Enantiocomplementary C–H Bond Hydroxylation through a Dual-Enzyme Catalyzed One-pot Two-step Process

Jian Xu , Pan Ma and Qing-song Chen

Supporting Information

1. General information

Unless otherwise noted, all starting materials and reagents were obtained from commercial suppliers and used without further purification. Horseradish peroxidase was purchased from Macklin (Shanghai, China). ^{1}H NMR spectra were recorded in $CDCl_3$ operating at 400 MHz. Proton chemical shifts are reported relative to the residual proton signals of the deuterated solvent $CDCl_3$ (7.26 ppm) or TMS. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), and multiple (m).

2. General procedure for the expression of carbonyl reductases

The cells were initially grown in 5 mL of LB medium supplemented with 50 μg/mL kanamycin at 37 °C and 220 rpm overnight. Afterwards, a 1% (v/v) seed culture was inoculated into TB medium with 50 μg/mL kanamycin at 37 °C and 220 rpm. Protein expression induction commenced upon reaching an OD600 of 0.6, with the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultivation was sustained for 16 hours at 20 °C and 220 rpm. The cells were then harvested by centrifugation, followed by resuspension in a 50 mM sodium phosphate buffer at pH 8.0 for the *Lb*ADH and pH 6.5 for *Ras*ADH reactions.

3. Synthesis of racemic alcohols

General procedures were adapted from literatures, the ketone (2 mmol) was reacted with $NaBH_4$ (5 mmol) in methanol (10 mL) at room temperature. The mixture was stirred until the ketone was fully converted, confirmed by TLC. The crude product was evaporated, dissolved in dichloromethane, and washed with water. The organic phase was concentrated under reduced pressure and purified using flash column chromatography to obtain the desired racemic alcohols.

4. General procedure for the one-pot two-step enantioselectivity C–H bond hydroxylation reaction

In a 25 mL flask with a magnetic stir bar, 200 μL of DMSO containing alkylarene **1** (0.01 mmol) and NHPI (0.01 mmol) were mixed. Next, 1.8 mL of 50 mM PBS buffer at pH 5.5 was added. Subsequently, 0.1 mmol H_2O_2 was slowly introduced. The reaction was carried out with the addition of an air balloon

and allowed to react at room temperature for 8-10 hour. If alcohol was produced as a byproduct, the addition of HRP could be continued to promote further reaction (monitored by thin-layer chromatography (TLC) or gas chromatography (GC)). Following this, whole-cell culture of carbonyl reductase (10 mL, with glucose 0.2 mmol) was introduced to the mixture, continuing the reaction overnight. The reaction was extracted three times with ethyl acetate. The stereochemistry and yield were evaluated using HPLC.

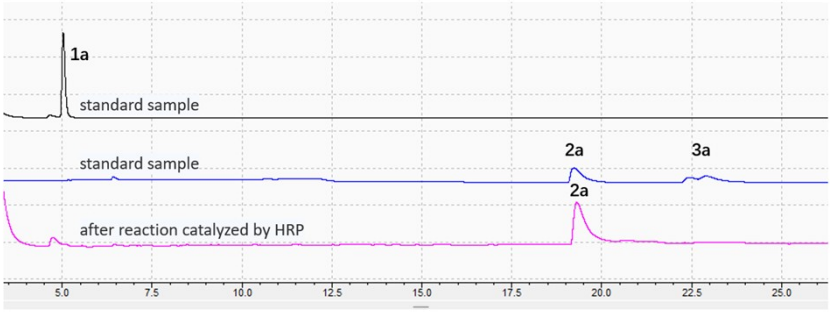
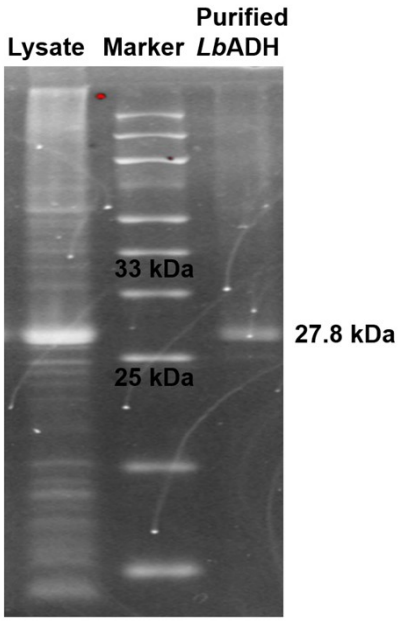


Figure S1. GC trace of HRP catalyzed oxidation

5. General procedure for the model reaction with purified *Lb*ADH

*Lb*ADH purification: The cells were subjected to three cycles of freezing and thawing before releasing the target proteins using sonication. The lysate was then clarified by centrifugation at 4000 rpm and 4°C for 30 minutes, and the resulting supernatant was applied to a 2 mL Ni-NTA agarose column. This column was washed with lysis buffer (100 mM PBS, pH 7.4, 500 mM NaCl, 5% glycerol) and eluted with five column volumes of elution buffer (100 mM PBS, pH 7.4, 500 mM NaCl, 5% glycerol, 50 mM imidazole). Finally, the collected fraction was desalted using ultrafiltration. The glucose dehydrogenase was purchased from Aladdin (Shanghai, China).



Reaction: In a 25 mL flask with a magnetic stir bar, 200 μL of DMSO containing alkylarene **1** (0.01

mmol) and NHPI (0.01 mmol) were mixed. Next, 1.8 mL of 50 mM PBS buffer at pH 5.5 was added. Subsequently, 0.1 mmol H_2O_2 was slowly introduced. The reaction was carried out with the addition of an air balloon and allowed to react at room temperature for 8-10 hour. Following this, purified *Lb*ADH (0.1 μm in 10 mL, with glucose dehydrogenase μm and 0.2 glucose 0.2 mmol) was introduced to the mixture, continuing the reaction overnight. The reaction was extracted three times with ethyl acetate. The stereochemistry and yield were evaluated using HPLC, to obtained (*R*)-3a in 20% yield and 90% ee.

6. Preparative scale reactions

In a 500 mL flask equipped with a magnetic stir bar, a mixture of 2 mL of DMSO containing alkylarene **1a** (0.1 mmol) and NHPI (0.1 mmol) was prepared. Following that, 18 mL of 50 mM PBS at pH 5.5 was added. Subsequently, 1.0 mmol H_2O_2 was slowly introduced. The reaction system was supplemented with an air balloon and proceeded at room temperature for 12 hours. If alcohol was produced as a byproduct, the addition of HRP could be continued to promote further reaction. Following this, whole-cell culture of carbonyl reductase (100 mL with glucose 2 mmol) was added to the mixture, and the reaction was left overnight. The reaction mixture was then extracted three times with ethyl acetate, concentrated under reduced pressure, and the resulting crude mixture was purified by flash column chromatography on silica gel.

7. Procedure for the one-pot two-step deracemization process of *rac*-3a

In a 10 mL flask with a magnetic stir bar, 200 μL of DMSO containing alkylarene **3a** (0.01 mmol) and NHPI (0.01 mmol) were mixed. Next, 1.8 mL of 50 mM PBS buffer at pH 5.5 was added. Subsequently, 0.8 mmol H_2O_2 was slowly introduced. The reaction was carried out with the addition of an air balloon and allowed to react at room temperature for about 6 hours. Following this, whole-cell culture carbonyl reductase (10 mL with glucose 0.2 mmol) was introduced to the mixture, continuing the reaction overnight. The reaction was extracted three times with ethyl acetate. The stereochemistry and yield were evaluated using chiral HPLC.

8. The protein sequence of carbonyl reductase.

*Ras*ADH
:

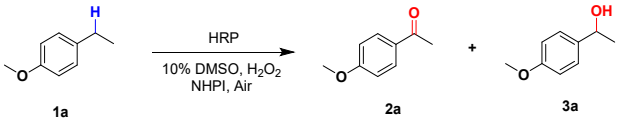
MGSSHHHHHHSSGLVPRGSHMYRLLNKTAVITGGNSGIGLATAKRFVAEGAYVFIVGRRRKE
LEQAAAEIGRNVTAVKADVTKLEDLDRLYAIVREQRGSIDVLFANSGAIEQKTLEEITPEHYDR
TFDVNVRGLIFTVQKALPLLRDGGSVILTSSVAGVLGLQAHDTYSAAKAAVRSLARTWTTELK
GRSIRVNAVSPGAIDTPIIENQVSTQEEADELRAKFAAATPLGRVGRPEELAAAVLFLASDDSS
YVAGIELFVDGGLTQV

*Lb*ADH:

MGHHHHHHGSGSNRLDGKVAIITGGTLGIGLAIATKFVEEGAKVMITGRHSDVGEKAAK
SVGTPDQIQFFQHDSSDEDGWTKLFDATEKAFGPVSTLVNNAGIAVNKSVEETTTAEWR
KLLAVNLDGVFFGTRLGIQRMKNKGLGASIINMSSIEGFVGDPSLGAYNASKGAVRIMSK
SAALDCALKDYDVRVNTVHPGYIKTPLVDDLPGAEEAMSQRTKTPMGHIGEPNDIAYICV

YLASNESKFATGSEFVVDGGYTAQ

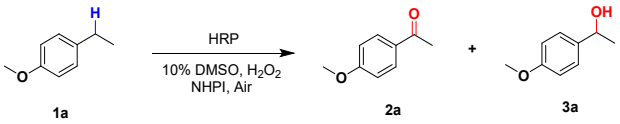
Table S1 Optimize the concentration of hydrogen peroxide



Entry	Variations of the standard conditions	Yield of **2a** (%) [b]
1	20 eq. H_2O_2 ,	80
2	15 eq. H_2O_2 ,	80
3	10 eq. H_2O_2 ,	78
4	8 eq. H_2O_2 ,	61
5	5 eq. H_2O_2 ,	50
6	2 eq. H_2O_2 ,	15

[a] Reaction conditions: The total reaction volume is 2 mL (contain 1.8 mL 50 mM PBS buffer, pH 5.5, and 200 μL DMSO), **1a** (0.01 mmol), NHPI (0.01 mmol)) and H_2O_2 under air. [b] Yields were determined by HPLC

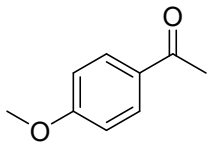
Table S2 Investigating the effects of oxygen



Entry	Variations of the standard conditions	Yield of **2a** (%) [b]
1	None	78
2	pure oxygen instead of air	79
3	without air	<10

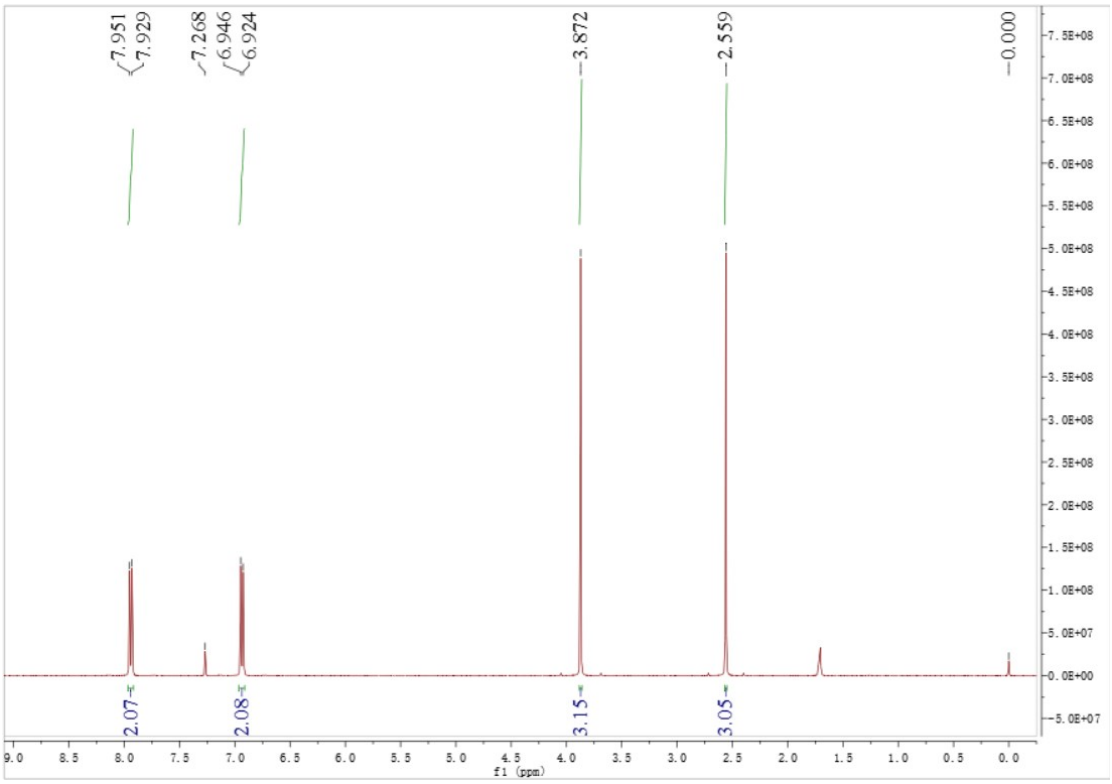
[a] Reaction conditions: The total reaction volume is 2 mL (contain 1.8 mL 50 mM PBS buffer, pH 5.5, and 200 μL DMSO), **1a** (0.01 mmol), NHPI (0.01 mmol)) and H_2O_2 (0.1 mmol) under air. [b] Yields were determined by HPLC

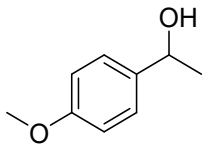
NMR-Spectra (from racemic standards)



2a

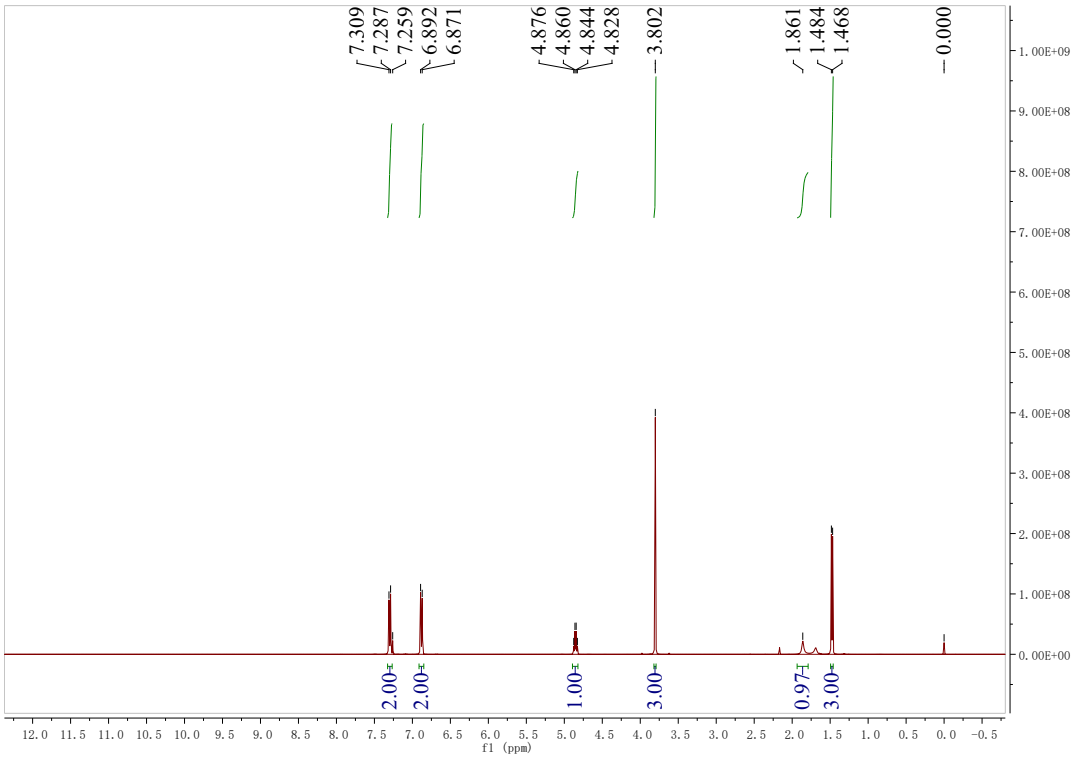
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96-7.92 (m, 2H, ArH), 6.96-6.92 (m, 2H, ArH), 3.88-3.86 (s, 3H, CH_3), 2.57-2.55 (s, 3H,CH_3).

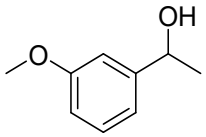




3a

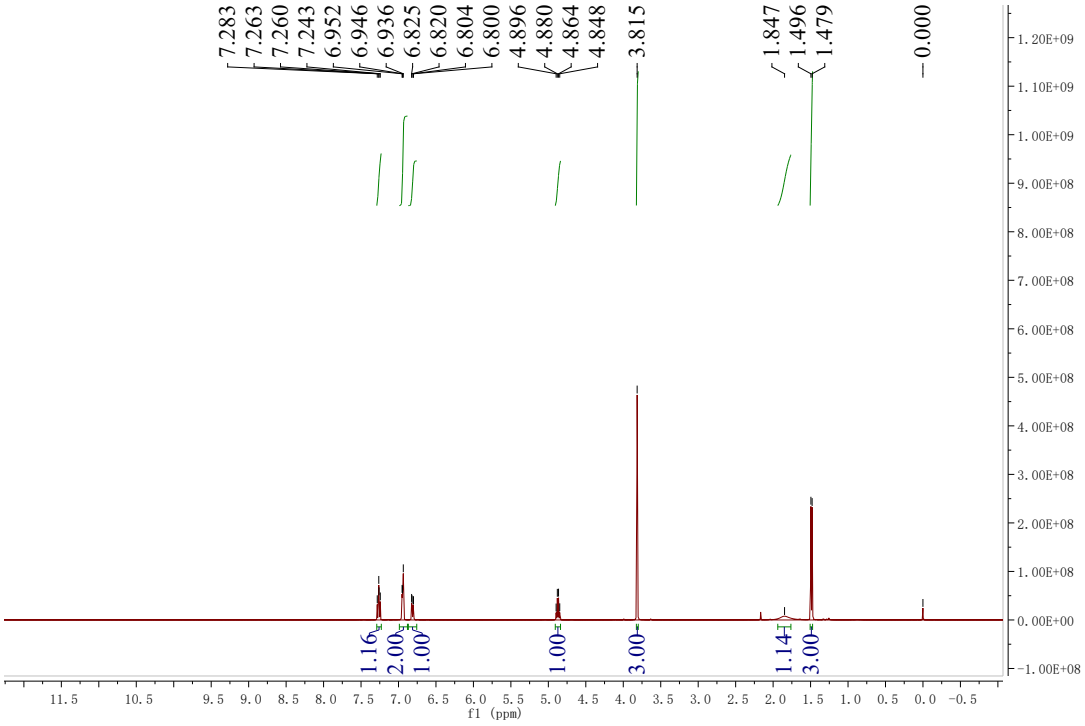
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (d, J = 8.8 Hz, 2H, ArH), 6.91-6.85 (m, 2H, ArH), 4.85 (q, J = 6.4 Hz, 1H, CH), 3.80 (s, 3H, CH_3), 1.86 (s, 1H, OH), 1.48 (d, J = 6.4 Hz, 3H, CH_3).

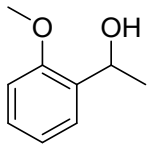

7.309
7.287
7.259
6.892
6.871
4.876
4.860
4.844
4.828
3.802
1.861
1.484
1.468
0.000
2.00
2.00
1.00
3.00
0.97
3.00
f1 (ppm)



3b

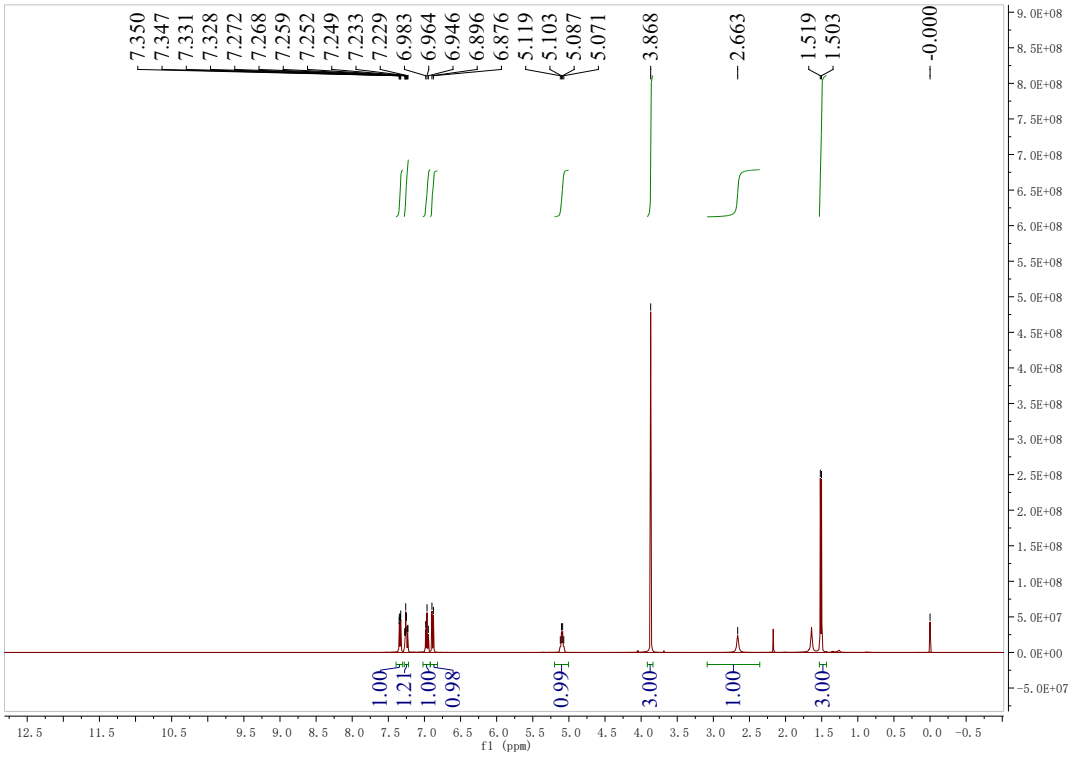
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29-7.23 (m, 1H, ArH), 6.99-6.88 (m, 2H, ArH), 6.87-6.76 (m, 1H, ArH), 4.87 (q, J = 6.4 Hz, 1H, CH), 3.82 (s, 3H, CH_3), 1.85 (s, 1H, OH), 1.49 (d, J = 6.8 Hz, 3H, CH_3).

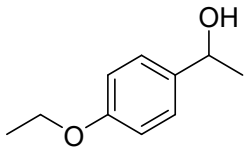




3c

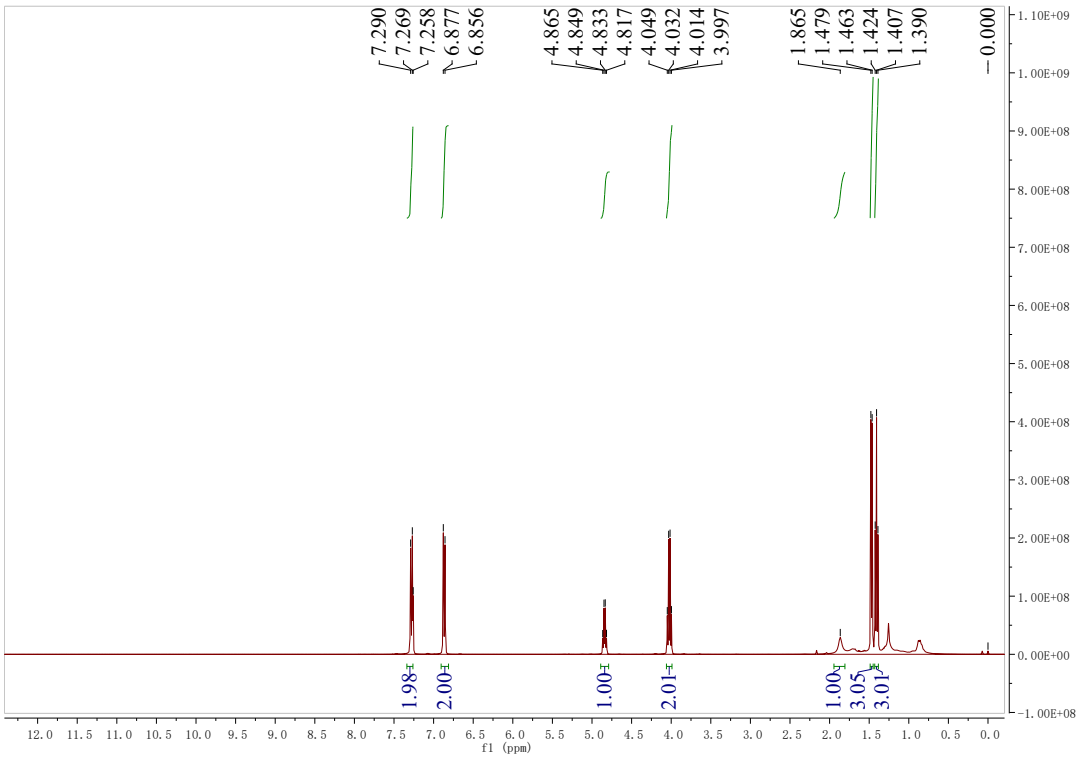
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (dd, J = 7.6, 1.2 Hz, 1H, ArH), 7.25 (td, J = 8.0, 1.6 Hz, 1H, ArH), 7.02-6.92 (m, 1H, ArH), 6.92-6.82 (m, 1H, ArH), 5.10 (q, J = 6.4 Hz, 1H, CH), 3.87 (s, 3H, CH_3), 2.66 (s, 1H, OH), 1.51 (d, J = 6.4 Hz, 3H, CH_3).

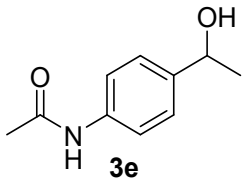




3d

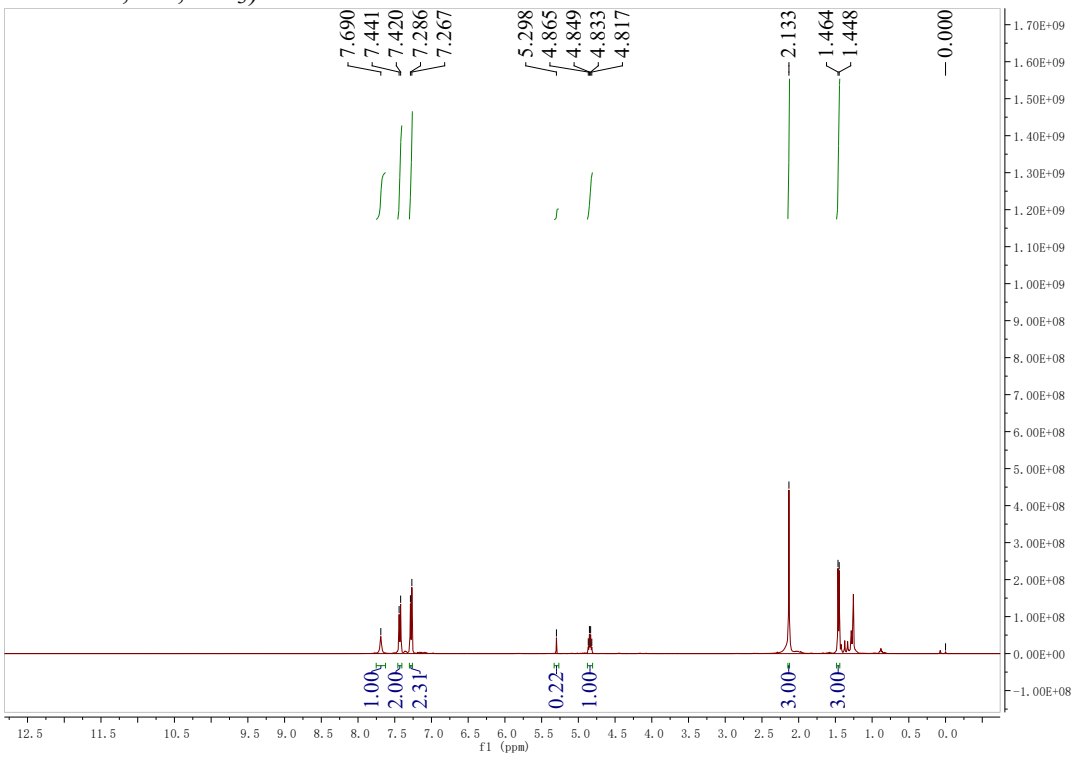
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34-7.26 (m, 2H, ArH), 6.91-6.61 (m, 2H, ArH), 4.84 (q, *J* = 6.4 Hz, 1H, CH), 4.02 (q, *J* = 7.2Hz, 2H, CH_2), 1.87 (s, 1H, OH), 1.47 (d, *J* = 6.4 Hz, 3H, CH_3).1.41 (t, *J* = 6.8 Hz, 3H, CH_3).

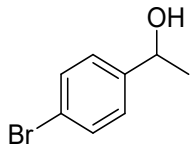




3e

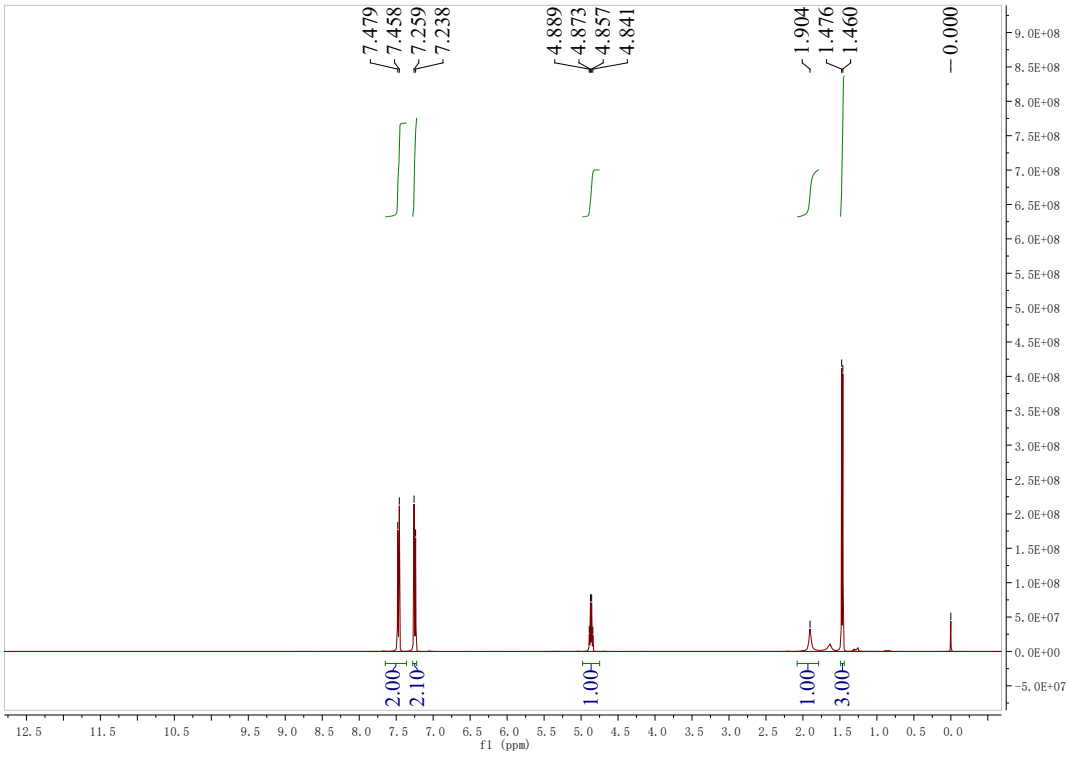
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (s, 1H, OH), 7.46-7.40 (m, 2H, ArH), 7.30-7.26 (m, 2H, ArH), 5.30 (s, 1H, NH), 4.84 (q, *J* = 6.4 Hz , 1H, CH), 2.13 (s, 3H, CH_3), 1.46 (d, *J*=6.4 Hz, 3H, CH_3).

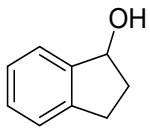




3f

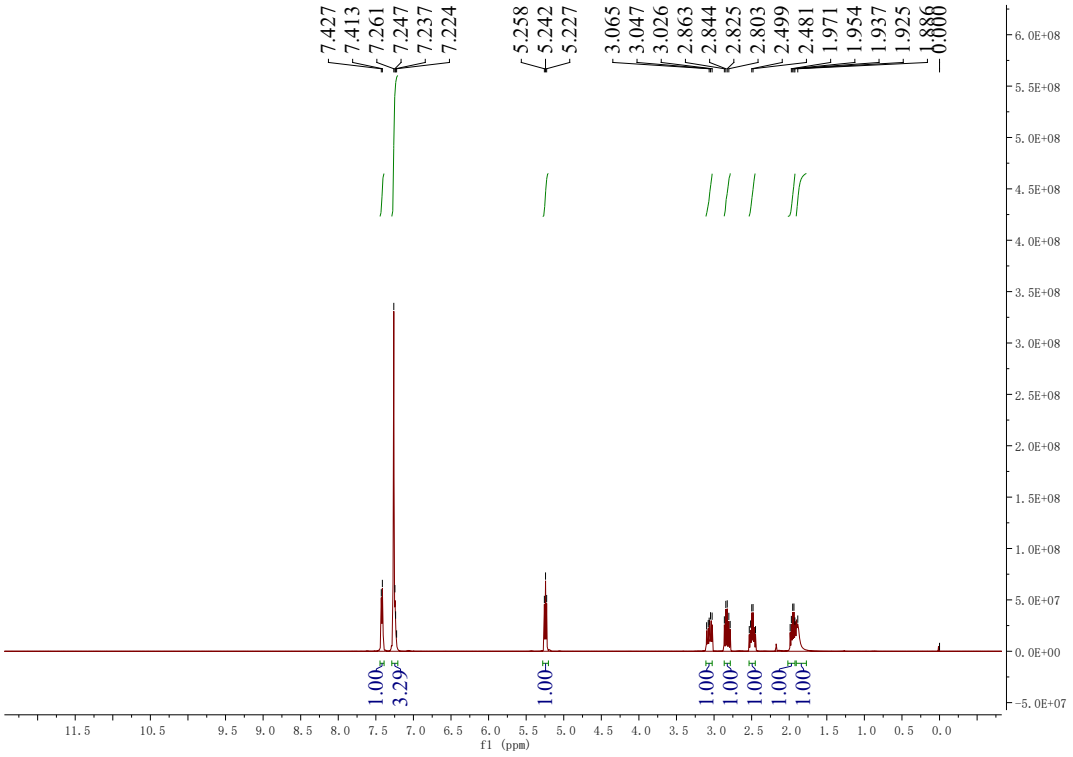
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65-7.36 (m, 2H, ArH), 7.28-7.22 (m, 2H, ArH), 4.87 (q, J = 6.4 Hz, 1H, CH), 1.90 (s, 1H, OH), 1.47 (d, J = 6.4 Hz, 3H, CH_3).

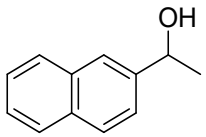




3g

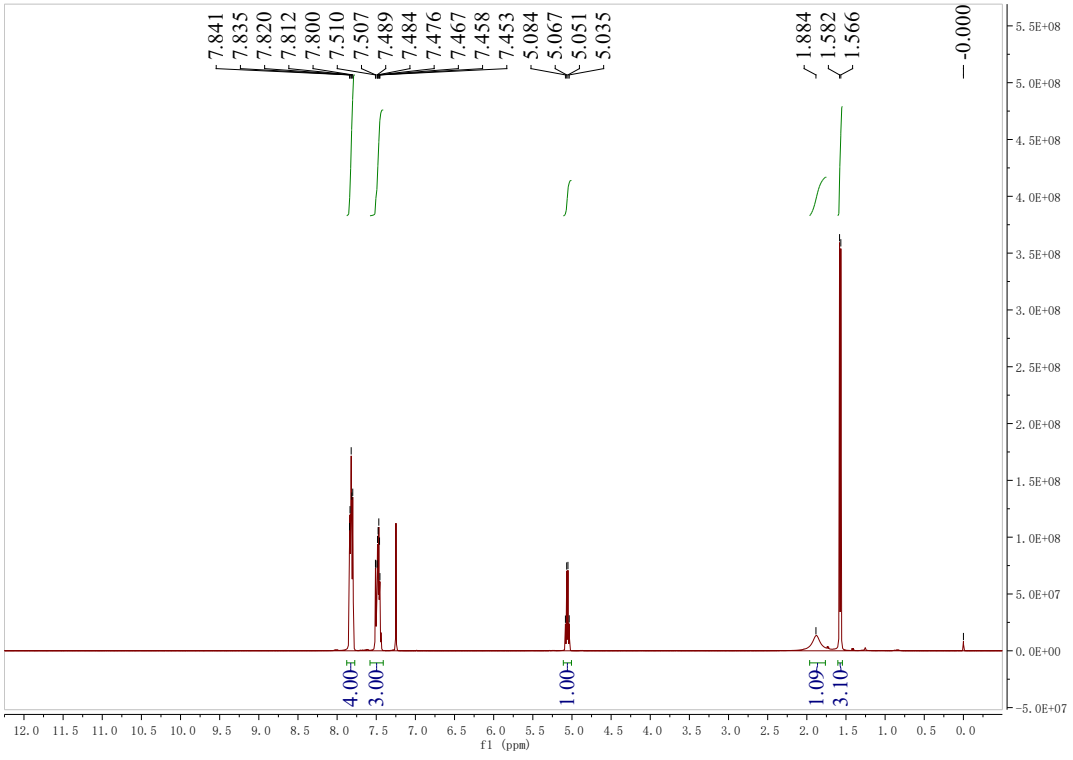
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45-7.39 (m, 1H, ArH), 7.29-7.21 (m, 2H, ArH), 5.24 (t, J = 6.4 Hz, 1H, CH), 3.11-3.03 (m, 1H, one proton of CH_2), 2.87-2.78 (m, 1H, one proton of CH_2), 2.54-2.45 (m, 1H, one proton of CH_2), 2.02-1.92 (m, 1H, one proton of CH_2), 1.89 (s, 1H, OH).

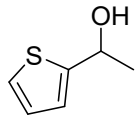




3h

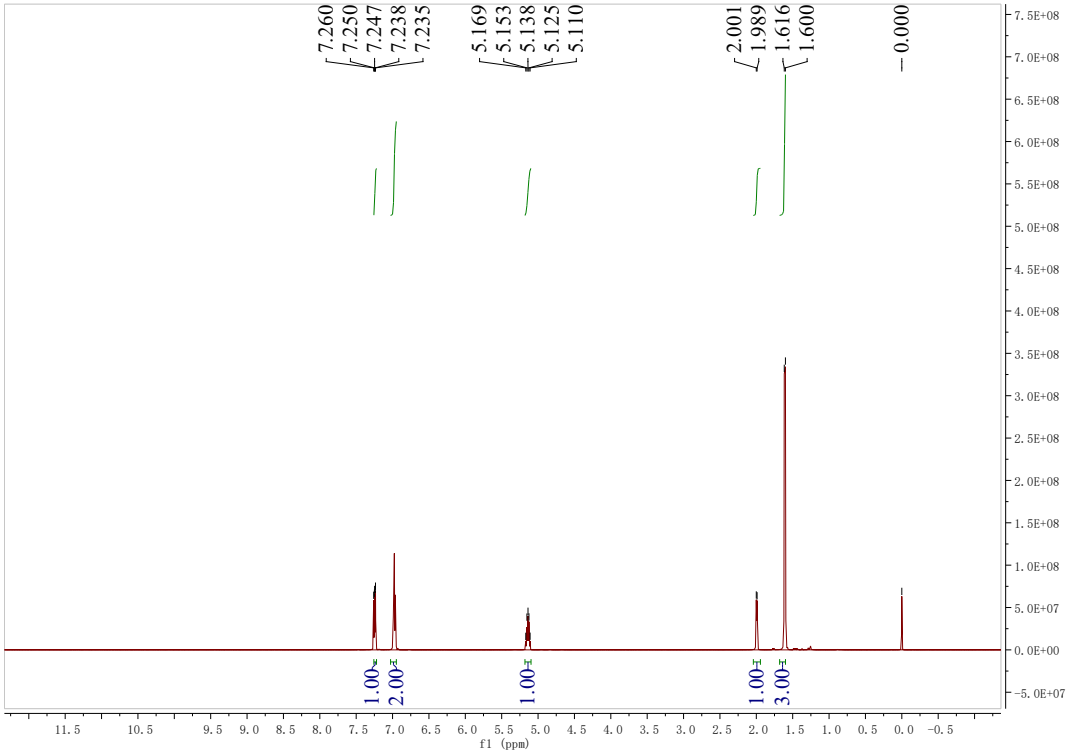
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88-7.77 (m, 4H, ArH), 7.58-7.41 (m, 3H, ArH), 5.06 (q, J = 6.4 Hz, 1H, CH), 1.88 (s,1H, OH), 1.57 (d, J =6.4 Hz, 3H, CH_3).

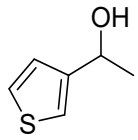




3i

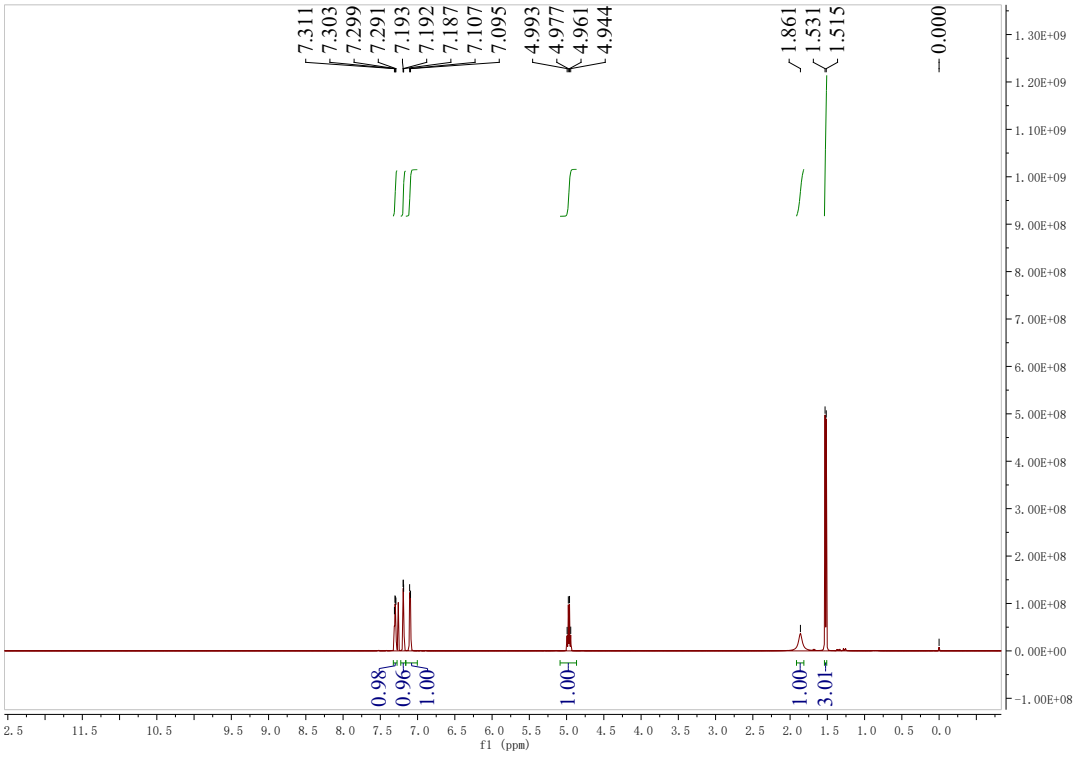
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26-7.22 (m, 1H, CH), 7.03-6.95 (m, 1H, CH), 5.18-5.10 (m, 1H, CH), 2.00 (d, J = 4.8 Hz, 1H, OH), 1.61 (d, J = 6.4 Hz, 3H, CH_3).

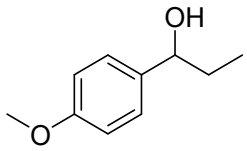




3j

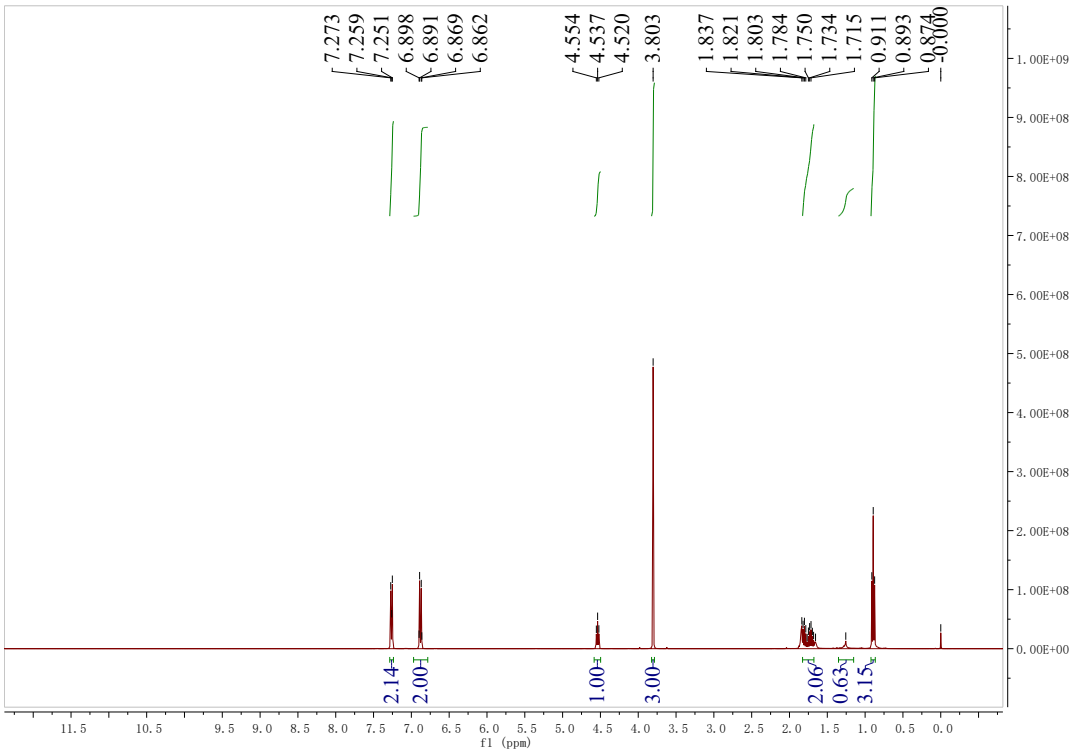
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33-7.28 (m, 1H, ArH), 7.23-7.16 (m, 1H, ArH), 7.16-7.00 (m, 1H, ArH), 4.97 (q, *J* =6.4 Hz ,1H, CH), 1.86 (s, 1H, OH), 1.52 (d, *J* =6.4 Hz, 3H, CH_3).

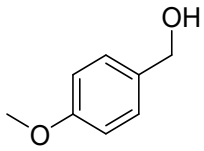




3k

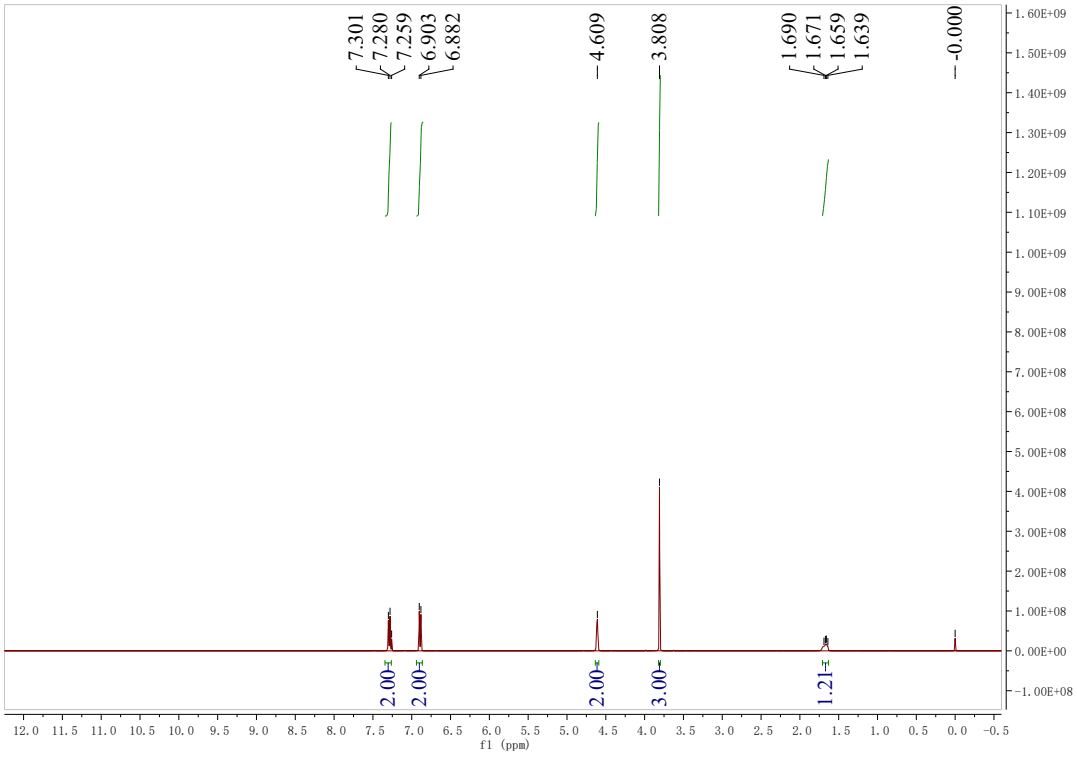
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27-7.25 (m, 2H, ArH), 6.90-6.86 (m, 2H, ArH), 4.54 (t, *J* = 6.8 Hz, 1H, CH), 3.80 (s, 3H, OCH_3), 1.84-1.66 (m, 2H, CH_2), 1.26 (s, 1H), 0.89 (t, *J* = 8.0 Hz, 3H).





3l

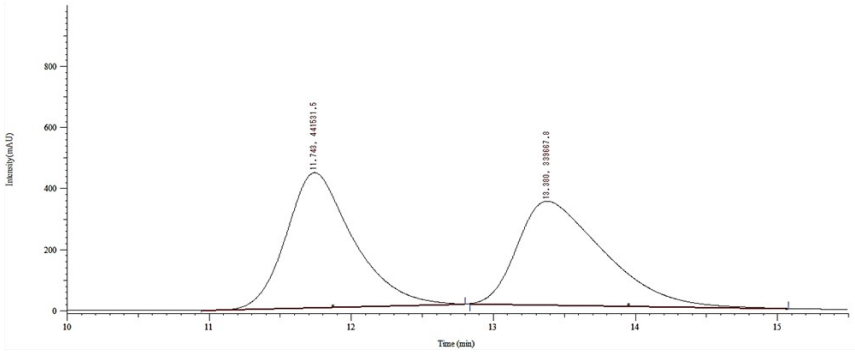
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34-7.26 (m, 2H, ArH), 6.94-6.86 (m, 2H, ArH), 4.61 (s, 1H, CH_2), 3.81 (s, 3H, CH_3), 1.71-1,63 (m, 1H, OH).



HPLC trace (Absolute configuration confirmed by comparison with literature values)[1]

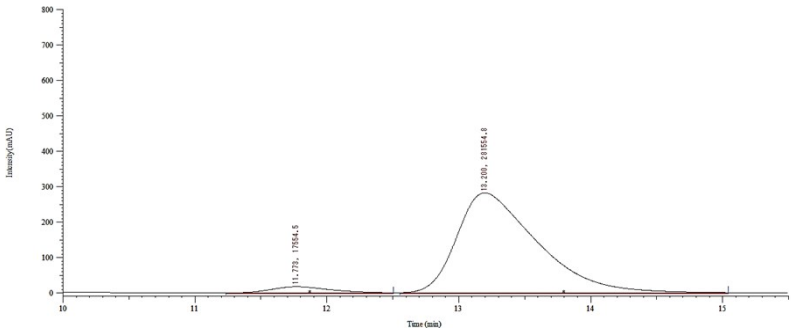
3a, HPLC analysis using a Chiralcel OD-H column. (HPLC: OD-H, 220 nm, *n*-hexane/isopropanol = 95:5, flow rate 1 mL/min)

***Rac*-3a**



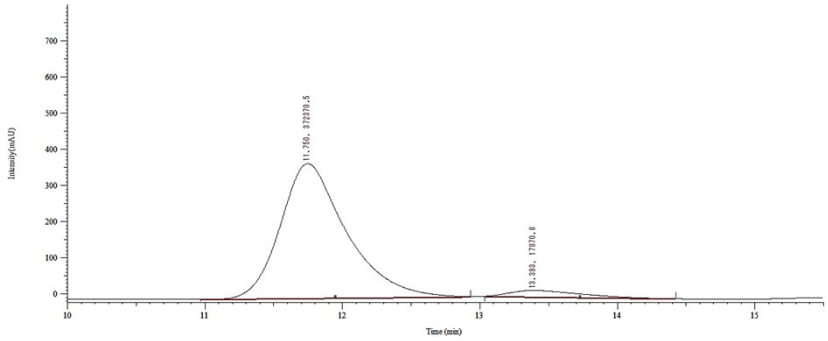
Peak	RT/min	Area	Area/%
1	11.743	14409349.6	50.304
2	13.380	14235240.9	49.696

(*S*)-3a



Peak	RT/min	Area	Area/%
1	11.773	538799.7	4.359
2	13.200	11821725.1	95.641

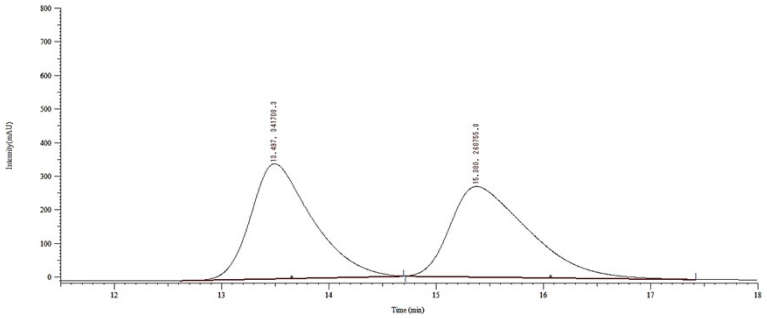
(*R*)-3a



Peak	RT/min	Area	Area/%
1	11.750	12417723.1	95.129
2	13.393	635771.6	4.871

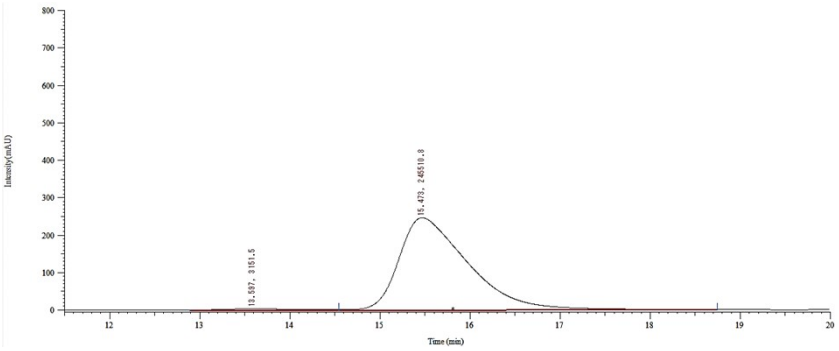
3b, HPLC analysis using a Chiralcel OD-H column. (HPLC: OD-H, 220 nm, *n*-hexane/isopropanol = 95:5, flow rate 1 mL/min)

***Rac*-3b**



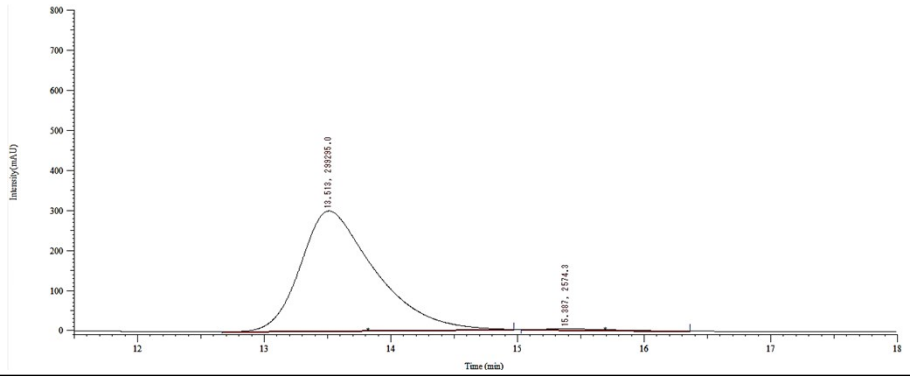
Peak	RT/min	Area	Area/%
1	13.497	13559822.7	50.208
2	15.380	13447510.9	49.792

(*S*)-3b



Peak	RT/min	Area	Area/%
1	13.597	126552.5	0.969
2	15.473	12939431.1	99.031

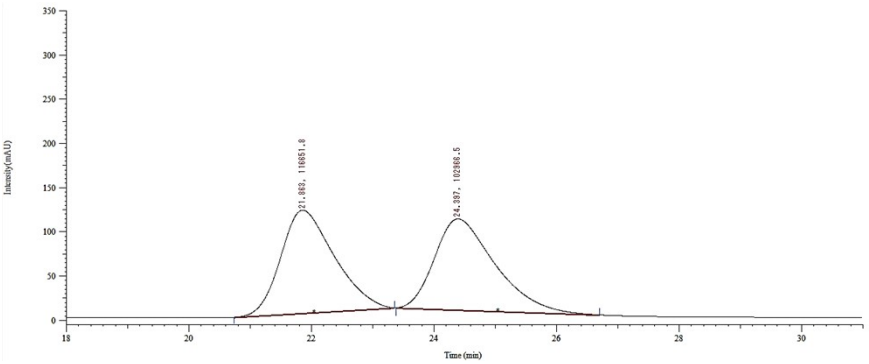
(*R*)-3b



Peak	RT/min	Area	Area/%
1	13.513	12191946.6	99.232
2	15.387	94367.0	0.768

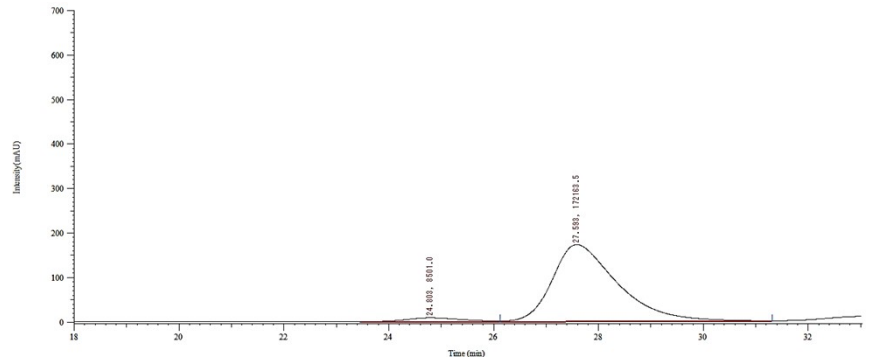
3c, HPLC analysis using a Chiralcel OD-H column. (HPLC: OD-H, 220 nm, *n*-hexane/isopropanol = 99:1, flow rate 1 mL/min)

***Rac*-3c**



Peak	RT/min	Area	Area/%
1	21.863	7068579.1	50.005
2	24.397	7067067.0	49.995

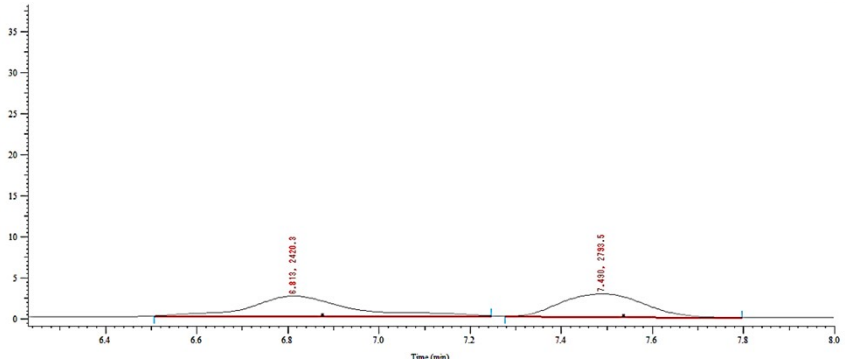
(*R*)-3c



Peak	RT/min	Area	Area/%
1	24.803	604014.7	3.934
2	27.593	14750297.1	96.066

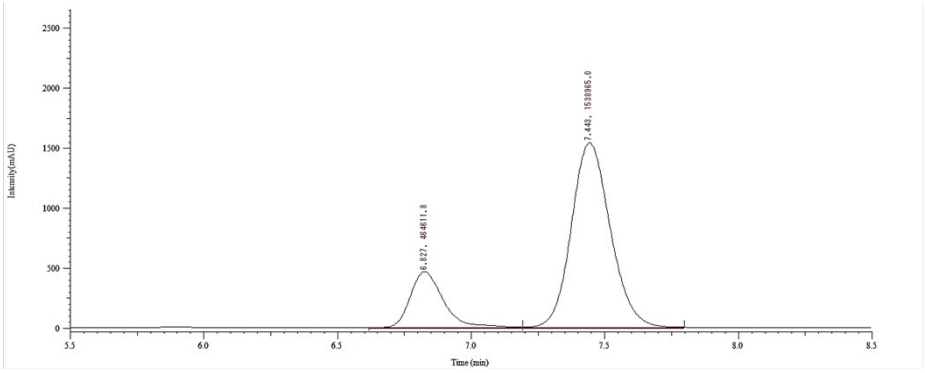
3d, HPLC analysis using a Chiralcel OD-H column. (HPLC: OD-H, 220 nm, *n*-hexane/isopropanol = 99:1, flow rate 1 mL/min)

***Rac*-3d**



Peak	RT/min	Area	Area/%
1	6.813	35391.4	50.042
2	7.490	35331.4	49.958

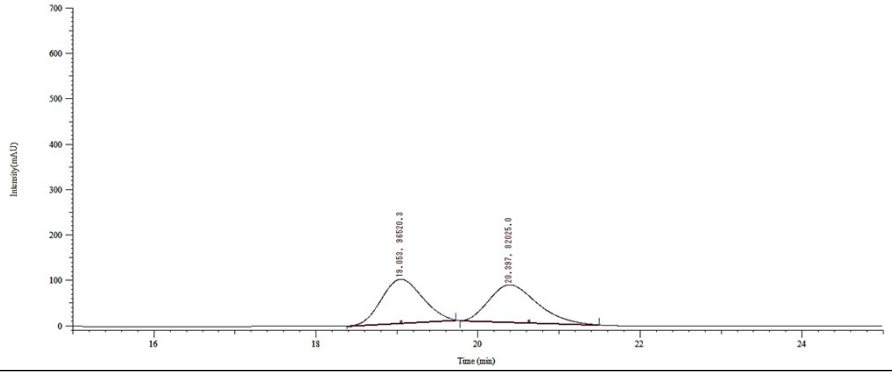
(*R*)-3d



Peak	RT/min	Area	Area/%
1	6.827	3991154.2	20.310
2	7.443	15659781.1	79.690

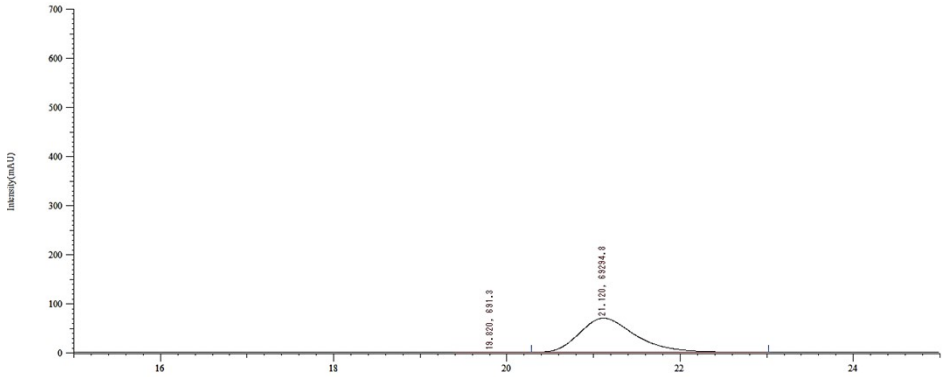
3e, HPLC analysis using a Chiralcel AD-H column (HPLC: AD-H, 220 nm, *n*-hexane/isopropanol = 90:10, flow rate 1 mL/min)

***Rac*-3e**



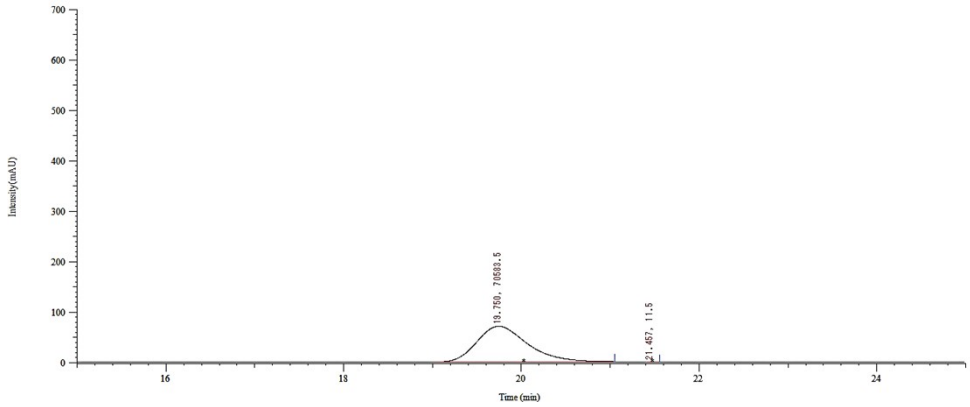
Peak	RT/min	Area	Area/%
1	19.053	3353296	49.930
2	20.397	3362719	50.070

(*S*)-3e



Peak	RT/min	Area	Area/%
1	19.820	18224	0.585
2	21.120	3098047	99.415

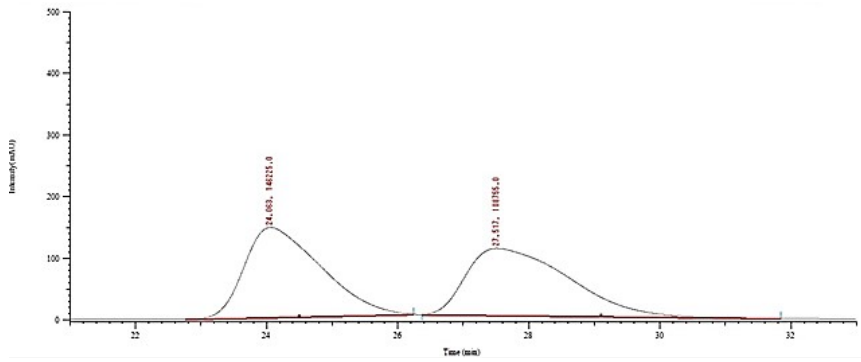
(*R*)-3e



Peak	RT/min	Area	Area/%
1	19.750	2784074	99.998
2	21.543	158	0.002

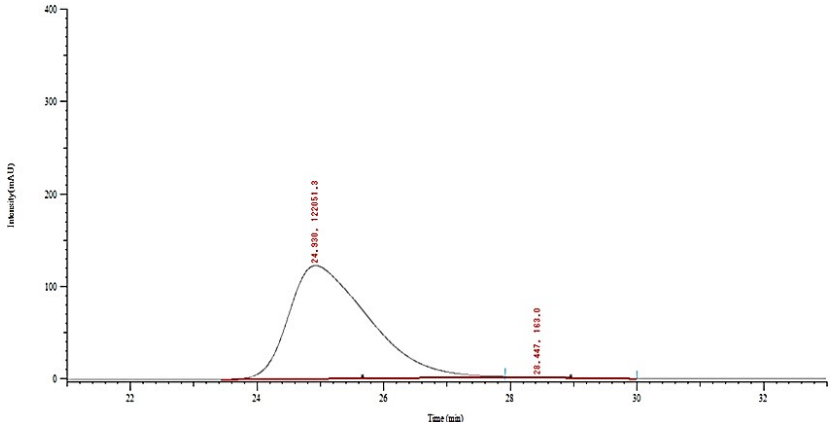
3f, HPLC analysis using a Chiralcel OD-H column, (HPLC: OD-H, 220 nm, *n*-hexane/isopropanol = 99:1, flow rate 1 mL/min)

***Rac*-3f**



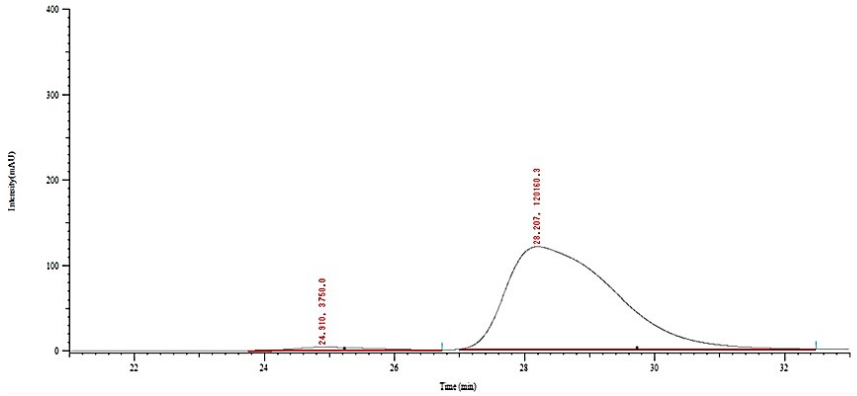
Peak	RT/min	Area	Area/%
1	24.063	11824582.8	50.067
2	27.517	11793034.8	49.933

(*S*)-3f



Peak	RT/min	Area	Area/%
1	24.930	10561947.2	99.932
2	28.447	7154.2	0.068

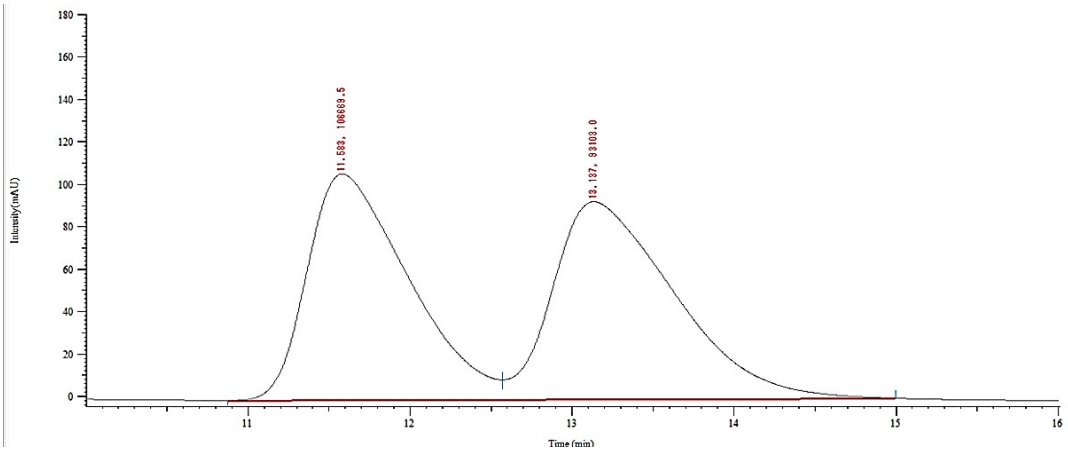
(*R*)-3f



Peak	RT/min	Area	Area/%
1	24.910	292422.1	2.076
2	28.207	13793305.3	97.924

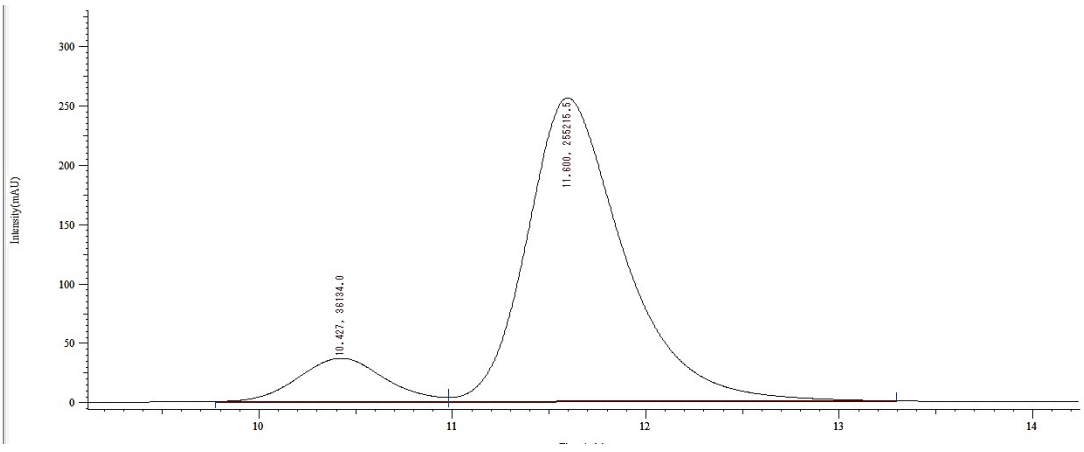
3g, HPLC analysis using a Chiralcel OD-H column (HPLC: OJ-H, 220 nm, *n*-hexane/isopropanol = 97:3)

***Rac*-3g**



Peak	RT/min	Area	Area/%
1	11.583	4545175.5	49.799
2	13.137	4581924.1	50.201

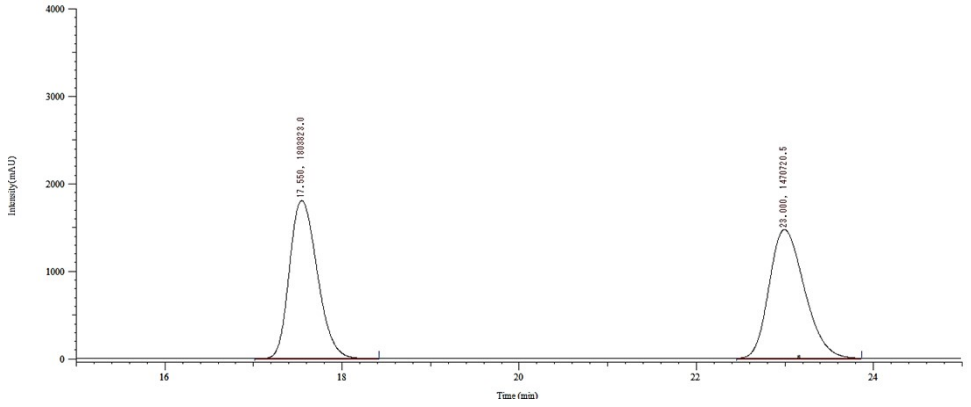
(*R*)-3g



Peak	RT/min	Area	Area/%
1	10.427	1117088.1	11.146
2	11.600	8905457.7	88.854

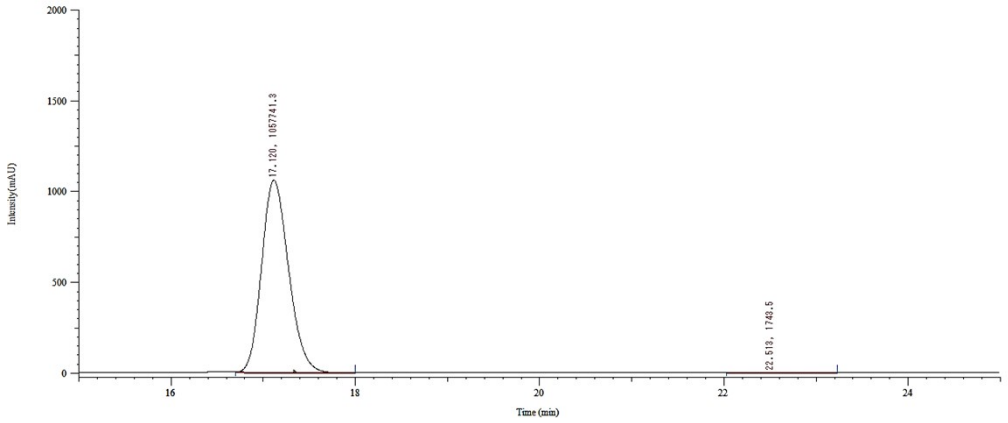
3h, HPLC analysis using a Chiralcel OJ-H column, (HPLC: OJ-H, 220 nm, *n*-hexane/isopropanol = 90:10, flow rate 1 mL/min)

***Rac*-3h**



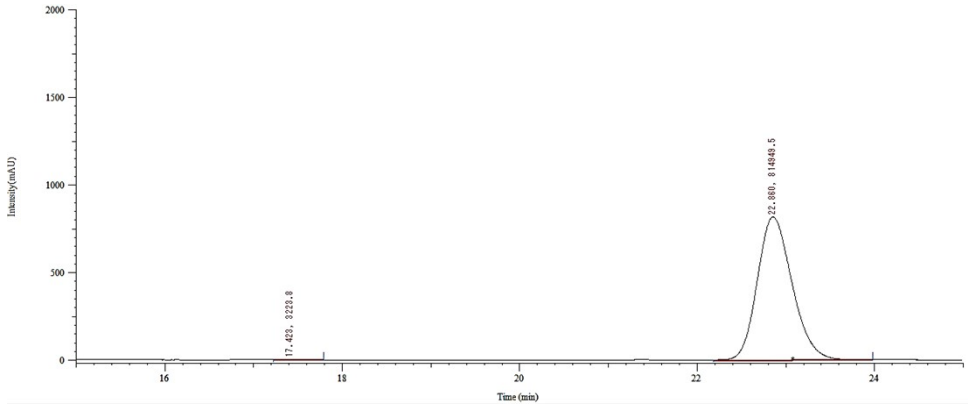
Peak	RT/min	Area	Area/%
1	17.550	39394692	49.165
2	23.000	40732411	50.835

(*S*)-3h



Peak	RT/min	Area	Area/%
1	17.120	21503870	99.794
2	22.513	44295	0.206

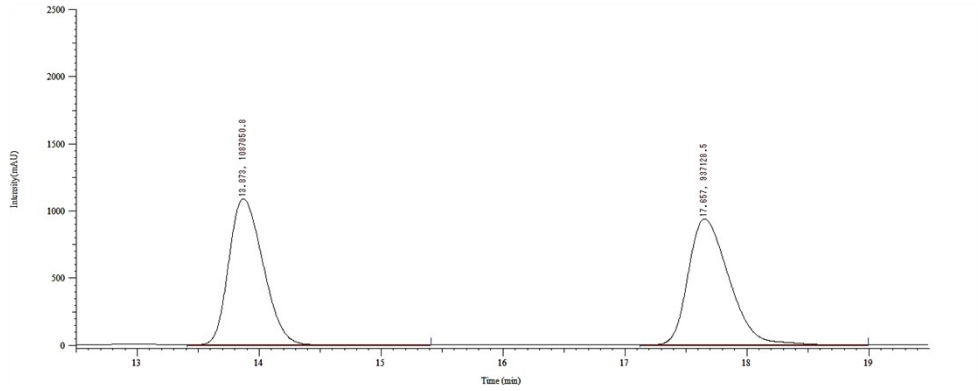
(*R*)-3h



Peak	RT/min	Area	Area/%
1	17.423	59362	0.268
2	22.860	22086474	99.732

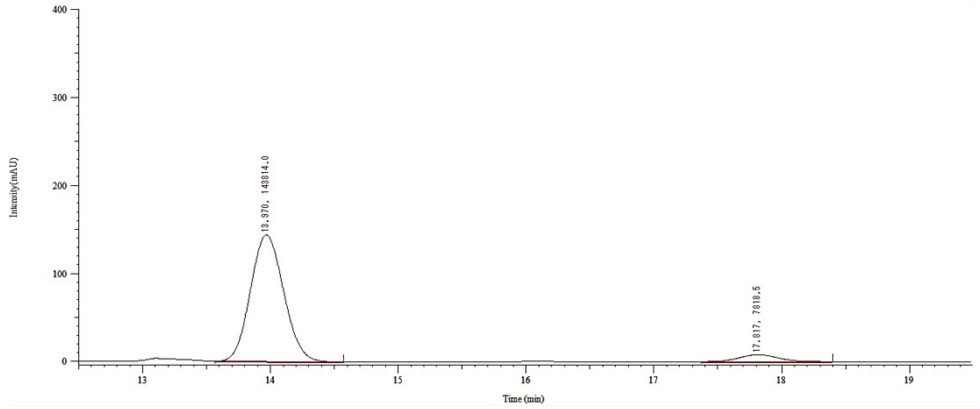
3i, HPLC analysis using a Chiralcel OJ-H column (HPLC: OJ-H, 220 nm, *n*-hexane/isopropanol = 95:5, flow rate 1 mL/min)

***Rac*-3i**



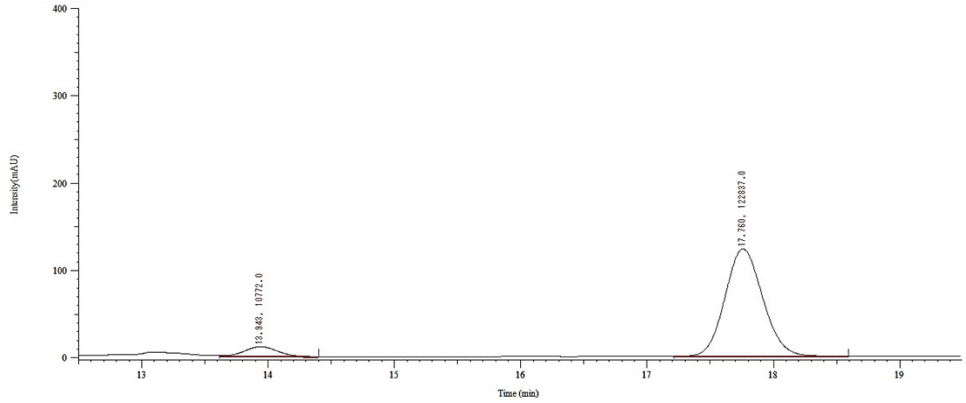
Peak	RT/min	Area	Area/%
1	13.873	21202334.3	49.375
2	17.657	21738819.4	50.625

(*S*)-3i



Peak	RT/min	Area	Area/%
1	13.970	2601458.9	94.973
2	17.817	151187.2	5.027

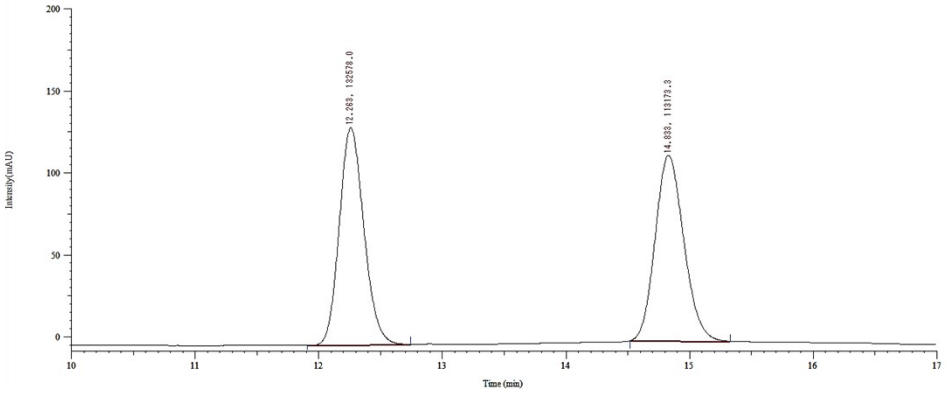
(*R*)-3i



Peak	RT/min	Area	Area/%
1	13.943	187348.7	6.858
2	17.760	2544631.9	93.142

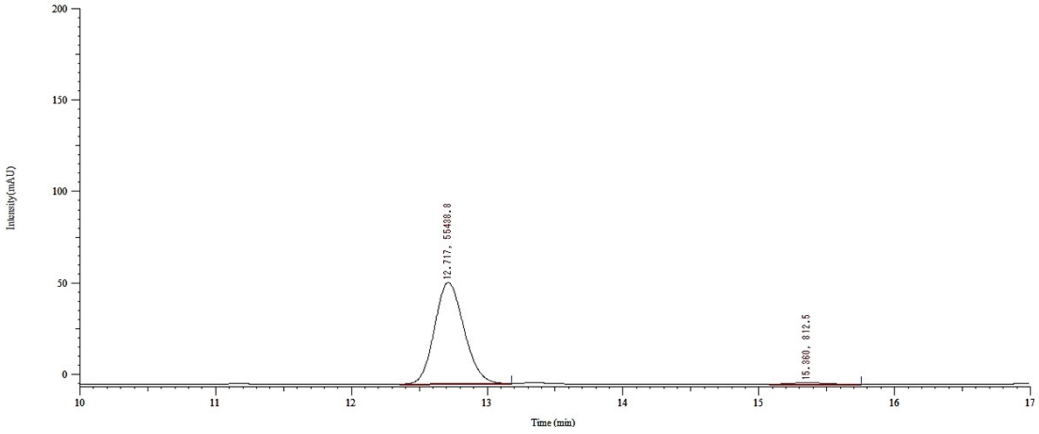
3j, HPLC analysis using a Chiralcel OJ-H column (HPLC: OJ-H, 220 nm, *n*-hexane/isopropanol = 95:5, flow rate 1 mL/min)

***Rac*-3j**



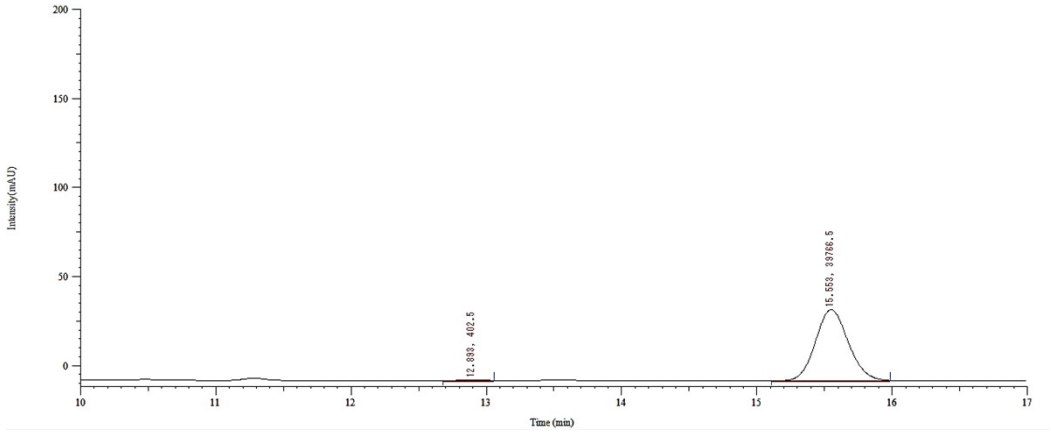
Peak	RT/min	Area	Area/%
1	12.263	1832259	50.105
2	14.833	1824587	49.895

(*S*)-3j



Peak	RT/min	Area	Area/%
1	12.717	831912	98.469
2	15.360	12931	1.531

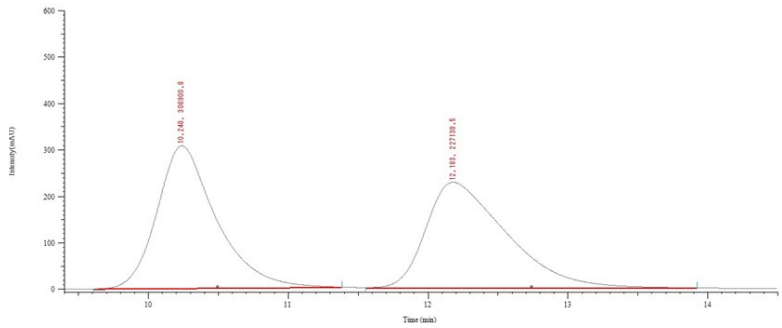
(*R*)-3j



Peak	RT/min	Area	Area/%
1	12.893	5322	0.782
2	15.553	675211	99.218

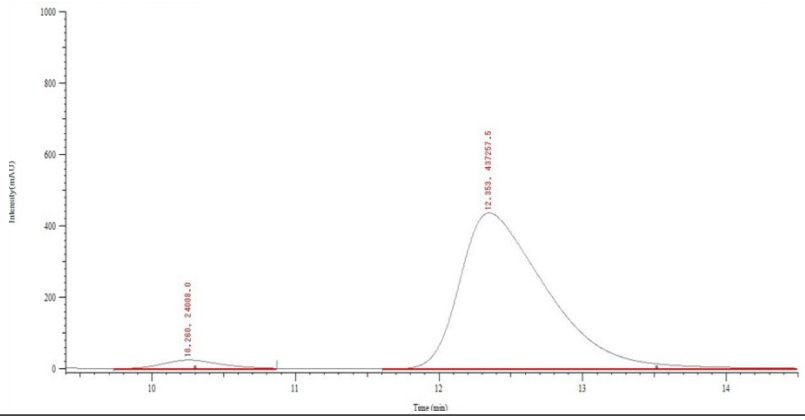
3k, HPLC analysis using a Chiralcel OD-H column (HPLC: OD-H, 220 nm, *n*-hexane/isopropanol = 95:5, flow rate 1 mL/min)

***Rac*-3k**



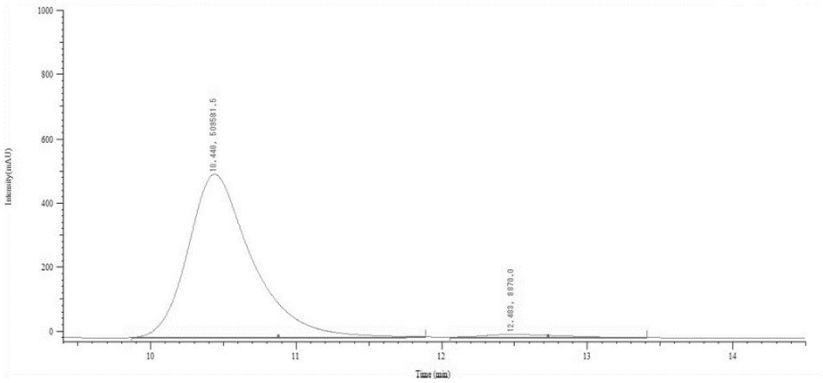
Peak	RT/min	Area	Area/%
1	10.240	9031933.6	49.994
2	12.183	9034193.1	50.006

(*S*)-3k



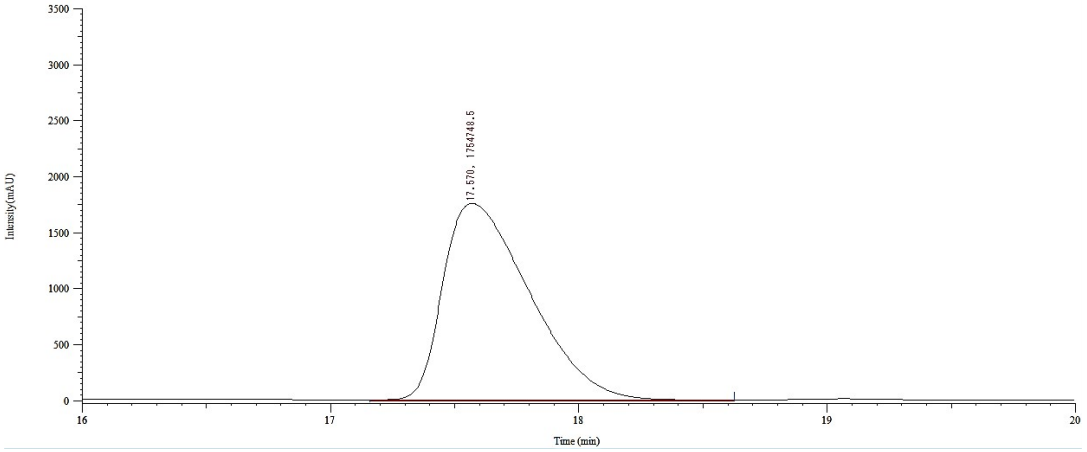
Peak	RT/min	Area	Area/%
1	10.260	655162.9	3.432
2	12.353	18432379.3	96.568

(*R*)-3k



Peak	RT/min	Area	Area/%
1	10.440	15528959.3	98.020
2	12.483	313758.0	1.980

2h, HPLC analysis using a Chiralcel OJ-H column (HPLC: OJ-H, 220 nm, *n*-hexane/isopropanol = 90:10, flow rate 1 mL/min)



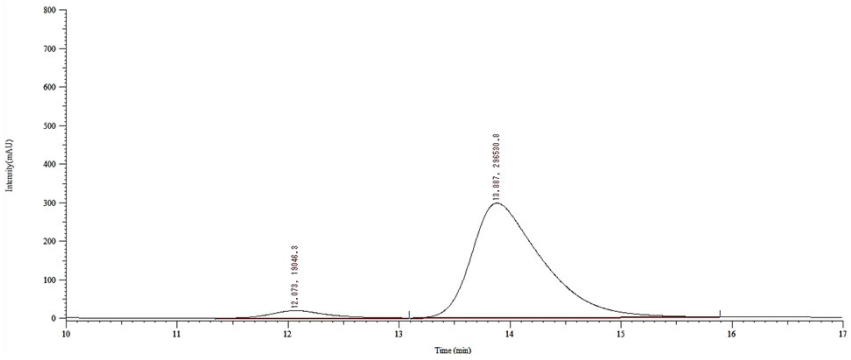
Peak	RT/min	Area	Area/%
1	17.560	37894036.2	100

Rac-**3a** as substrate, HPLC analysis using a Chiralcel OD-H column (HPLC: OD-H, 220 nm, *n*-hexane/isopropanol = 95:5, flow rate 1 mL/min).

***Rac*-3a**

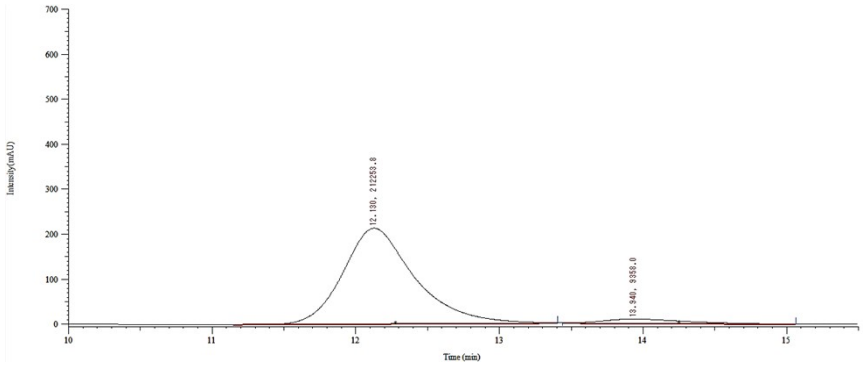
Peak	RT/min	Area	Area/%
1	11.743	14409349.6	50.304
2	13.380	14235240.9	49.696

(*S*)-3a



Peak	RT/min	Area	Area/%
1	12.073	640389.4	4.679
2	13.887	13045612.9	95.321

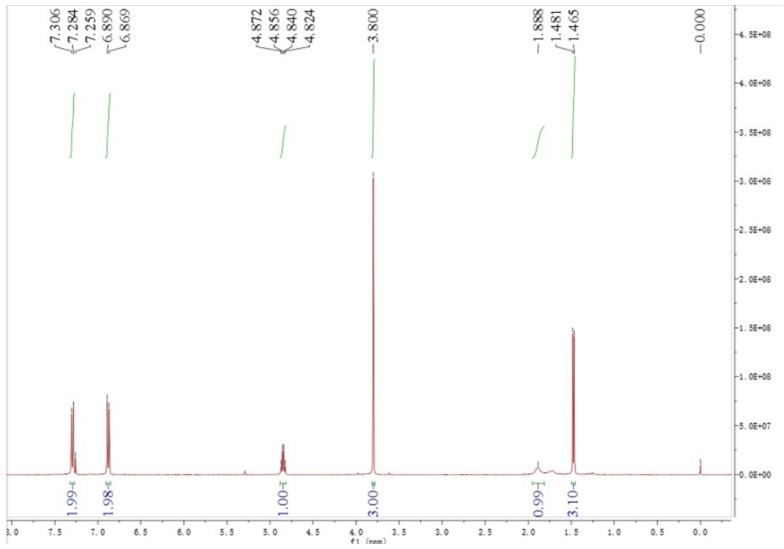
(*R*)-3a

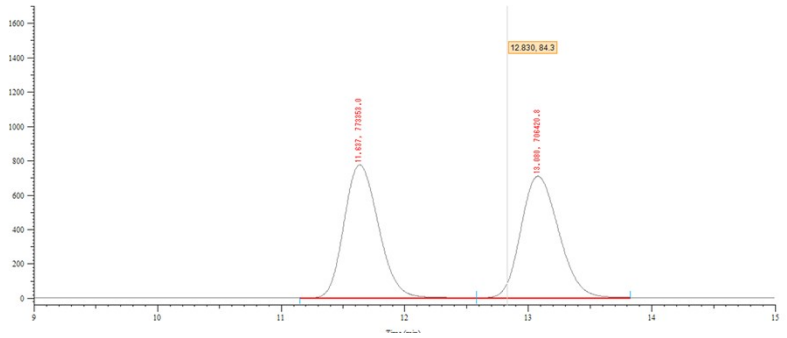


Peak	RT/min	Area	Area/%
1	12.130	7318188.7	95.409
2	13.940	352144.1	4.591

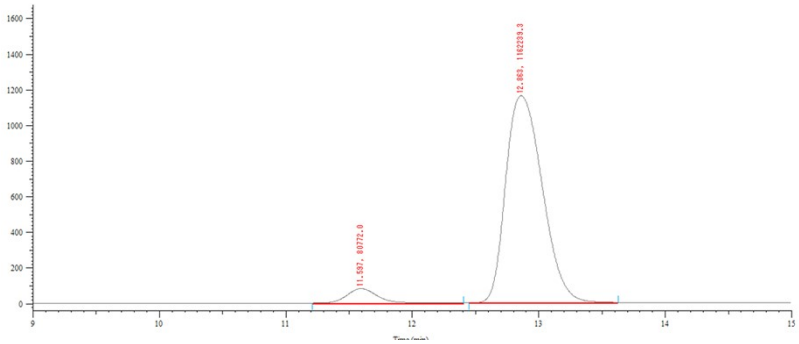
Spectral data for the enzymatic products

(*S*)-**3a**



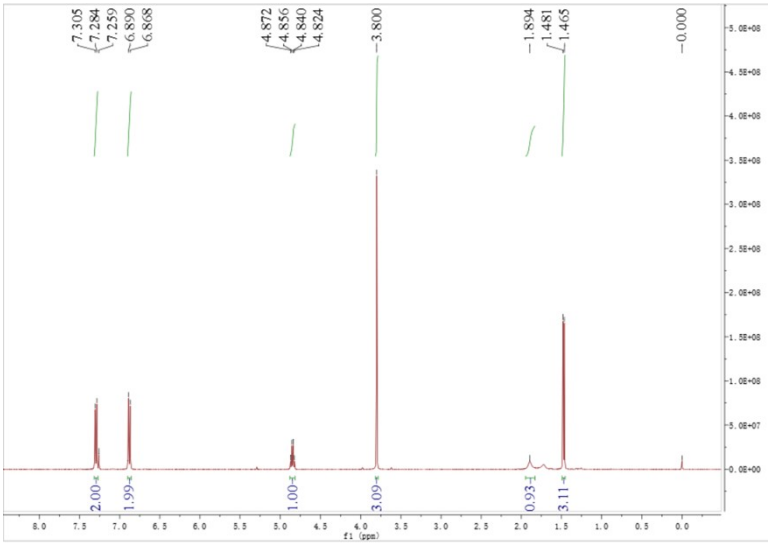


Peak	RT/min	Area	Area/%
1	11.637	14942332.3	49.937
2	13.080	14979756.3	50.063

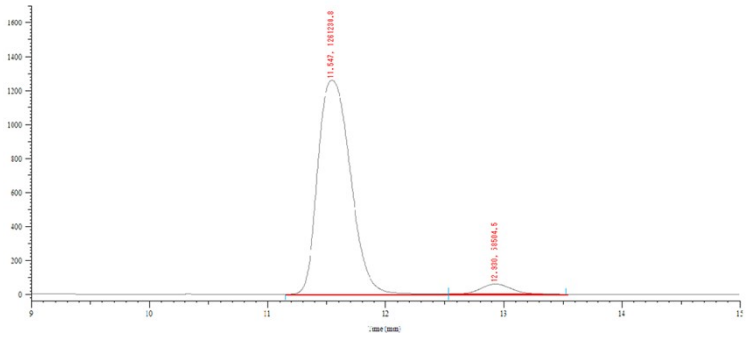


Peak	RT/min	Area	Area/%
1	11.597	1302062.2	5.261
2	12.863	23403523.0	94.729

(*R*)-**3a**



Peak	RT/min	Area	Area/%
1	11.637	14942332.3	49.937
2	13.080	14979756.3	50.063



Peak	RT/min	Area	Area/%
1	11.547	24214722.7	95.907
2	12.930	1033479.6	5.493

1. Niu, L.-X. Liu, B. Wu, and Y.-.Zhou, *J. Org. Chem.* 2023, **88**, 7863−7871