Supporting information for

Enantioselective bioreduction of benzo-fused cyclic ketones with engineered *Candida glabrata* ketoreductase 1 - A promising synthetic route to ladostigil (TV3326)

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Supplementary Materials and Methods

General chemicals and equipment

All the compounds (including **4a**, isatin) and biochemical materials were purchased from commercial sources. The ketones **1a-3a** and **5a** were synthesized according to literature methods and characterized by LC-MS and ^{1}H-NMR spectra. The synthetic routes towards other related compounds are as stated in referenced literatures. The racemic alcohol standards were prepared by reduction of ketones with sodium borohydride. The molecular biological reagents were purchased from TaKaRa (Dalian, China) and Tiangen (Beijing, China). HPLC-grade methanol, hexane and 2-propanol were purchased from Dikma (Beijing, China).

T100 Thermal Cycler (Bio-Rad) was used for polymerase chain reaction (PCR). Chiral HPLC analysis was performed on a JASCO LC-1500 system using chiral columns (Daicel Chiral Technologies Co., LTD, Shanghai, China) with a JASCO UV-2075 detector. ^{1}H NMR spectra were obtained at 400 MHz with a Bruker AVANCE-400 spectrometer. The specific optical rotation was tested by an Anton Paar MCP200 modular circular polarimeter.

Expression of CgKR1

To express of CgKR1 with His_6-tag, its gene was amplified from vector pET-22b-CgKR1 (without His_6-tag) using primers CgKR1_Nde I_F and CgKR1_Xho I_R (**Table S1**). After digestion by *Nde* I and *Xho*

I, the DNA fragment was ligated to vector pET-22b that digested by the same enzymes. Expression of CgKR1 was accomplished using *E. coli* strain Rosetta2 (DE3). An overnight culture of *E. coli* harboring the expression vector was inoculated into 1 L LB medium, containing 100 μg mL^{-1} ampicillin. The cells were grown to an OD600 nm of 0.8-1.0, and then chilled on ice for 0.5 h before induction with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). Expression of CgKR1 was performed at 20 °C for 20 h. The cells were harvested by centrifugation at 4000 rpm and 4 °C for 30 min. Cell pellets were washed twice by 0.9 % NaCl solution, resuspended in PBS buffer (100 mM, pH 6.0).

Molecular docking

Docking calculations were performed using AutoDock Vina 1.1.2 program.[1] For the docking algorithm, the ligand and residues Tyr175, Ser134 were set as flexibility while the other of protein were set as rigid. The grid box dimensions and center were set as follows: center_x, y, z = 19.898, 8.897, 112.426, size_x, y, z = 20, 20, 20. Docking runs were carried out using the standard parameters of the program and docking results were sorted by the lowest binding energy of the most populated cluster in cases of convergence. Substrates docked with the lowest energies were chosen for analyses.

The optimized homology model structure of CgKR1 with a closed conformation based on the crystal structure of NADPH-complex Gre2 (PDB ID: 4PVD), which was proved to be useful and sufficient as a basis for further analyses,[2] was applied in this study.

Site-directed mutation

The mutations at the corresponding sites were created by the overlap extension PCR method[3] with the template plasmid pET22b containing the wild-type CgKR1 gene. The primers were listed in **Table S1**. The 1st step PCR was carried out with ExTaq DNA polymerase (TaKaRa) under the following conditions: started at 95 °C for10 min, followed by 30 cycles: 94°C for 30 s, 45°C for 30s, 72 °C for 1 min, with a final extension at 72 °C for 10 min. Then the purified gene fragments were extended by primers CgKR1_Nde I_F and CgKR1_Xho I_R using Taq DNA polymerase (TaKaRa). And the PCR program was: 94ºC for 10 min followed by 30 cycles: 94 ºC for 30 s, 45 ºC for 30 s, 72ºC for 1 min, and finally 72 ºC for 10 min. The gene fragments were purified and digested with *Nde* I and *Xho* I and then ligated to the vector pET-22b and transformed into *E. coli* Rosetta2 (DE3) cells. Expressions of the mutants were performed similarly as the WT of CgKR1.

Coexpression of CgKR1 variants and GDH

The genes of GDH and CgKR1 variants were amplified by PCR with the template plasmids pET22b containing the GDH gene and CgKR1 mutants' gene, using primers CgKR1_Nde I_F/CgKR1_Xho I_R and GDH_Nco I_F/GDH_Hind III_R respectively. The recombinant plasmid pRSFDuet-1-GDH was constructed by inserting GDH gene between the *Nco* I and *Hind* III sites of pRSFDuet-1 plasmid. Then the genes of CgKR1 mutants were ligated into pRSFDuet-1-GDH between *Nde* I and *Xho* I sites to generate the recombinant plasmid pRSFDuet-1-GDH-CgKR1 mutants. The recombinant plasmids pRSFDuet-1-GDH-CgKR1 mutants were transformed into *E. coli* Rosetta2 (DE3) cells. The expression of CgKR1 mutants and GDH in one cell were similar as the expression of CgKR1. The cells were harvested by centrifugation (4000 rpm, 10 min), the medium was decanted, and the cells were resuspended in 8% sucrose solution (W/V = 1 : 17), shock-frozen (liquid nitrogen) and lyophilized. Before use, the lyophilized cells were rehydrated in PBS buffer (100 mM, pH 6.0).

Synthesis of ketone 1a-3a and 5a

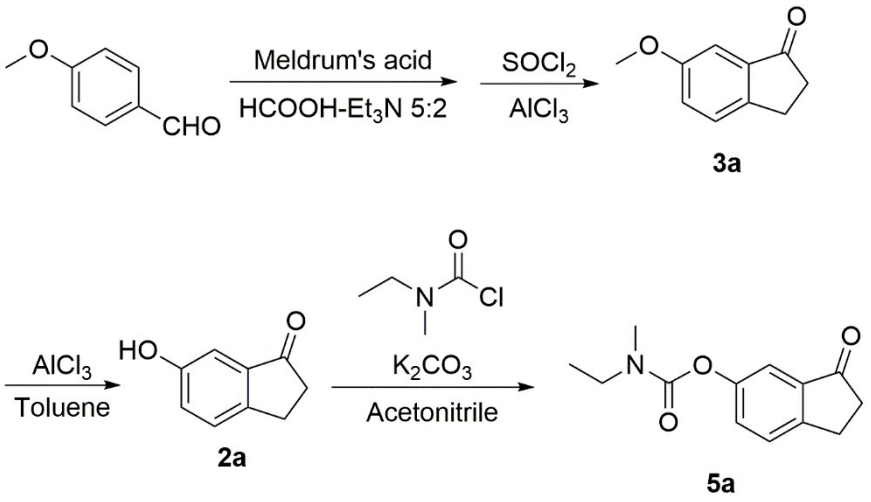


Figure S1. Synthesis of **2a, 3a and 5a**.

2,3-Dihydro-1*H*-inden-1-one (1a).

1a was prepared using 3-phenylpropionic and polyphosphoric acid, as reported in our previous work (yield: 98.2%).[4] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.77 (d, *J* = 7.6 Hz, 1H), 7.59 (td, *J* = 7.5, 1.2 Hz, 1H), 7.49 (d, *J* = 7.6 Hz, 1H), 7.38 (t, *J* = 7.2 Hz, 1H), 3.21 – 3.10 (m, 2H), 2.74 – 2.66 (m, 2H).

6-Methoxy-2,3-dihydro-1*H*-inden-1-one (3a).

A mixture of *p*-methoxybenzaldehyde (34.0 g, 0.25 mol), Meldrum's acid (36.0 g, 0.25 mol), triethylammonium formate (70 mL, prepared from triethylamine (41.7 mL, 0.30 mol) and formic acid (28.3 mL, 0.75 mol)), and dry DMF (50 mL) was stirred at room temperature overnight. The reaction mixture was then refluxed at 100℃ for 2 h, cooled to room temperature and poured into 750 mL water, which was then adjusted to pH 2 with 2N aqueous HCl. After stirred in an ice bath for 10 minutes, the solid was collected by filtration, washed twice with cold water. The filtrate was added to 500 mL water and the mixture was adjusted to pH 8~9 with 30% NaOH before treated with activated charcoal. 3-(4-Methoxyphenyl)propanoic acid was obtained via acidification (pH 2, adjusted with 2N aqueous HCl), filtration, washing with cold water and drying (white solid, 37.1 g, 82.4% based on *p*-methoxybenzaldehyde), mp. 102~103℃.

To a stirred solution of 3-(4-methoxyphenyl)propanoic acid (18 g, 0.1 mol) in 500 mL dry CH_2Cl_2, 80 mL thionyl chloride (0.78 mol) was added dropwise in a cold bath, along with 1 mL dry DMF. The reaction mixture was heated at reflux for 2 h. The solvent and the excess of $SOCl_2$ were distilled off under reduced pressure and the residue was diluted with dry CH_2Cl_2 (500 mL). To the mixture, $AlCl_3$ (15 g, 0.11 mol) was slowly added in batches at 0-5℃, and then the suspension obtained was stirred at room temperature for 1 h. After that, the suspension was poured into 1000 mL cold saturated brines under stirring. The organic layer was separated and the aqueous layer was extracted by CH_2Cl_2 (300 mL × 2). Then the combined organic layer was washed by 3% NaOH (1000 mL× 2) and saturated brine (1000 mL× 1) in turn, and dried over Na_2SO_4. After the solvent was evaporated, the crude product was purified by recrystallization from mathanol to obtain compound **3a** (12.3 g, 75.6% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.40 – 7.35 (m, 1H), 7.22 – 7.16 (m, 2H), 3.84 (s, 3H), 3.11 – 3.04 (m, 2H), 2.75 – 2.69 (m, 2H).

6-Hydroxy-2,3-dihydro-1*H*-inden-1-one (2a).

6-Hydroxy-2,3-dihydro-1*H*-inden-1-one was prepared with **3a** and $AlCl_3$ as reported in literature[5], and purified by recrystallization from mathanol with a yield of 80.5%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.76 (s, 1H), 7.38 (d, J = 8.2 Hz, 1H), 7.10 (dd, J = 8.2, 2.5 Hz, 1H), 6.93 (d, J = 2.4 Hz, 1H), 3.02 – 2.92 (m, 2H), 2.65 – 2.56 (m, 2H).

***N*-Ethyl-*N*-methyl-2-(3-oxo-2,3-dihydro-1*H*-inden-5-yl)acetamide (5a).**

5a was prepared using a literature method[6] (white crystals, 83.5% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.50 – 7.42 (m, 2H), 7.36 (dt, J = 8.5, 2.5 Hz, 1H), 3.45 (dp, J = 28.1, 7.0 Hz, 2H), 3.17 – 2.95 (m, 5H), 2.78 – 2.66 (m, 2H), 1.22 (dt, J = 22.6, 7.1 Hz, 3H). LC/MS (EI+APCI) m/z: $[M+H]^+$ 234.3.

Asymmetric reduction of ketones (1a-5a) by whole cell biocatalysts

A solution of ketones (**1a-5a**) with different concentrations in DMSO (100 μL) was added to PBS buffer (100 mM, pH 6.0, 900 μL) containing glucose (7 eq.), $NADP^+$ (0.5 mM), and lyophilized whole cells harboring GDH and CgKR1 mutants (40 gL^{-1}). The reaction mixture was stirred at 37°C and the pH of the mixture were maintained at 6.0 by automatically adding 2.0 M NaOH. The reactions were monitored by TLC. After the reaction finished, the reaction mixtures were extracted by EtOAc (3 × 1 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, concentrated in vacuum and purified by silica column chromatography. The products were analyzed by HPLC.

Scale-up asymmetric reduction of 5a by whole cell biocatalysts

A 100 mM solution of **5a** (1.51 g, 6.5 mmol) in DMSO (6.5 mL) was added to PBS buffer (100 mM, pH 6.0, 58.5 mL) containing glucose (9 g, 45.5 mmol), $NADP^+$ (27 mg, 0.5 mM), and lyophilized whole cells harboring GDH and CgKR1 mutants (2.6 g, 40 gL^{-1}). The reaction mixture was magnetically stirred at 37°C for 9h and the pH of the mixture were maintained at 6.0 by automatically adding 2.0 M NaOH. After the reaction finished, the reaction mixtures were extracted by EtOAc (3 × 65 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, concentrated in vacuum and purified by silica column chromatography to afford (*S*)-**5b** as a gray oil (1.24 g, 81.4%). Analysis of this material by chiral HPLC indicated it to be over 99.9% *ee*.

(*R*)-2,3-Dihydro-1*H*-inden-1-ol ((*R*)-1b). Combined products by different variants were purified by silica column chromatography and then used for the optical rotation test. $[\alpha]_D^{20}$=-21.15 (c 0.156, $CHCl_3$), 60.7% *ee* (*R*). Lit.:[7] $[\alpha]_D^{20}$=-32.1 (*c* 0.9, $CHCl_3$), 98.0% *ee* (*R*). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.45 – 7.39 (m, 1H), 7.30 – 7.21 (m, 3H), 5.30 – 5.20 (m, 1H), 3.06 (ddd, J = 16.1, 8.5, 4.8 Hz, 1H), 2.82 (ddd, J = 15.6, 8.3, 6.7 Hz, 1H), 2.49 (dddd, J = 13.2, 8.4, 6.9, 4.8 Hz, 1H), 1.95 (dddd, J = 13.5, 8.5, 6.6, 5.3 Hz, 1H).

(*S*)-2,3-Dihydro-1*H*-indene-1,6-diol ((*S*)-2b). Combined products by different variants were purified by silica column chromatography and then used for the optical rotation test. $[\alpha]_D^{25}$=7.36 (c 0.734, CH_3CN), 99.0% *ee* (*S*). Lit.:[8] $[\alpha]_D^{25}$=6.1 (*c* 1.0, CH_3CN), 98.8% *ee* (*S*). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (s, 1H), 6.97 (d, J = 8.0 Hz, 1H), 6.72 (d, J = 2.3 Hz, 1H), 6.58 (dd, J = 8.1, 2.4 Hz, 1H), 5.13 (d, J = 6.1 Hz, 1H), 4.93 (q, J = 6.6 Hz, 1H), 2.76 (ddd, J = 15.2, 8.7, 3.6 Hz, 1H), 2.56 (dt, J = 15.4, 7.9 Hz, 1H), 2.36 – 2.21 (m, 1H), 1.72 (dtd, J = 12.6, 8.3, 6.6 Hz, 1H).

(*S*)-6-Methoxy-2,3-dihydro-1*H*-inden-1-ol ((*S*)-3b). Combined products by different variants were purified by silica column chromatography and then used for the optical rotation test. $[\alpha]_D^{25}$=3.10 (c 0.452, $CHCl_3$), 9.8% *ee* (*S*). Lit.:[9] $[\alpha]_D^{25}$=20.8 (*c* 1.0, $CHCl_3$), 95% *ee* (*S*). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.14 (d, *J* = 8.2 Hz, 1H), 6.96 (d, *J* = 2.5 Hz, 1H), 6.82 (dd, *J* = 8.3, 2.5 Hz, 1H), 5.21 (t, *J* = 6.2 Hz, 1H), 3.81 (s, 3H), 2.98 (ddd, *J* = 15.6, 8.5, 4.5 Hz, 1H), 2.75 (dt, *J* = 15.3, 7.5 Hz, 1H), 2.51 (dddd, *J* = 12.9, 8.2, 6.9, 4.5 Hz, 1H), 1.94 (dddd, *J* = 13.1, 8.6, 6.8, 5.5 Hz, 1H).

3-Hydroxyindolin-2-one (4b). Combined products by different variants were purified by silica column chromatography and then used for the optical rotation test. $[\alpha]_D^{20}$=-1.28 (c 0.078, CH_3OH), 6.2% *ee*. Lit.:[10] $[\alpha]_D^{20}$=7 (*c* 1, CH_3OH) (*R*). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.24 (s, 1H), 7.28 (d, *J* = 7.3 Hz, 1H), 7.20 (tt, *J* = 7.7, 1.0 Hz, 1H), 6.96 (td, *J* = 7.5, 1.0 Hz, 1H), 6.78 (d, *J* = 7.7 Hz, 1H), 6.17 (d, *J* = 7.6 Hz, 1H), 4.82 (d, *J* = 7.6 Hz, 1H). LC/MS (EI+APCI) m/z: $[M+Na]^+$ 172.1.

(*S*)-3-Hydroxy-2,3-dihydro-1*H*-inden-5-yl ethyl(methyl)carbamate ((*S*)-5b). Combined products by different variants were purified by silica column chromatography and then used for the optical rotation test. $[\alpha]_D^{20}$=21.54 (c 3.5, $CHCl_3$), ＞99.9% *ee* (*S*). Lit.:[6] $[\alpha]_D^{20}$=27.2 (*c* 2.38, $CHCl_3$), 98% *ee* (*S*). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.20 (d, *J* = 8.1 Hz, 1H), 7.15 (s, 1H), 6.98 (d, *J* = 8.2 Hz, 1H), 5.19 (t, *J* = 6.2 Hz, 1H), 3.53 – 3.35 (m, 3H), 3.11 – 2.95 (m, 4H), 2.77 (dt, *J* = 15.6, 7.6 Hz, 1H), 2.50 (dddd, *J* = 12.8, 8.2, 6.9, 4.5 Hz, 1H), 1.95 (dddd, *J* = 12.9, 8.5, 6.9, 5.6 Hz, 1H), 1.21 (dt, *J* = 21.7, 6.9 Hz, 4H). LC/MS (EI+APCI) m/z: $[M+H]^+$ 236.3.

Substrate and product inhibition of CgKR1 in the reduction of 5a

With the increasement of substrate concentration, obvious inhibition was observed in the reduction of **5a**, which could be attributed to substrate inhibition. But product inhibition cannot be excluded. Thus, a test reaction was performed by comparing the peak area of **5a** in reactions (by F92V/Y208I) started from 100 mM **5a** but supplemented with 25 mM, 50 mM, 75 mM and 100mM (*S*)-**5b** respectively after 0.5 h. The peak area of **5a** gradually increased with the concentration of additional (*S*)-**5b**, which indicates the product inhibition of CgKR1.

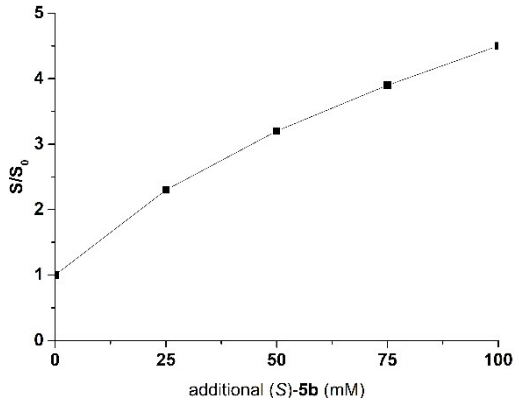


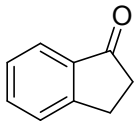
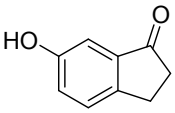
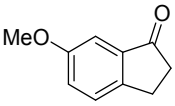
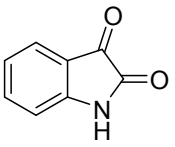
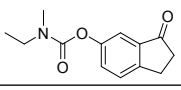
Figure S2. Product inhibition of F92V/Y208I in the reduction of **5a**. 'S_0' refers to the peak area in the case of 100 mM **5a**; 'S' refers to the peak area in the case of 100 mM **5a** with additional (*S*)-**5b.** S/S_0 indicates the inhibitory effect of products.

Table S1. Primers used in this study.

Primers[a]	Sequence (5’ to 3’)
CgKR1_Nde I_F	GGAATTCCATATGGCTTCTGATAACAGCAAC
CgKR1_Xho I_R	CCGCTCGAGATTAGAGTTCTTCTCGGC
GDH_Nco I_F	CATGCCATGGGCTATCCGGATTTAAAAGGA
GDH_Hind III_R	CCCAAGCTTTTAACCGCGGCCTGCCTGGAATG
CgKR1_144/145_F	TCCACAGTTGACSYTSYTTACGCTAAGGAT
CgKR1_144/145_R	ATCCTTAGCGTAARSARSGTCAACTGTGGA

[a]Primers of F92A, F92V, F92V/Y208I and F92V/Y208T variants are written in the supporting information of our previous work.[2]

Table S2. Chiral HPLC methods and retention times for pro-chiral ketones and chiral alcohols.

Ketone	Method	$Rt_{(Sub.)}$ min	$Rt_{((R)\text{-Prod.})}$ min	$Rt_{((S)\text{-Prod.})}$ min	Reference[a]
	Chiralcel OD-H, hexane/2-propanol (95 : 5) flowing at 0.8 mL min^{-1}, UV 254 nm, column temperature: 30 °C	9.3	12.6	11.4	11
	Chiralcel OD-H, hexane/2-propanol /TFA (85 : 15: 0.1) flowing at 0.8 mL min^{-1}, UV 220 nm	6.6	11.0	9.5	8
	Chiralcel OD-H, hexane/2-propanol (95 : 5) flowing at 0.8 mL min^{-1}, UV 254 nm, column temperature: 30 °C	10.1	16.1	14.5	11
	Chiralcel OB-H, hexane/2-propanol/TFA (85 : 15: 0.1) flowing at 0.8 mL min^{-1}, UV 210 nm, column temperature: 30 °C	17.4	9.3 ; 10.7		12
	(1) Chiralcel OB-H, hexane/2-propanol (60 : 40) flowing at 0.5 mL min^{-1}, UV 254 nm, column temperature: 40 °C	18.5	31.7	17.8	6

	(2) Platisil ODS, methanol/H_2O(50 : 50) flowing at 0.8 mL min^{-1}, UV 254 nm	21.6	23.7	6

[a]References used to identify the absolute configurations of resultant alcohols by comparing the retention times of chiral HPLC.

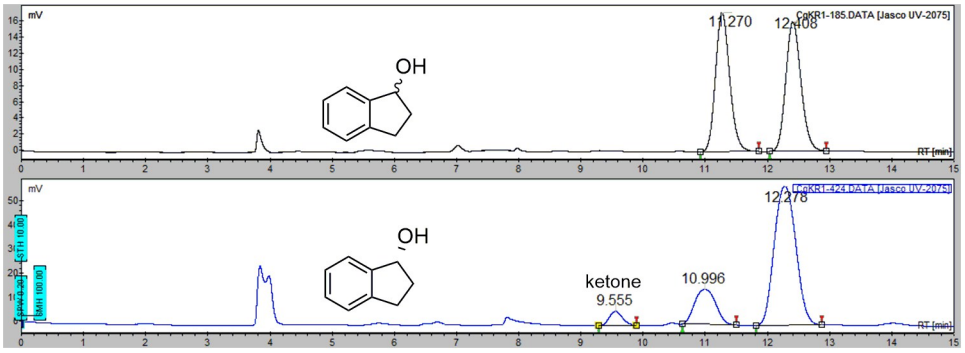


Figure S3. HPLC traces of (*rac*)-**1b** and (*S*)-**1b** product.

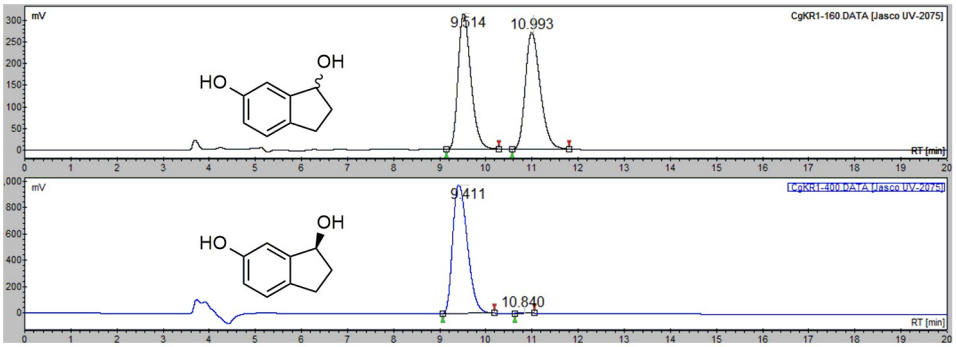


Figure S4. HPLC traces of (*rac*)-**2b** and (*S*)-**2b** product.

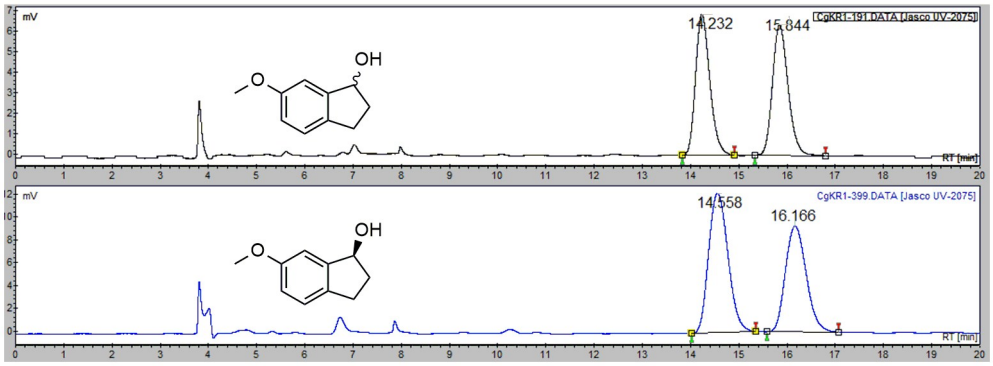


Figure S5. HPLC traces of (*rac*)-**3b** and (*S*)-**3b** product.

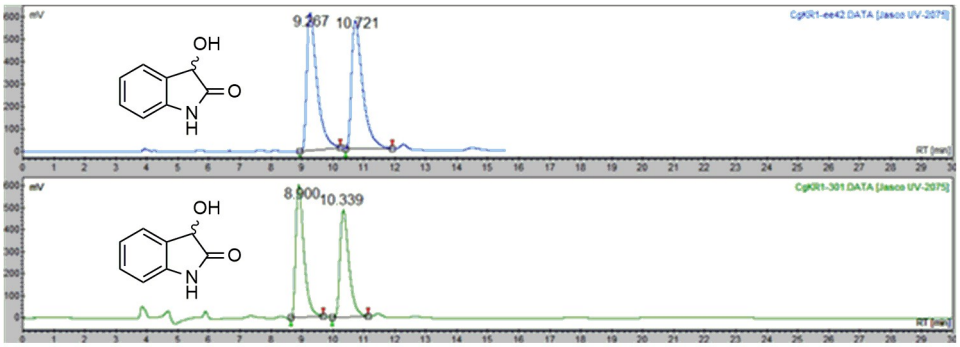


Figure S6. HPLC traces of (*rac*)-**4b** and **4b** product.

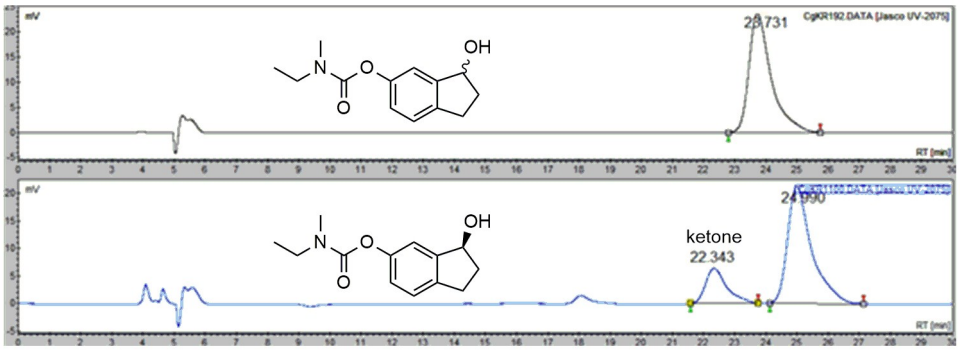


Figure S7. HPLC traces (ODS) of (*rac*)-**5b** and the product without purification.

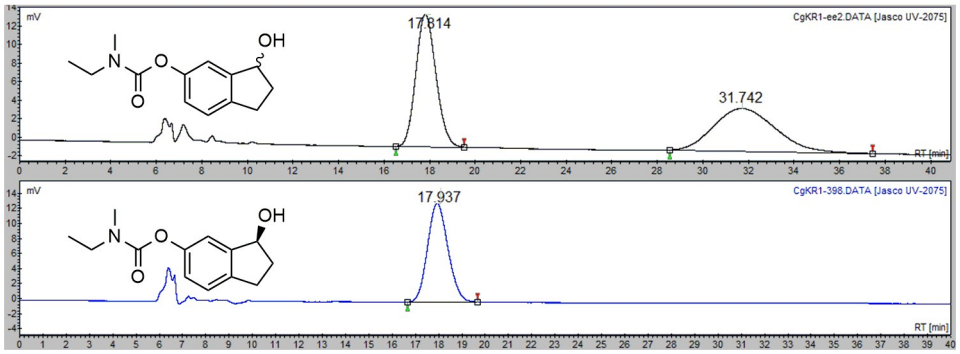


Figure S8. HPLC traces (OB-H) of (*rac*)-**5b** and (*S*)-**5b** product after purification.

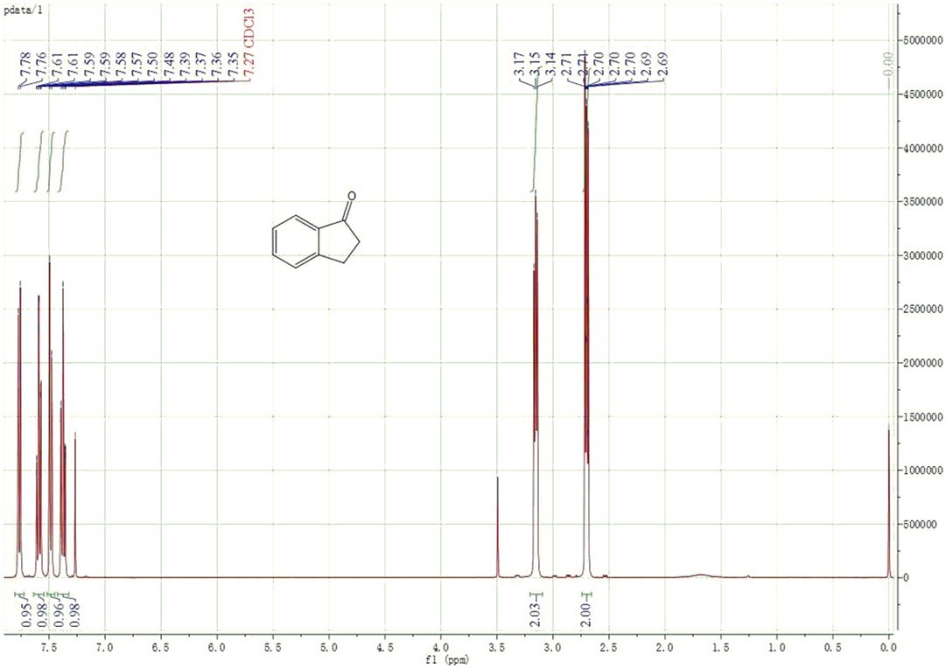


Figure S9. ^{1}H NMR spectrum of **1a** ($CDCl_3$, 400MHz).

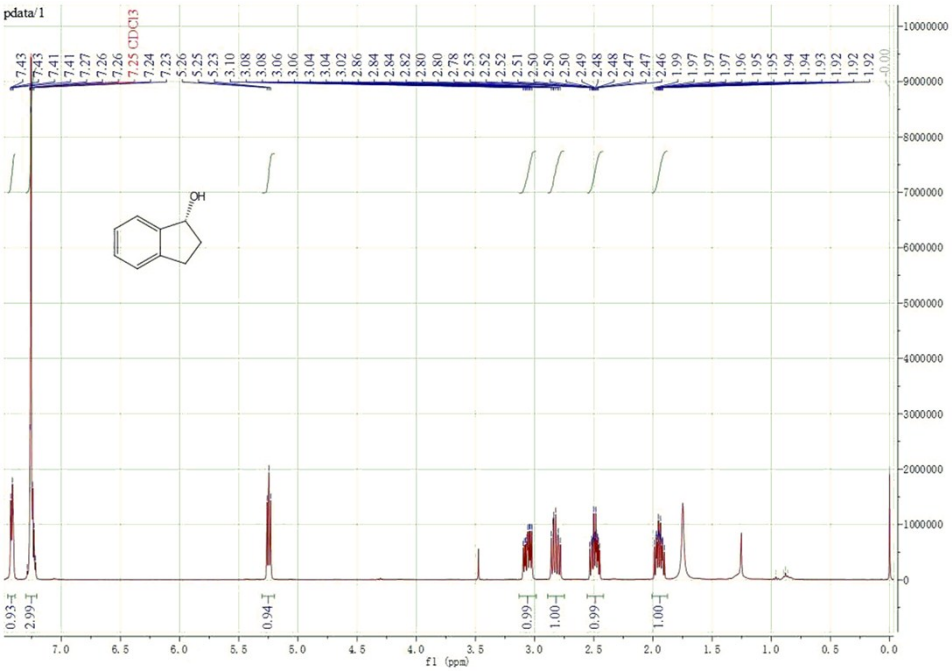


Figure S10. ^{1}H NMR spectrum of **1b** ($CDCl_3$, 400MHz).

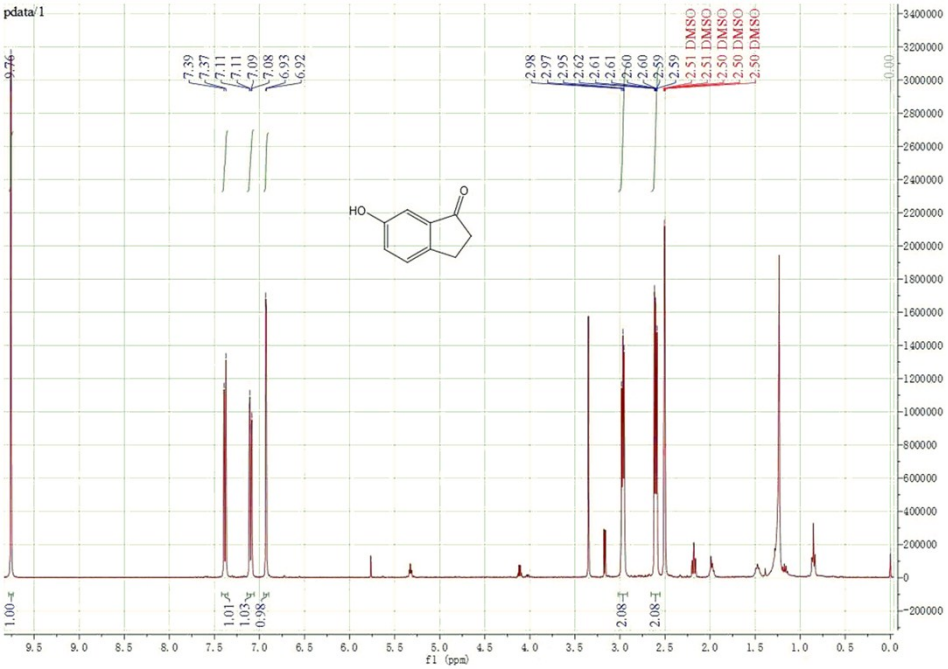


Figure S11. ^{1}H NMR spectrum of **2a** (DMSO-d_6, 400MHz).

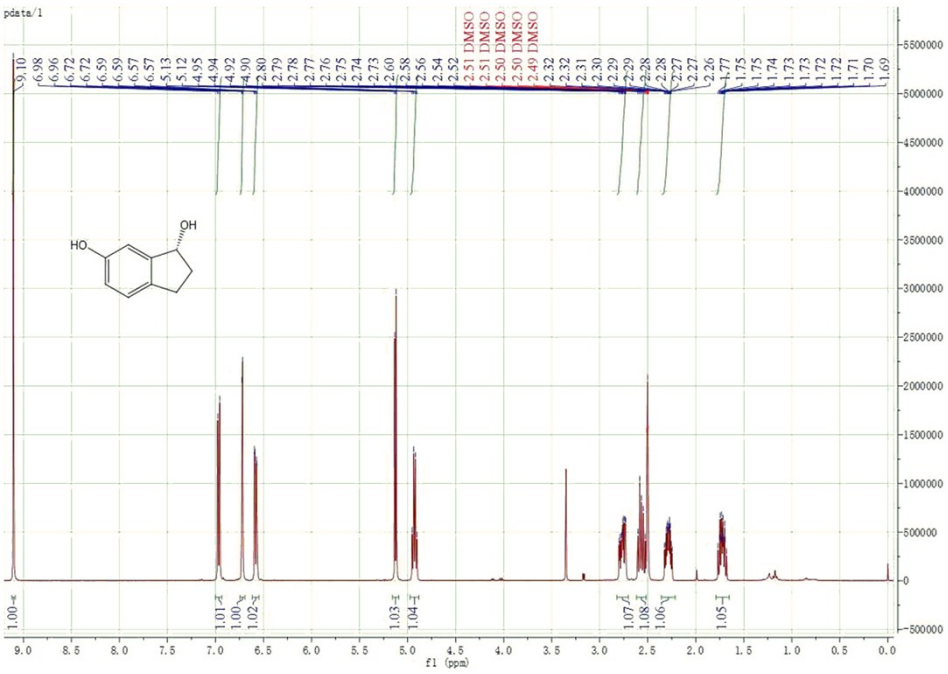


Figure S12. ^{1}H NMR spectrum of **2b** (DMSO-d_6, 400MHz).

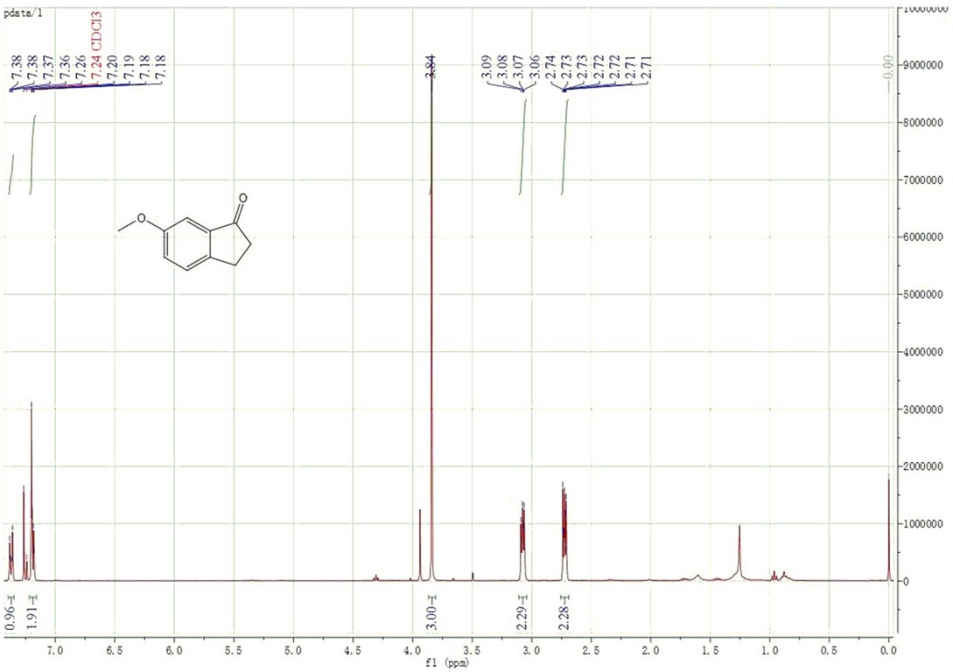


Figure S13. ^{1}H NMR spectrum of **3a** ($CDCl_3$, 400MHz).

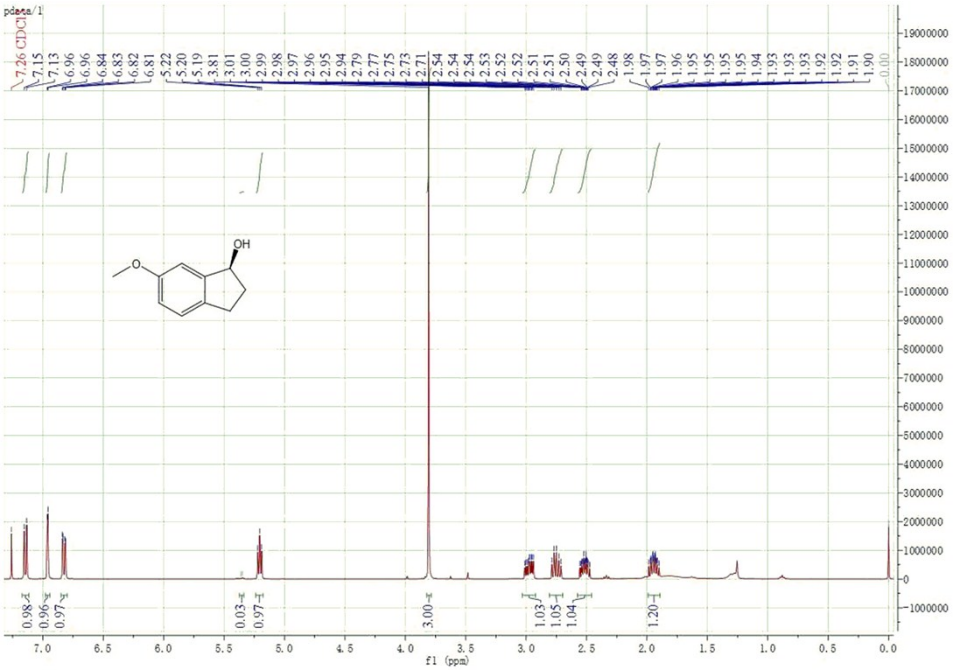


Figure S14. ^{1}H NMR spectrum of **3b** ($CDCl_3$, 400MHz).

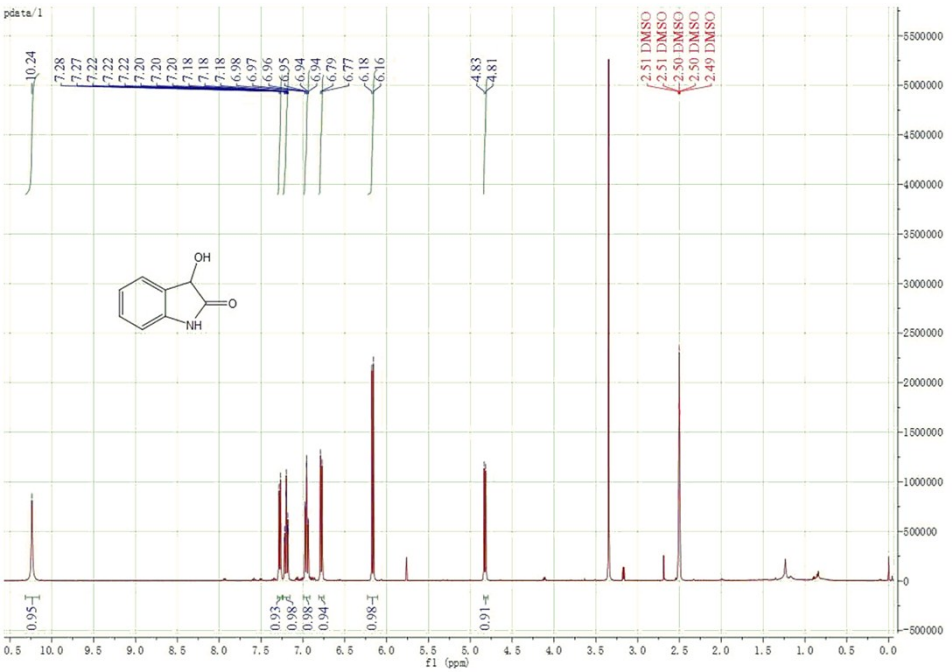


Figure S15. ^{1}H NMR spectrum of **4b** (DMSO-d_6, 400MHz).

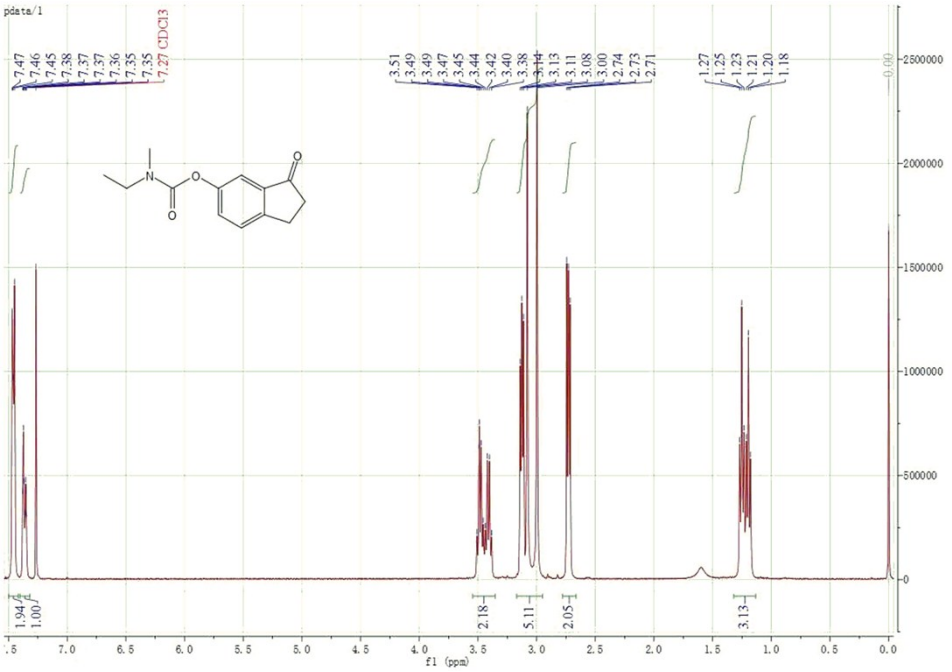


Figure S16. ^{1}H NMR spectrum of **5a** ($CDCl_3$, 400MHz).

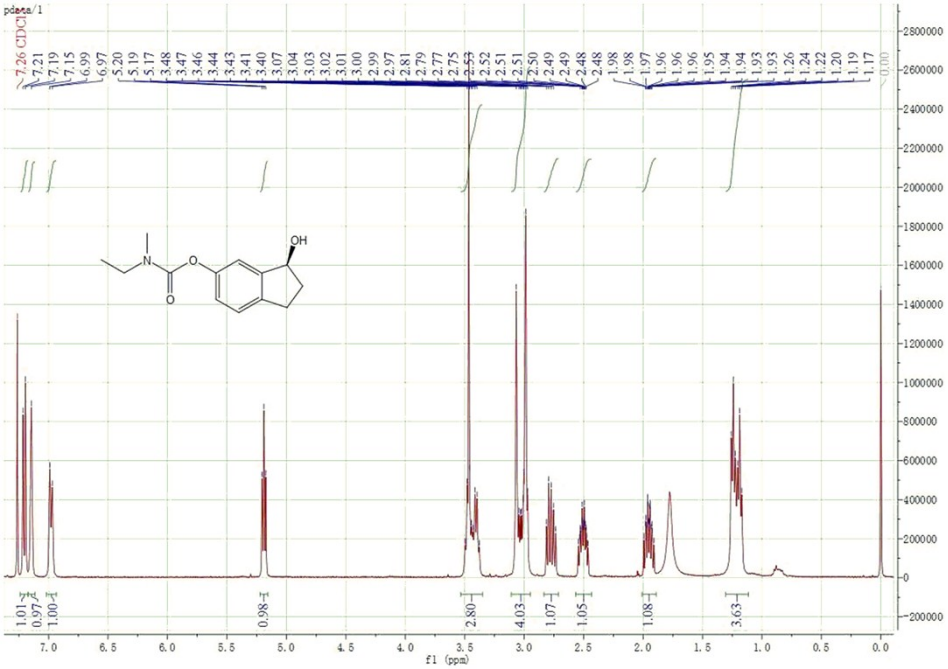


Figure S17. ^{1}H NMR spectrum of **5b** ($CDCl_3$, 400MHz).

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