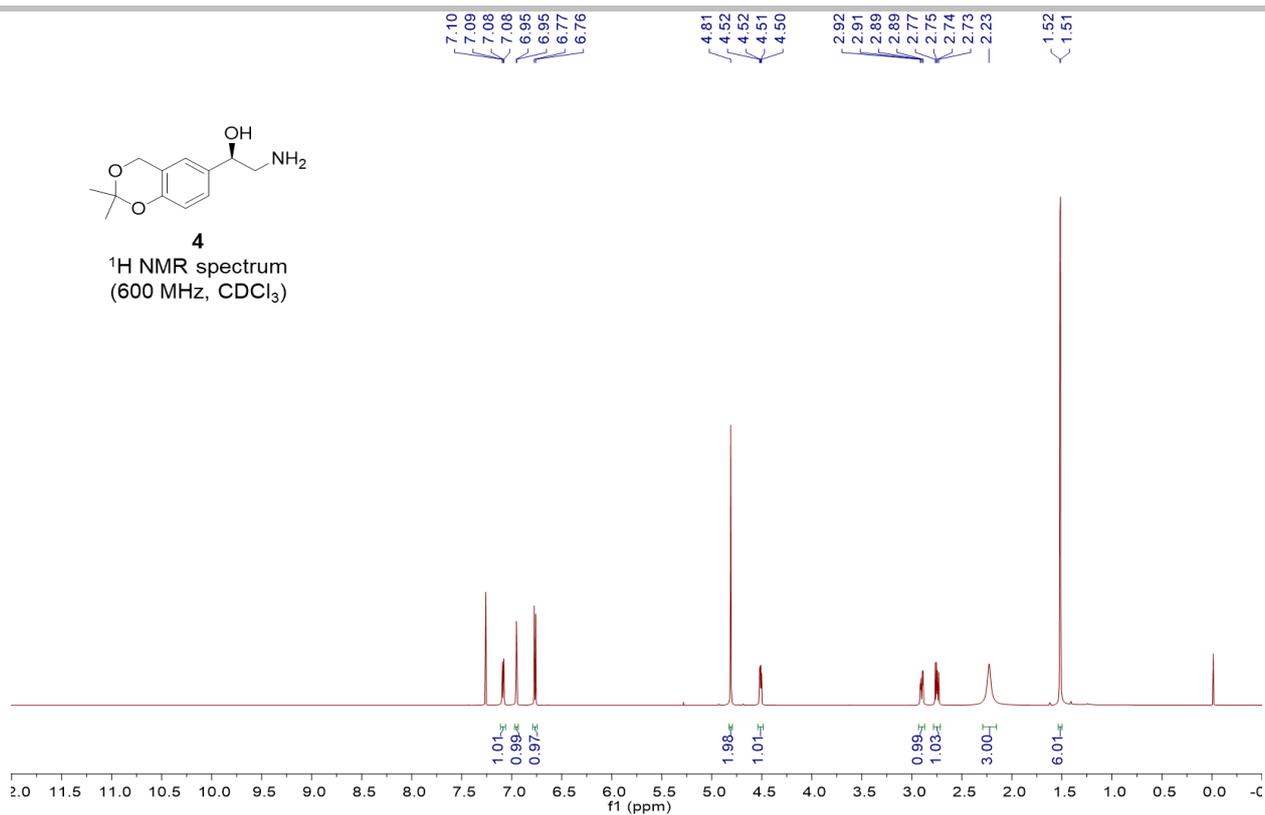


Figure S62. ¹H NMR trace of **4** prepared for (*R*)-salmeterol synthesis.

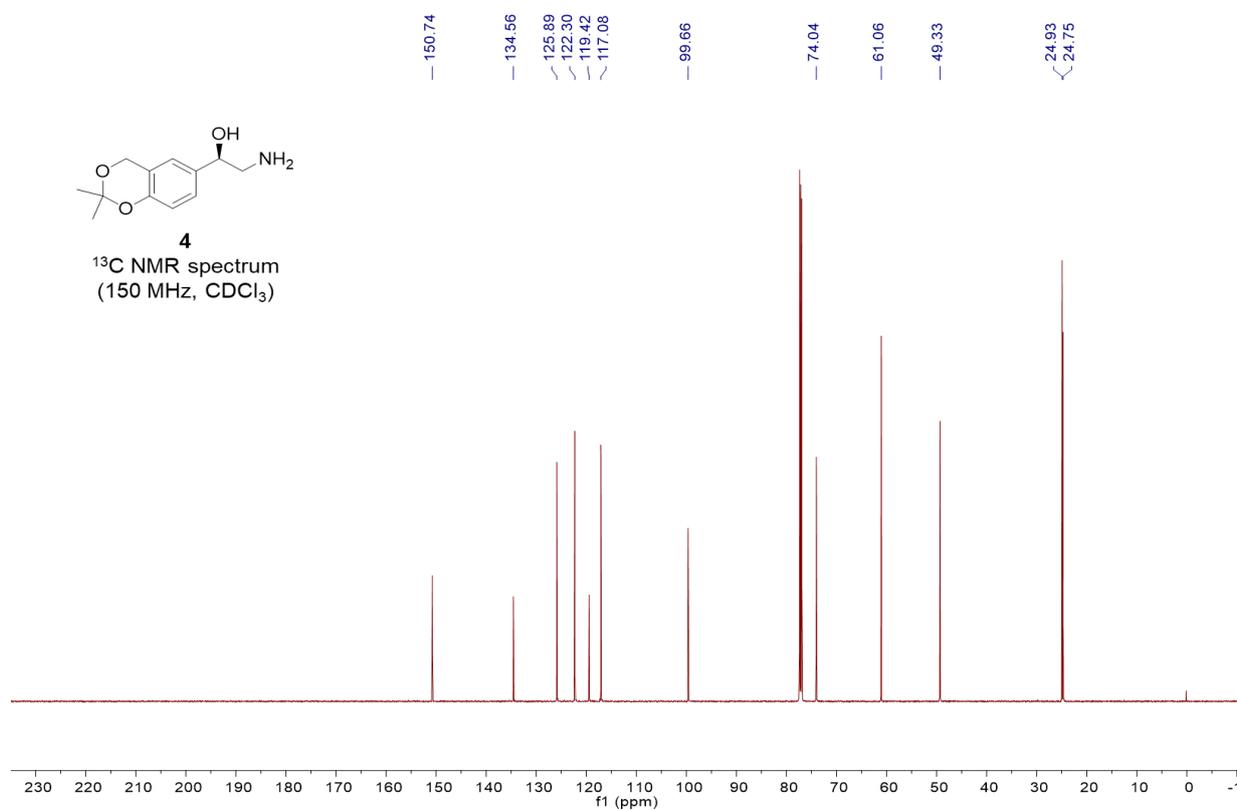


Figure S63. ¹³C NMR trace of **4** prepared for (*R*)-salmeterol synthesis.

SUPPORTING INFORMATION

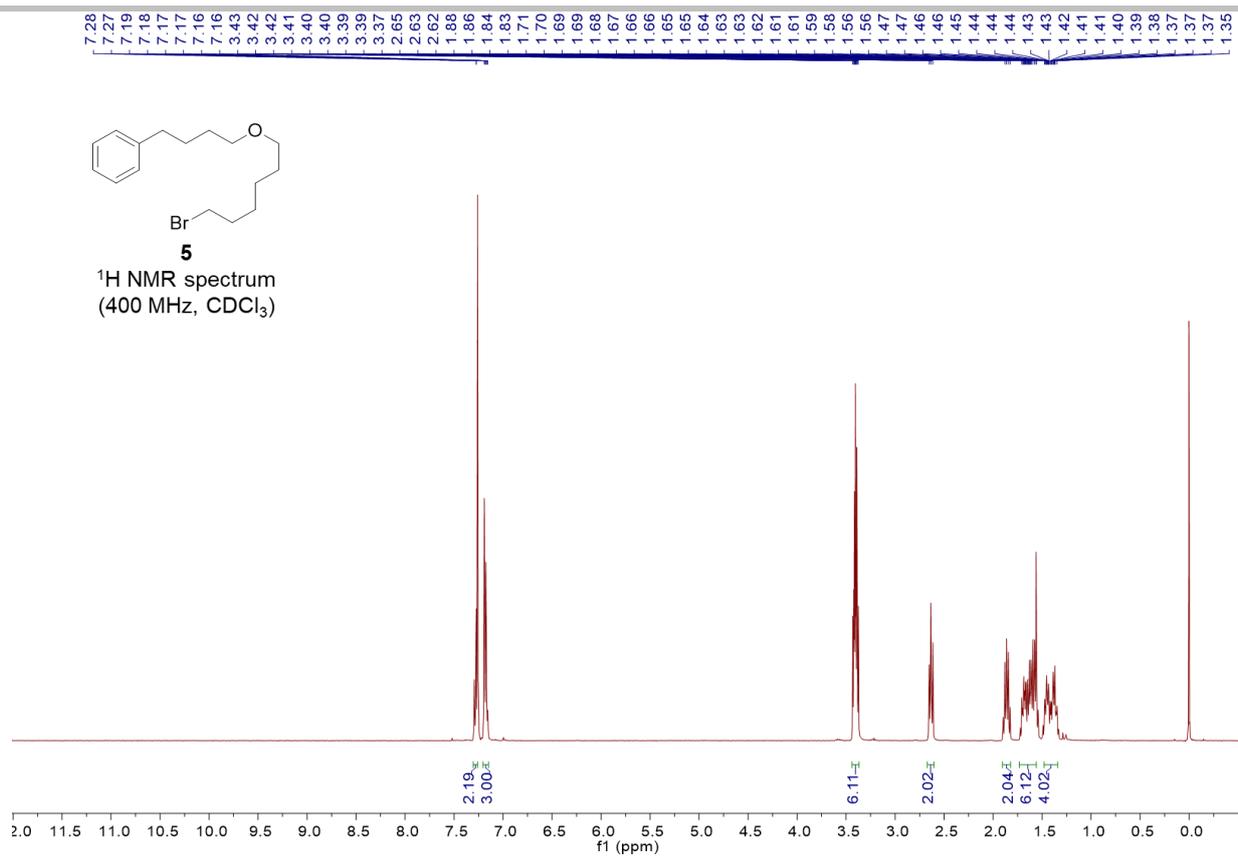


Figure S64. ¹H NMR trace of **5** synthesized for (*R*)-salmeterol synthesis.

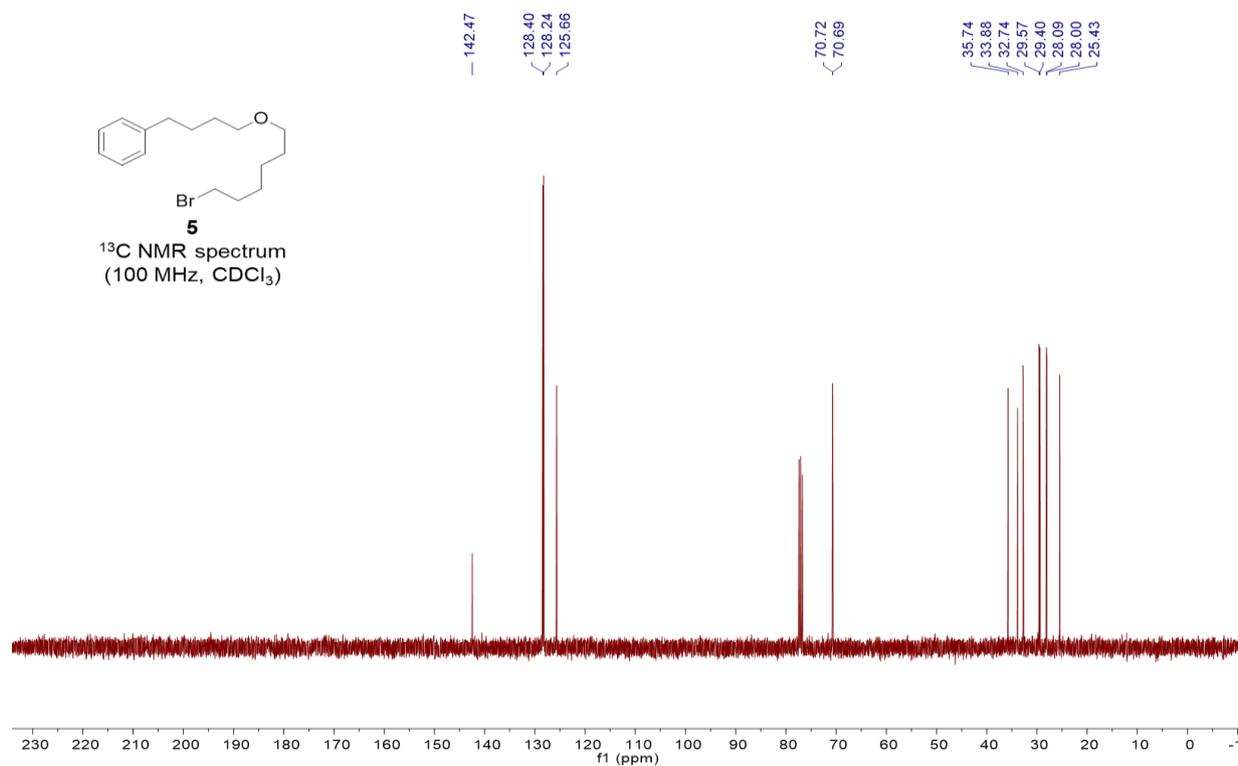


Figure S65. ¹³C NMR trace of **5** synthesized for (*R*)-salmeterol synthesis.

SUPPORTING INFORMATION

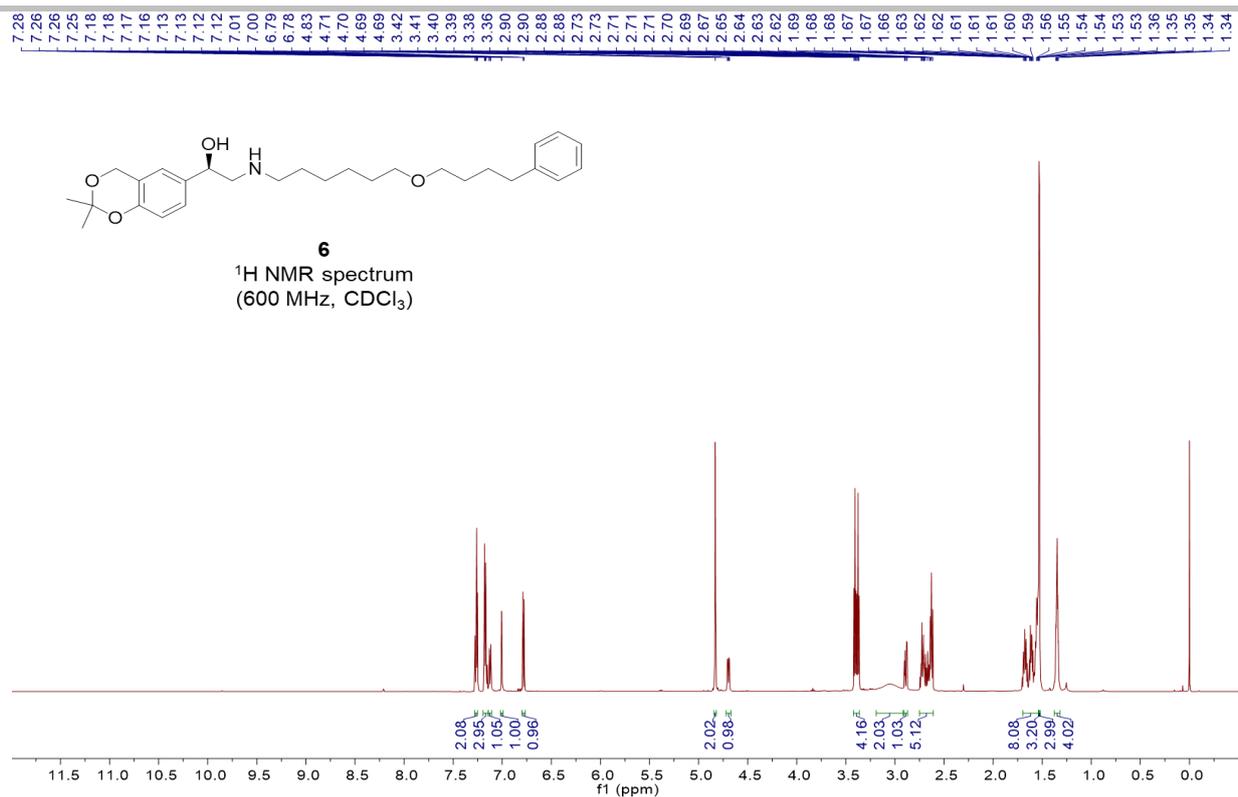


Figure S66. ¹H NMR trace of **6** synthesized for (*R*)-salmeterol synthesis.

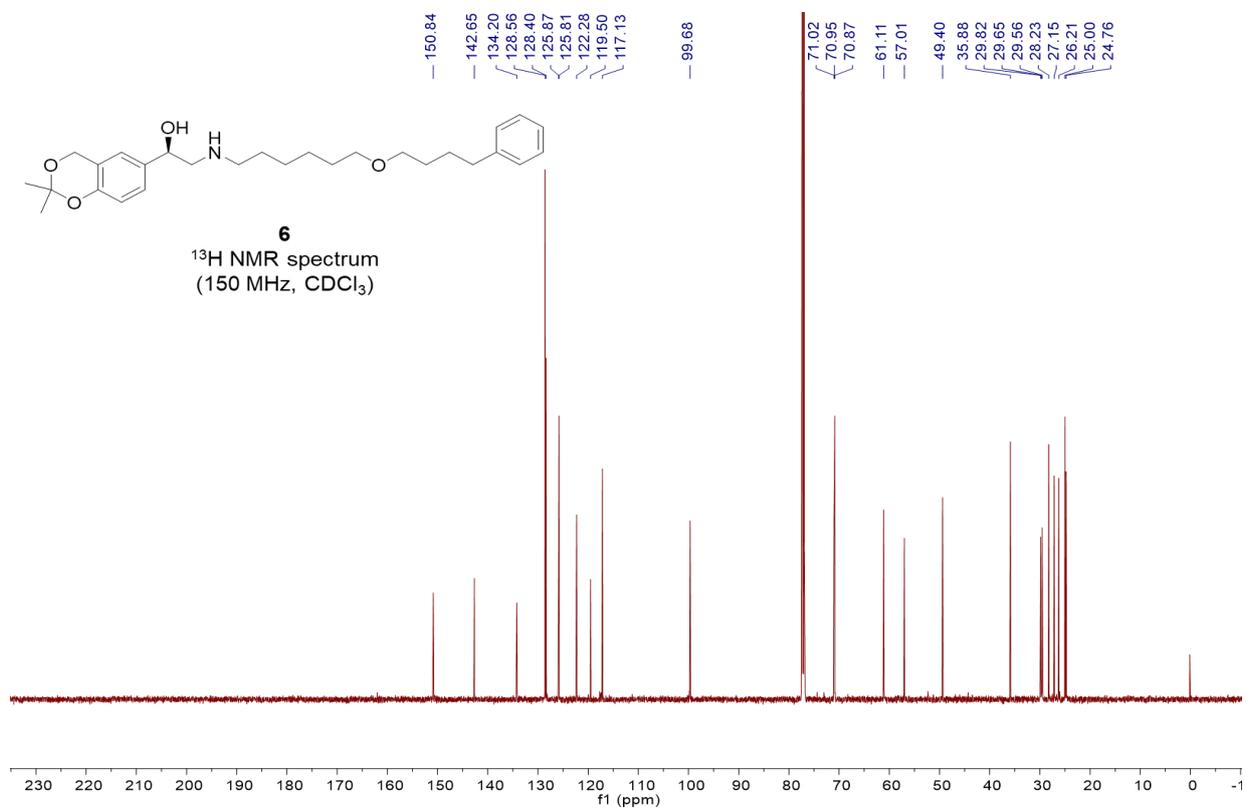


Figure S67. ¹³C NMR trace of **6** synthesized for (*R*)-salmeterol synthesis.

SUPPORTING INFORMATION

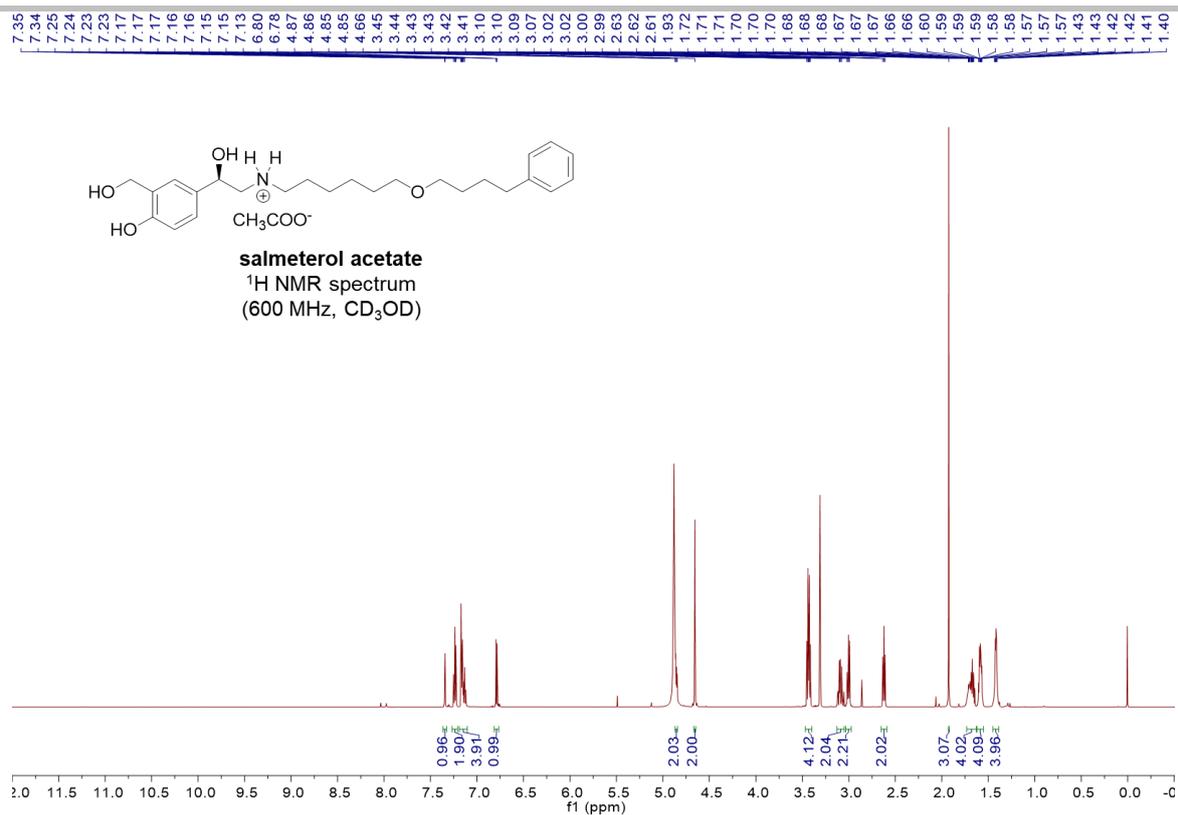


Figure S68. ¹H NMR trace of (*R*)-salmeterol acetate prepared in this study.

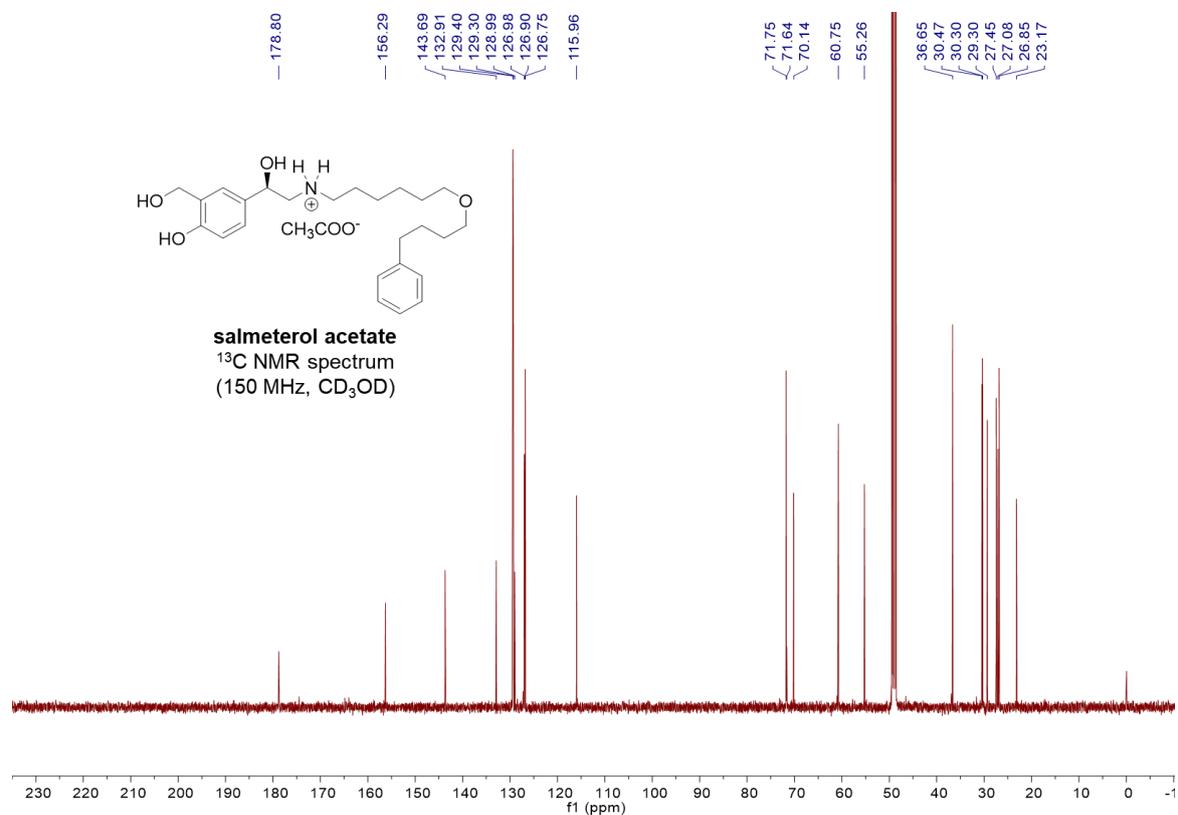


Figure S69. ¹³C NMR trace of (*R*)-salmeterol acetate prepared in this study.

SUPPORTING INFORMATION

2.2 HRMS spectra

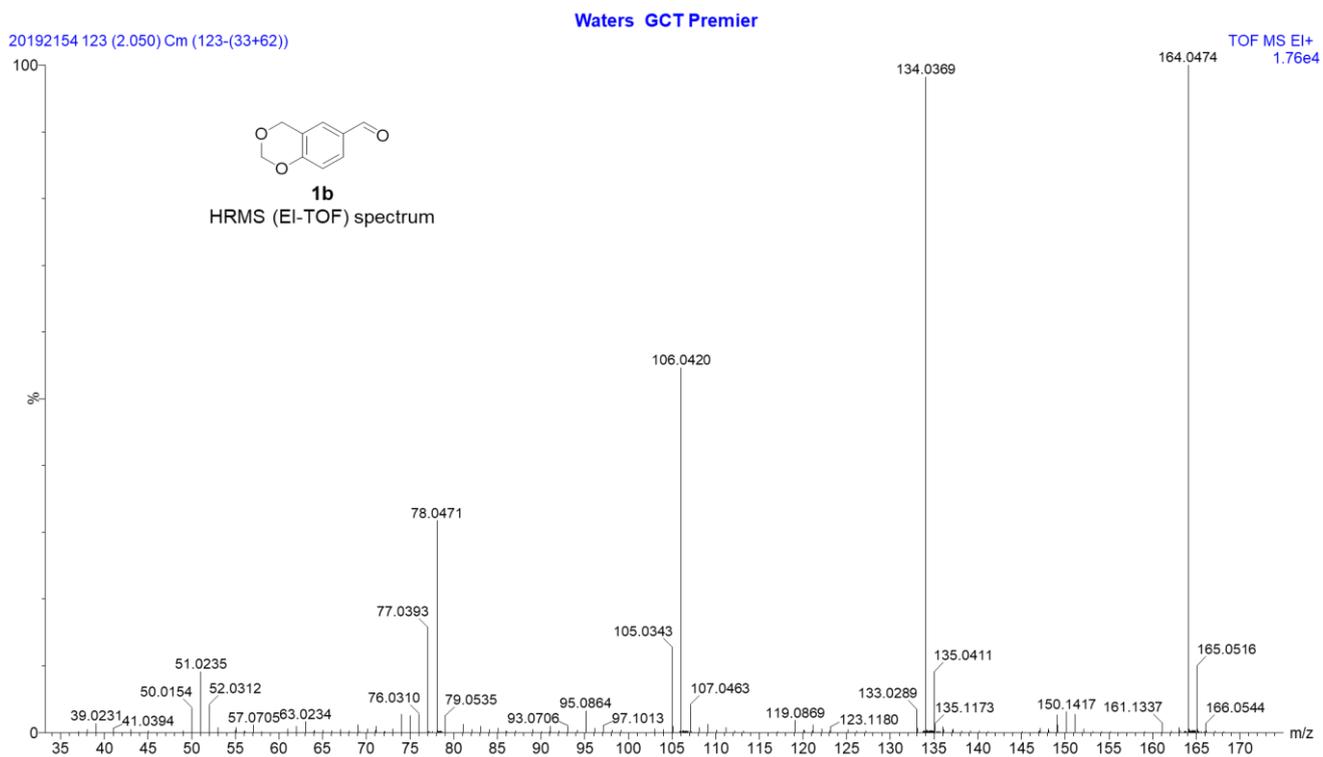


Figure S70. HRMS (EI-TOF) trace of **1b** synthesized for activity determination.

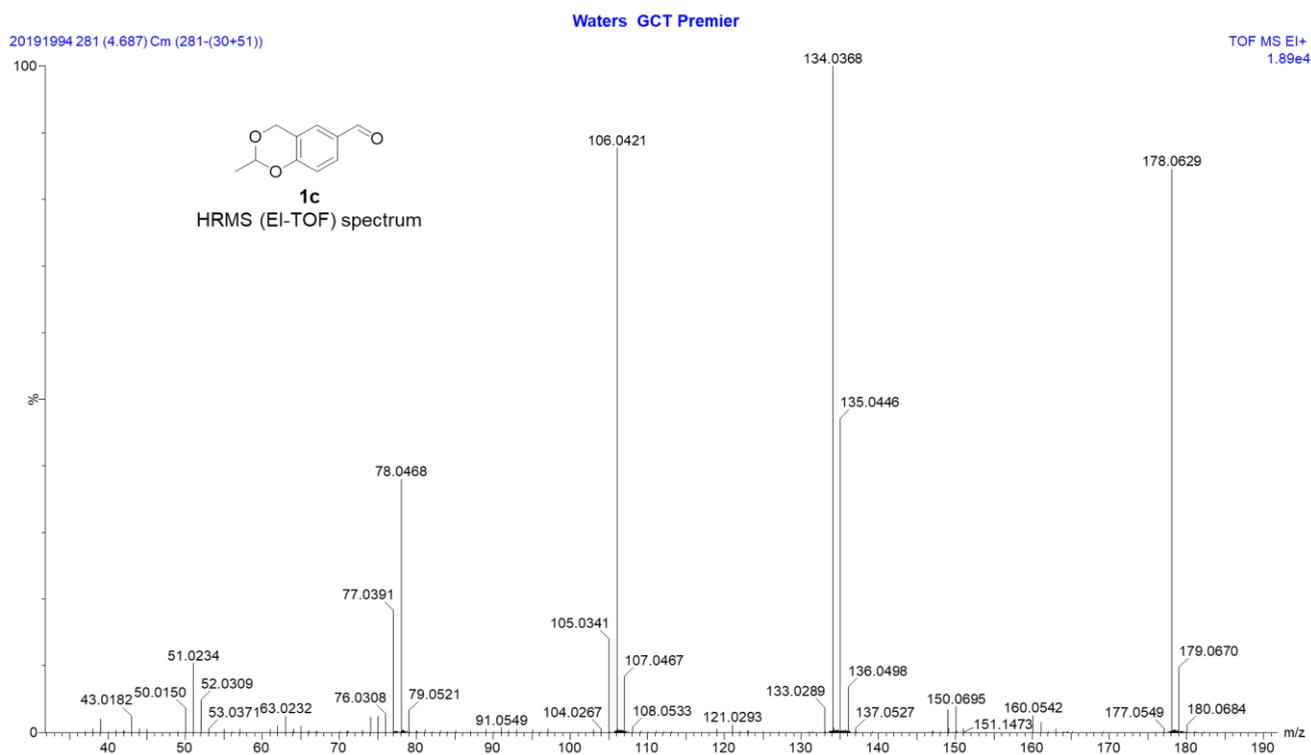


Figure S71. HRMS (EI-TOF) trace of **1c** synthesized for activity determination.

SUPPORTING INFORMATION

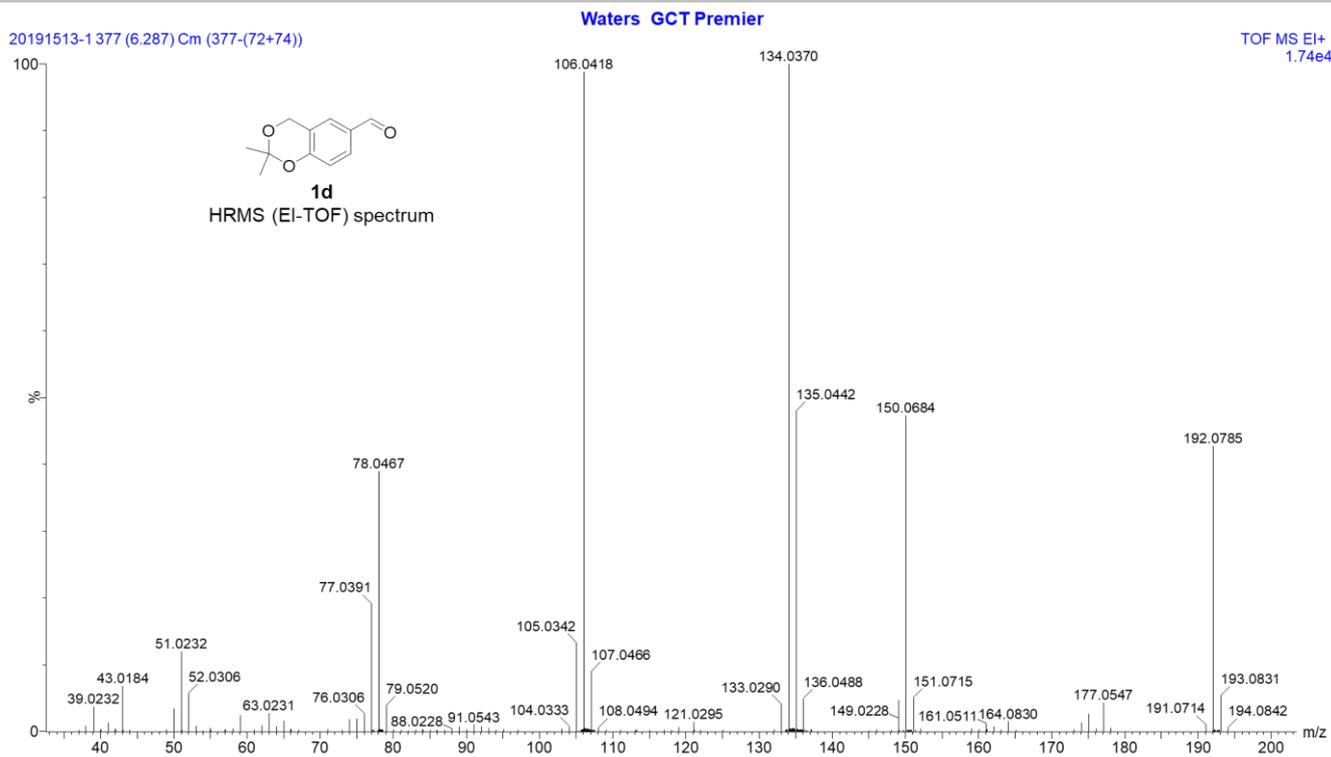


Figure S72. HRMS (EI-TOF) trace of **1d** synthesized for activity determination.

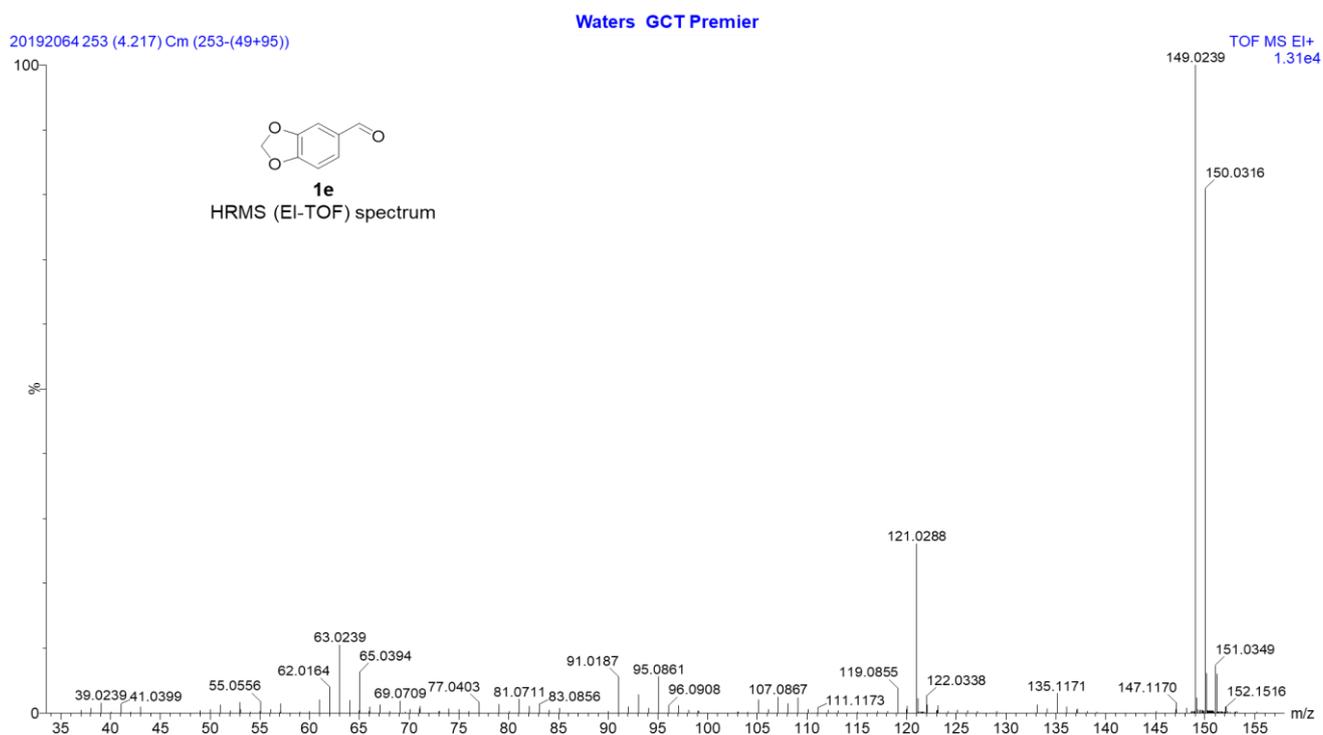


Figure S73. HRMS (EI-TOF) trace of **1e** synthesized for activity determination.

SUPPORTING INFORMATION

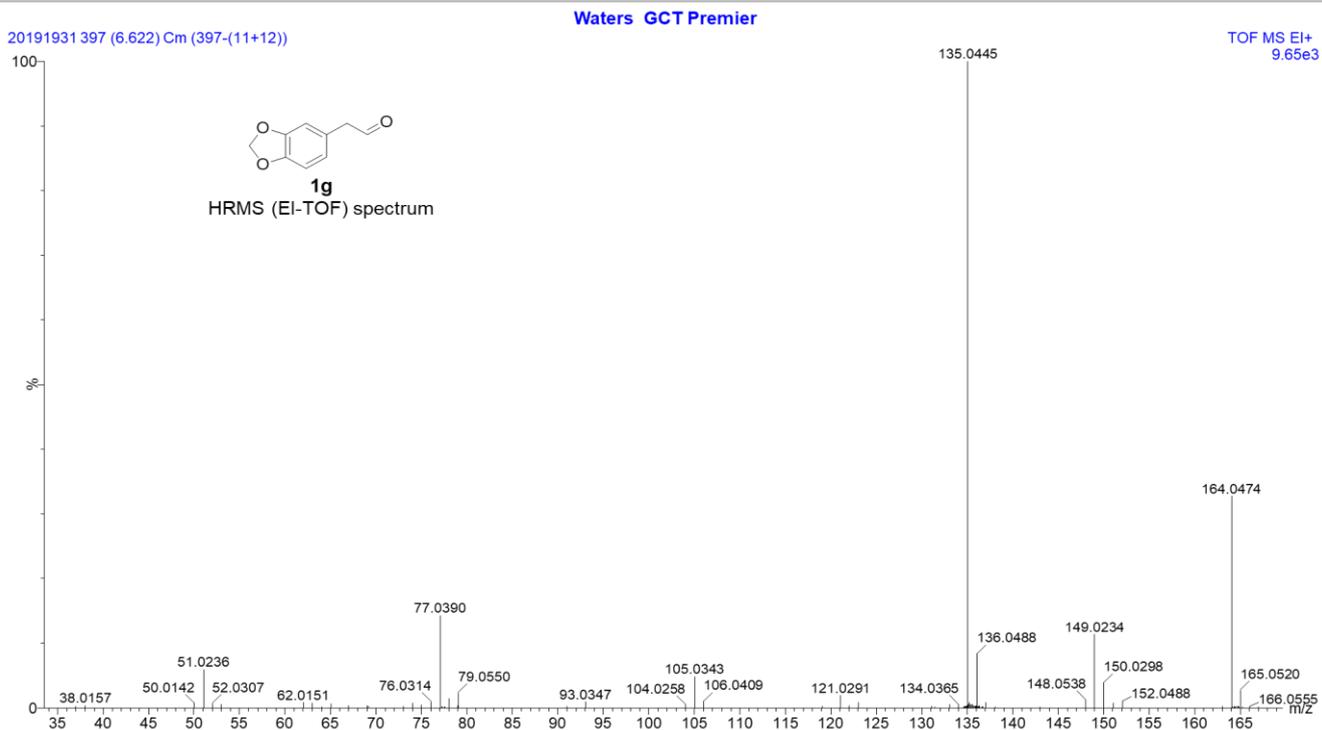


Figure S74. HRMS (EI-TOF) trace of **1g** synthesized for activity determination.

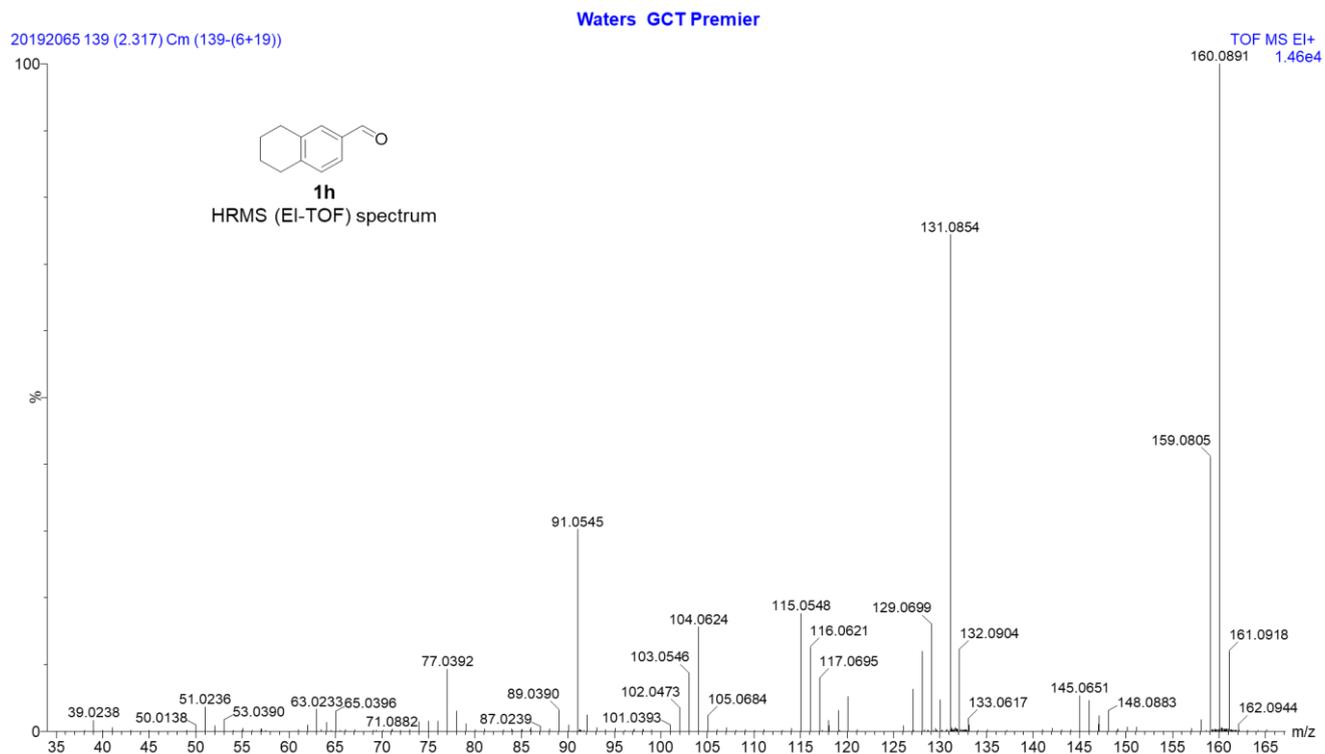


Figure S75. HRMS (EI-TOF) trace of **1h** synthesized for activity determination.

SUPPORTING INFORMATION

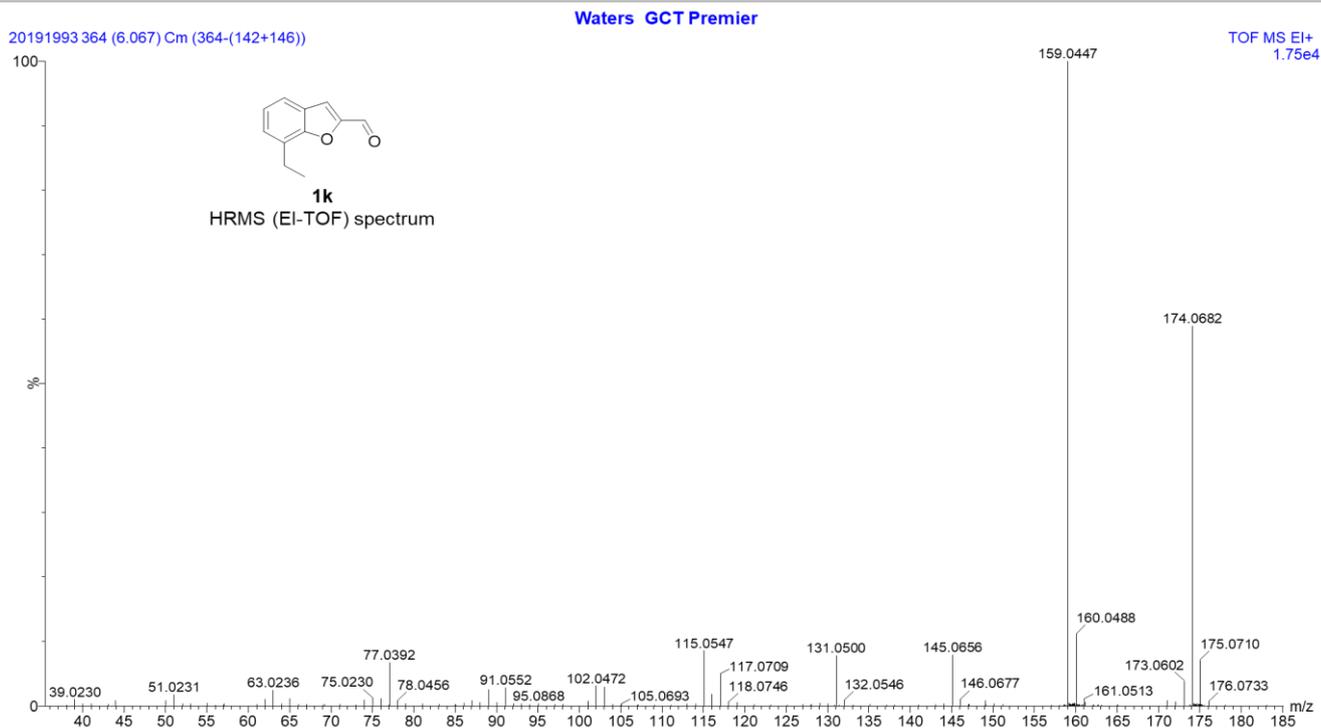


Figure S76. HRMS (EI-TOF) trace of **1k** synthesized for activity determination.

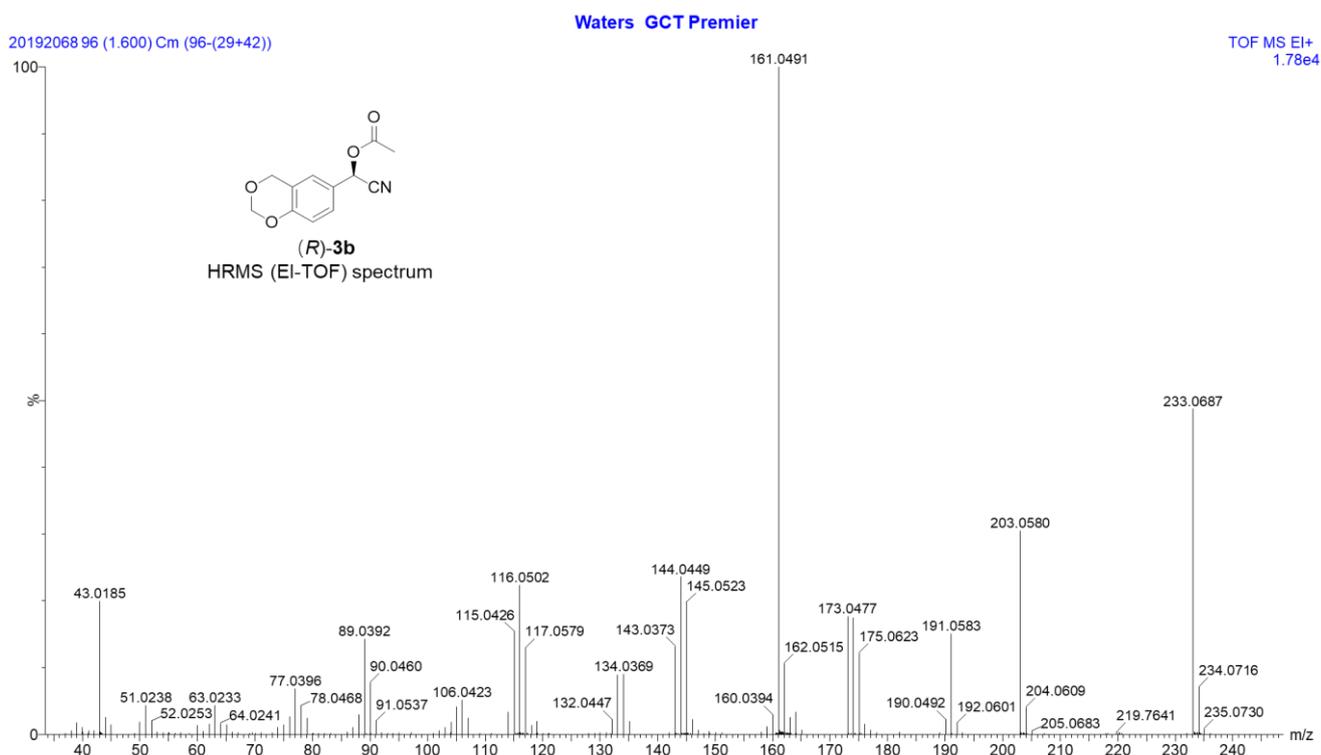


Figure S77. HRMS (EI-TOF) trace of **(R)-3b** prepared in this study.

SUPPORTING INFORMATION

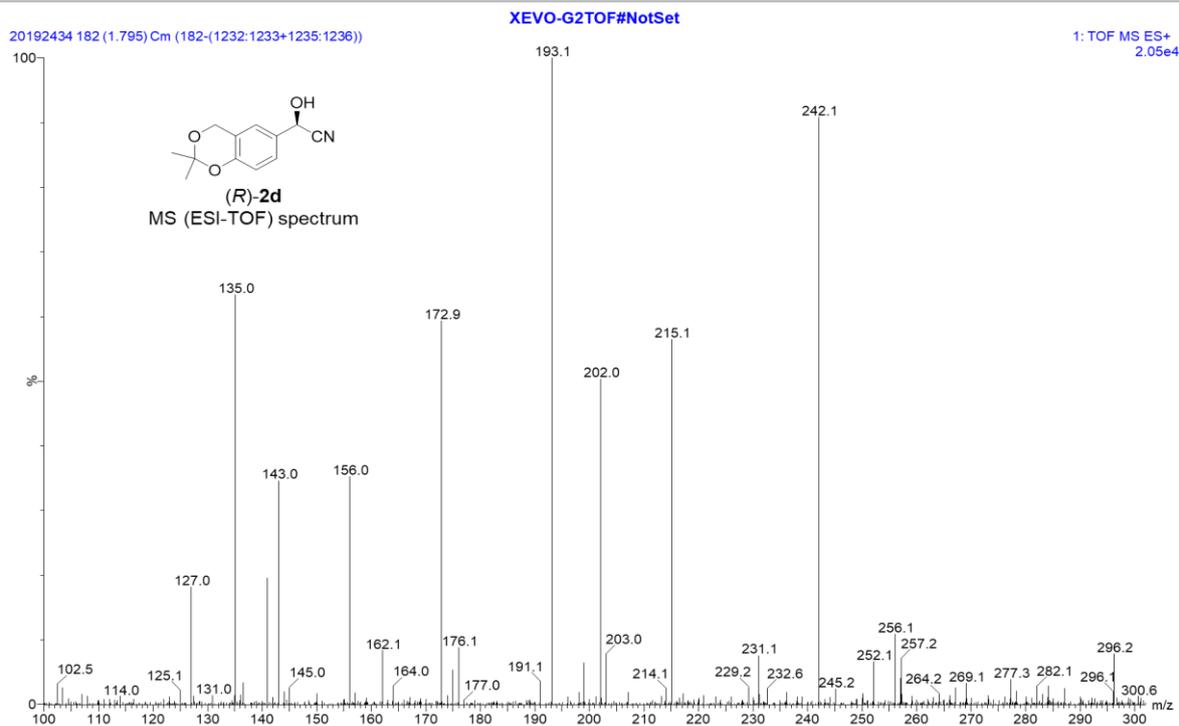


Figure S78. MS (ESI-TOF) trace of (*R*)-**2d** prepared in this study.

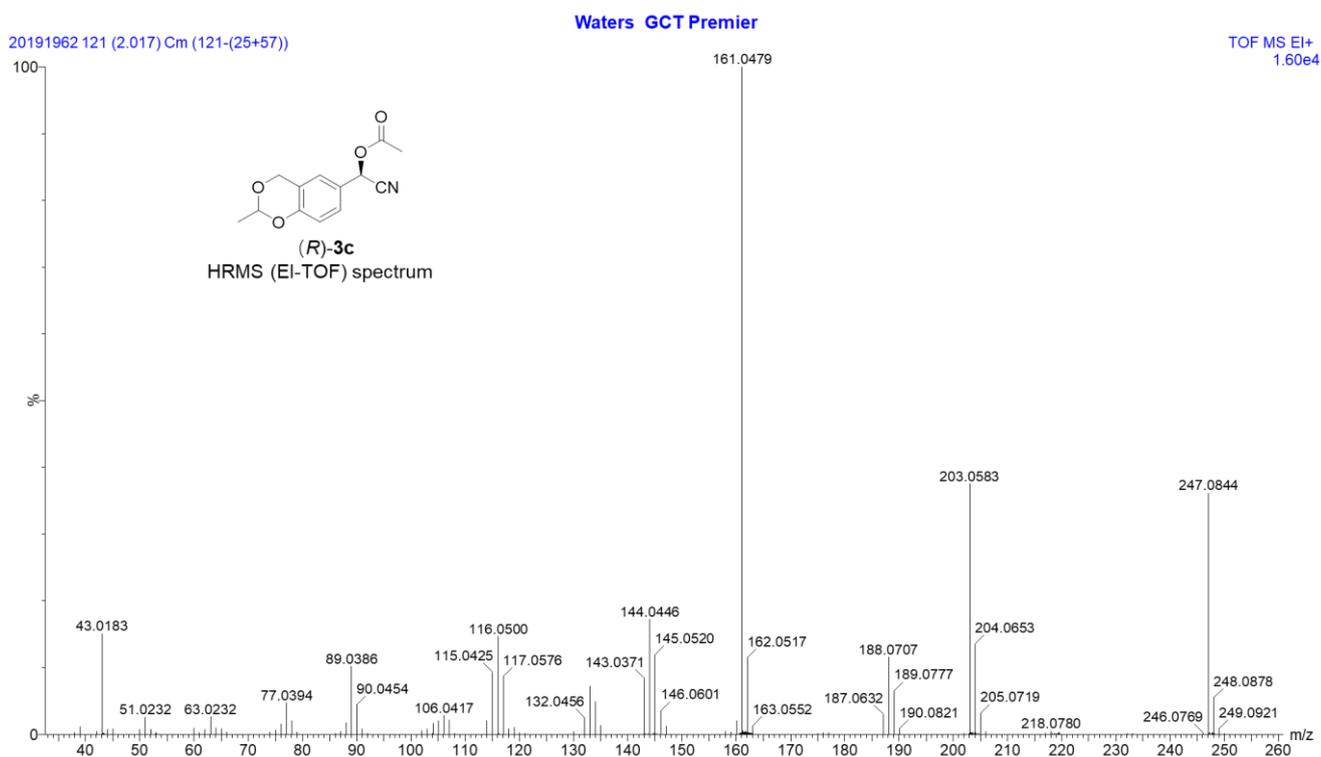


Figure S79. HRMS (EI-TOF) trace of (*R*)-**3c** prepared in this study.

SUPPORTING INFORMATION

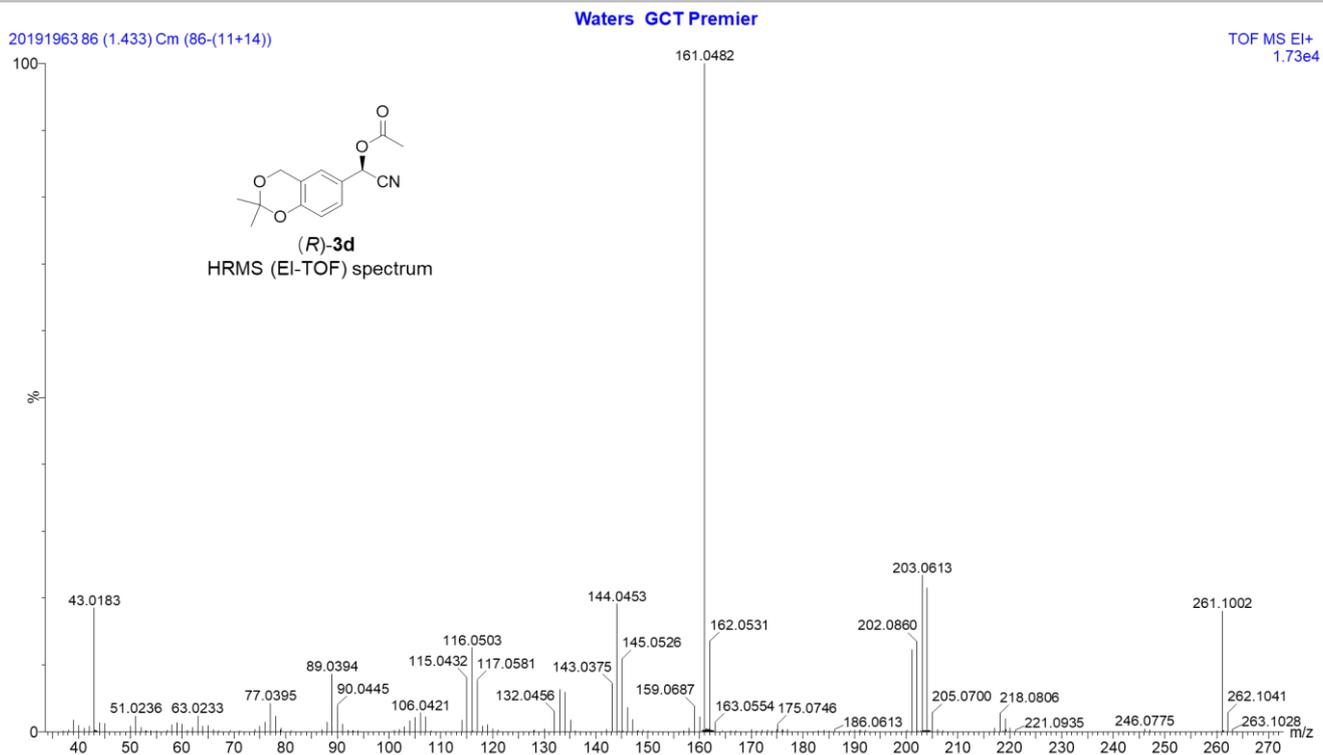


Figure S80. HRMS (EI-TOF) trace of (R)-3d prepared in this study.

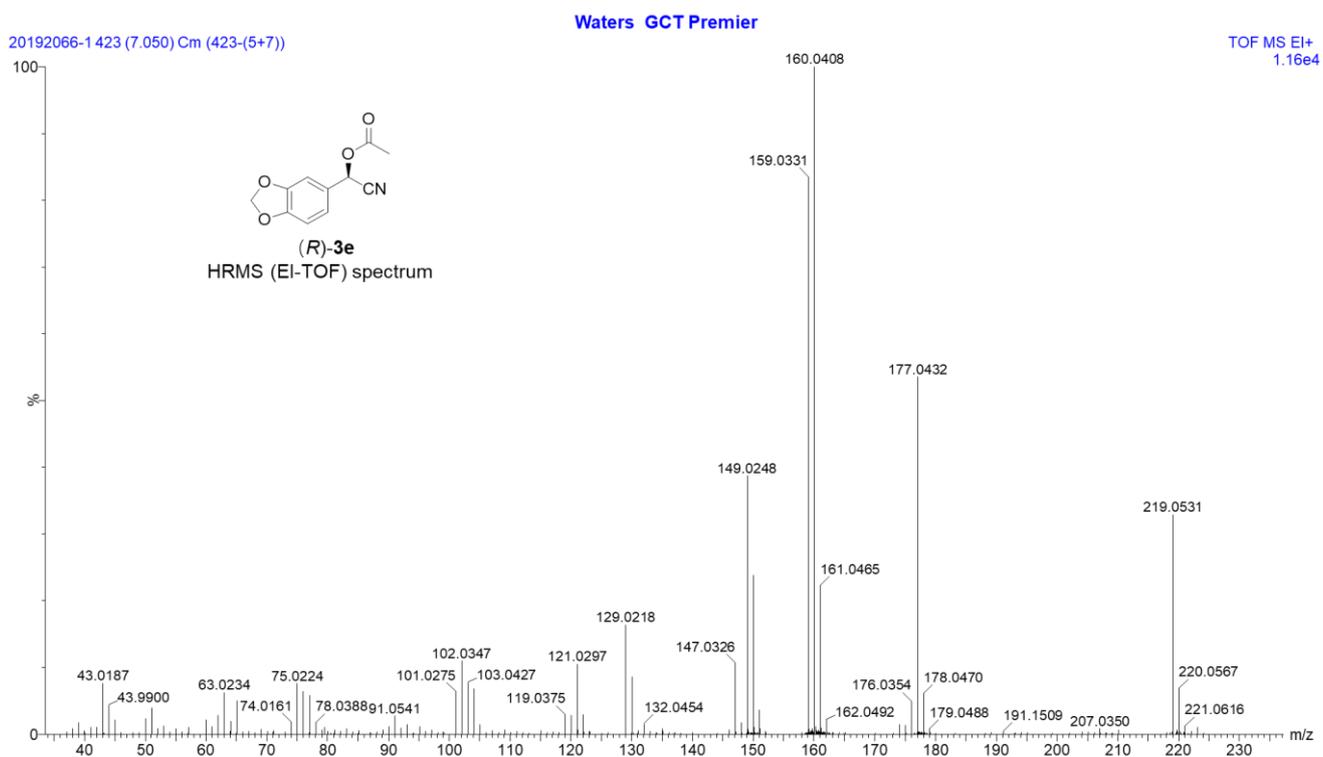


Figure S81. HRMS (EI-TOF) trace of (R)-3e prepared in this study.

SUPPORTING INFORMATION

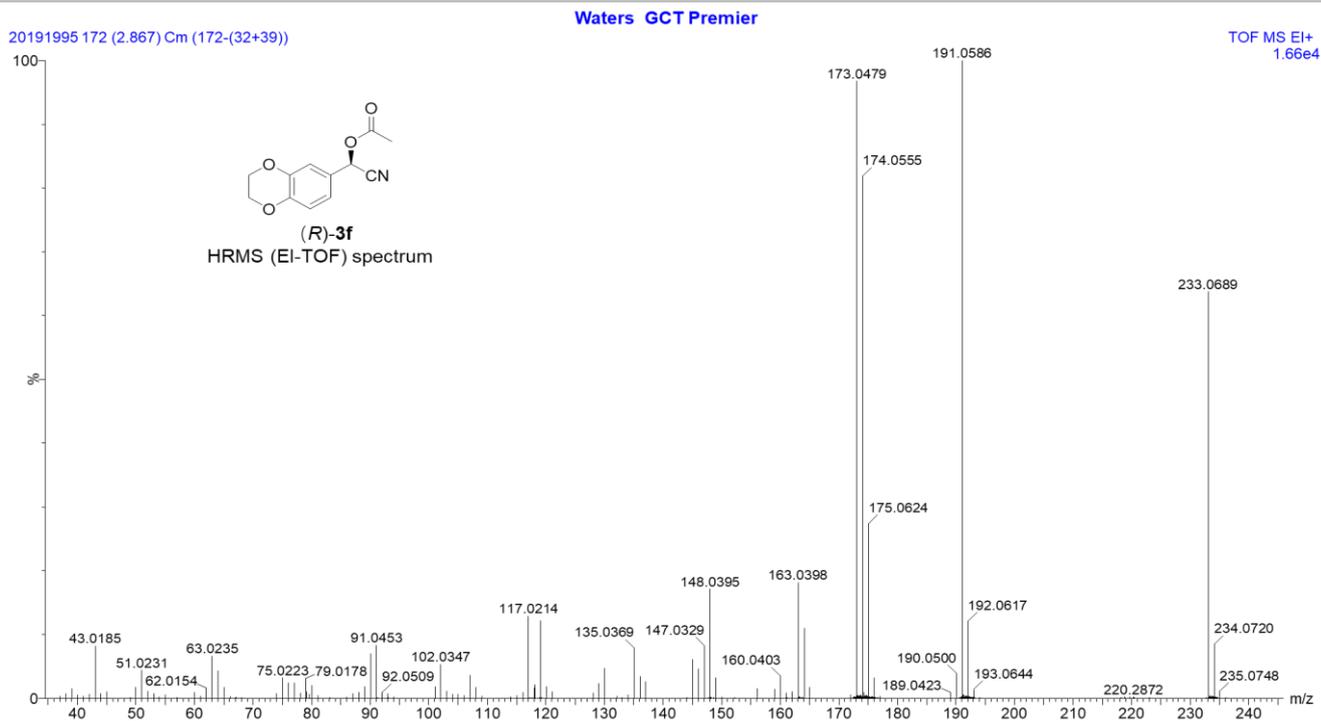


Figure S82. HRMS (EI-TOF) trace of *(R)*-**3f** prepared in this study.

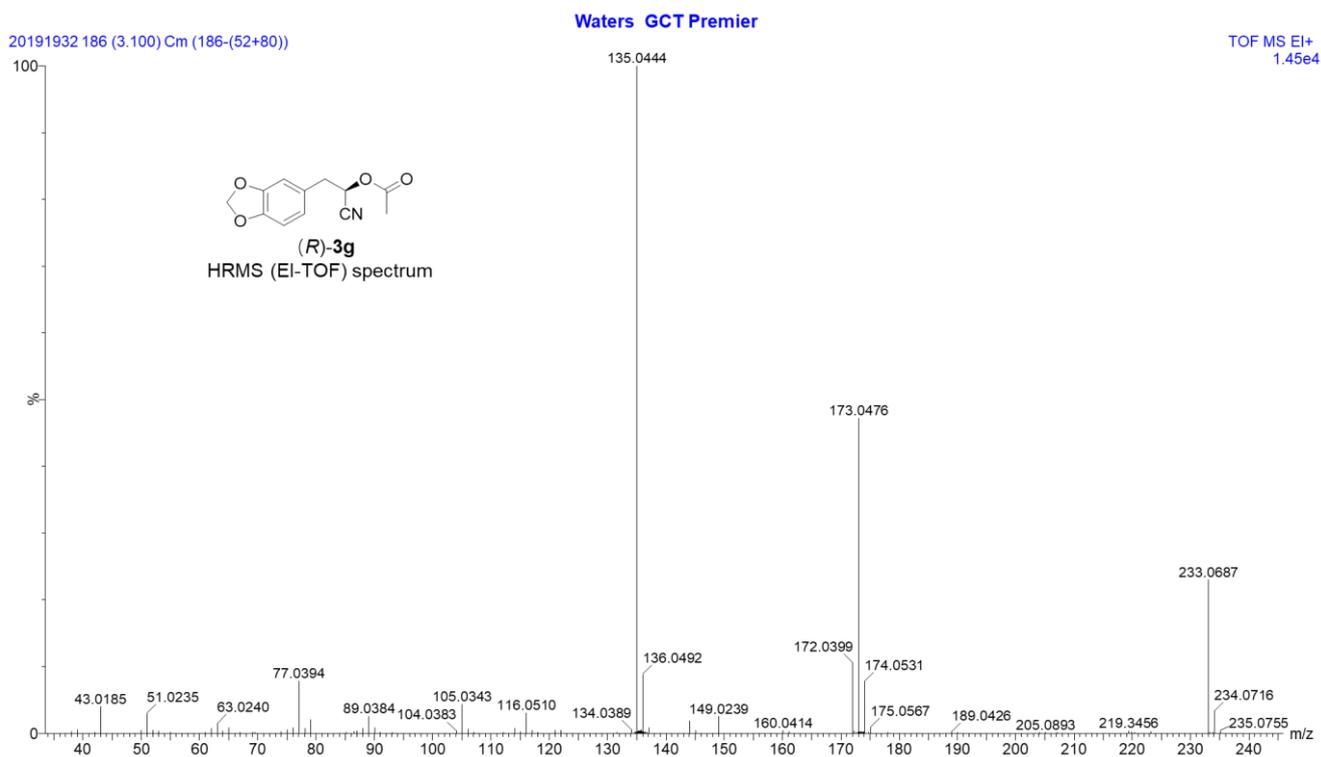


Figure S83. HRMS (EI-TOF) trace of *(R)*-**3g** prepared in this study.

SUPPORTING INFORMATION

Waters GCT Premier

20192067-1 15 (0.250) Cm (15-(323+325))

TOF MS EI+
1.03e4

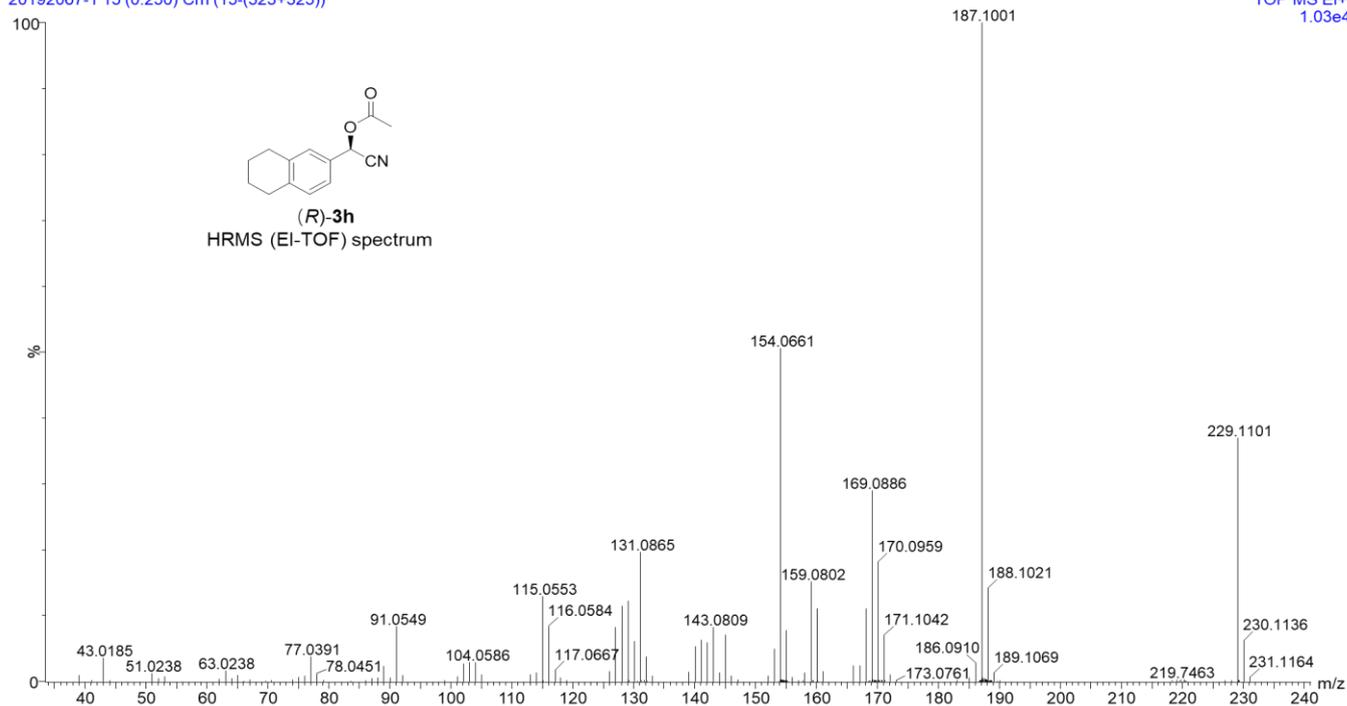


Figure S84. HRMS (EI-TOF) trace of (R)-3h prepared in this study.

Waters GCT Premier

20191933-1 335 (5.583) Cm (335-(34+57))

TOF MS EI+
1.34e4

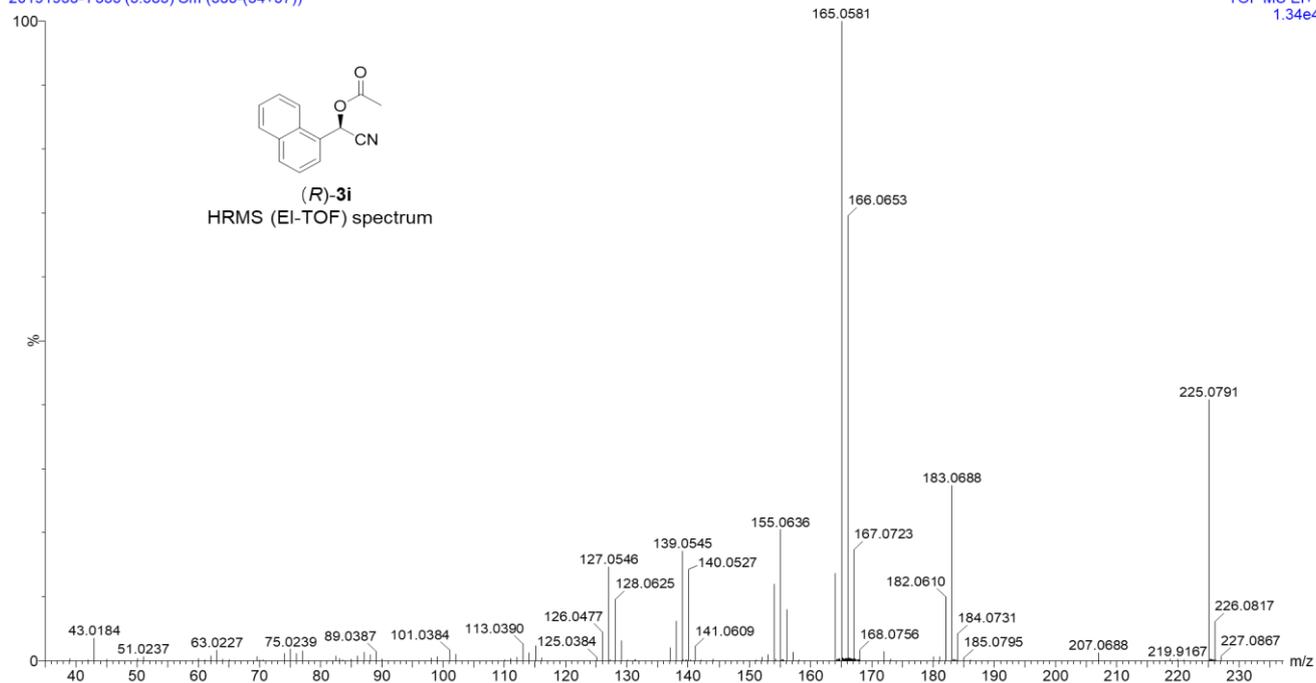


Figure S85. HRMS (EI-TOF) trace of (R)-3i prepared in this study.

SUPPORTING INFORMATION

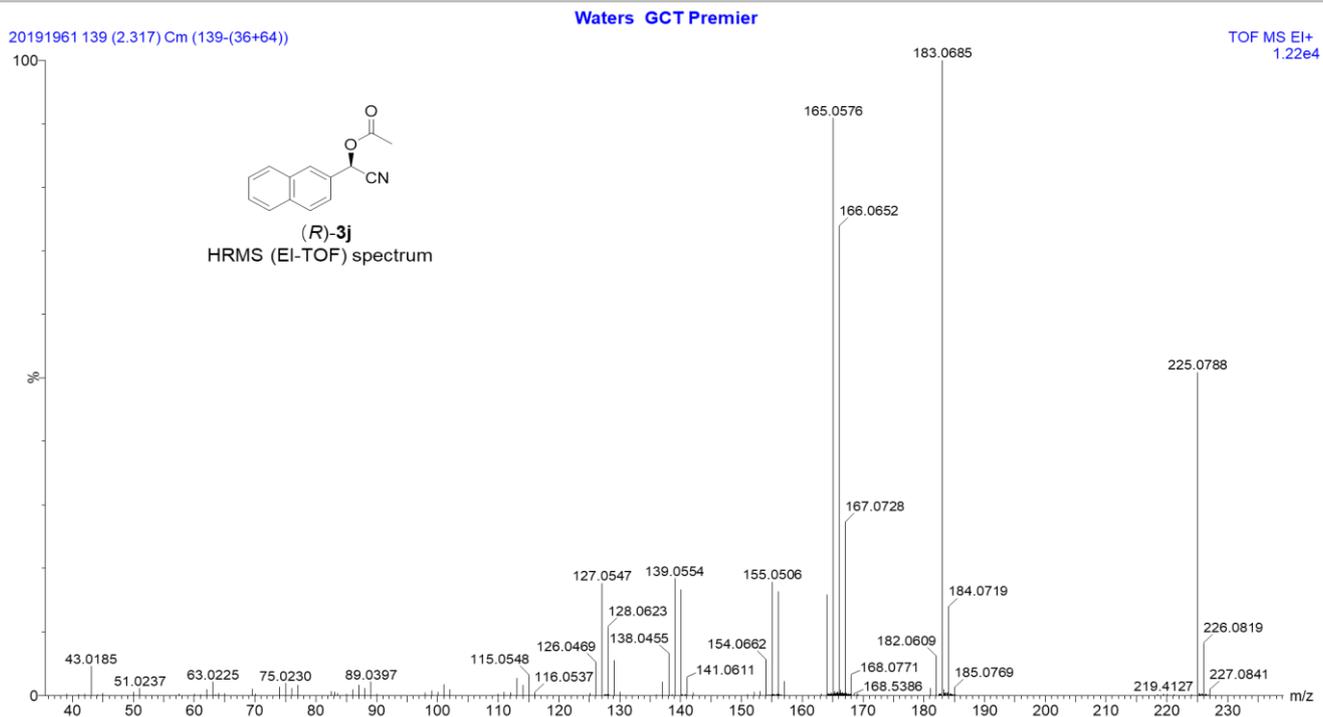


Figure S86. HRMS (EI-TOF) trace of (R)-3j prepared in this study.

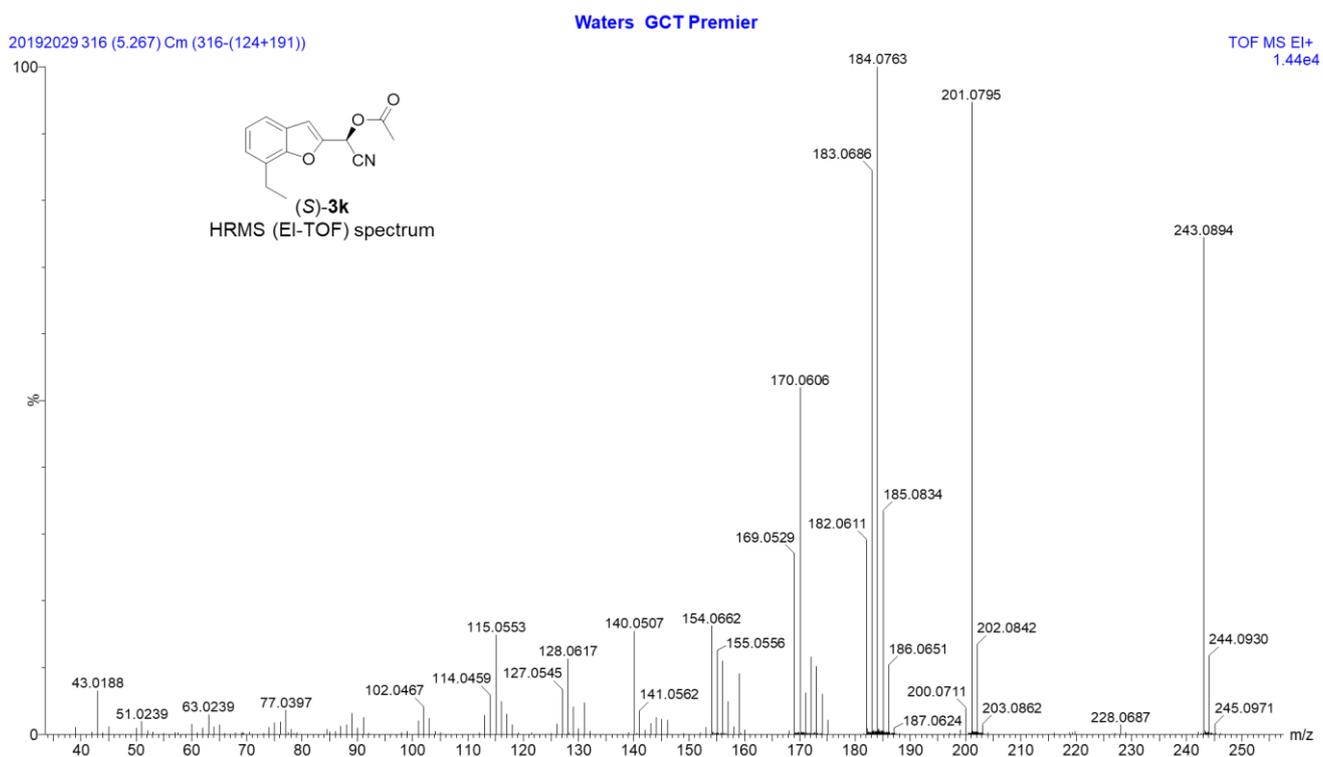


Figure S87. HRMS (EI-TOF) trace of (S)-3k prepared in this study.

SUPPORTING INFORMATION

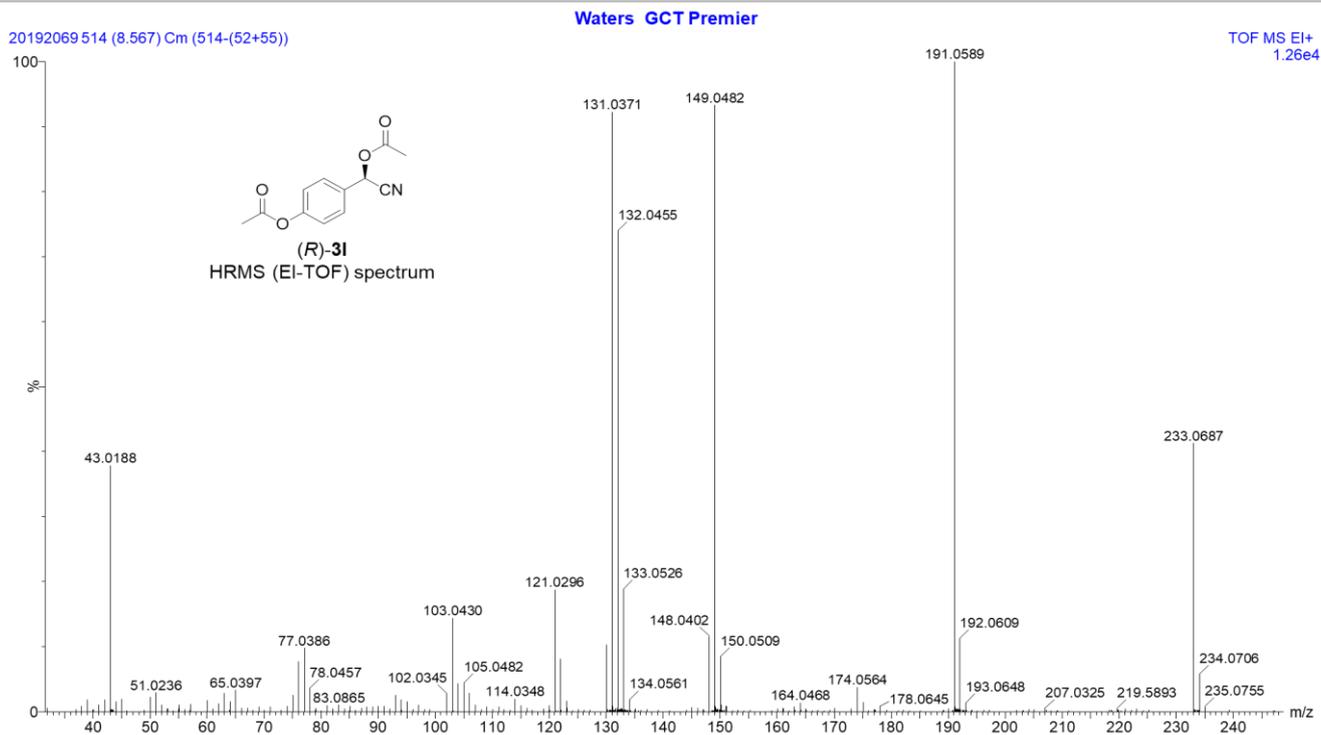


Figure S88. HRMS (EI-TOF) trace of *(R)*-**3l** prepared in this study.

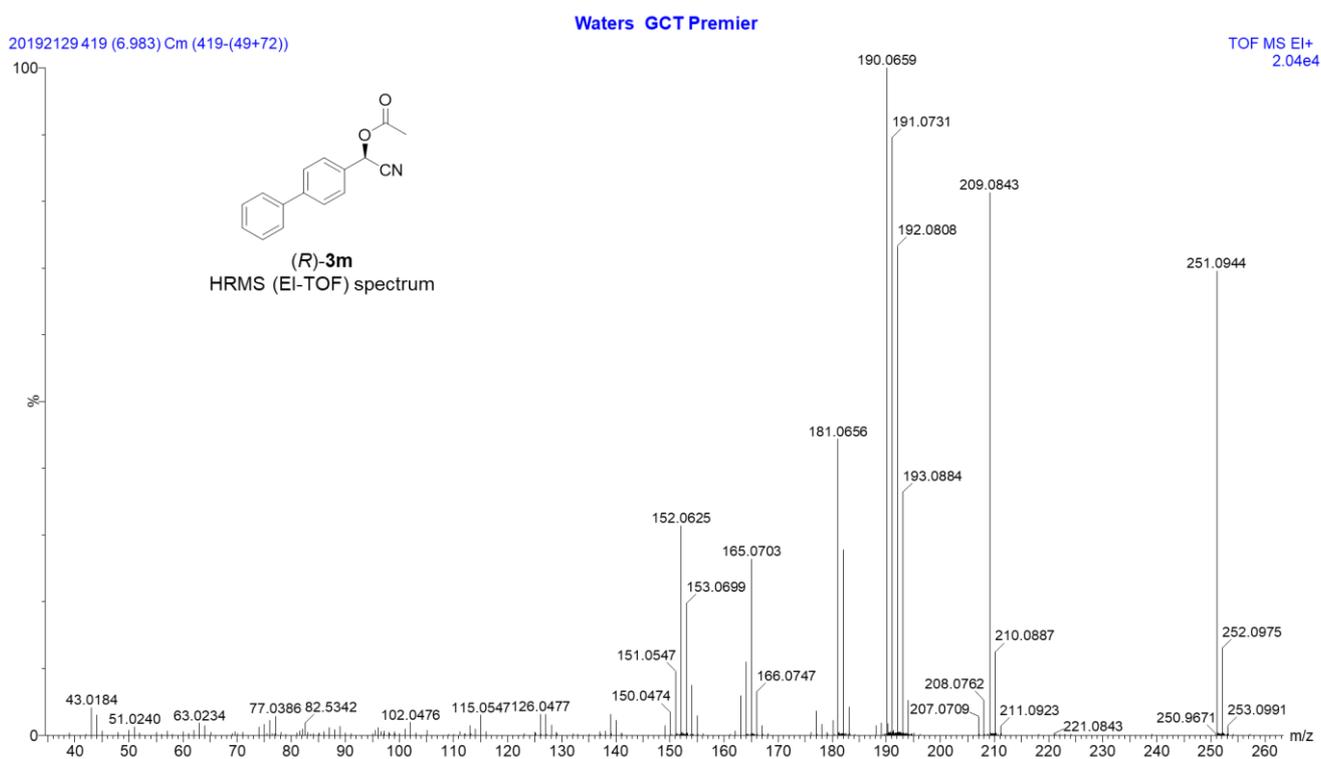


Figure S89. HRMS (EI-TOF) trace of *(R)*-**3m** prepared in this study.

SUPPORTING INFORMATION

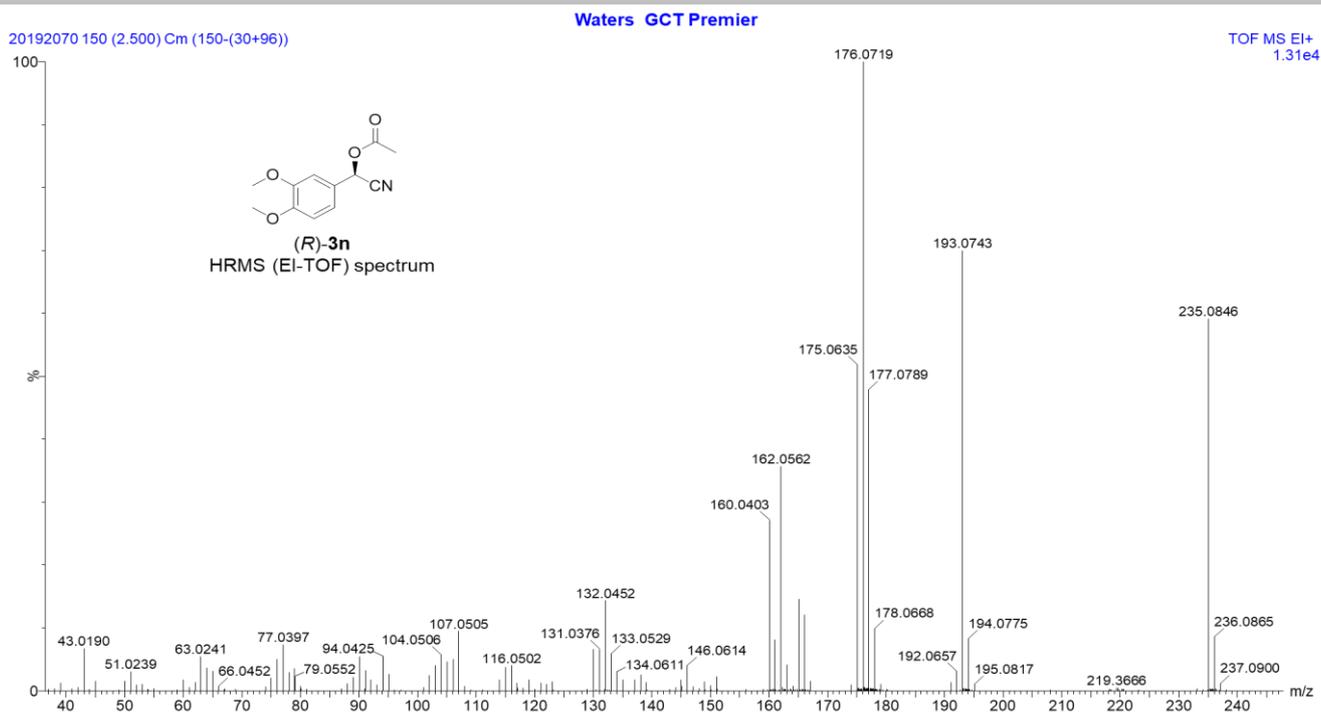


Figure S90. HRMS (EI-TOF) trace of (R)-3n prepared in this study.

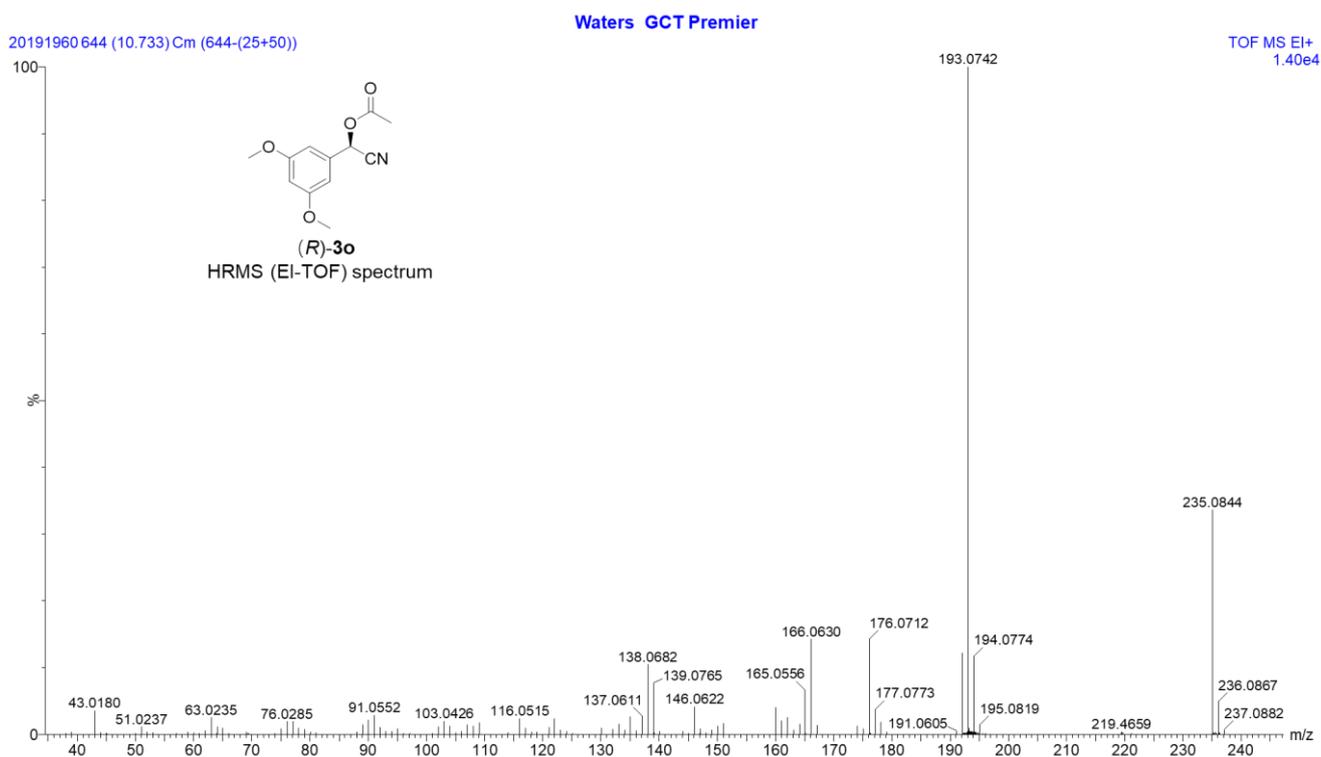


Figure S91. HRMS (EI-TOF) trace of (R)-3o prepared in this study.

SUPPORTING INFORMATION

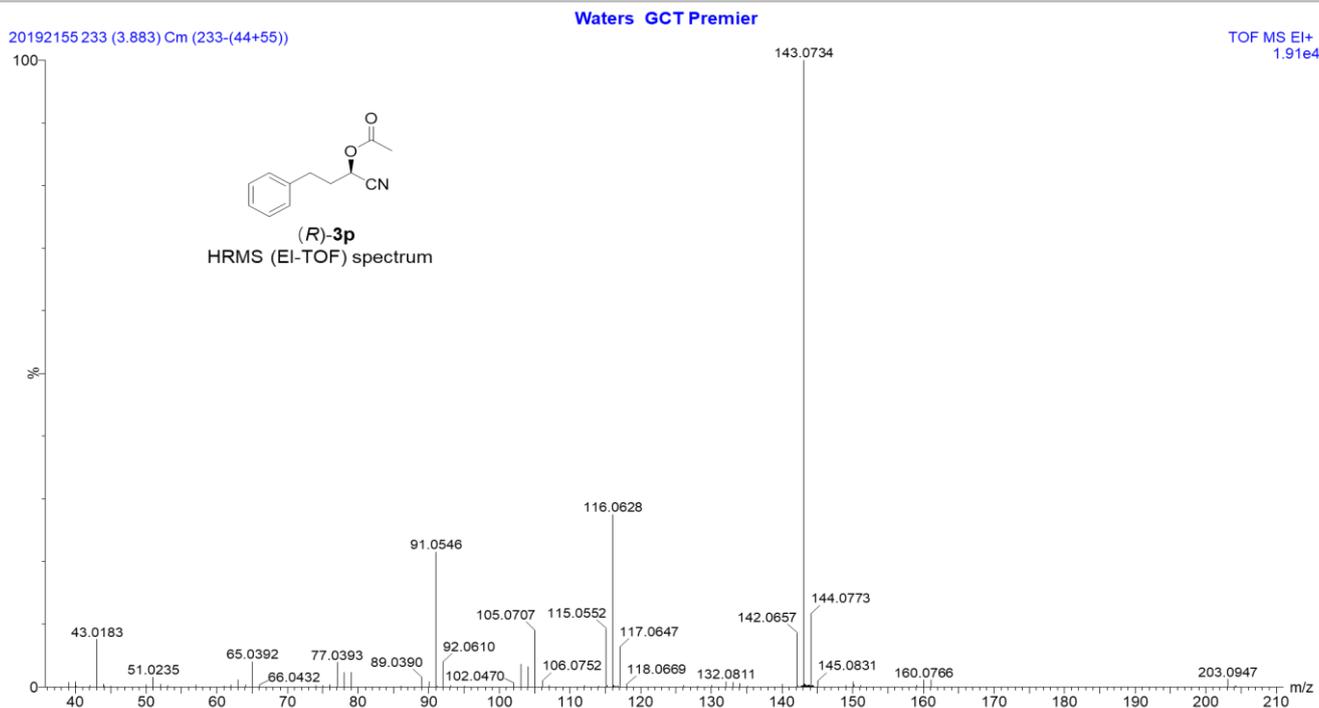


Figure S92. HRMS (EI-TOF) trace of (R)-3p prepared in this study.

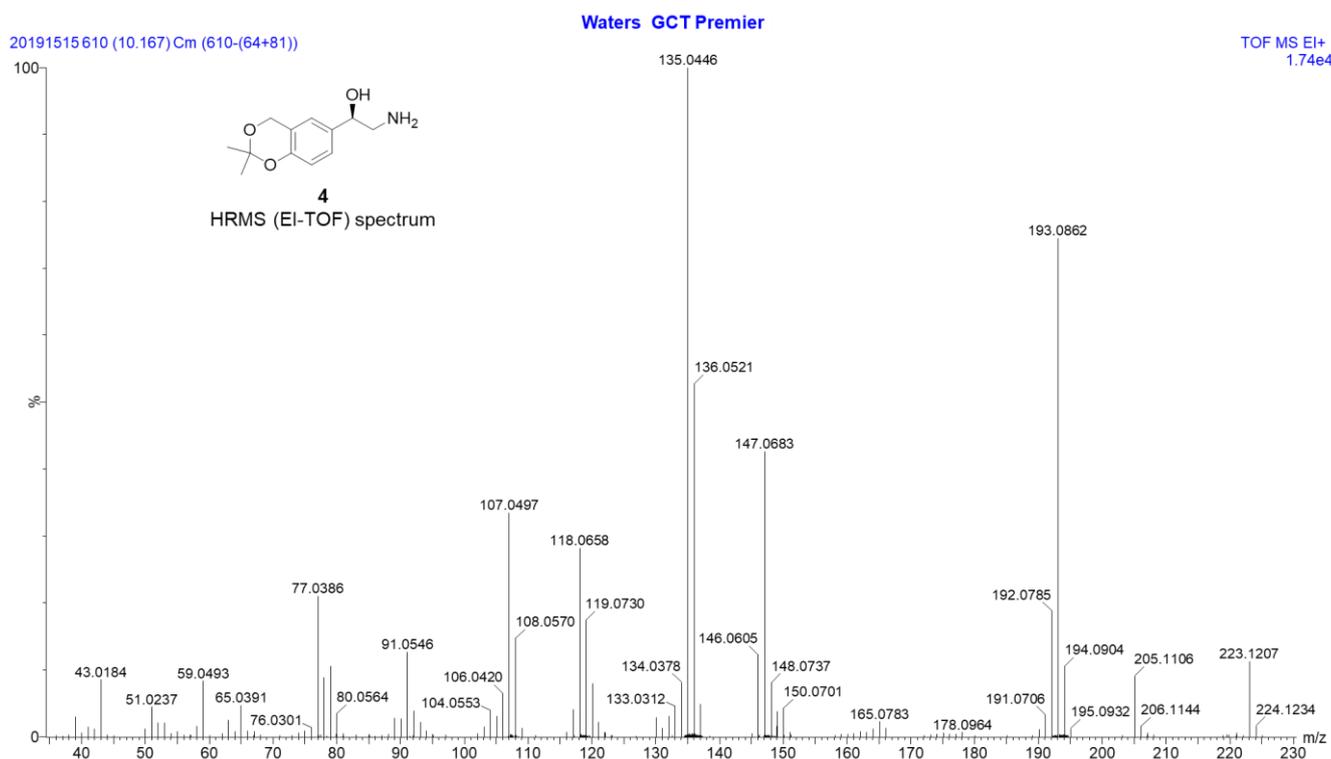


Figure S93. HRMS (EI-TOF) trace of 4 for (R)-salmeterol synthesis.

SUPPORTING INFORMATION

Waters GCT Premier

20191516261 (4.350) Cm (261-(21+53))

TOF MS EI+
1.57e4

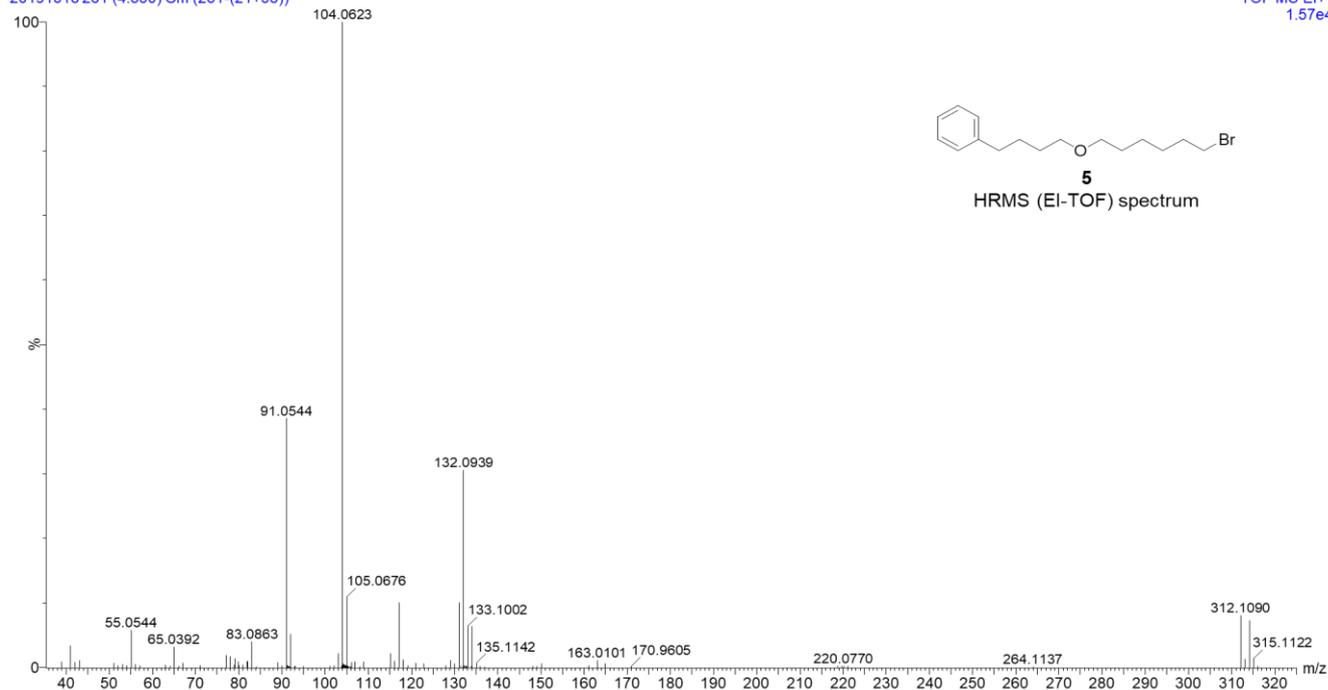


Figure S94. HRMS (EI-TOF) trace of **5** for (R)-salmeterol synthesis.

XEVO-G2TOF#NotSet

20191562133 (1.316) Cm (133-(20:22+37:40))

1: TOF MS ES+
1.42e6

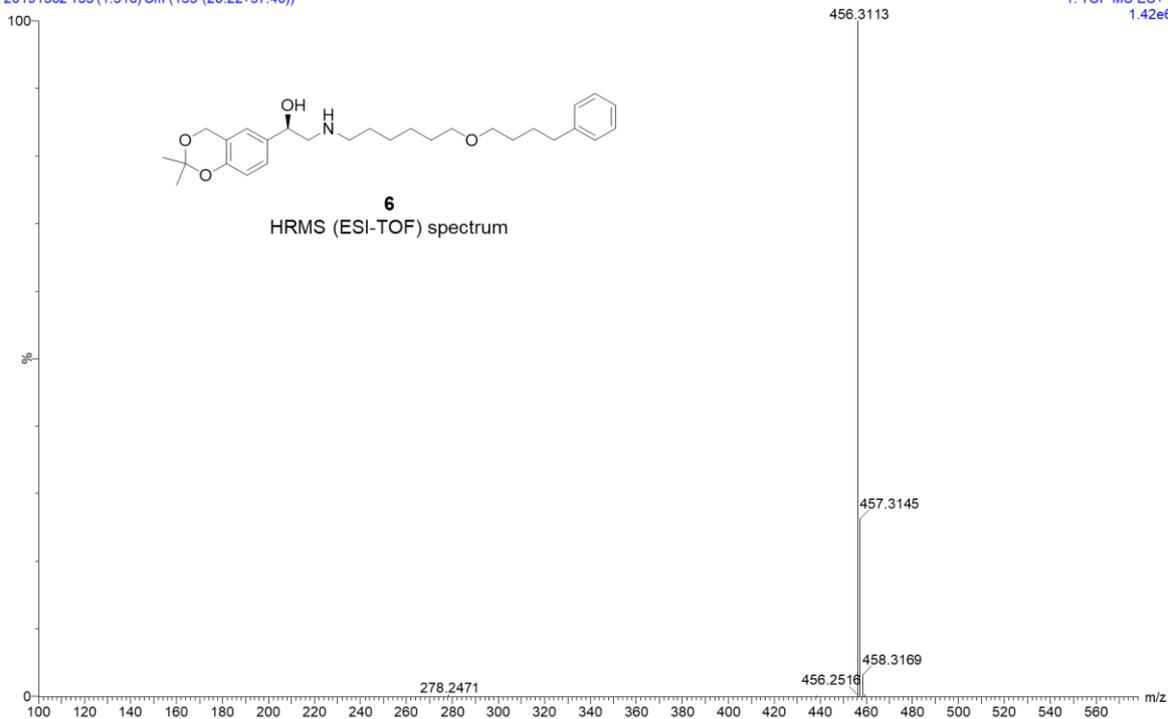


Figure S95. HRMS (ESI-TOF) trace of **6** for (R)-salmeterol synthesis.

SUPPORTING INFORMATION

XEVO-G2TOF#NotSet

20191561 42 (0.437) Cm (42-(601+606))

1: TOF MS ES+
2.35e5

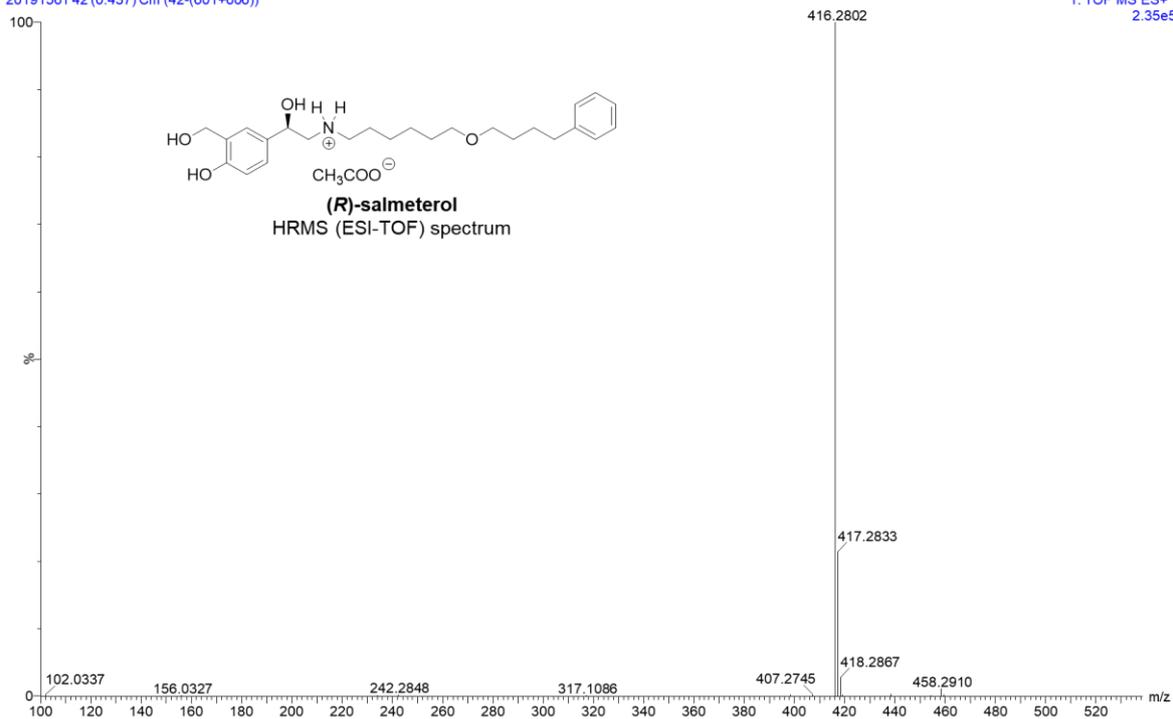
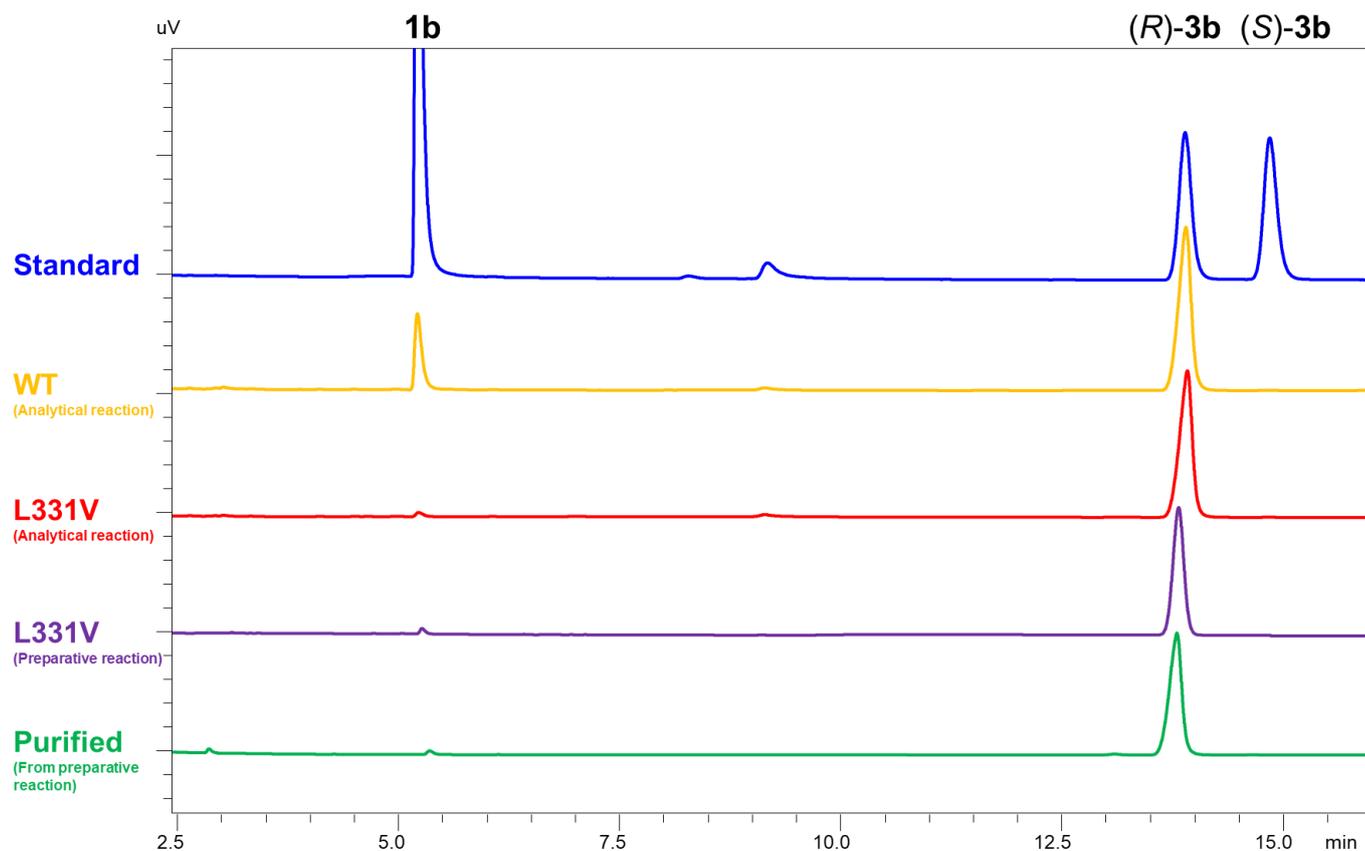


Figure S96. HRMS (ESI-TOF) trace of (*R*)-salmeterol acetate.

SUPPORTING INFORMATION

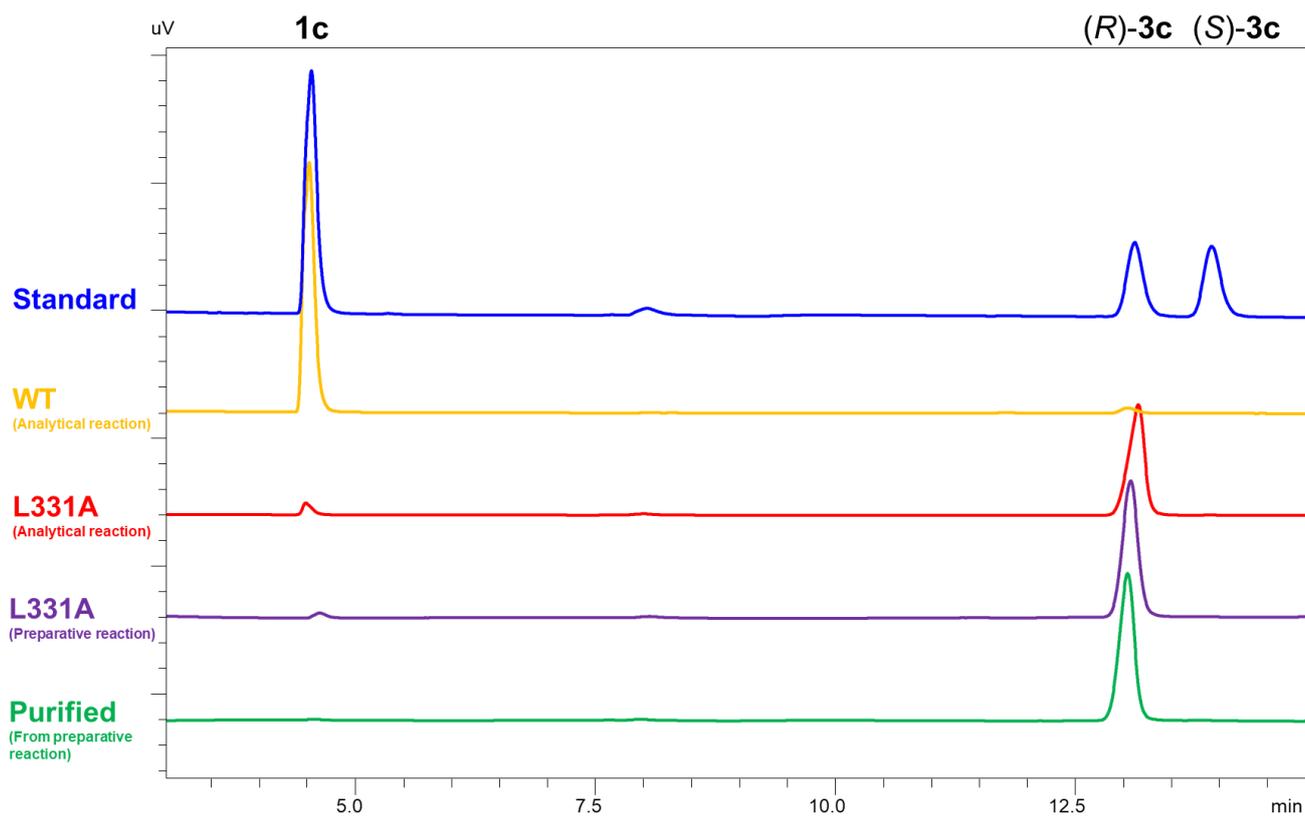
2.3 GC spectra



Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	
Standard	5.218	13.897	14.854	109696	29921	28778	695021	305691	305951	49.979	50.021	
WT (Analytical reaction)	5.215	13.904	14.824	39200	84289	265	224365	863345	2694	99.689	0.331	
L331V (Analytical reaction)	5.229	13.921	14.834	3021	100369	140	18391	1037249	1361	99.869	0.131	
L331V (Preparative reaction)	5.146	13.702	14.619	1148	26732	37	4808	236357	315	99.867	0.133	
Purified (From preparative reaction)	-	13.826	14.880	-	90401	168	-	909963	1562	99.829	0.171	

Figure S97. GC spectrum of bio-asymmetric hydrocyanation of **1b** by PcHNL5 variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1b** and *rac*-**3b**. Yellow line: analytical reaction employed by PcHNL5 wild-type. Red line: analytical reaction employed by PcHNL5_{L331A}. Purple line: preparative reaction employed by PcHNL5_{L331A} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3b** from preparative reaction. The detailed data of the peak table was shown as follows.

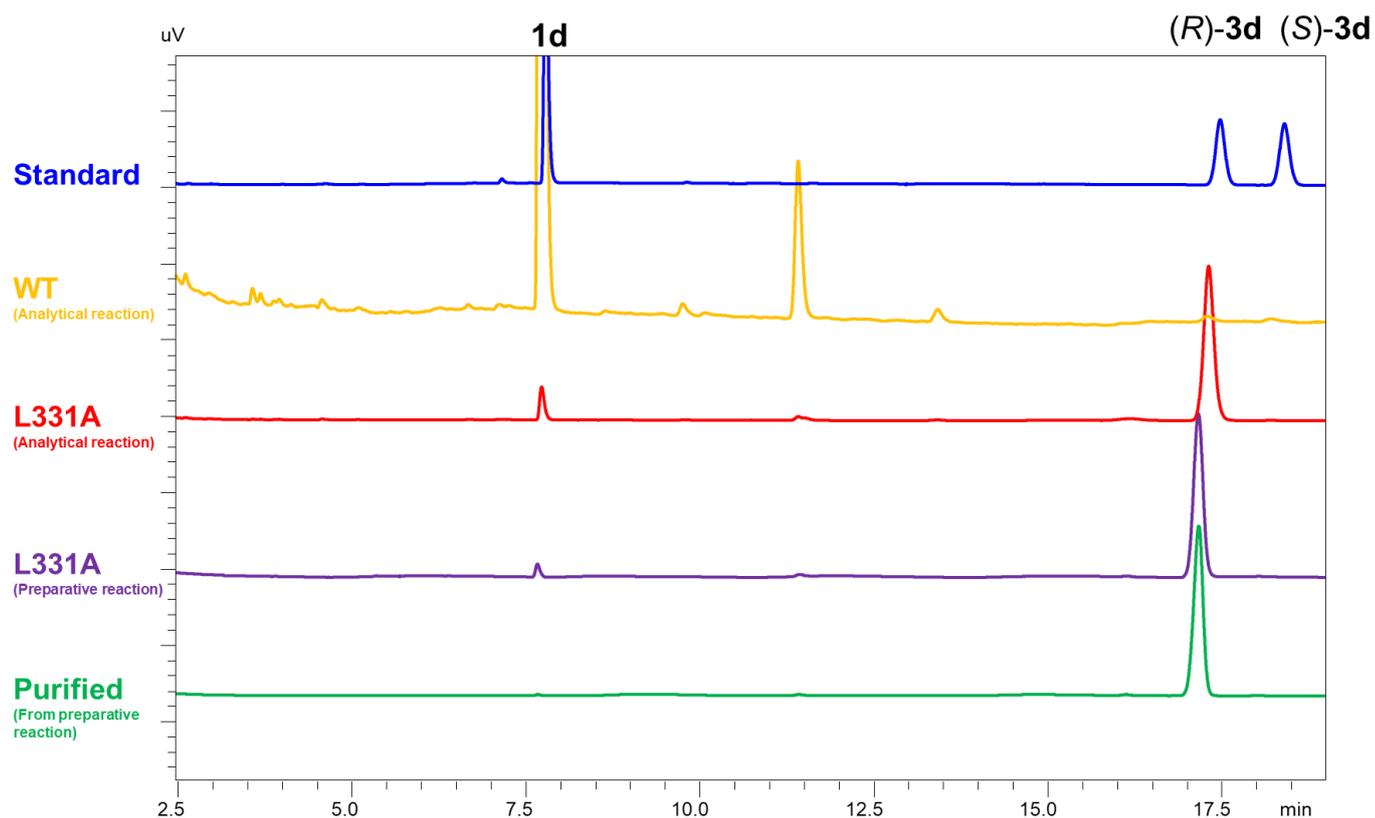
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	4.538	13.136	13.926	101970	31534	30844	837757	378213	376330	50.125	49.875
WT (Analytical reaction)	4.520	13.043	13.877	94570	1950	121	777501	22587	1292	94.590	5.410
L331A (Analytical reaction)	4.483	13.156	13.898	11095	103902	186	79596	1237049	2161	99.826	0.174
L331A (Preparative reaction)	4.468	12.916	13.703	1189	34433	87	10646	400678	984	99.745	0.245
Purified (From preparative reaction)	-	12.999	13.765	-	51110	127	-	571264	1270	99.778	0.222

Figure S98. GC spectrum of bio-asymmetric hydrocyanation of **1c** by PcHNL5 variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1c** and *rac*-**3c**. Yellow line: analytical reaction employed by PcHNL5 wild-type. Red line: analytical reaction employed by PcHNL5_{L331A}. Purple line: preparative reaction employed by PcHNL5_{L331A} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3c** from preparative reaction. The detailed data of the peak table was shown as follows.

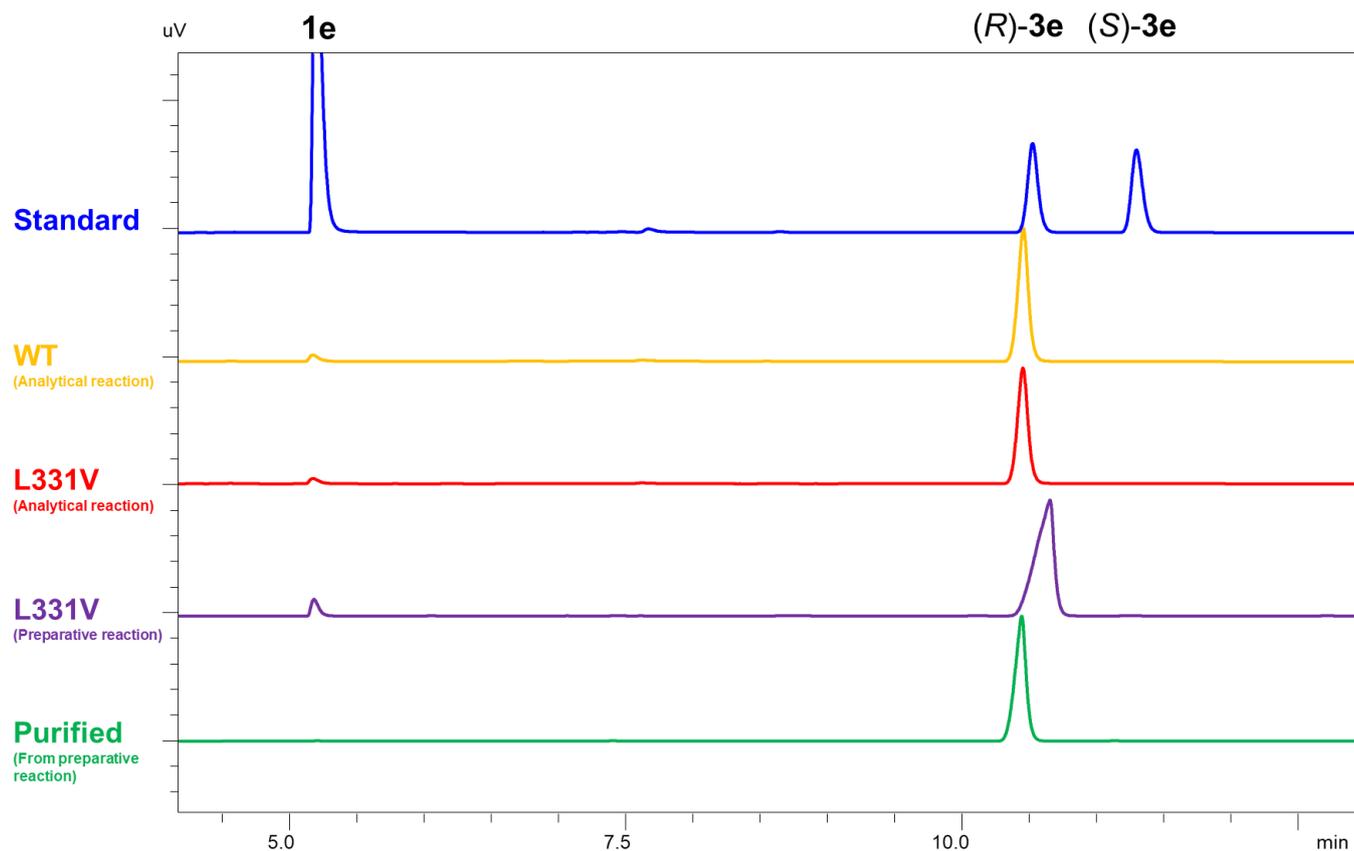
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	7.785	17.479	18.403	38718	8527	8042	169312	80356	80022	50.104	49.896
WT (Analytical reaction)	7.711	17.301	18.214	69241	108	53	348561	1091	539	66.944	33.056
L331A (Analytical reaction)	7.727	17.313	18.218	9110	42491	63	43303	443784	562	99.874	0.126
L331A (Preparative reaction)	7.668	17.165	18.028	2738	34443	73	12110	330629	739	99.777	0.223
Purified (From preparative reaction)	-	17.171	18.014	-	68141	134	-	645887	1317	99.796	0.204

Figure S99. GC spectrum of bio-asymmetric hydrocyanation of **1d** by *PchNL5* variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1d** and *rac*-**3d**. Yellow line: analytical reaction employed by *PchNL5* wild-type. Red line: analytical reaction employed by *PchNL5*_{L331A}. Purple line: preparative reaction employed by *PchNL5*_{L331A} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3d** from preparative reaction. The detailed data of the peak table was shown as follows.

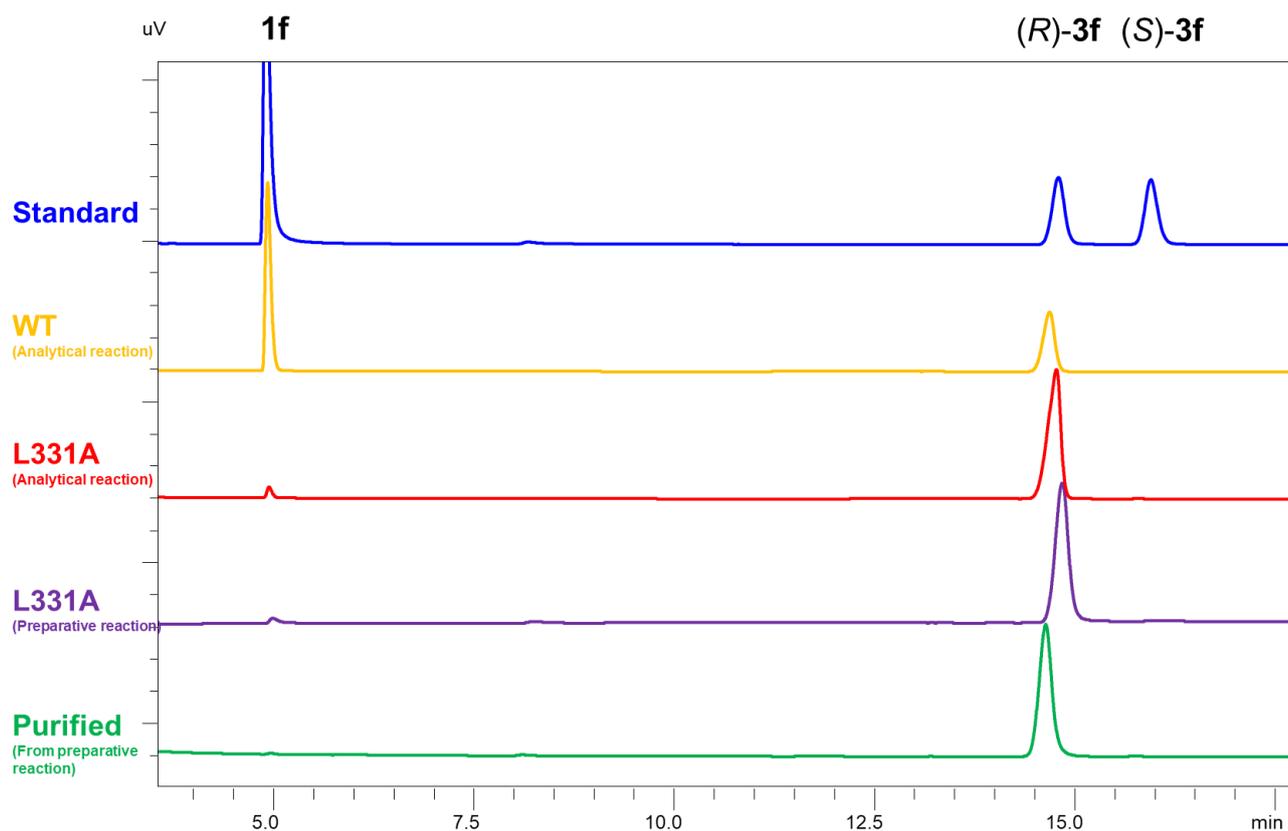
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV-s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	5.200	10.528	11.301	135904	39483	36602	661081	217621	216891	50.092	49.908
WT (Analytical reaction)	5.174	10.460	11.221	5115	104201	202	25471	583413	1117	99.809	0.191
L331V (Analytical reaction)	5.175	10.456	11.224	4222	90598	197	21173	510684	1166	99.772	0.228
L331V (Preparative reaction)	5.143	10.618	11.210	63480	446549	1810	255536	4288376	10259	99.761	0.239
Purified (From preparative reaction)	-	10.347	11.038	-	185170	608	-	1105177	3434	99.690	0.310

Figure S100. GC spectrum of bio-asymmetric hydrocyanation of **1e** by *PcHNL5* variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1e** and *rac*-**3e**. Yellow line: analytical reaction employed by *PcHNL5* wild-type. Red line: analytical reaction employed by *PcHNL5*_{L331V}. Purple line: preparative reaction employed by *PcHNL5*_{L331V} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3f** from preparative reaction. The detailed data of the peak table was shown as follows.

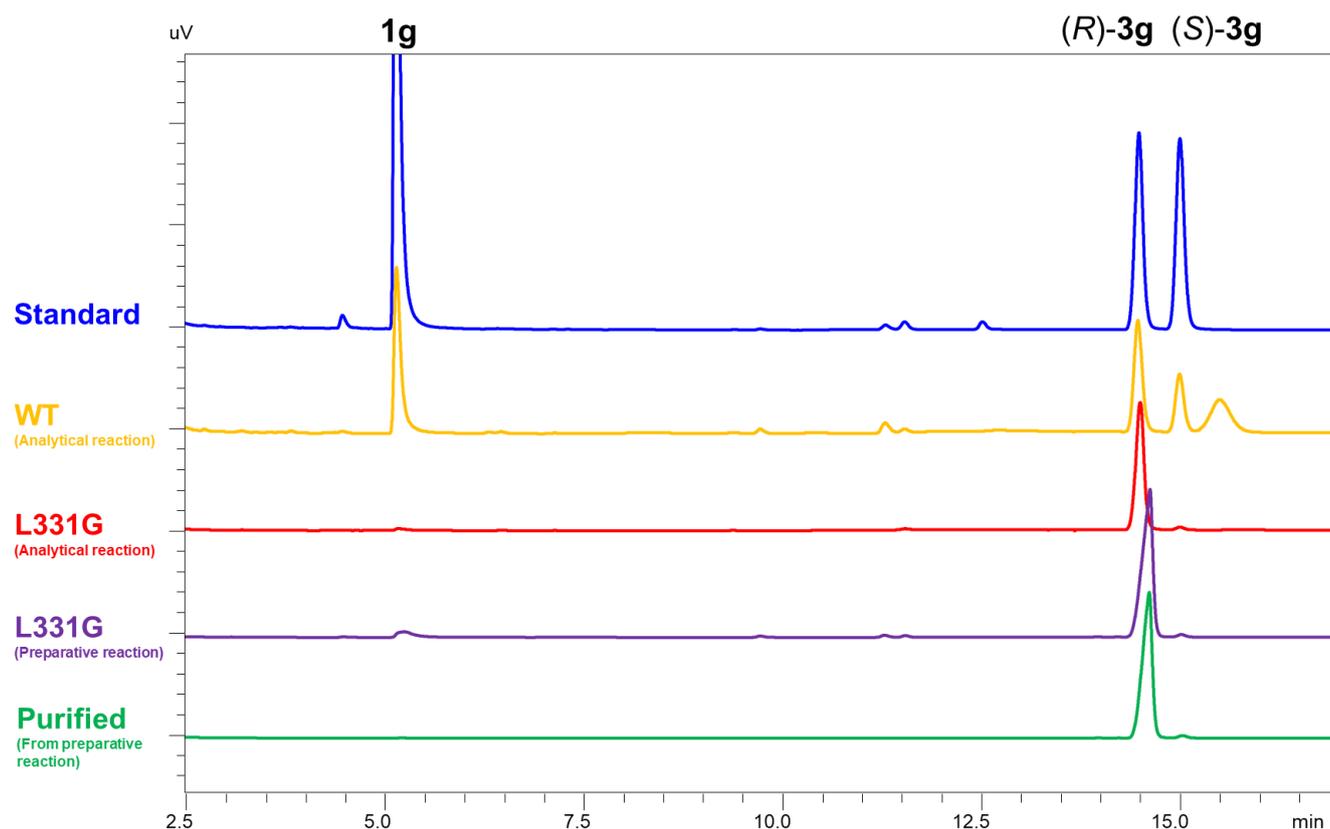
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	5.037	14.928	16.082	92360	20900	20144	583508	229729	228594	50.124	49.876
WT (Analytical reaction)	4.932	14.691	15.764	118735	37714	143	568976	387579	1744	99.552	0.448
L331A (Analytical reaction)	4.950	14.775	15.806	7448	86252	122	33879	982669	1263	99.872	0.128
L331A (Preparative reaction)	5.030	14.880	16.060	1133	32331	138	8395	382264	1698	99.558	0.442
Purified (From preparative reaction)	-	14.641	15.774	-	30739	80	-	355392	869	99.756	0.244

Figure S101. GC spectrum of bio-asymmetric hydrocyanation of **1f** by PcHNL5 variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1f** and *rac*-**3f**. Yellow line: analytical reaction employed by PcHNL5 wild-type. Red line: analytical reaction employed by PcHNL5_{L331A}. Purple line: preparative reaction employed by PcHNL5_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3f** from preparative reaction. The detailed data of the peak table was shown as follows.

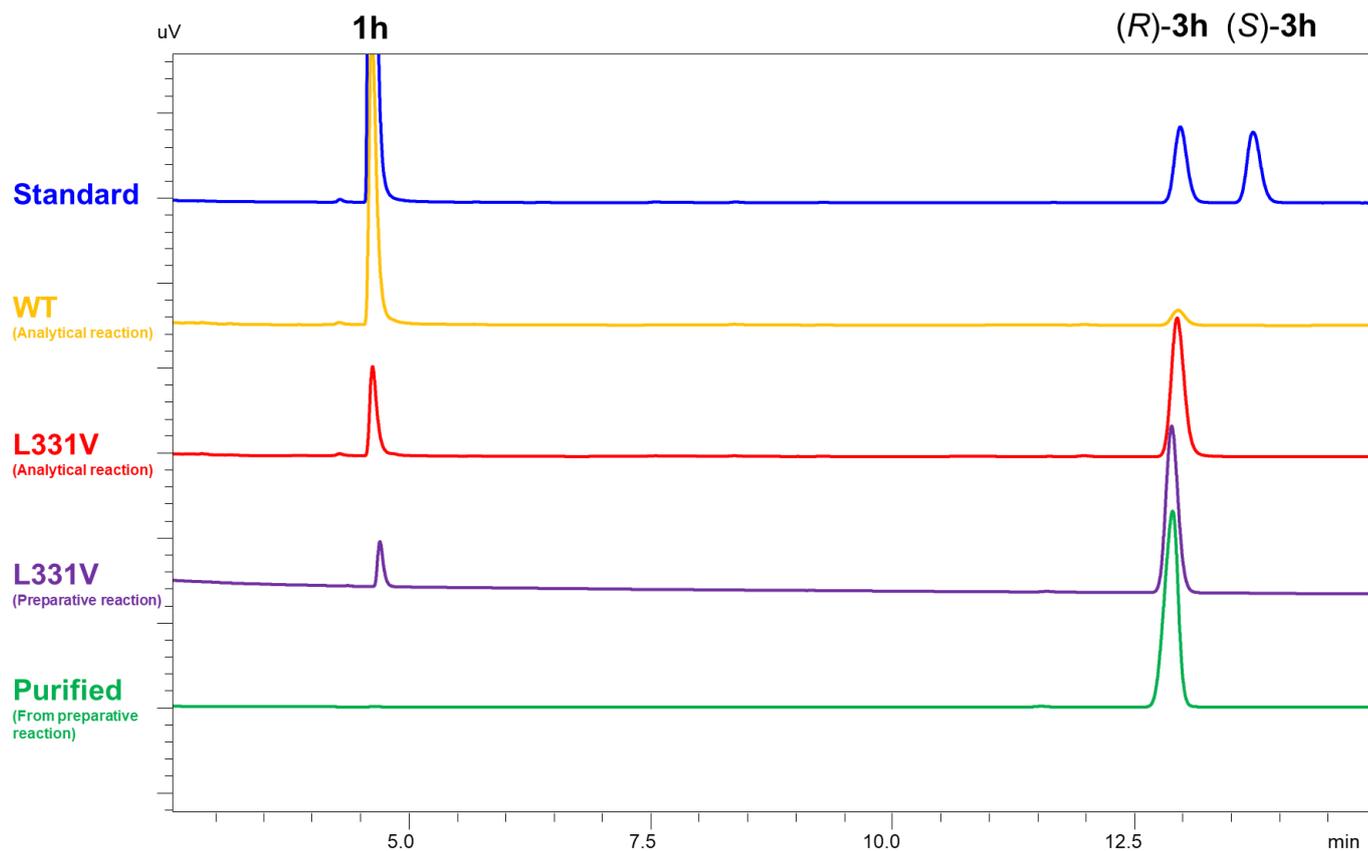
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	5.137	14.485	15.000	83019	29666	28749	513099	217094	216789	50.035	49.965
WT (Analytical reaction)	5.135	14.560	15.034	89930	128758	90429	670640	1047505	652637	61.613	38.387
L331G (Analytical reaction)	5.166	14.499	14.999	809	51655	1180	7976	386319	9133	97.690	2.310
L331G (Preparative reaction)	5.240	14.622	15.016	7466	193089	3645	123973	1792206	29049	98.459	1.541
Purified (From preparative reaction)	-	15.613	15.032	-	167761	2838	-	1390456	21816	98.455	1.545

Figure S102. GC spectrum of bio-asymmetric hydrocyanation of **1g** by *PcHNL5* variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1g** and *rac*-**3g**. Yellow line: analytical reaction employed by *PcHNL5* wild-type. Red line: analytical reaction employed by *PcHNL5*_{L331G}. Purple line: preparative reaction employed by *PcHNL5*_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3g** from preparative reaction. The detailed data of the peak table was shown as follows.

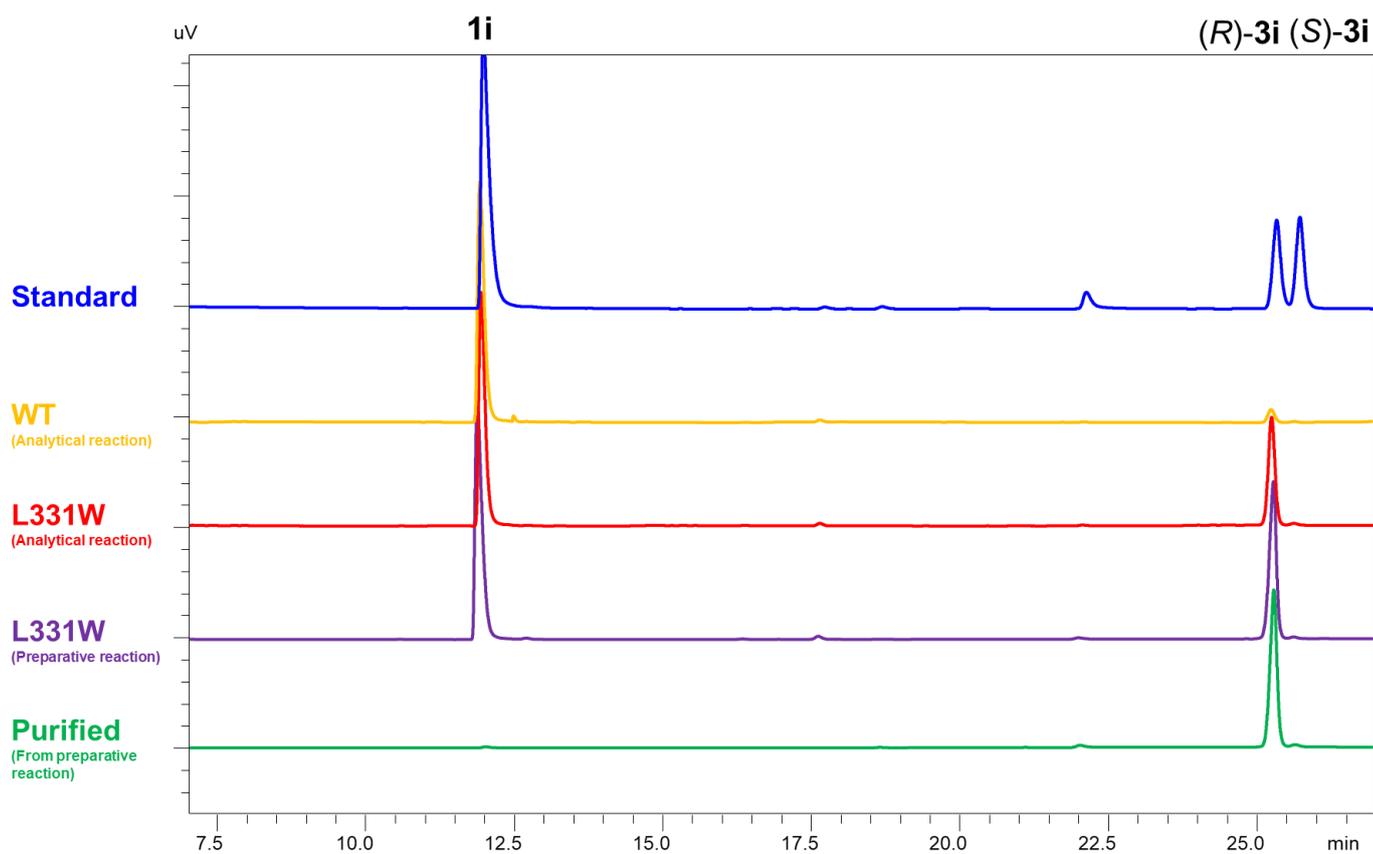
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV-s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	4.611	12.976	13.729	268528	29118	27083	1425519	270701	269022	50.156	49.844
WT (Analytical reaction)	4.620	12.952	13.701	61379	3206	22	333482	32075	185	99.425	0.575
L331V (Analytical reaction)	4.624	12.943	13.769	21492	33400	16	117876	322697	101	99.969	0.031
L331V (Preparative reaction)	4.533	12.719	13.443	13524	50145	38	56579	448330	250	99.944	0.056
Purified (From preparative reaction)	-	12.812	13.494	-	440273	274	-	4453619	2077	99.953	0.047

Figure S103. GC spectrum of bio-asymmetric hydrocyanation of **1h** by PcHNL5 variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1h** and *rac*-**3h**. Yellow line: analytical reaction employed by PcHNL5 wild-type. Red line: analytical reaction employed by PcHNL5_{L331V}. Purple line: preparative reaction employed by PcHNL5_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3h** from preparative reaction. The detailed data of the peak table was shown as follows.

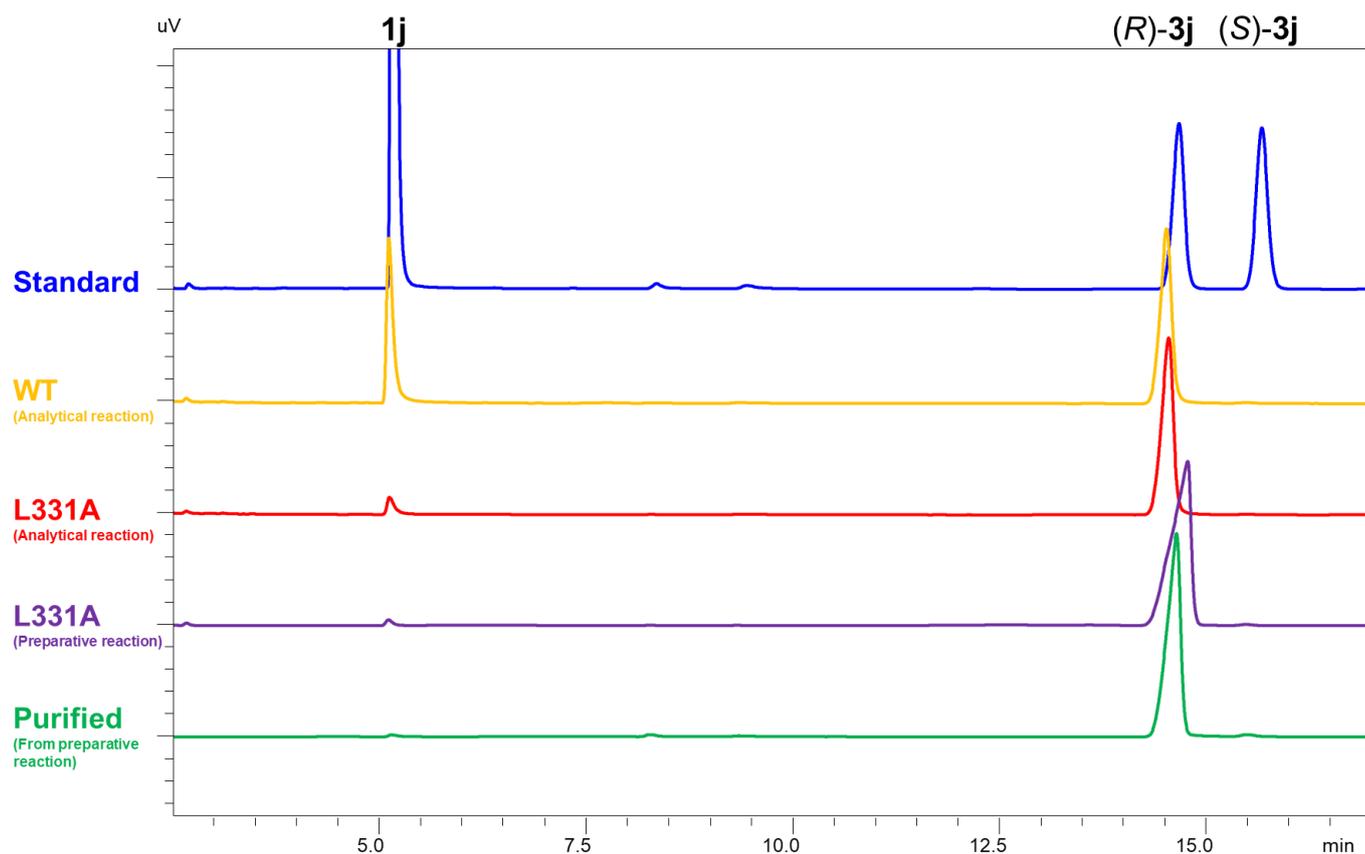
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV-s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	11.982	25.335	25.726	109465	34389	35541	1144461	334214	346651	49.087	50.913
WT (Analytical reaction)	11.923	25.240	25.627	73481	3972	290	583979	32901	2101	93.997	6.003
L331W (Analytical reaction)	11.942	25.249	25.624	52765	24414	382	398989	196075	3208	98.390	1.610
L331W (Preparative reaction)	11.880	25.279	25.618	97168	65128	752	833127	546860	7048	98.727	1.273
Purified (From preparative reaction)	-	25.286	25.642	-	46017	715	-	383873	6440	98.350	1.650

Figure S104. GC spectrum of bio-asymmetric hydrocyanation of **1i** by *PcHNL5* variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1i** and *rac*-**3i**. Yellow line: analytical reaction employed by *PcHNL5* wild-type. Red line: analytical reaction employed by *PcHNL5*_{L331W}. Purple line: preparative reaction employed by *PcHNL5*_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3i** from preparative reaction. The detailed data of the peak table was shown as follows.

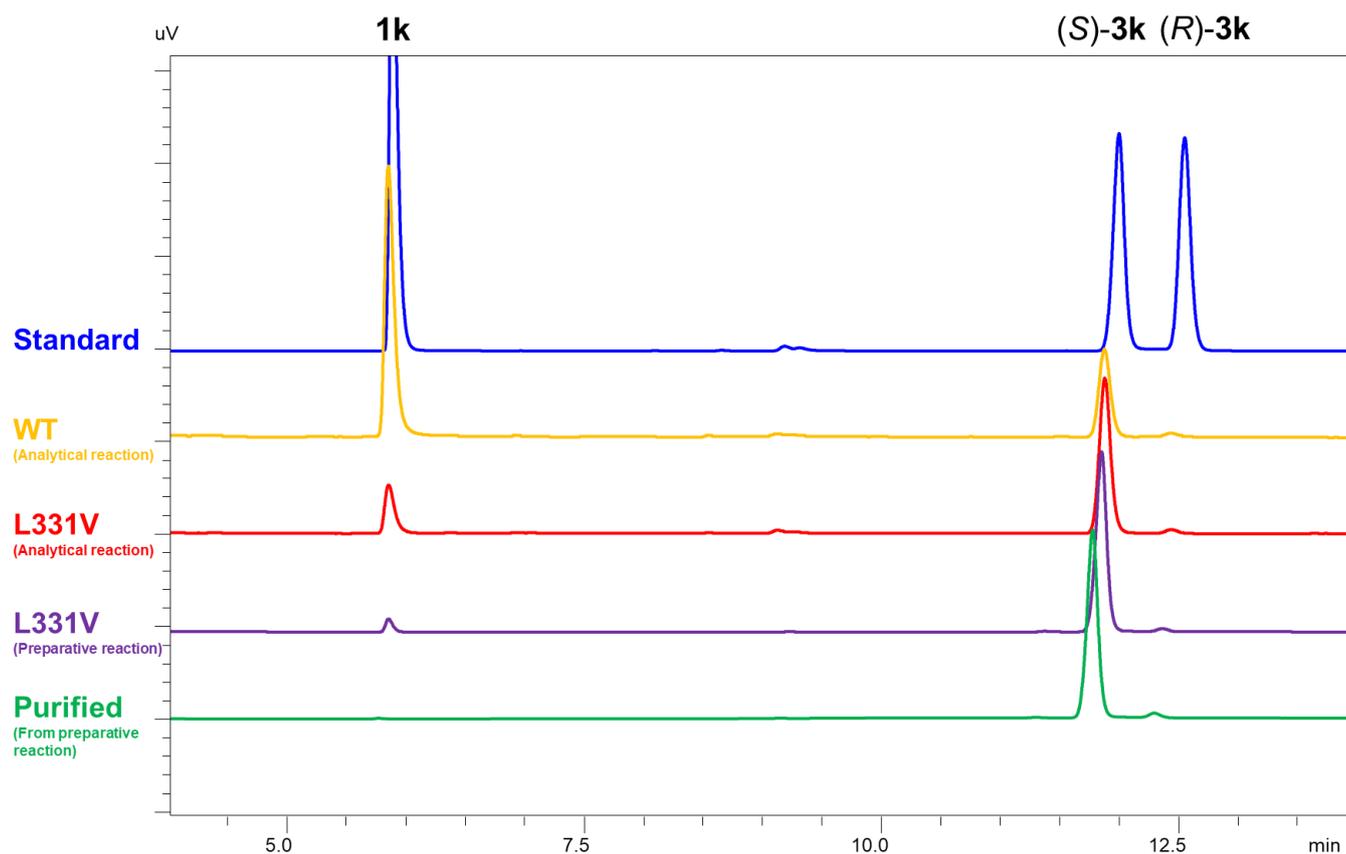
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	5.167	14.680	15.680	238176	39452	38421	1171151	390784	389349	50.092	49.908
WT (Analytical reaction)	5.121	14.529	15.489	55155	58606	241	330625	584864	2603	99.557	0.443
L331A (Analytical reaction)	5.128	14.555	15.490	7724	79026	225	51759	814584	2531	99.690	0.310
L331A (Preparative reaction)	5.115	14.786	15.485	10014	283074	2093	62291	4622219	21542	99.536	0.464
Purified (From preparative reaction)	-	14.651	15.504	-	144102	1322	-	1686958	13644	99.197	0.803

Figure S105. GC spectrum of bio-asymmetric hydrocyanation of **1j** by PcHNL5 variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1j** and *rac*-**3j**. Yellow line: analytical reaction employed by PcHNL5 wild-type. Red line: analytical reaction employed by PcHNL5_{L331A}. Purple line: preparative reaction employed by PcHNL5_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3j** from preparative reaction. The detailed data of the peak table was shown as follows.

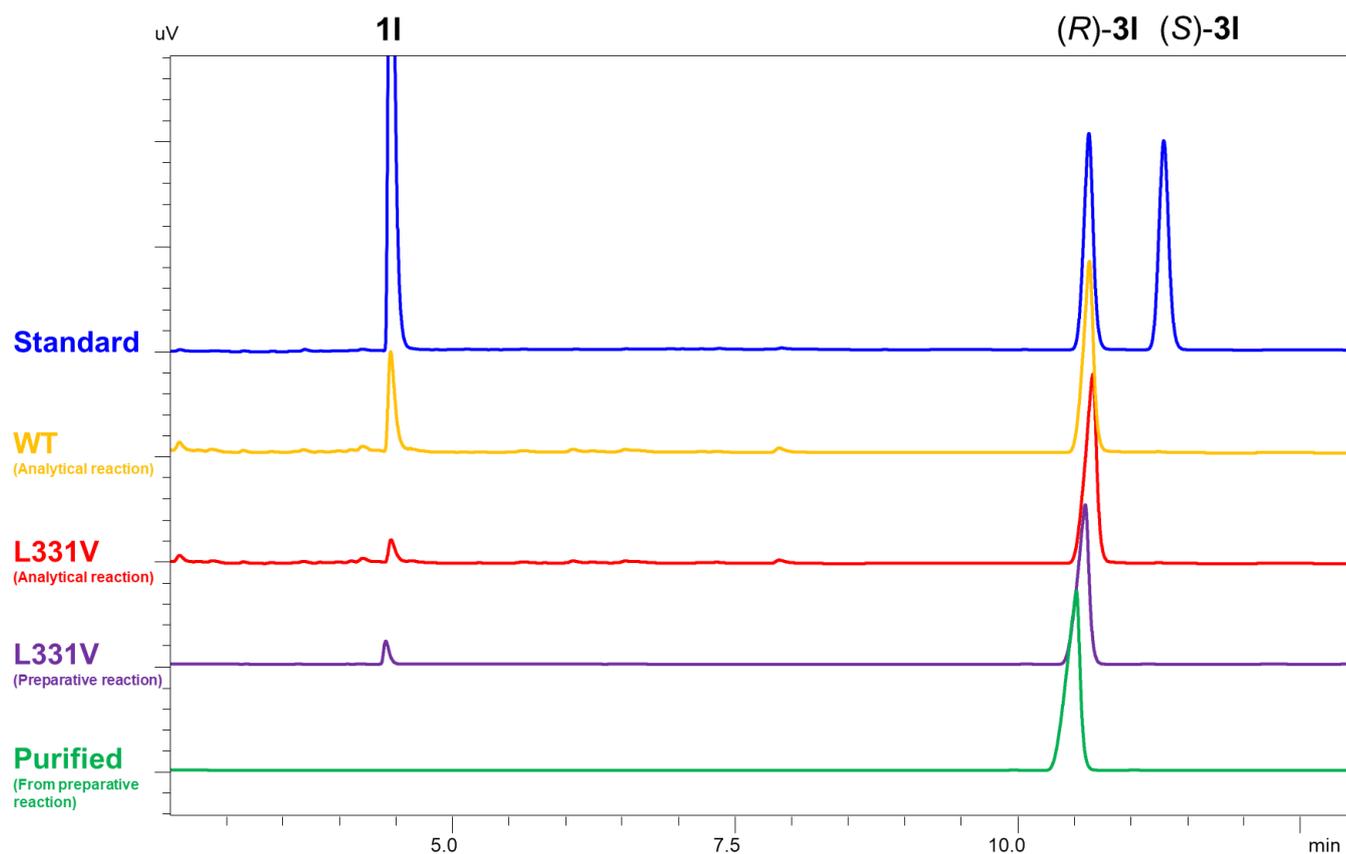
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV-s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	5.892	12.000	12.552	188335	94837	92919	935064	647318	646245	50.041	49.959
WT (Analytical reaction)	5.856	11.877	12.437	26018	8414	297	148343	58430	2063	96.590	3.410
L331V (Analytical reaction)	5.859	11.880	12.439	7869	25184	562	45990	170546	4086	97.661	2.339
L331V (Preparative reaction)	5.746	11.742	12.253	7139	97606	1581	32077	641048	12172	98.137	1.863
Purified (From preparative reaction)	-	11.778	12.295	-	78658	1815	-	518187	11612	97.808	2.192

Figure S106. GC spectrum of bio-asymmetric hydrocyanation of **1k** by *PcHNL5* variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1k** and *rac*-**3k**. Yellow line: analytical reaction employed by *PcHNL5* wild-type. Red line: analytical reaction employed by *PcHNL5*_{L331V}. Purple line: preparative reaction employed by *PcHNL5*_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3k** from preparative reaction. The detailed data of the peak table was shown as follows.

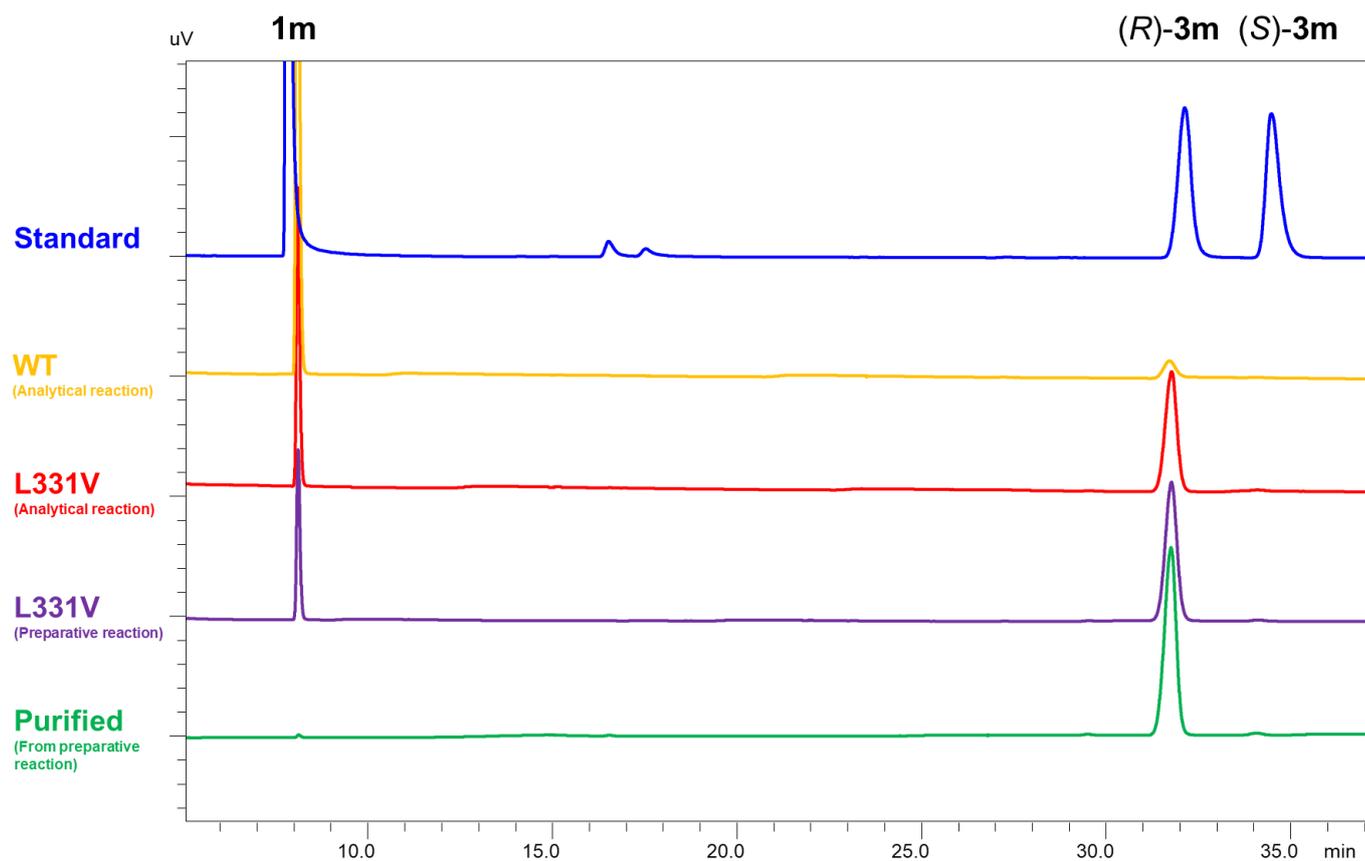
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV-s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	4.458	10.630	11.292	162717	61712	59793	631376	369239	369133	50.007	49.993
WT (Analytical reaction)	4.457	10.634	11.246	63039	122164	494	262823	803867	2967	99.632	0.368
L331V (Analytical reaction)	4.460	10.665	11.249	19416	165640	436	81684	1212761	2529	99.792	0.208
L331V (Preparative reaction)	4.413	10.598	11.152	26957	184819	670	95544	1366999	3984	99.709	0.291
Purified (From preparative reaction)	-	10.502	11.029	-	235804	831	-	2094162	4918	99.766	0.234

Figure S107. GC spectrum of bio-asymmetric hydrocyanation of **11** by *PcHNL5* variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **11** and *rac*-**31**. Yellow line: analytical reaction employed by *PcHNL5* wild-type. Red line: analytical reaction employed by *PcHNL5*_{L331V}. Purple line: preparative reaction employed by *PcHNL5*_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**31** from preparative reaction. The detailed data of the peak table was shown as follows.

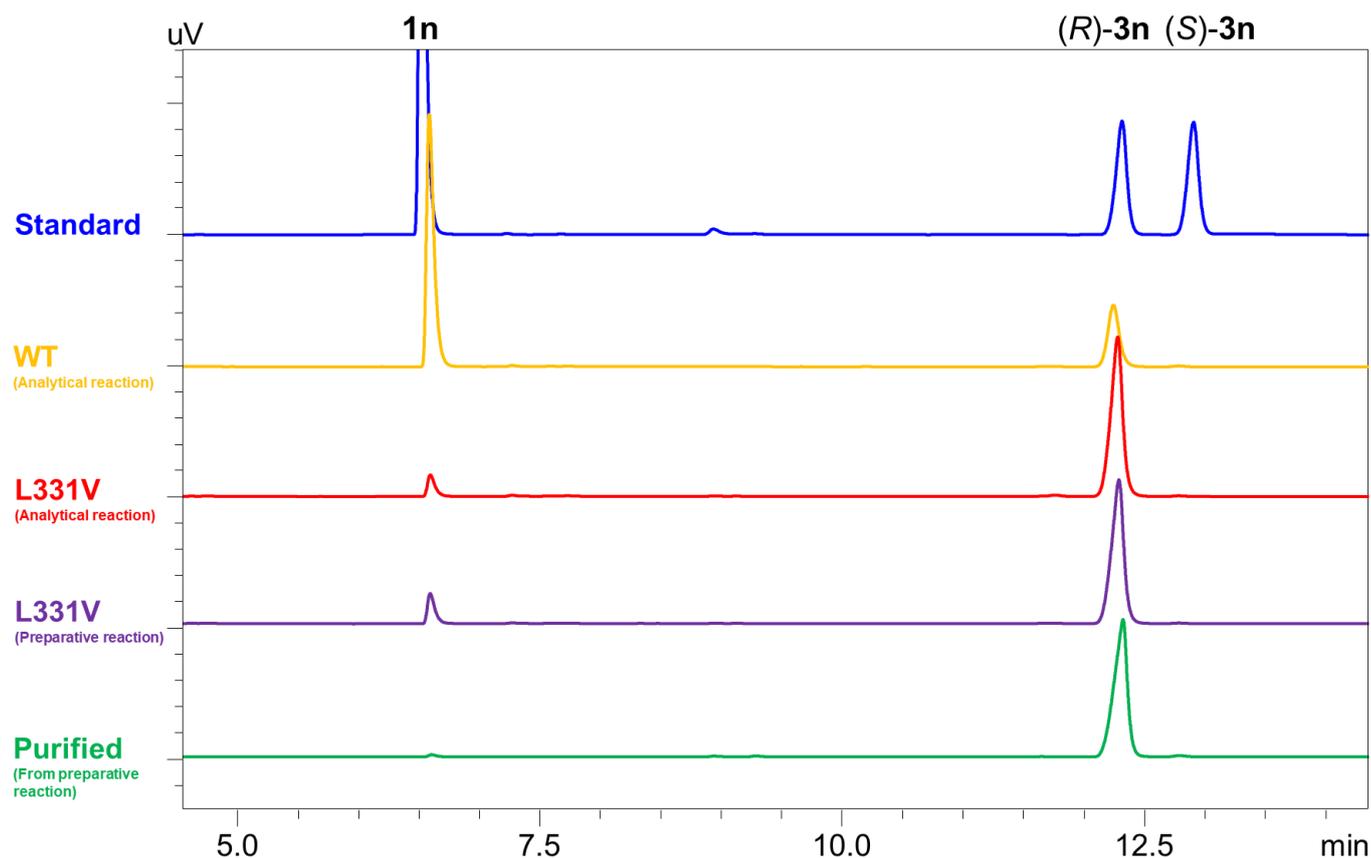
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	8.287	32.605	34.958	257768	36422	34991	2443718	944511	937298	50.192	49.808
WT (Analytical reaction)	8.098	31.725	34.080	149969	3492	98	996418	78788	2478	96.950	3.050
L331V (Analytical reaction)	8.117	31.783	34.096	62263	25049	183	406076	561859	4239	99.251	0.749
L331V (Preparative reaction)	8.125	31.778	34.129	25916	21153	133	166114	475757	2767	99.422	0.578
Purified (From preparative reaction)	-	31.765	34.077	-	34788	289	-	788566	5654	99.288	0.712

Figure S108. GC spectrum of bio-asymmetric hydrocyanation of **1m** by PchNL5 variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1m** and *rac*-**3m**. Yellow line: analytical reaction employed by PchNL5 wild-type. Red line: analytical reaction employed by PchNL5_{L331V}. Purple line: preparative reaction employed by PchNL5_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3m** from preparative reaction. The detailed data of the peak table was shown as follows.

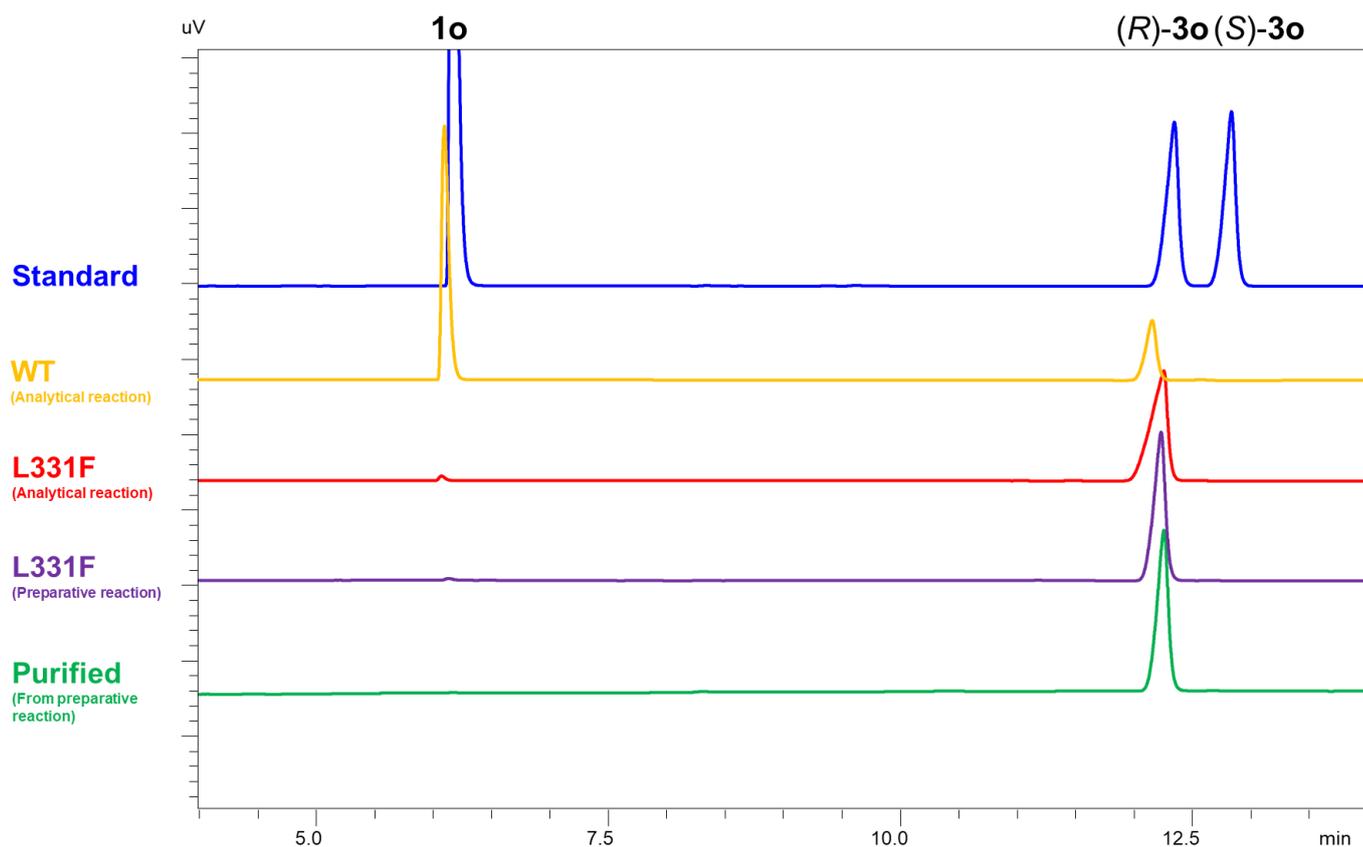
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV·s]		Area [%]		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
Standard	6.710	12.506	13.097	213480	43200	42719	903794	281146	279969	50.105	49.895
WT (Analytical reaction)	6.586	12.245	12.784	128217	31375	533	563901	216107	3544	98.386	1.614
L331V (Analytical reaction)	6.595	12.281	12.784	8655	64702	276	38483	457204	1757	99.617	0.383
L331V (Preparative reaction)	6.594	12.290	12.786	15585	74426	2172	69391	540132	2172	99.599	0.401
Purified (From preparative reaction)	-	12.324	12.789	-	102321	1009	-	801769	6783	99.161	0.839

Figure S109. GC spectrum of bio-asymmetric hydrocyanation of **1n** by *PcHNL5* variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1n** and *rac*-**3n**. Yellow line: analytical reaction employed by *PcHNL5* wild-type. Red line: analytical reaction employed by *PcHNL5*_{L331V}. Purple line: preparative reaction employed by *PcHNL5*_{L331G} acidified fermentation clear portion after high cell density fermentation in 5-L bioreactor. Green line: isolated (*R*)-**3n** from preparative reaction. The detailed data of the peak table was shown as follows.

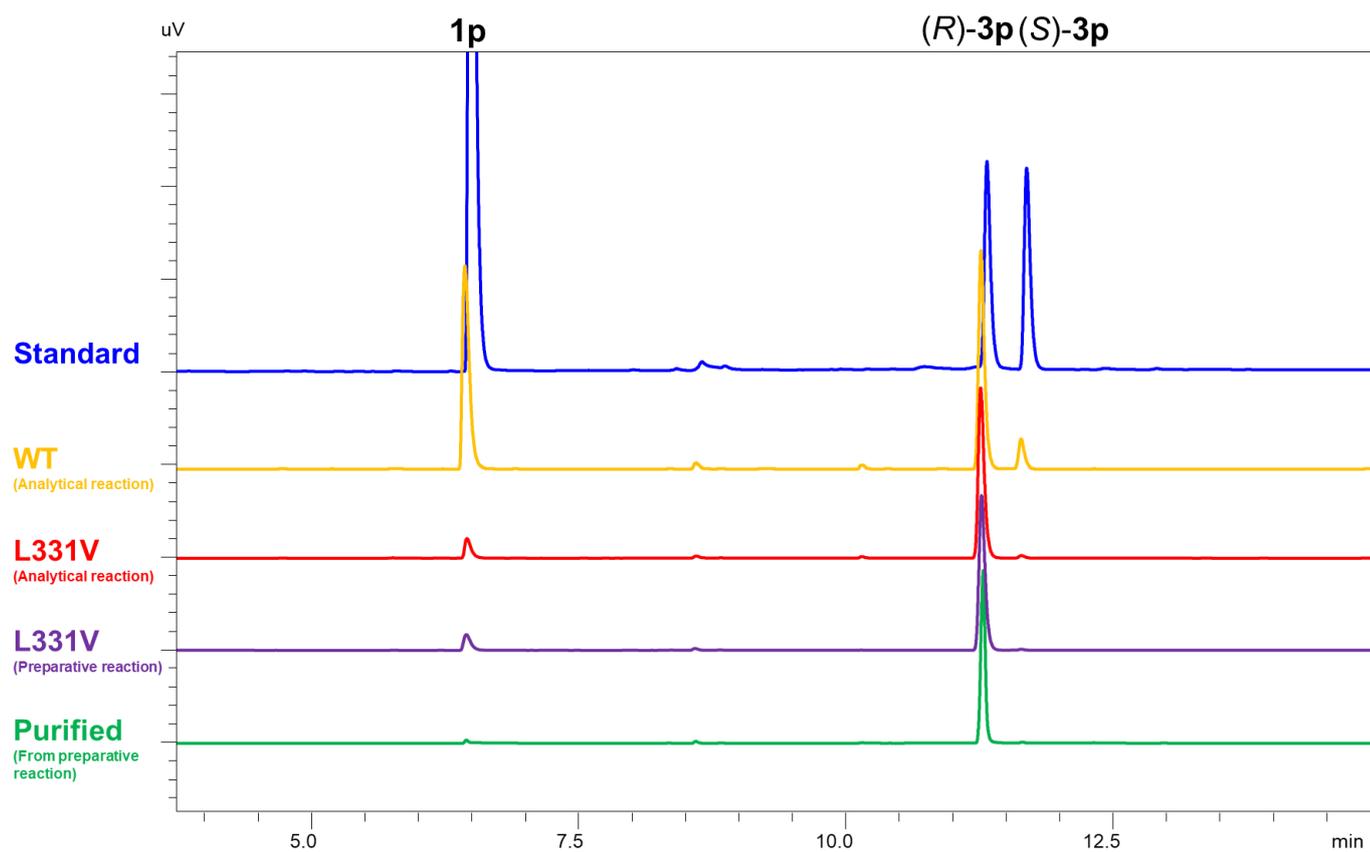
SUPPORTING INFORMATION



Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	
Standard	6.172	12.347	12.836	362146	93407	99545	1693793	699111	700023	49.967	50.033	
WT (Analytical reaction)	6.176	12.267	12.726	128560	39218	985	621557	280076	7814	97.286	2.714	
L331F (Analytical reaction)	6.196	12.361	12.735	807	125878	373	3823	1050903	2762	99.738	0.262	
L331F (Preparative reaction)	6.134	12.233	12.646	797	75887	193	4149	536070	1254	99.767	0.233	
Purified (From preparative reaction)	-	12.257	12.677	-	71589	212	-	492421	1362	99.724	0.276	

Figure S110. GC spectrum of bio-asymmetric hydrocyanation of **1o** by PcHNL5 variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1o** and *rac*-**3o**. Yellow line: reactions employed by PcHNL5 wild-type. Red line: reactions employed by PcHNL5_{L331F}. The detailed data of the peak table was shown as follows.

SUPPORTING INFORMATION

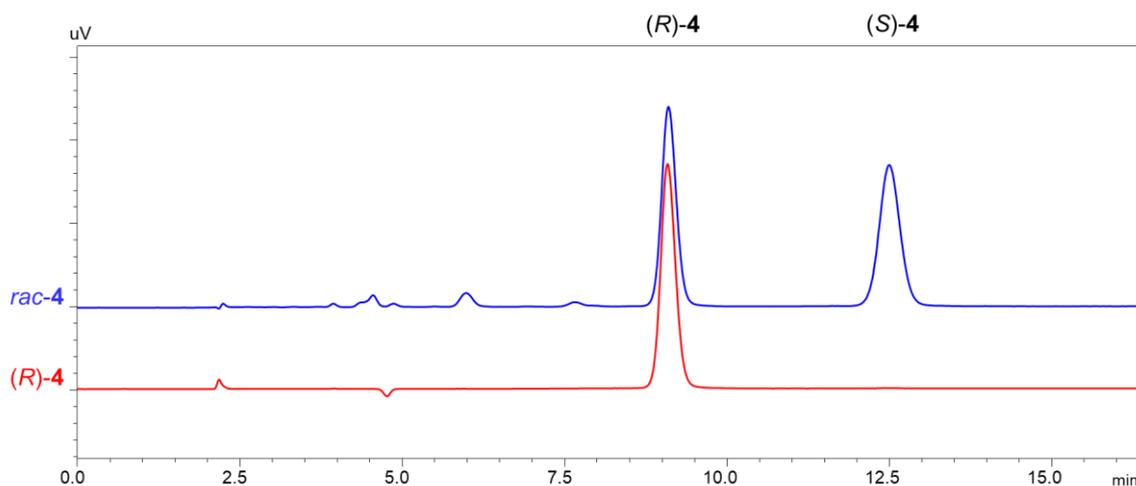


Entry	Ret. time [min]			Height [mV]			Area [mV·s]			Area [%]	
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3	Peak 2	Peak 3
Standard	6.495	11.322	11.693	111920	31222	30368	541133	123549	122884	50.135	49.865
WT (Analytical reaction)	6.436	11.266	11.641	76980	82481	11128	356013	328723	43450	88.325	11.675
L331V (Analytical reaction)	6.456	11.263	11.644	21826	190145	2906	92745	763667	11943	98.460	1.540
L331V (Preparative reaction)	6.449	11.271	11.641	28163	276228	1697	132989	1121858	6976	99.382	0.618
Purified (From preparative reaction)	-	11.285	11.654	9336	485147	2993	-	1442815	8136	99.439	0.561

Figure S111. GC spectrum of bio-asymmetric hydrocyanation of **1p** by PchNL5 variants in MTBE/citrate buffer systems. Cyanohydrin product was *O*-acetylated to corresponding ester **3** before GC analysis. Blue line: aldehyde **1p** and *rac*-**3p**. Yellow line: reactions employed by PchNL5 wild-type. Red line: reactions employed by PchNL5_{L331V}. The detailed data of the peak table was shown as follows.

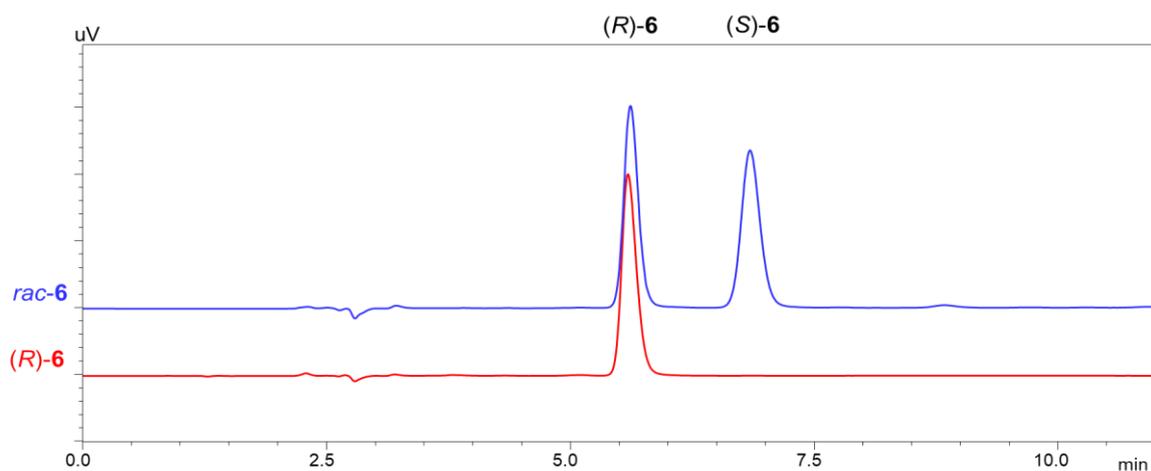
SUPPORTING INFORMATION

2.4 HPLC spectra



Entry	Ret. time [min]		Height [mV]		Area [mV·s]		Area [%]	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
<i>rac</i> -4	9.097	12.496	417.257	294.927	6684.159	6686.040	49.993	50.007
(<i>R</i>)-4	9.083	12.613	509.316	0.541	8214.370	8.969	99.891	0.109

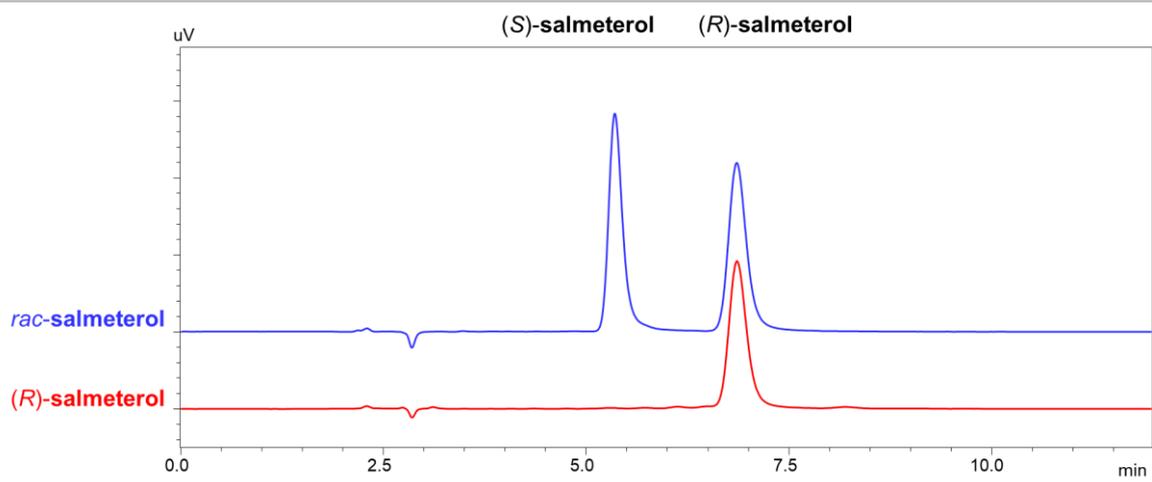
Figure S112. HPLC spectrum of compound **4** by LiAlH₄ reduction of cyanohydrin **2d**. The detailed data of the peak table was shown as follows.



Entry	Ret. time [min]		Height [mV]		Area [mV·s]		Area [%]	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
<i>rac</i> -6	5.613	6.838	331.703	258.514	3519.485	3528.799	49.993	50.007
(<i>R</i>)-6	5.586	6.876	970.595	4.101	10490.941	13.373	99.873	0.127

Figure S113. HPLC spectrum of compound **6** by *N'*-alkylation of compound **4**. The detailed data of the peak table was shown as follows.

SUPPORTING INFORMATION



Entry	Ret. time [min]		Height [mV]		Area [mV·s]		Area [%]	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
<i>rac</i> -salmeterol	5.348	6.852	282.668	218.373	3475.258	3473.765	50.011	49.989
(<i>R</i>)-salmeterol	5.297	6.855	1.861	433.151	21.284	6733.824	0.315	99.695

Figure S114. HPLC spectrum of salmeterol by deprotection of compound **6**. The detailed data of the peak table was shown as follows.

SUPPORTING INFORMATION

2.5 A summary of literatures reporting the synthetic applications of chiral cyanohydrins created in this work

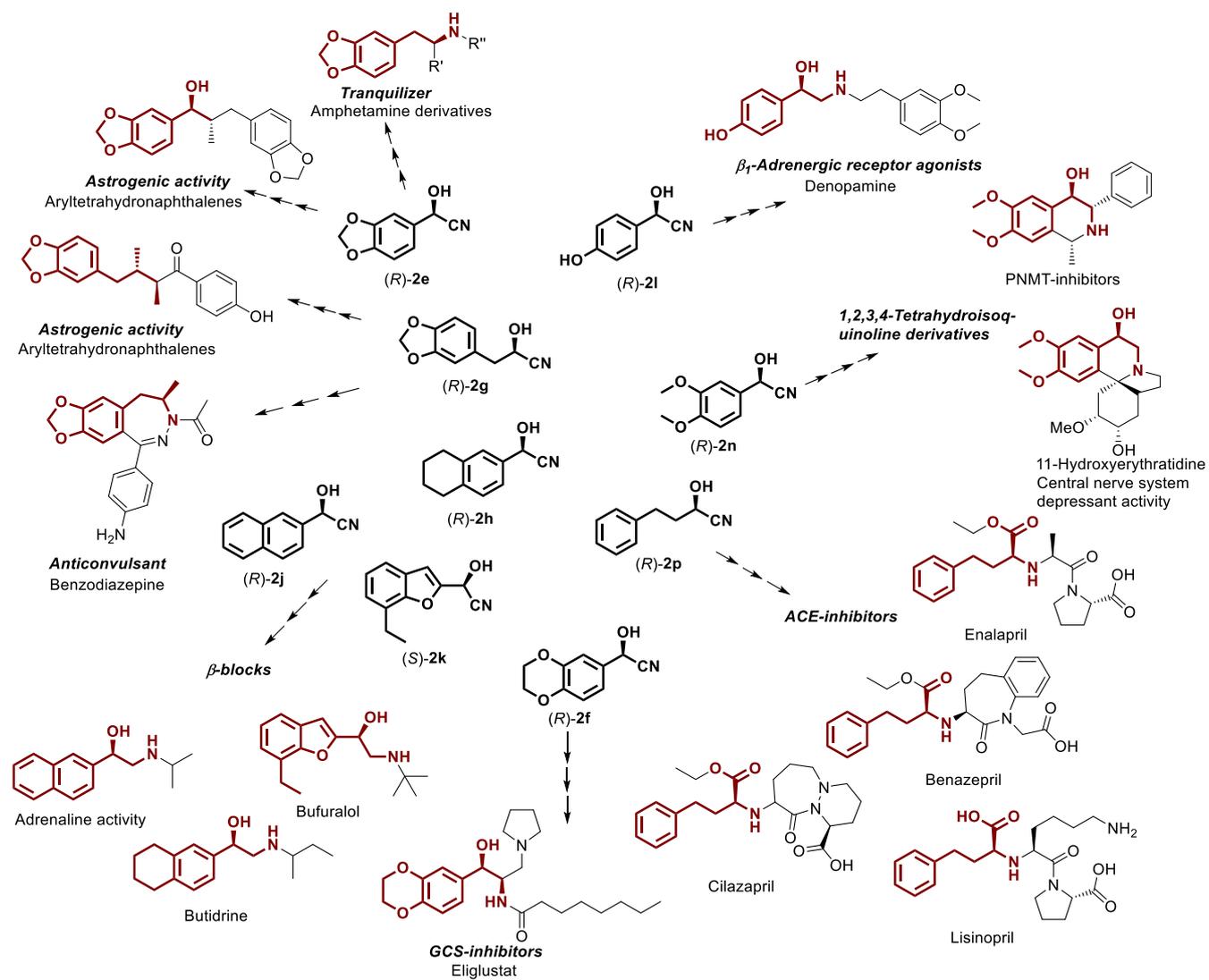


Figure S115. Examples of unnatural cyanohydrins converted by PchNL5 L331 mutant library that have been developed as drugs or presented as precursors in natural products that possess important biological activity.

SUPPORTING INFORMATION

2.6 Kinetic curves of enzymatic reactions

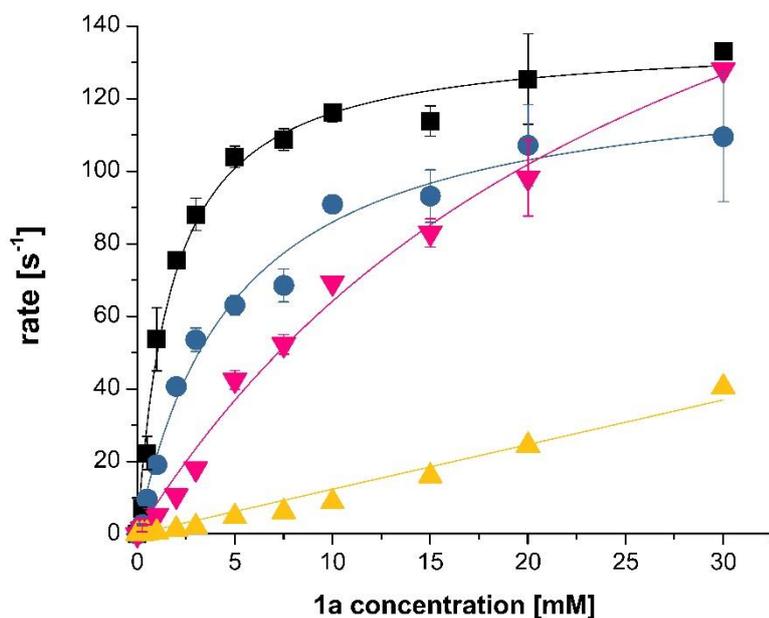


Figure S116. Michaelis-Menten curve for PcHNL5 variants with benzaldehyde (**1a**) as substrate.

PcHNL5 variants: PcHNL5 wild-type (■); L331V (●); L331A (▼); L331G (▲).

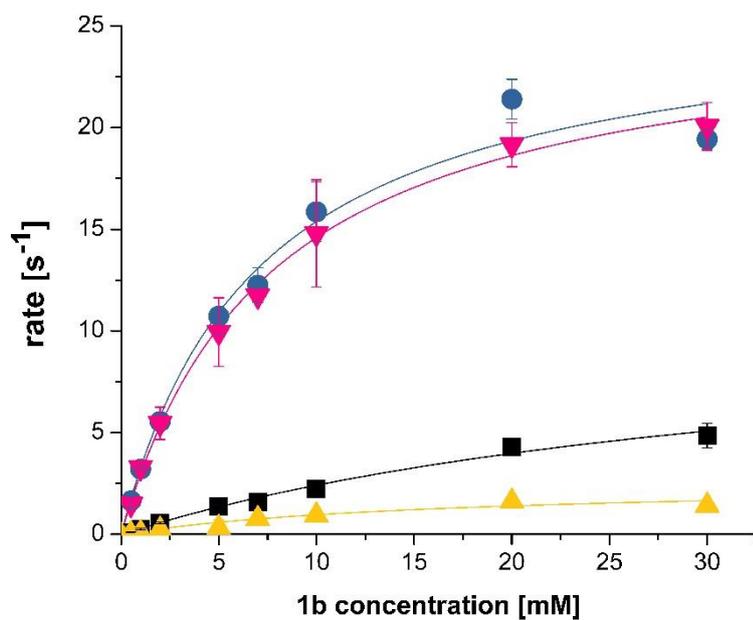


Figure S117. Michaelis-Menten curve for PcHNL5 variants with benzo-aldehyde (**1b**) as substrate.

PcHNL5 variants: PcHNL5 wild-type (■); L331V (●); L331A (▼); L331G (▲).

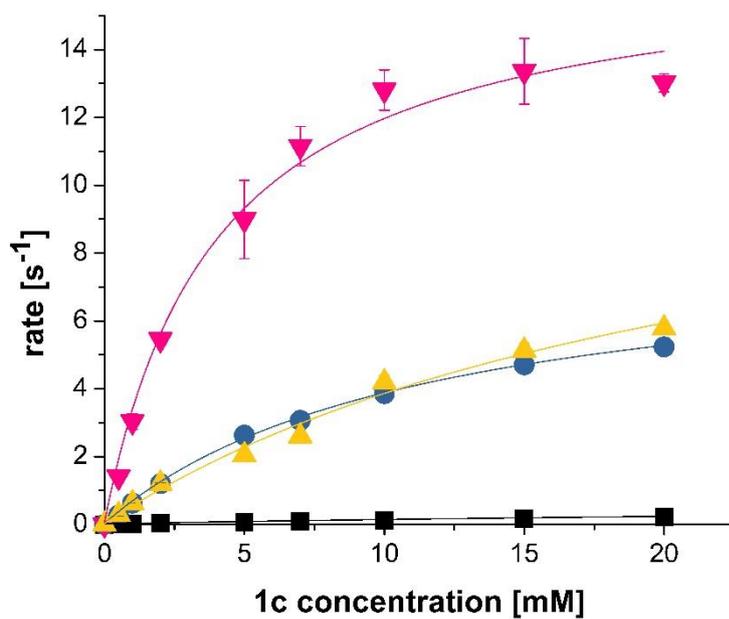


Figure S118. Michaelis-Menten curve for PcHNL5 variants with benzo-aldehyde (**1c**) as substrate.

PcHNL5 variants: PcHNL5 wild-type (■); L331V (●); L331A (▼); L331G (▲).

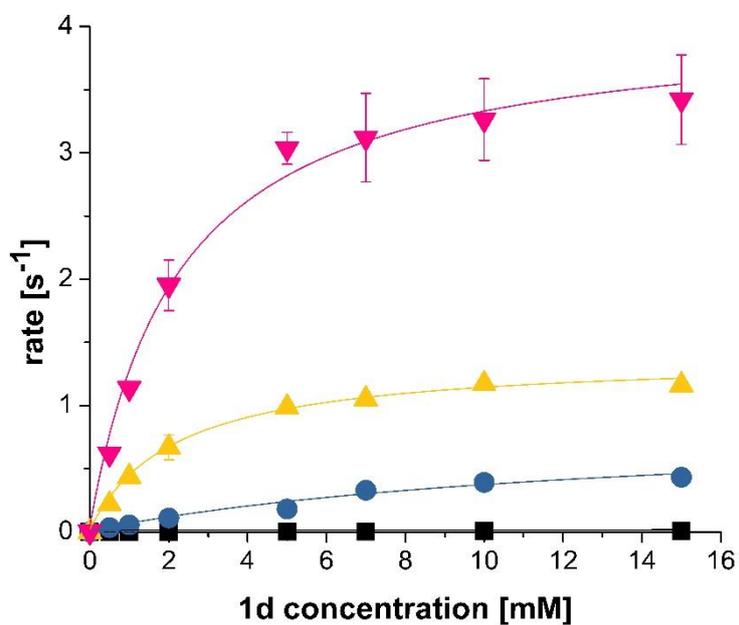


Figure S119. Michaelis-Menten curve for PcHNL5 variants with benzo-aldehyde (**1d**) as substrate.

PcHNL5 variants: PcHNL5 wild-type (■); L331V (●); L331A (▼); L331G (▲).

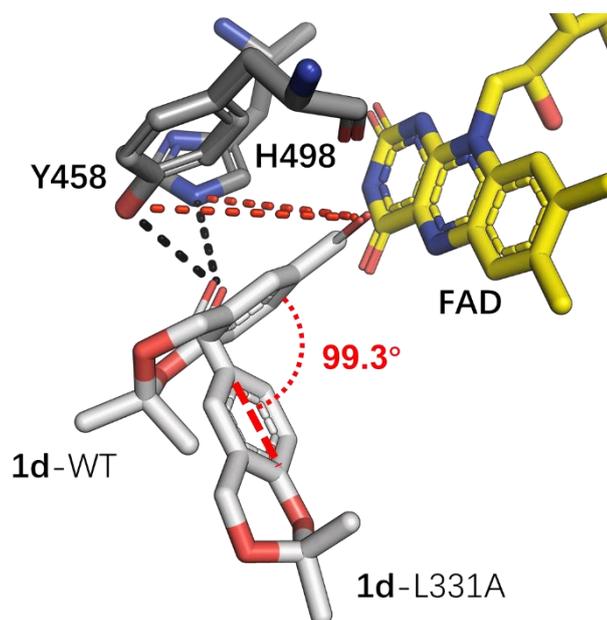


Figure S120. The structural insights of of substrate binding pose of **1d** with *PcHNL5* wild type and mutant L331A, respectively.
The angle between two aromatic planes was highlighted.

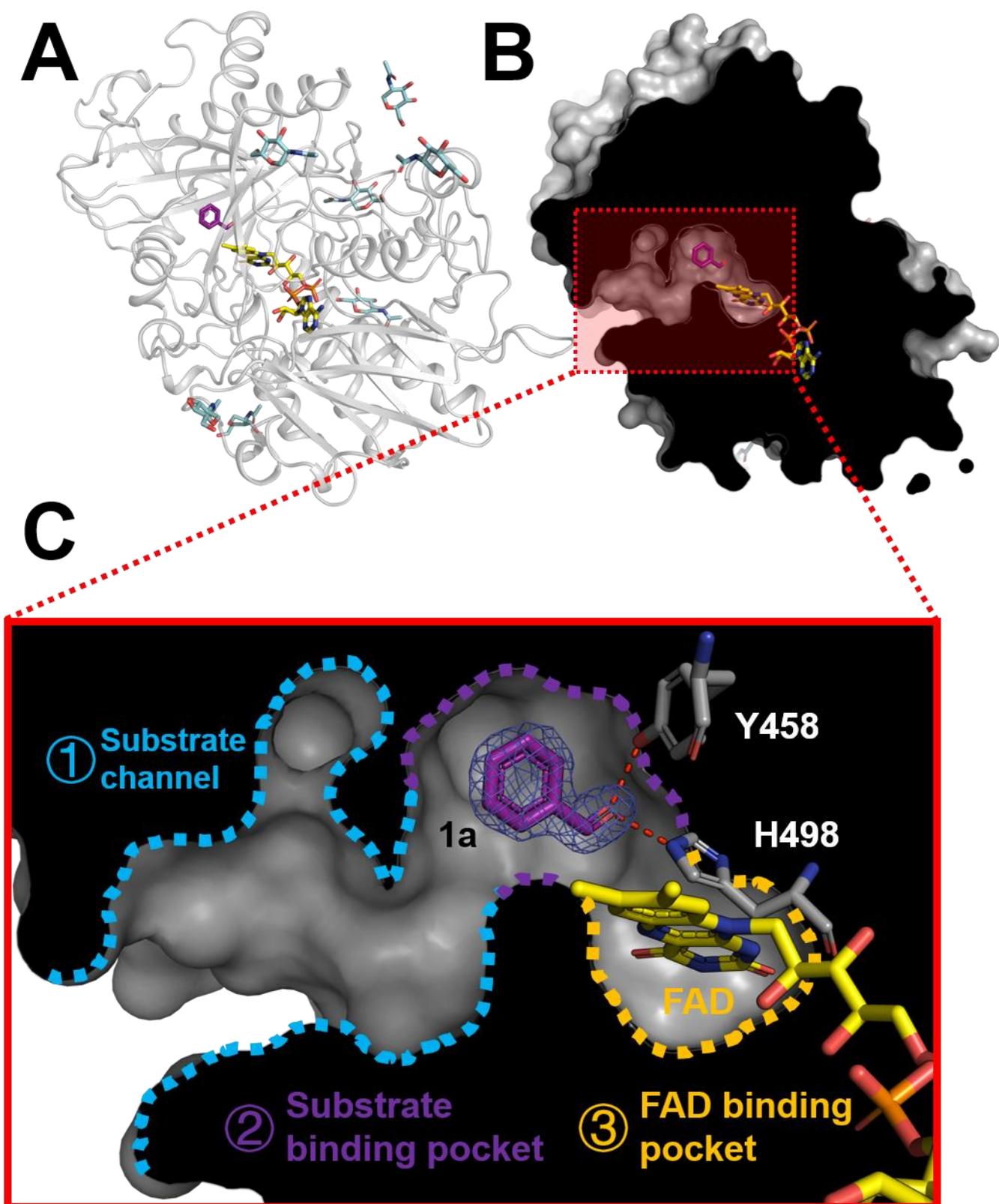


Figure S121. (A) Overall structure of PcHNL5, the bound benzaldehyde (1a), FAD, NAG residues were coloured as purple, yellow, cyan sticks, respectively. (B) sectional view of PcHNL5, high-lightening the deep buried substrate binding pocket and the winding substrate channel. (C) Supervised view of the binding pocket and the substrate channel of PcHNL5, the electron densities correspond to the F_0-F_c and $2F_0-F_c$ maps at levels of 2.5σ and 1σ , respectively.

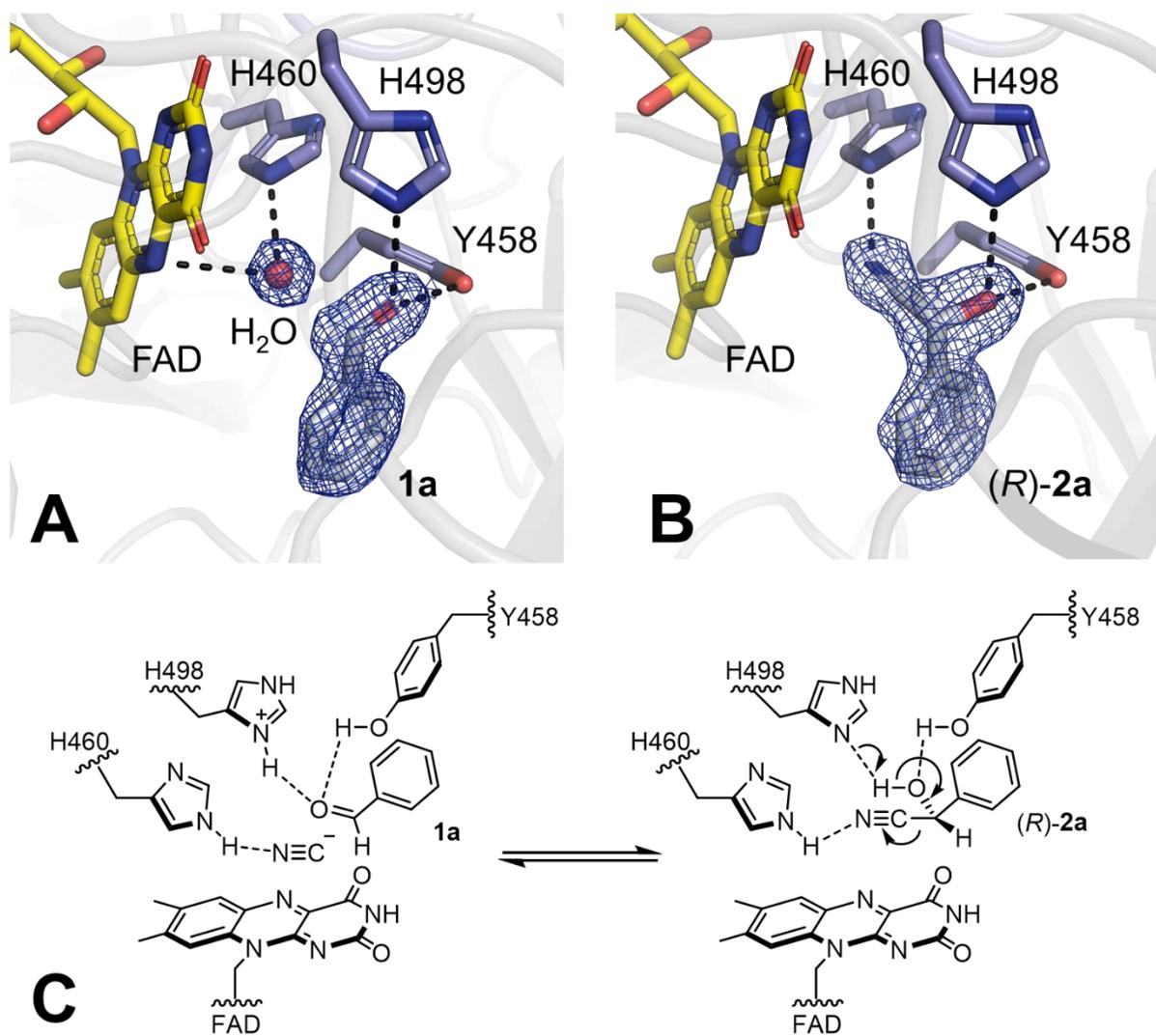


Figure S122. Structural insights of the crystal complex of PcHNL5 wild-type with **1a** (A) and *(R)*-**2a** (B), the hydrogen interactions between ligands in the active pocket were presented as black dashes, the electron densities correspond to the $F_o - F_c$ and $2F_o - F_c$ maps at levels of 2.5σ and 1σ , respectively. (C) Proposed catalytic mechanism of PcHNL5.

SUPPORTING INFORMATION

2.8 High-cell density fermentation curves

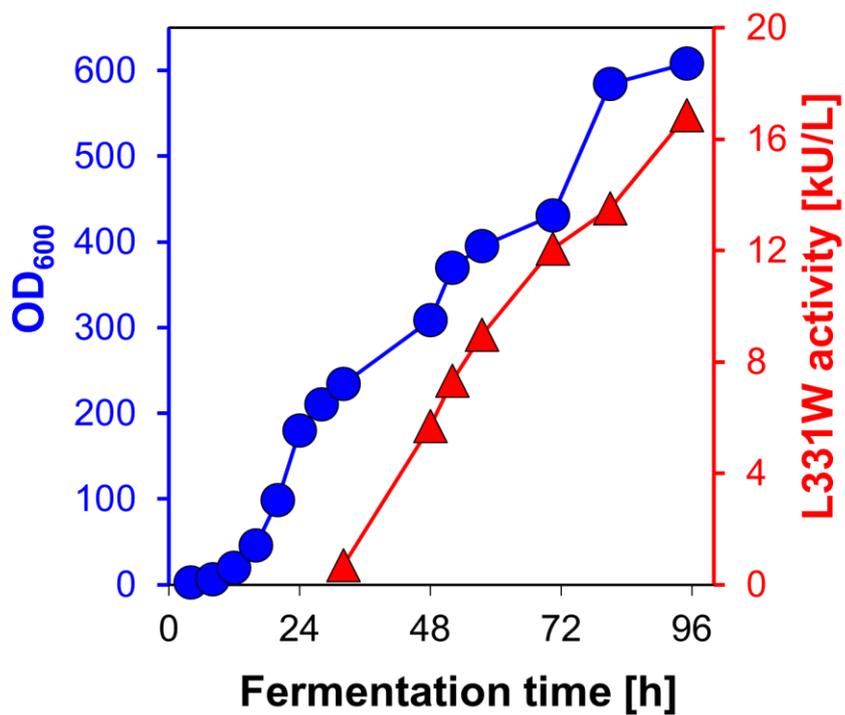


Figure S123. Time course of high-cell-density fermentation using strain *Pichia pastoris* X33/pPICZ α A-PcHNL5_{L331W} in 5-L bioreactor.

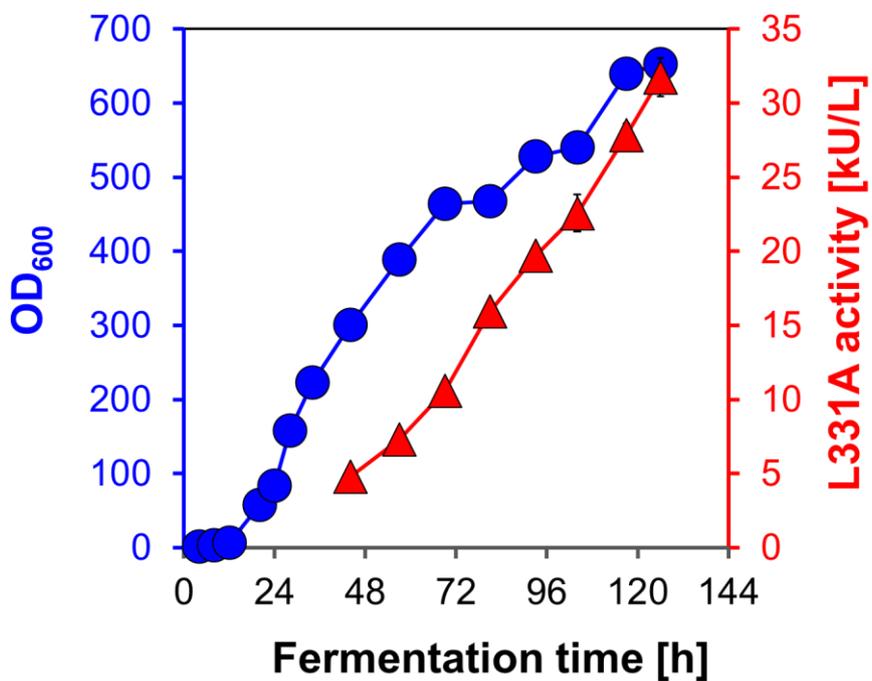


Figure S124. Time course of high-cell-density fermentation using strain *Pichia pastoris* X33/pPICZ α A-PcHNL5_{L331A} in 5-L bioreactor.

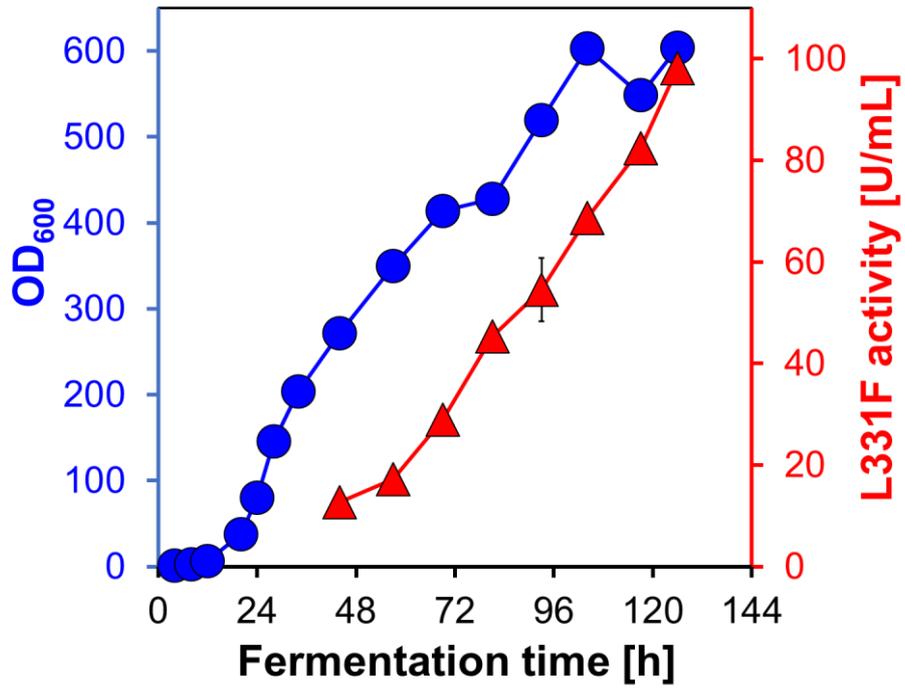


Figure S125. Time course of high-cell-density fermentation using strain *Pichia pastoris* X33/pPICZαA-PcHNL5_{L331F} in 5-L bioreactor.

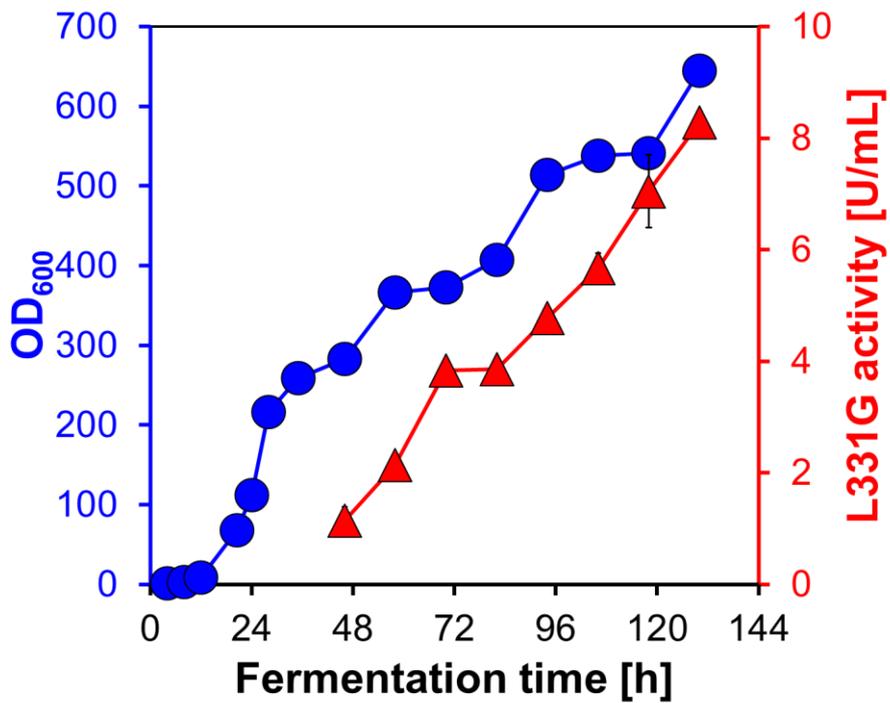


Figure S126. Time course of high-cell-density fermentation using strain *Pichia pastoris* X33/pPICZαA-PcHNL5_{L331G} in 5-L bioreactor.

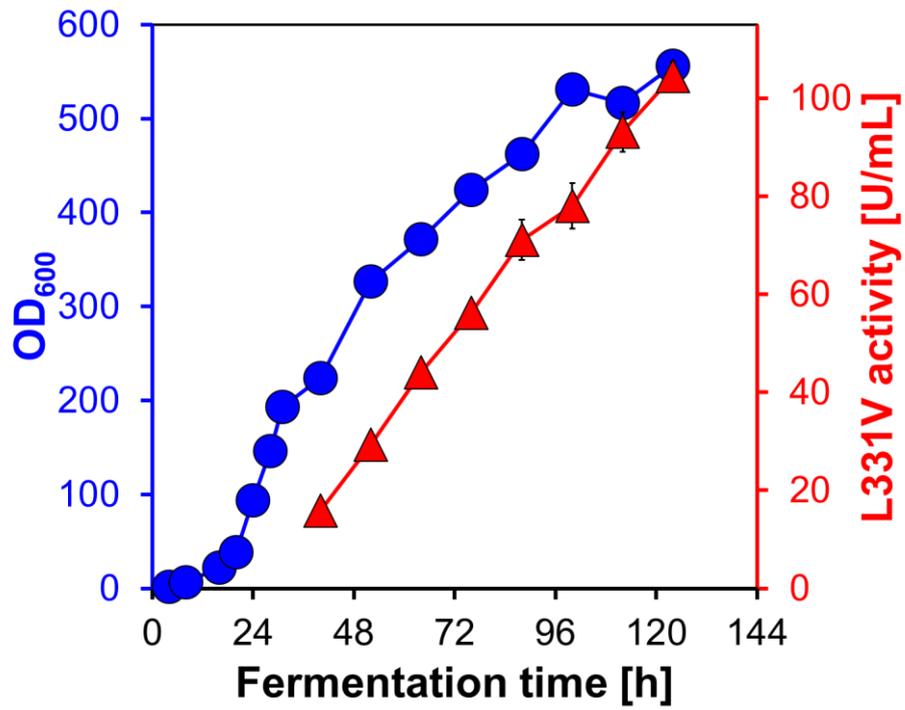


Figure S127. Time course of high-cell-density fermentation using strain *Pichia pastoris* X33/pPICZ α A-PcHNL5_{L331V} in 5-L bioreactor.