Semi-rational design of an imine reductase for asymmetric synthesis of alkylated *S*-4-azepanamines

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1. General

HPLC analysis was performed on Waters 2695 using C18 analytical column (Phenomenex Gemini, 4.6×250 mm, 5 μm). LC-MS analysis was performed on Agilent 1100 with a mass spectrum detector (MSD) using an analytical column (Ultimate XB-C18, 2.1×100 mm, 3.0 μm). Chiral HPLC analysis was performed on Waters 2695 using chiral analytical columns (CHIRALPAK AD/AY-H, 4.6×250 mm, 5 μm). ^{1}H, ^{13}C were recorded on Bruker AV 400 MHz instrument at 400 MHz (^{1}H NMR), 100 MHz (^{13}C NMR) or Bruker AV 500 MHz instrument at 500 MHz (^{1}H NMR), 125 MHz (^{13}C NMR). Silica gel plates pre-coated on glass were used for thin-layer chromatography using UV light, or 7% ethanolic phosphomolybdic acid or potassium permanganate solution and heating as the visualizing methods. Silica gel was used for flash column chromatography with mixed CH_2Cl_2 and MeOH or ethyl acetate (EtOAc) and hexane as the eluting solvents.

2. Materials

All commercially available chemicals and reagents, including ketone **1**, amines **a**-**g**, and $NADP^+$, were purchased from Meryer (Shanghai, China), Macklin (Shanghai, China), J&K Scientific (Beijing, China), or Sigma-Aldrich (Poole, Dorset, UK) unless stated otherwise. All HPLC or LC-MS grade solvents, including acetonitrile, *n*-hexane, and ethanol, were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Sodium phosphate buffer (0.1 M, pH 7.0) was prepared in-house.

3. Preparation of racemic amine and chiral amine standards

A dry methanol (10 mL) solution of the cyclic ketone (2.0 mmol), the corresponding amine (3.0 mmol), and acetic acid (300 μL) was stirred for 1.5 h at r.t under an N_2 atm. The reaction was placed in an ice-H_2O bath and sodium cyanoborohydride (0.377 g, 6.0 mmol) was added. Then the reaction mixture gradually warmed to r.t. and stirred overnight. The reaction progress was monitored by TLC and following completion was quenched with saturated $NaHCO_3$ solution (10 mL) was used and stirred for an additional 30 minutes. The mixture was extracted with EtOAc (3 × 10 mL) and the combined organic phase was concentrated under reduced pressure after drying by anhydrous sodium sulfate (Na_2SO_4). Column chromatography on silica afforded the corresponding racemic amine.
Using the same method, the optical pure standards can be prepared by the reduction amination reaction between the chiral amine and the corresponding ketone or aldehyde.

4. NMR data of amine products

4-benzylamino-1-boc-azepane, 1a

Colourless oil. ^{1}H NMR δ_H (500 MHz, CD_3OD) 7.44-7.52 (5H, overlap), 4.25 (2H, m), 3.65 (1H, m), 3.46 (1H, m), 3.41 (1H, m), 3.31 (1H, m), 3.21 (1H, m), 2.34 (1H, m), 2.23 (1H, m), 1.99 (1H, m), 1.78 (1H, m), 1.66 (2H, m), 1.47 (9H, s). ^{13}C NMR δ_C (125 MHz, CD_3OD) 157.1, 157.0, 132.8, 130.9, 130.6, 130.3, 81.38, 8.3, 59.5, 59.4, 49.9, 49.7, 47.39, 46.7, 43.6, 43.0, 32.4, 32.1, 30.6, 30.3, 28.7, 25.6, 25.4. HRMS (ESI) calculated for $C_{18}H_{28}N_2O_2$ $[M + H]^+$ 305.2229, found: 305.2309.

4-propylamino-1-boc-azepane, 1b

Colorless oil. ^{1}H NMR δ_H (400 MHz, $CDCl_3$) 3.53 (2H, m), 3.22 (2H, m), 2.97 (1H, s), 2.80 (2H, t J = 8.0 Hz), 2.32 – 2.06 (2H, m), 1.99 – 1.49 (6H, overlap), 1.42 (9H, s), 0.94 (3H, t, J = 7.4 Hz). ^{13}C NMR δ_C (100 MHz, $CDCl_3$) 155.37, 155.29, 79.87, 58.20, 57.84, 46.50, 45.72, 42.89, 42.39, 31.17, 30.87, 30.11, 29.42, 28.53, 25.21, 24.84, 19.81, 11.45. HRMS (ESI) calculated for $C_{14}H_{28}N_2O_2$ $[M + H]^+$ 257.2229, found: 257.2305.

4-amylamino-1-boc-azepane, 1c

Colorless oil. ^{1}H NMR δ_H (400 MHz, $CDCl_3$) 3.53 (2H, s), 3.39 – 3.14 (2H, m), 2.99 (1H, d, J = 11.1 Hz,), 2.84 (2H, t, J = 8.2 Hz), 2.21 (2H, m), 1.99 – 1.51 (6H, overlap), 1.44 (9H, s), 1.30 (4H, s), 0.88 (3H, s). ^{13}C NMR δ_C (100 MHz, $CDCl_3$) 155.38, 155.29, 79.95, 58.29, 57.99, 46.48, 45.74, 44.98, 42.87, 42.38, 31.13, 30.81, 30.18, 29.46, 28.98, 28.55, 25.95, 25.22, 24.83, 22.25, 13.92. HRMS (ESI) calculated for $C_{16}H_{32}N_2O_2$ $[M + H]^+$ 285.2542, found: 285.2616.

4-cyclopropylamino-1-boc-azepane, 1d

Colorless oil. ^{1}H NMR δ_H (400 MHz, CDC_{l3}) 3.65 – 3.40 (2H, m), 3.36 – 3.14 (2H, m), 3.03 (1H, s), 2.26 (3H, overlap), 1.98 – 1.83 (1H, m), 1.77 (1H, m), 1.60 (2H, m), 1.43 (9H, s), 0.90 (2H, d, J = 4.7 Hz), 0.71 (2H, d, J = 7.1 Hz). ^{13}C NMR δ_C (100 MHz, CDC_{l3}) 155.44, 79.76, 59.58, 59.28, 46.45, 45.71, 42.81, 42.24, 31.95, 31.71, 30.66, 29.93, 28.55, 27.92, 25.02, 24.69, 4.32, 4.20, 4.12, 4.03. HRMS (ESI) calculated for $C_{14}H_{26}N_2O_2$ $[M + H]^+$ 255.2073, found: 255.2150.

4-cyclobutylamino-1-boc-azepane, 1e

Colorless oil. ^{1}H NMR δ_H (400 MHz, CDC_{l3}) 3.65 (1H, t, J = 8.4 Hz), 3.49 (2H, m), 3.24 (2H, m), 2.99 – 2.89 (1H, m), 2.39 – 2.04 (6H, overlap), 1.88 (2H, m), 1.78 (2H, m), 1.60 (2H, m), 1.43 (9H, s). ^{13}C NMR δ_C (100 MHz, CDC_{l3}) 155.41, 155.31, 79.90, 56.88, 56.72, 49.85, 49.78, 46.54, 45.79, 42.74, 42.22, 31.51, 31.22, 30.33, 29.65, 28.55, 27.60, 27.46, 27.35, 25.12, 24.76, 15.58. HRMS (ESI) calculated for $C_{15}H_{28}N_2O_2$ $[M + H]^+$ 269.2229, found: 269.2300.

4-phenethylamino-1-boc-azepane, 1f

Colorless oil. ^{1}H NMR δ_H (400 MHz, CDC_{l3}) δ 7.34 – 7.20 (5H, overlap), 3.56 (2H, m), 3.29 (2H, m), 3.19 – 3.02 (5H, overlap), 2.23 (2H, m), 1.90 (2H, m), 1.72 (1H, m), 1.59 (1H, m), 1.47 (9H, s). ^{13}C NMR δ_C (100 MHz, CDC_{l3}) 155.39, 155.28, 136.80, 128.99, 128.76, 127.23, 79.97, 77.48, 77.36, 77.16, 76.84, 58.57, 58.24, 46.52, 46.41, 45.68, 42.80, 42.30, 32.81, 31.49, 31.14, 30.33, 29.71, 28.55, 25.06, 24.72. HRMS (ESI) calculated for $C_{19}H_{30}N_2O_2$ $[M + H]^+$ 319.2386, found:

319.2488.

4-phenylpropylamino-1-boc-azepane, 1g

Colorless oil. ^{1}H NMR δ_H (400 MHz, CDC_{l3}) 7.30 (2H, overlap), 7.21 (3H, overlap), 3.51 (2H, s), 3.21 (2H, m), 2.99 (1H, t, J = 10.6 Hz), 2.94 – 2.82 (2H, m), 2.67 (2H, t, J = 7.6 Hz), 2.29 – 1.99 (4H, overlap), 1.92 – 1.72 (2H, m), 1.65 (2H, m), 1.47 (9H, s). ^{13}C NMR δ_C (100 MHz, CDC_{l3}) 155.39, 140.05, 128.77, 128.40, 126.53, 80.00, 77.48, 77.36, 77.16, 76.84, 58.66, 58.34, 46.39, 45.64, 44.61, 42.71, 42.24, 32.90, 31.11, 30.83, 30.21, 29.53, 28.56, 27.82, 25.09, 24.72. HRMS (ESI) calculated for $C_{20}H_{32}N_2O_2$ $[M + H]^+$ 333.2542, found: 333.2636.

5. HPLC and chiral HPLC analysis

All biotransformation products were analyzed by HPLC or LC-MS and the products were confirmed by ESI-MS or UV spectrum compared with standards. The chiral products and chiral amine standards were analyzed by chiral HPLC with different chiral columns and different solvent ratios of *n*-hexane and ethanol containing 0.02% diethylamine (Supplementary Figs. 1-7). The conversions were calculated according to the standard curves. Standard curves were plotted for varying concentrations of products using the UV detection wavelength of 210 nm (**1a**, **1f**-**1g**) or 204 nm (**1b**-**1e**), which were analyzed by Waters 2695 Separation Module and Wates 2996 Photodiode Array Detector with gradient method (1.0 mL/min, 20 min, H_2O/MeCN, 90/10→0/100, v/v) using C-18 analytic column (Phenomenex Gemini, 4.6×250 mm, 5 μm).

6. Expression and purification of M5 and variants

The plasmids containing the genes of IR-G36-M5 and its mutants were used to transform to *E. coli* BL21(DE3) competent cells for gene expression. Pre-cultures were grown in LB-medium (10 mL) containing 50 μg/mL kanamycin overnight at 37 °C with shaking at 220 rpm. 1L LB cultures were inoculated with the pre-culture (10 mL) and incubated at 37°C, with shaking at 220 rpm to an OD_{600} of 0.6-0.8. Gene expression was induced by the addition of 0.1 mM isopropyl-*β*-*D*-thiogalactopyranoside (IPTG) and shaking was continued for 16 h at 18 °C, 180 rpm. The cells were then harvested by centrifugation at 5000 rpm for 30 min and resuspended in binding buffer (50 mM Tris-HCl buffer pH 8.0, 300 mM NaCl, containing 20 mM imidazole). Cells were disrupted by ultrasonication for 30 min, 5 s on, 9 s off cycles, and the suspension was centrifuged at 12,000 rpm for 25 min to yield a clear lysate. The *N*-terminal His-tagged proteins were purified using the Ni-NTA column. In each case, the lysate was loaded onto a pre-equilibrated Ni-NTA column, followed by washing with 40~60 mL washing buffer (50 mM Tris-HCl buffer pH 8.0, 300 mM NaCl, containing 40 mM imidazole). The bound protein was eluted with elution buffer containing 250 mM

imidazole. Proteins were concentrated, and used for biotransformation reactions.

7. Triple code saturation mutagenesis library construction and screening procedure

Triple code saturation mutagenesis (A/L/F) library were constructed using Fast Mutagenesis System (TransGen Biotech). The primers of the mutation site were referred to in Supplementary Table 1. Cell-free extract of variants of M5 were used to measuring the enantioselectivity for ketone **1** and amine **a**. A typical 5 mL reaction mixture contained 30 mM ketone **1**, 33 mM amine **a** (1.1 eq),1 mM $NADP^+$, cell-free extracts (10 g/L, wet cells weight), 45 mM *D*-glucose (1.5 eq), 1 mg/mL glucose dehydrogenase (GDH), and 10% DMSO in sodium phosphate buffer (100 mM, pH 7.0). All reactions were incubated at 30 ℃ with shaking at 220 rpm for 24 h, after which they were quenched by 1 mL saturated sodium carbonate ($NaHCO_3$) and extracted with ethyl acetate (3×5 mL). The combined organic extracts were finally dried using anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The extracts were dissolved in ethanol and subjected to HPLC analysis for measurement of the ee values.

8. Site-saturation mutagenesis libraries construction and screening procedure

Site-saturation mutagenesis libraries were also constructed using Fast Mutagenesis System (TransGen Biotech). The codon of the mutation site was replaced by the NNK degeneracy codon (Supplementary Table 1). Colonies were picked up in 96-well plates containing 600 μL LB medium with 50 μg/mL kanamycin and cultured overnight at 37 ℃ with 800 rpm. The 19 diverse variants with the mutation site respectively replaced by the remaining AA residues were obtained through DNA sequencing. Pre-cultures of the variants were grown in LB-medium (5 mL) containing 50 μg/mL kanamycin overnight at 37 °C with shaking at 220 rpm. An aliquot of 500 μL was transferred to a new culture flask containing 50 mL LB medium with 50 μg/mL kanamycin and cultured at 37 ℃ with shaking at 220 rpm to an OD_{600} of 0.6~0.8. The cells were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 20 °C, 220 rpm. The cells were harvested and washed twice with 10 mL potassium phosphate buffer (100 mM, pH 7.0) and centrifuged for 30 min with 4000 rpm. Then, the pellets were resuspended in 3 mL of the same buffer containing 6 U DNase I and 1 mg/mL lysozyme for breaking the cell at 30 °C, 200 rpm for 2 h. Then, 2 mL stock solution containing ketone **1** (30 mM, 1eq), amine **a** (33 mM, 1.1eq), $NADP^+$ (1 mM), *D*-glucose (45 mM, 1.5 eq), 1 mg/mL glucose dehydrogenase (GDH), and 20% v/v DMSO was added. After being shaken for 24 h at 30 °C with 200 rpm, reactions were quenched by 1 mL saturated $NaHCO_3$ and extracted with ethyl acetate (3×5 mL). The combined organic extracts were dried by anhydrous

Na_2SO_4 and evaporated under reduced pressure for further detecting the e.e. values by HPLC analysis.

9. Biotransformation for purification enzyme

For analyzing the reductive aminations of ketone **1** and amines **a-g**, enzyme of M5-I149Y/L200H/W234K was purified. A typical 5 mL reaction mixture contained 30-80 mM ketone **1** with amine **a** (1.1 eq), or 60 mM amine **1** with amine **b-g** (1.1 eq), 1 mM $NADP^+$, purification enzyme (5 mg/mL), D-glucose (1.5 eq), 1 mg/mL glucose dehydrogenase (GDH), and 20% DMSO in sodium phosphate buffer (100 mM, pH 7.0). All reactions were incubated at 30 ℃ with shaking at 220 rpm for 24 h, and were quenched by 1 mL saturated $NaHCO_3$. The liquids were extracted with ethyl acetate (3×5 mL) and the combined organic extracts were evaporated under reduced pressure after drying by anhydrous Na_2SO_4. The extracts were dissolved in ethanol and subjected to HPLC analysis for measurement of the conversions and e.e. values.

10. Molecular docking

Molecular docking was performed by using the CDOCKER procedure of Discovery Studio Client. The previously reported crystal structure of M5 (PDB: 7WNW) was used as the receptor target in this study, which was a complex with $NADP^+$. The docking of imine intermediate of **1a'** into M5 was performed using a flexible docking protocol. Docking runs were carried out using the standard parameters of the program. The conformations with the lowest energy were chosen for the analysis of substrate-enzyme interactions and distance between carbonyl group of the substrate and C_4-H of NADPH.

11. Sequence of IR-G36-M5 and the triple-mutant

DNA sequence of IR-G36-M5

ATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCCGG
AATCTACCACCCCGAGTACCGCCACCCCGGTGACCATCATCGGTCTTGGTGCAATGGGCACCGCCCT
GGCAAACGCATTCCTCGATGCAGGTCATAGTACCACCGTTTGGAATCGTACCGCAGCACGCGCCACC
GCATTAGCCGCACGCGGCGCACATCATGCAGAAACCGTGACCGAAGCCATTGCAGCCTCTCCGTTAG
TGATTGCCTGTGTGCTGGATTATGATGCCTTTCATGAAACCTTAGCCCCGGCTACAGACGCGCTGGCA
GGTCGCGCCCTGGTTAATCTGACCACAGGTACCCCGAAACAGGCACGCGAAACCGCCTCTTGGGCAG
CCGATCATCGTATTGATTATCTGGATGGCAAAATTATGGCCATTCCGCCGGGTATTGCAACCCCGGAT
AGTTTTATTCTGTATAGCGGTCCGTTAGGTACCTTTGAAGCACATCGCTCAACCTTAGAAGTGCTGGG
CGCAGCAAATCATGTGGGTACCGATGCAGGTTTGGCGAGCTTACATGATATTGCACTGCTGACCGGT
ATGTATGGCATGATTGCAGGCATTTTACAGGCCTTTGCCTTAATTGATAGTGAAGGTATTCCGGCAGG

CGATCTGGCCCCGATGTTAACCAATTGGTTAACCGGCGCAGCACATAGCGTGGCCCATTATGCCCAG
CAGATTGATACCGGCGATTATGAAACCGGTGTTGTGTTTAATTTAGCACATCAGAGCCATGGCTTTGC
AAAATTAGTTCAGGCCGGTGAAGATCAGGGTGTGGATGTGGGCTTACTGCGTCCGCTGTTTGAACTG
ATGCGTCATCAGGTTGCCGCAGGCTATGGTAATGGTGATGTTGCCTCAGTTATTGAACTGATTCGTCG
CGAAGAACGTCGTCAGCCGGCCAAAAGTCCGGGCGCAGATAAAATTACCCGTGCACGTCGTCCGTA
A

Amino acid sequence IR-G36-M5

MGSSHHHHHHSSGLVPRGSHMPESTTPSTATPVTIIGLGAMGTALANAFLDAGHSTTVWNRTAARATAL
AARGAHHAETVTEAIAASPLVIACVLDYDAFHETLAPATDALAGRALVNLTTGTPKQARETASWAADHR
IDYLDGKIMAIPPGIATPDSFILYSGPLGTFEAHRSTLEVLGAANHVGTDAGLASLHDIALLTGMYGMIAGI
LQAFALIDSEGIPAGDLAPMLTNWLTGAAHSVAHYAQQIDTGDYETGVVFNLAHQSHGFAKLVQAGED
QGVDVGLLRPLFELMRHQVAAGYGNGDVASVIELIRREERRQPAKSPGADKITRARRP*

DNA sequence of M5-I149Y-L200H-W234K

ATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCCGG
AATCTACCACCCCGAGTACCGCCACCCCGGTGACCATCATCGGTCTTGGTGCAATGGGCACCGCCCT
GGCAAACGCATTCCTCGATGCAGGTCATAGTACCACCGTTTGGAATCGTACCGCAGCACGCGCCACC
GCATTAGCCGCACGCGGCGCACATCATGCAGAAACCGTGACCGAAGCCATTGCAGCCTCTCCGTTAG
TGATTGCCTGTGTGCTGGATTATGATGCCTTTCATGAAACCTTAGCCCCGGCTACAGACGCGCTGGCA
GGTCGCGCCCTGGTTAATCTGACCACAGGTACCCCGAAACAGGCACGCGAAACCGCCTCTTGGGCAG
CCGATCATCGTATTGATTATCTGGATGGCAAAATTATGGCCTATCCGCCGGGTATTGCAACCCCGGAT
AGTTTTATTCTGTATAGCGGTCCGTTAGGTACCTTTGAAGCACATCGCTCAACCTTAGAAGTGCTGGG
CGCAGCAAATCATGTGGGTACCGATGCAGGTTTGGCGAGCTTACATGATATTGCACTGCATACCGGT
ATGTATGGCATGATTGCAGGCATTTTACAGGCCTTTGCCTTAATTGATAGTGAAGGTATTCCGGCAGG
CGATCTGGCCCCGATGTTAACCAATAAATTAACCGGCGCAGCACATAGCGTGGCCCATTATGCCCAG
CAGATTGATACCGGCGATTATGAAACCGGTGTTGTGTTTAATTTAGCACATCAGAGCCATGGCTTTGC
AAAATTAGTTCAGGCCGGTGAAGATCAGGGTGTGGATGTGGGCTTACTGCGTCCGCTGTTTGAACTG
ATGCGTCATCAGGTTGCCGCAGGCTATGGTAATGGTGATGTTGCCTCAGTTATTGAACTGATTCGTCG
CGAAGAACGTCGTCAGCCGGCCAAAAGTCCGGGCGCAGATAAAATTACCCGTGCACGTCGTCCGTA
A

Amino acid sequence M5-I149Y-L200H-W234K

MGSSHHHHHHSSGLVPRGSHMPESTTPSTATPVTIIGLGAMGTALANAFLDAGHSTTVWNRTAARATAL
AARGAHHAETVTEAIAASPLVIACVLDYDAFHETLAPATDALAGRALVNLTTGTPKQARETASWAADHR
IDYLDGKIMAYPPGIATPDSFILYSGPLGTFEAHRSTLEVLGAANHVGTDAGLASLHDIALHTGMYGMIAG
ILQAFALIDSEGIPAGDLAPMLTNKLTGAAHSVAHYAQQIDTGDYETGVVFNLAHQSHGFAKLVQAGED

QGVDVGLLRPLFELMRHQVAAGYGNGDVASVIELIRREERRQPAKSPGADKITRARRP*

Supplementary Tables

Table 1. List of primers in this study.

Primer	mutant	Sequences (5′→3′)
F	S241A	AGCACATGCCGTGGCCCATTATGCCCA
R	S241A	GGGCCACGGCATGTGCTGCGCCGGTTAA
F	S241L	AGCACATTTAGTGGCCCATTATGCCCAG
R	S241L	GGGCCACTAAATGTGCTGCGCCGGTTAA
F	S241F	GCGCAGCACATTTTGTGGCCCATTATGCCCAGCAGATT
R	S241F	ATAATGGGCCACAAAATGTGCTGCGCCGGTTAACCAATT
F	M203A	ACCGGTGCATATGGCATGATTGCAGGCATT
R	M203A	GCCATATGCACCGGTCAGCAGTGCAATAT
F	M203L	ACCGGTTTATATGGCATGATTGCAGGCATT
R	M203L	GCCATATAAACCGGTCAGCAGTGCAATATC
F	M203F	ACCGGTTTTTATGGCATGATTGCAGGCATT
R	M203F	GCCATAAAAACCGGTCAGCAGTGCAATATC
F	I149A	TATGGCCGCACCGCCGGGTATTGCAACCC
R	I149A	CCCGGCGGTGCGGCCATAATTTTGCCATC
F	I149L	TATGGCCTTACCGCCGGGTATTGCAACCC
R	I149L	CCCGGCGGTAAGGCCATAATTTTGCCATCC
F	I149F	TATGGCCTTTCCGCCGGGTATTGCAACCC
R	I149F	CCCGGCGGAAAGGCCATAATTTTGCCATCC
F	W234A	TTAACCAATGCATTAACCGGCGCAGCACAT
R	W234A	CCGGTTAATGCATTGGTTAACATCGGGGCC
F	W234L	TTAACCAATTTATTAACCGGCGCAGCACAT
R	W234L	CCGGTTAATAAATTGGTTAACATCGGGGCC
F	W234F	TTAACCAATTTTTAACCGGCGCAGCACAT
R	W234F	CCGGTTAAAAAATTGGTTAACATCGGGGCC
F	L200A	GCACTGGCCACCGGTATGTATGGCATG
R	L200A	CATACCGGTGGCCAGTGCAATATCATG
F	L200F	GCACTGTTTACCGGTATGTATGGCATG
R	L200F	CATACCGGTAAACAGTGCAATATCATG
F	A238L	AACCGGCCTGGCACATAGCGTGGCCCATTA
R	A238L	ATGTGCCAGGCCGGTTAACCAATTGGTTAA
F	A238F	AACCGGCTTTGCACATAGCGTGGCCCATTA

R	A238F	ATGTGCAAAGCCGGTTAACCAATTGGTTAA
F	W234NNK	ACCAATNNKTTAACCGGCGCAGCACATAGC
R	W234NNK	GCCGGTTAAMNNATTGGTTAACATCGGGGC
F	L200NNK	GCACTGNNKACCGGTATGTATGGCATGA
R	L200NNK	CCGGTMNNCAGTGCAATATCATGTAAGC
F	I149NNK	TATGGCCNNKCCGCCGGGTATTGCAACCC
R	I149NNK	CCGGCGGMNNGGCCATAATTTTGCCATCC

Table 2. Enantioselectivity of the mutants generated by Triple code saturation mutagenesis (A/L/F) of residues S241, M203, and A238 over M5.

Mutants	ee value	Mutants	ee value	Mutants	ee value
S241A	38%, *S*	M203A	57%, *S*	A238L	42%, *S*
S241L	30%, *S*	M203L	22%, *S*	A238F	31%, *S*
S241F	9%, *S*	M203F	48%, *S*		

Table 3. Enantioselectivity of the mutants generated by site-saturation mutagenesis of residue W234 over M5.

Mutants	ee value	Mutants	ee value	Mutants	ee value
W234H	48%, *S*	W234M	48%, *S*	W234N	76%, *S*
W234R	83%, *S*	W234P	71%, *S*	W234Q	71%, *S*
W234K	89%, *S*	W234W	-	W234Y	62%, *S*
W234A	75%, *S*	W234G	75%, *S*	W234D	74%, *S*
W234L	56%, *S*	W234C	59%, *S*	W234E	70%, *S*
W234I	48%, *S*	W234S	74%, *S*	W234F	37%, *S*
W234V	58%, *S*	W234T	62%, *S*		

Table 4. Enantioselectivity of the mutants generated by site-saturation mutagenesis of residue I149 over M5.

Mutants	ee value	Mutants	ee value	Mutants	ee value
I149H	81%, *S*	I149M	38%, *S*	I149N	72%, *S*
I149R	77%, *S*	I149P	70%, *S*	I149Q	48%, *S*
I149K	69%, *S*	I149W	85%, *S*	I149Y	91%, *S*
I149A	49%, *S*	I149G	66%, *S*	I149D	71%, *S*
I149L	42%, *S*	I149C	50%, *S*	I149E	47%, *S*
I149I	-	I149S	55%, *S*	I149F	88%, *S*
I149V	20%, *S*	I149T	48%, *S*		

Table 5. Enantioselectivity of the mutants generated by site-saturation mutagenesis of residue L200 over M5.

Mutants	ee value	Mutants	ee value	Mutants	ee value
L200H	71%, *S*	L200M	51%, *S*	L200N	0%, *S*
L200R	N.D.	L200P	N.D.	L200Q	28%, *S*
L200K	N.D.	L200W	7%, *R*	L200Y	0%, *S*
L200A	24%, *S*	L200G	25%, *S*	L200D	16%, *S*
L200L	-	L200C	24%, *R*	L200E	25%, *S*
L200I	3%, *S*	L200S	6%, *S*	L200F	23%, *R*
L200V	23%, *R*	L200T	27%, *R*		

N.D. was not determined because the mutants were almost inactivated.

Chiral HPLC Chromatograms

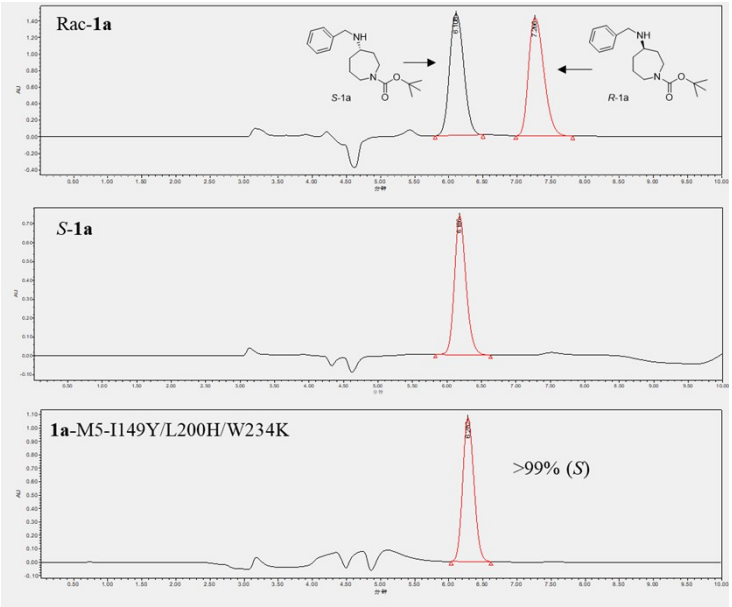

Figure 1. Chiral HPLC analysis of racemic standard of **1a** (up), optically pure standard of (*S*)-**1a** (middle)**,** and M5-I149Y/L200H/W234K catalytic product **1a** (bottom). HPLC conditions: CHIRALPAK AD-H column with a mobile phase of *n*-hexane/ethanol (90:10, v/v, 0.2‰ diethylamine), flow rate 1.0 mL/min, 30 °C, UV detection at 210 nm.

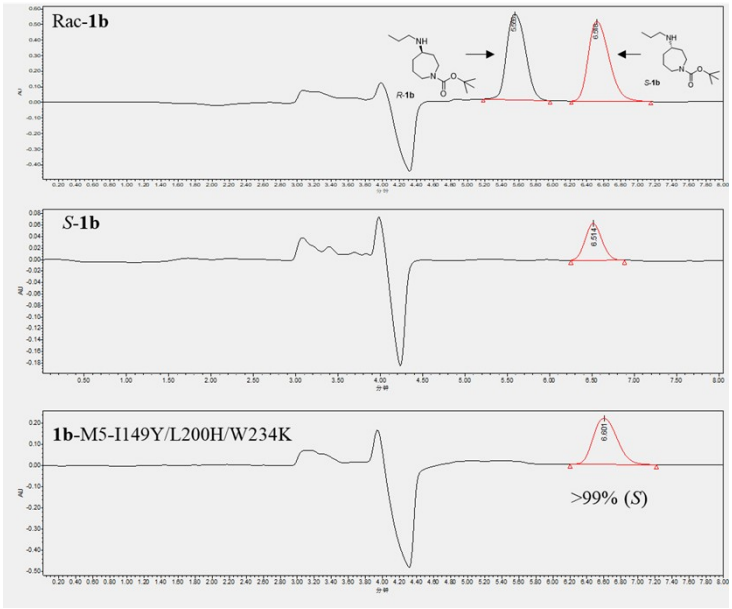

Figure 2. Chiral HPLC analysis of racemic standard of **1b** (up), optically pure standard of (*S*)-**1b** (middle)**,** and M5-I149Y/L200H/W234K catalytic product **1b** (bottom). HPLC conditions: CHIRALPAK IG column with a mobile phase of *n*-hexane/ethanol (80:20, v/v, 0.2‰ diethylamine), flow rate 1.0 mL/min, 30 °C, UV detection at 204 nm.

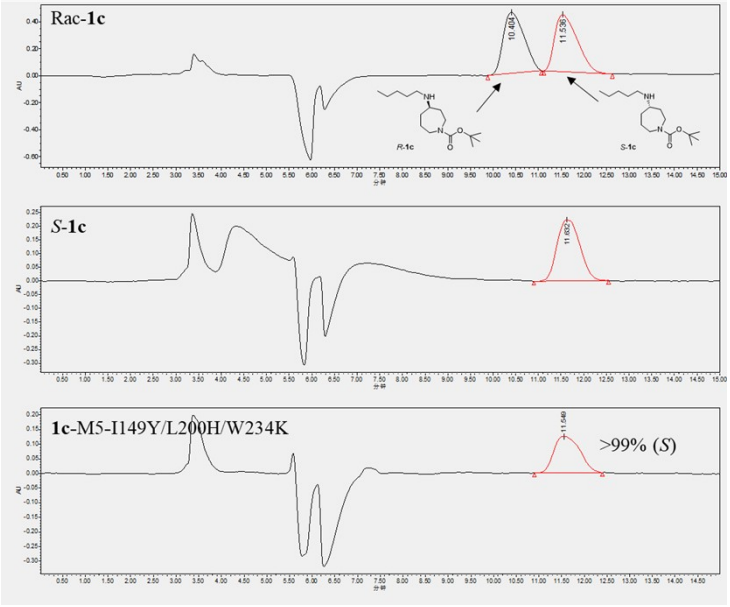

Figure 3. Chiral HPLC analysis of racemic standard of **1c** (up), optically pure standard of (*S*)-**1c** (middle)**,** and M5-I149Y/L200H/W234K catalytic product **1c** (bottom). HPLC conditions: CHIRALPAK IG column with a mobile phase of *n*-hexane/ethanol (95:5, v/v, 0.2‰ diethylamine), flow rate 1.0 mL/min, 30 °C, UV detection at 204 nm.

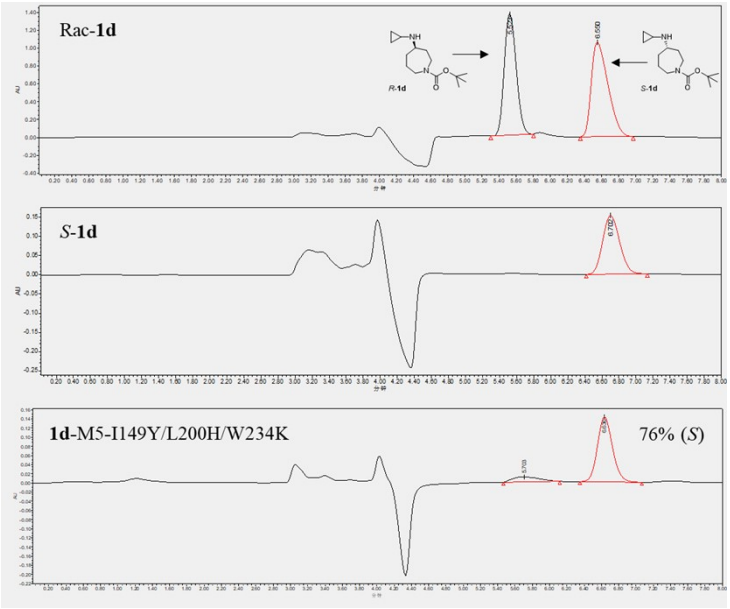

Figure 4. Chiral HPLC analysis of racemic standard of **1d** (up), optically pure standard of (*S*)-**1d** (middle)**,** and M5-I149Y/L200H/W234K catalytic product **1d** (bottom). HPLC conditions: CHIRALPAK IG column with a mobile phase of *n*-hexane/ethanol (80:20, v/v, 0.2‰ diethylamine), flow rate 1.0 mL/min, 30 °C, UV detection at 204 nm.

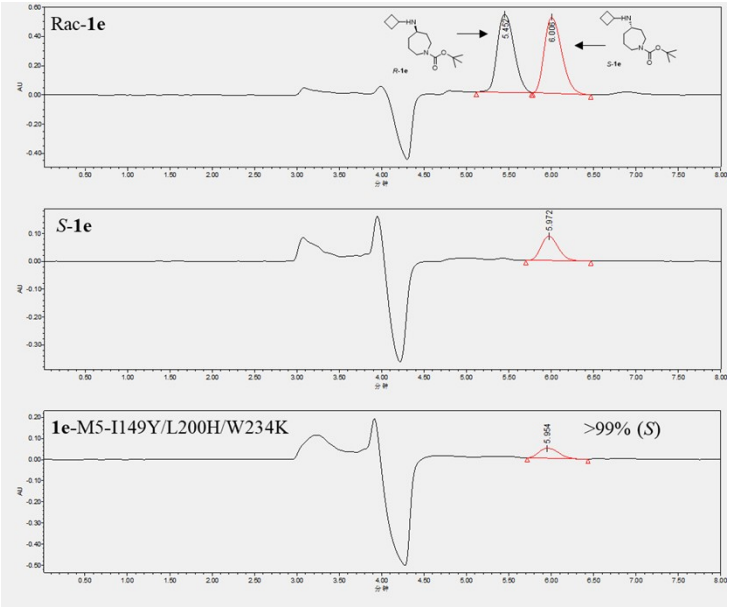

Figure 5. Chiral HPLC analysis of racemic standard of **1e** (up), optically pure standard of (*S*)-**1e** (middle)**,** and M5-I149Y/L200H/W234K catalytic product **1e** (bottom). HPLC conditions: CHIRALPAK IG column with a mobile phase of *n*-hexane/ethanol (80:20, v/v, 0.2‰ diethylamine), flow rate 1.0 mL/min, 30 °C, UV detection at 204 nm.

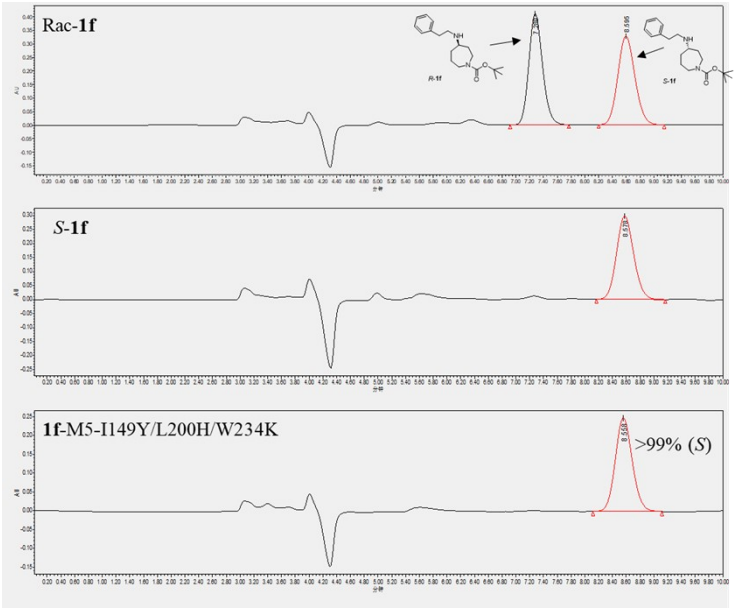

Figure 6. Chiral HPLC analysis of racemic standard of **1f** (up), optically pure standard of (*S*)-**1f** (middle)**,** and M5-I149Y/L200H/W234K catalytic product **1f** (bottom). HPLC conditions: CHIRALPAK IG column with a mobile phase of *n*-hexane/ethanol (80:20, v/v, 0.2‰ diethylamine), flow rate 1.0 mL/min, 30 °C, UV detection at 210 nm.

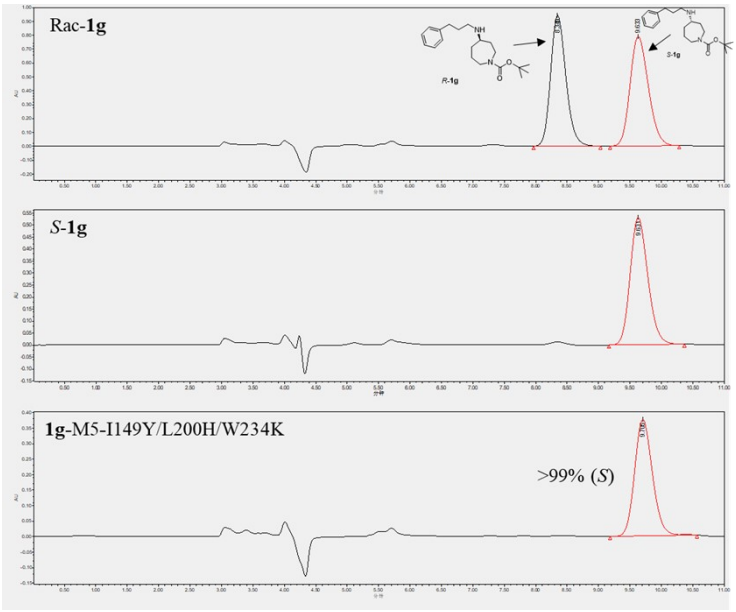

Figure 7. Chiral HPLC analysis of racemic standard of **1g** (up), optically pure standard of (*S*)-**1g** (middle)**,** and M5-I149Y/L200H/W234K catalytic product **1g** (bottom). HPLC conditions: CHIRALPAK IG column with a mobile phase of *n*-hexane/ethanol (80:20, v/v, 0.2‰ diethylamine), flow rate 1.0 mL/min, 30 °C, UV detection at 210 nm.

NMR spectra of amine products

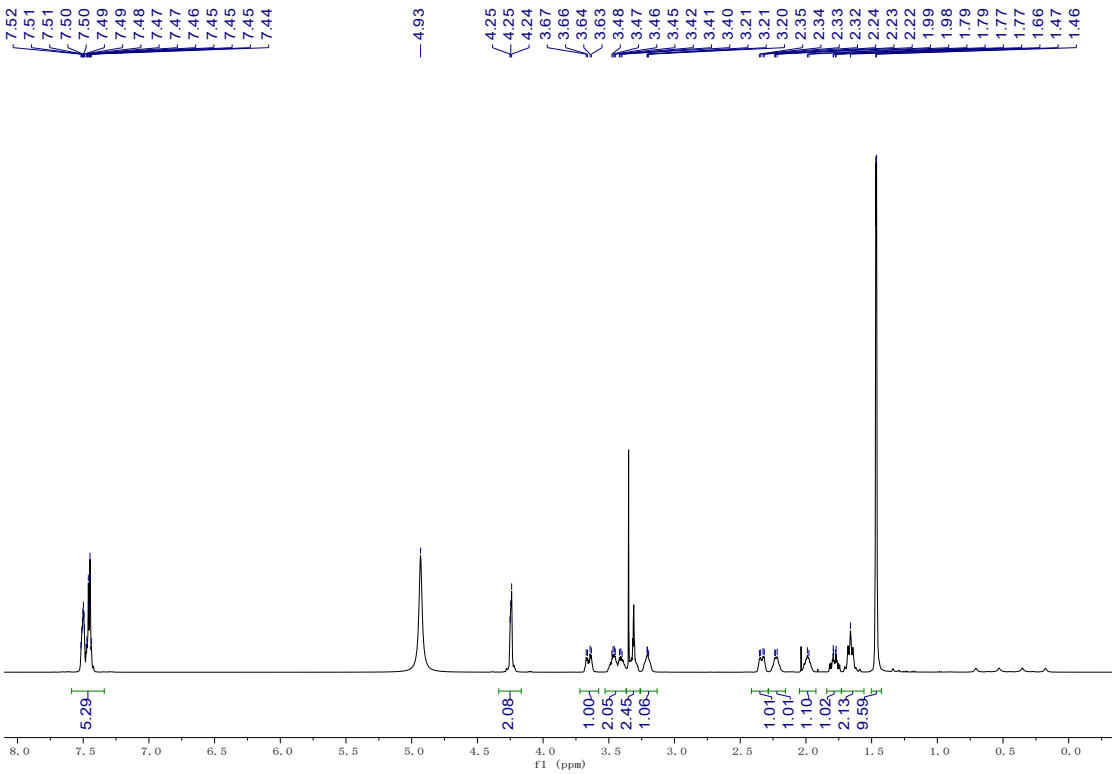

Figure 8. The ^{1}H NMR spectrum of **1a** in methanol-d_4 (500 MHz)

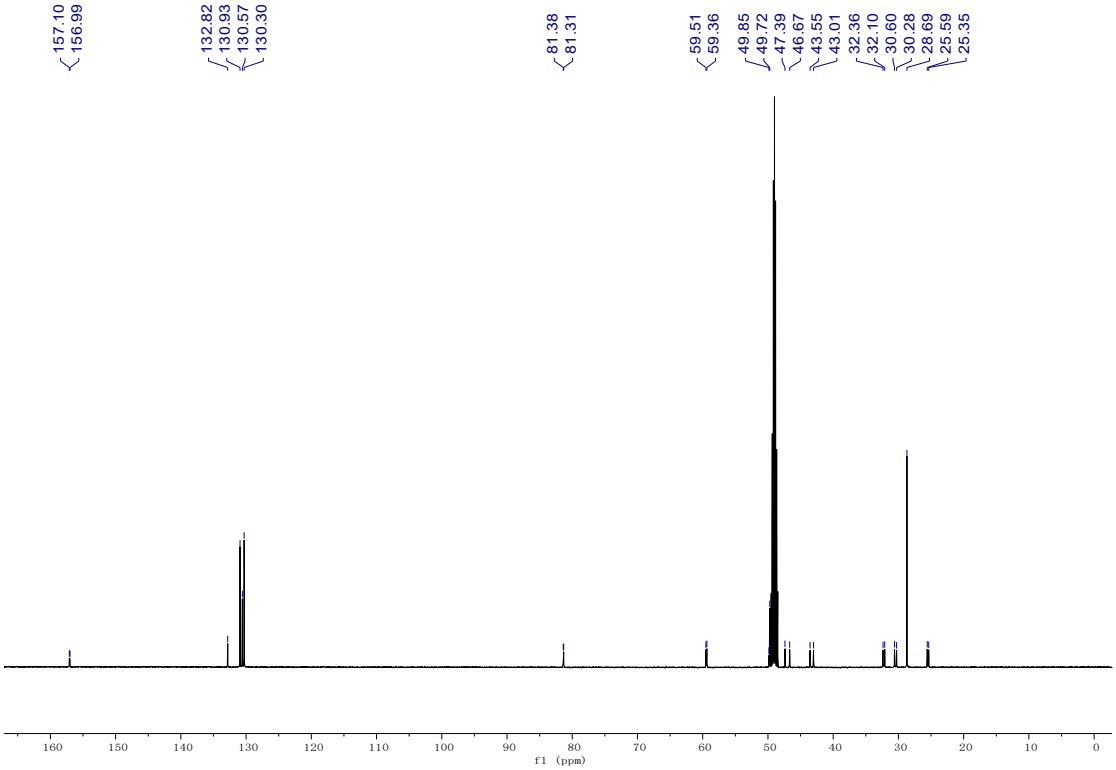

Figure 9. The ^{13}C NMR spectrum of **1a** in methanol-d_4 (125 MHz)

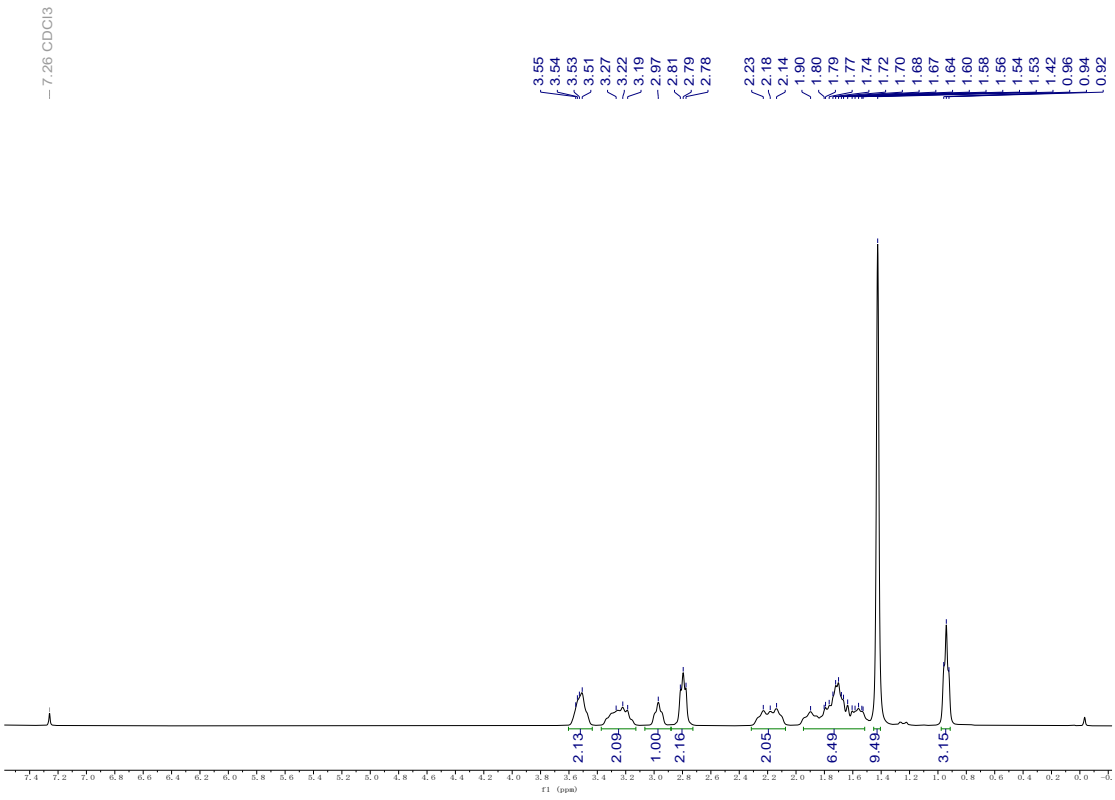

Figure 10. The ^{1}H NMR spectrum of **1b** in chloroform-*d* (400 MHz)

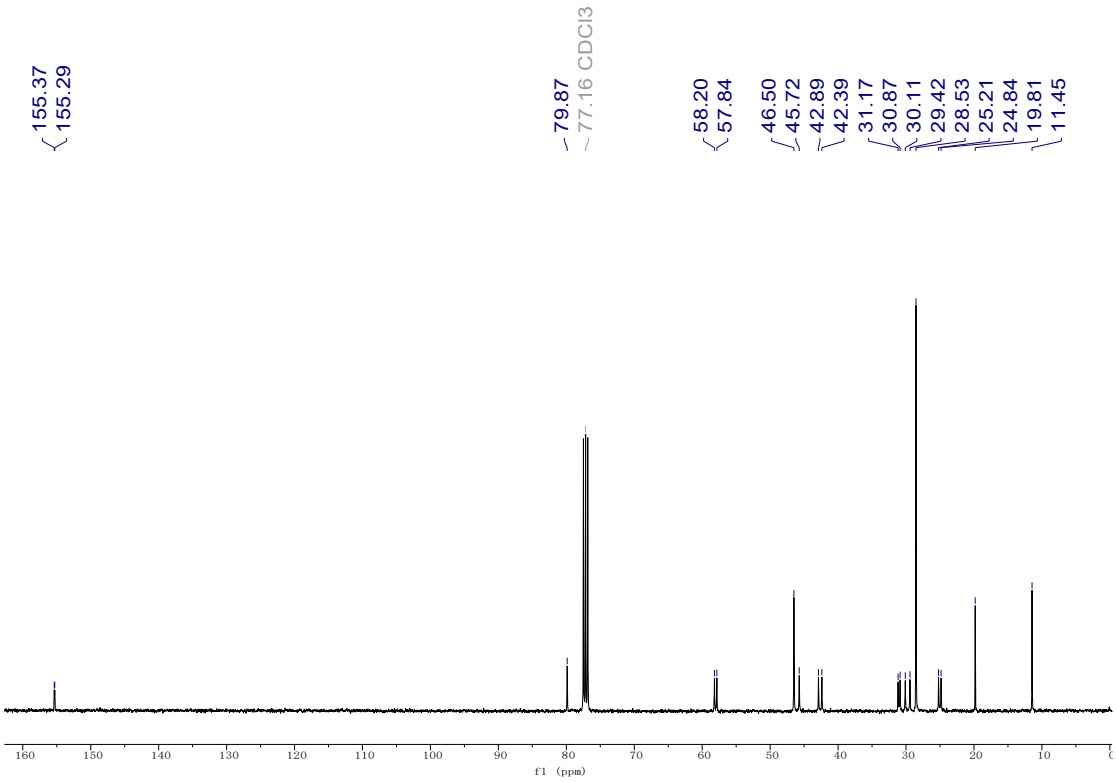

Figure 11. The ^{13}C NMR spectrum of **1b** in chloroform-*d* (100 MHz)

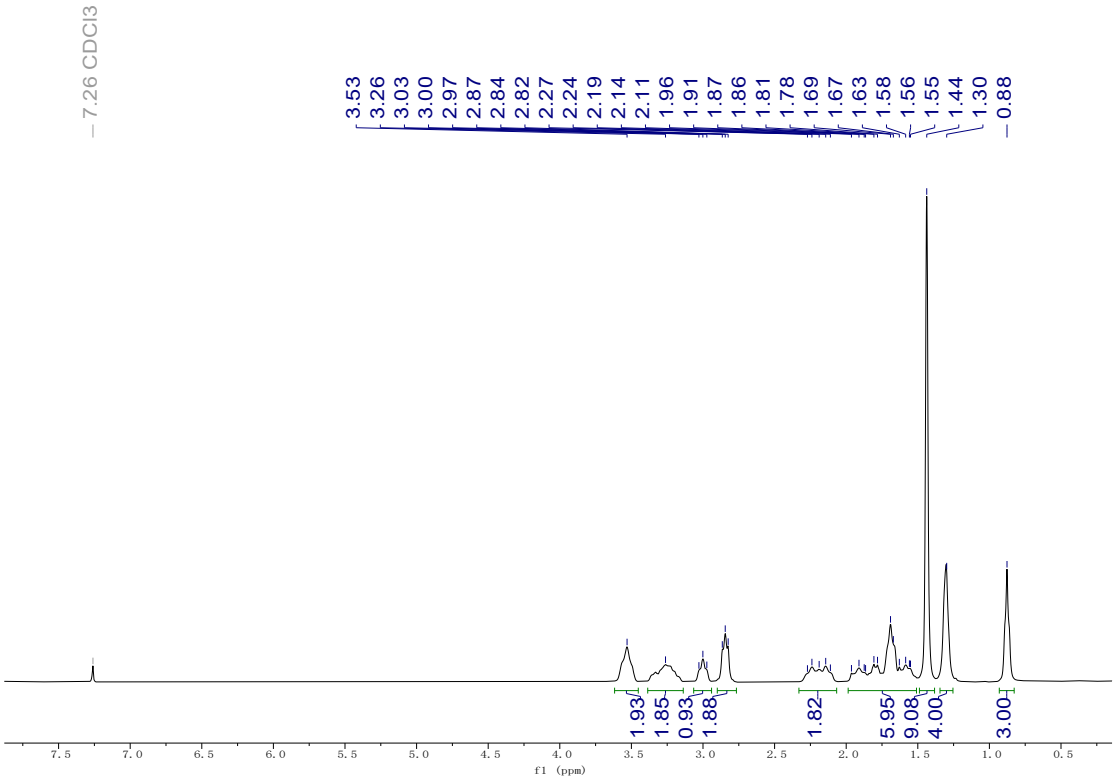

Figure 12. The ^{1}H NMR spectrum of **1c** in chloroform-*d* (400 MHz)

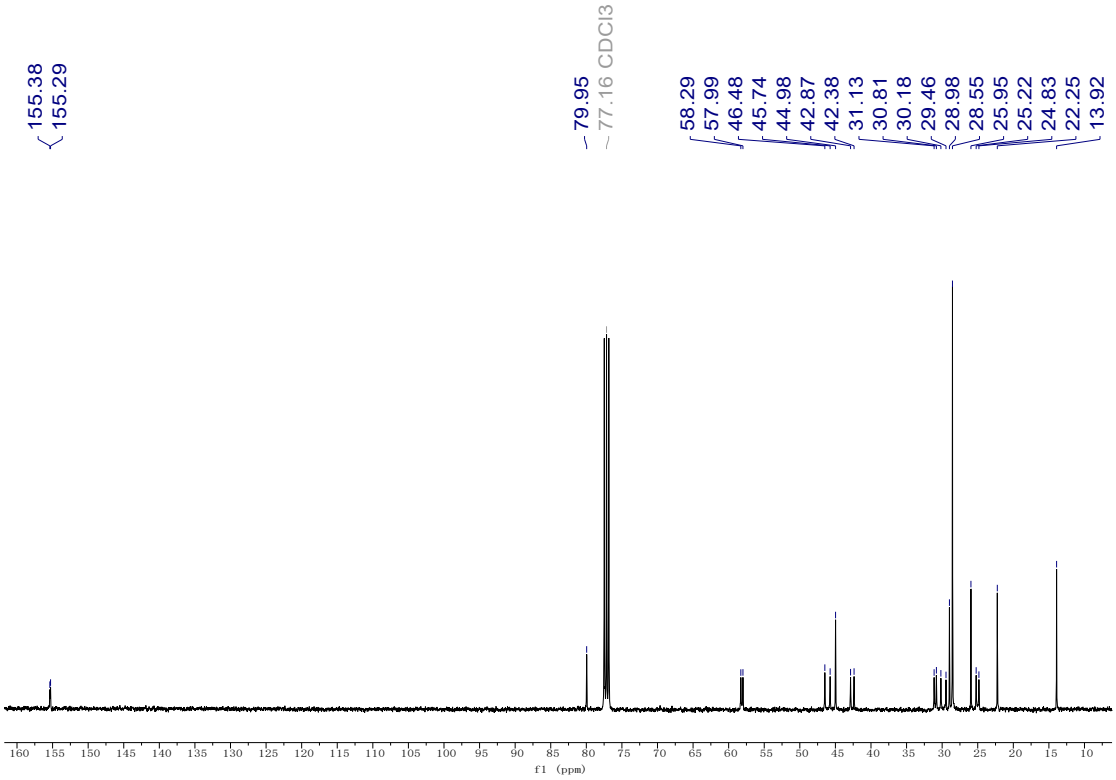

Figure 13. The ^{13}C NMR spectrum of **1c** in chloroform-*d* (100 MHz)

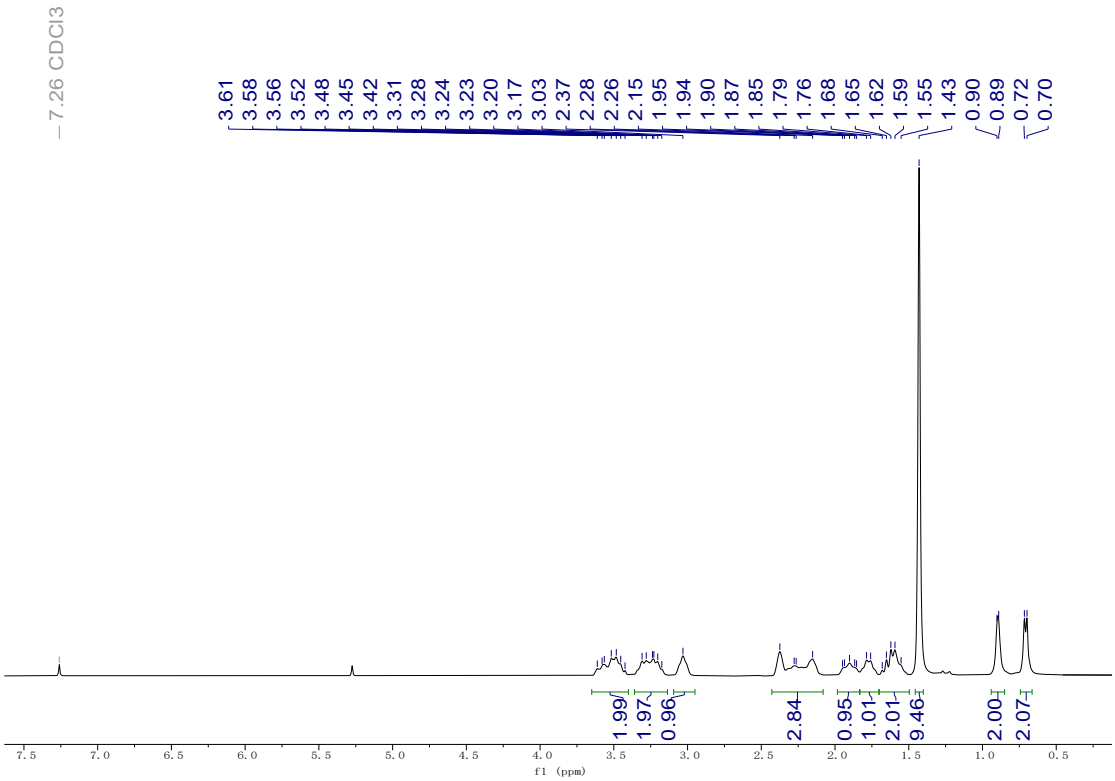

Figure 14. The ^{1}H NMR spectrum of **1d** in chloroform-*d* (400 MHz)

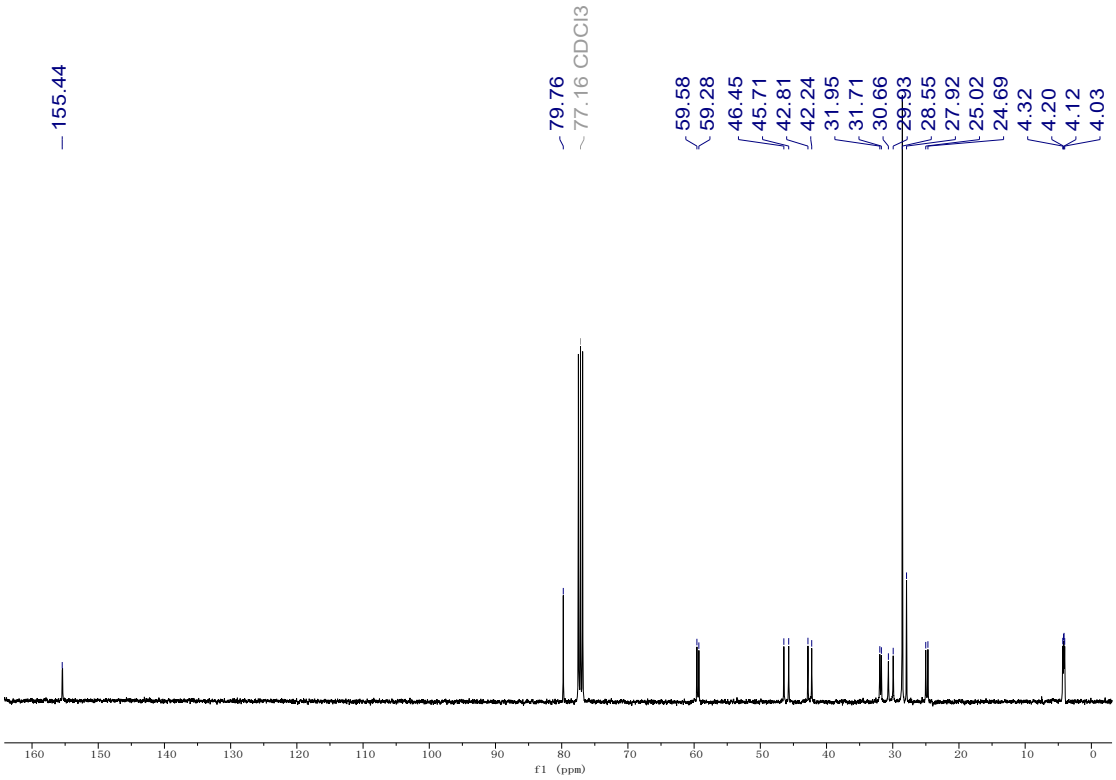

Figure 15. The ^{13}C NMR spectrum of **1d** in chloroform-*d* (100 MHz)

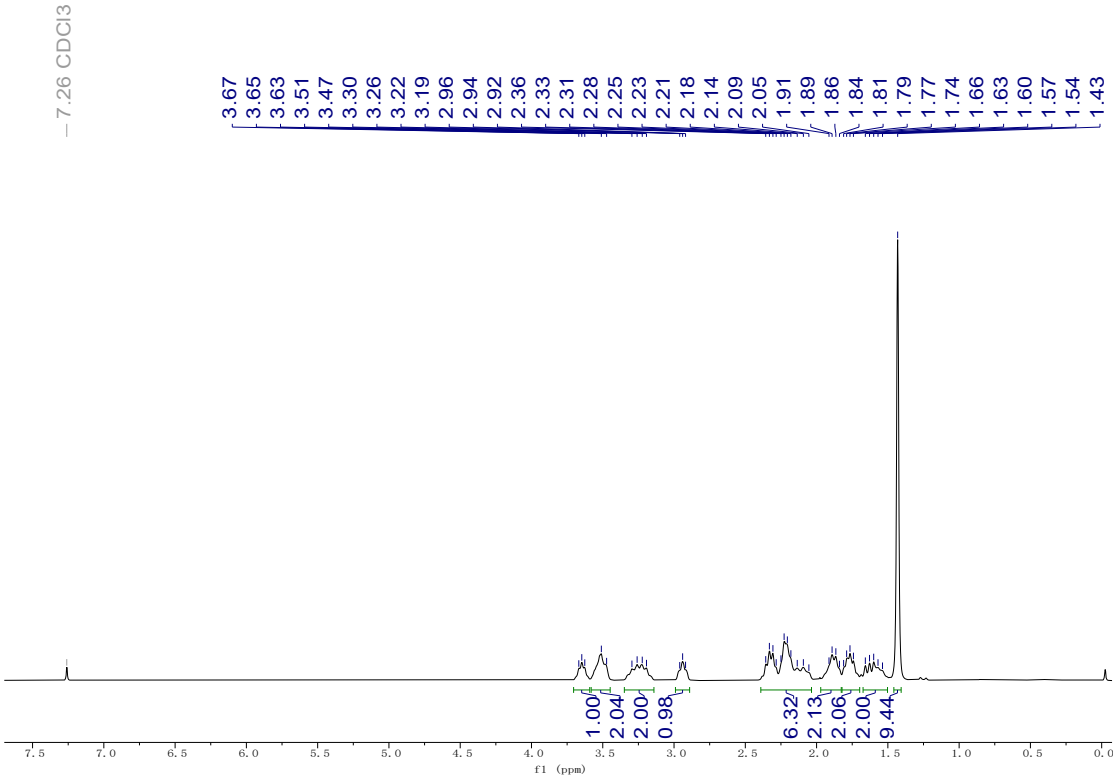

Figure 16. The ^{1}H NMR spectrum of **1e** in chloroform-*d* (400 MHz)

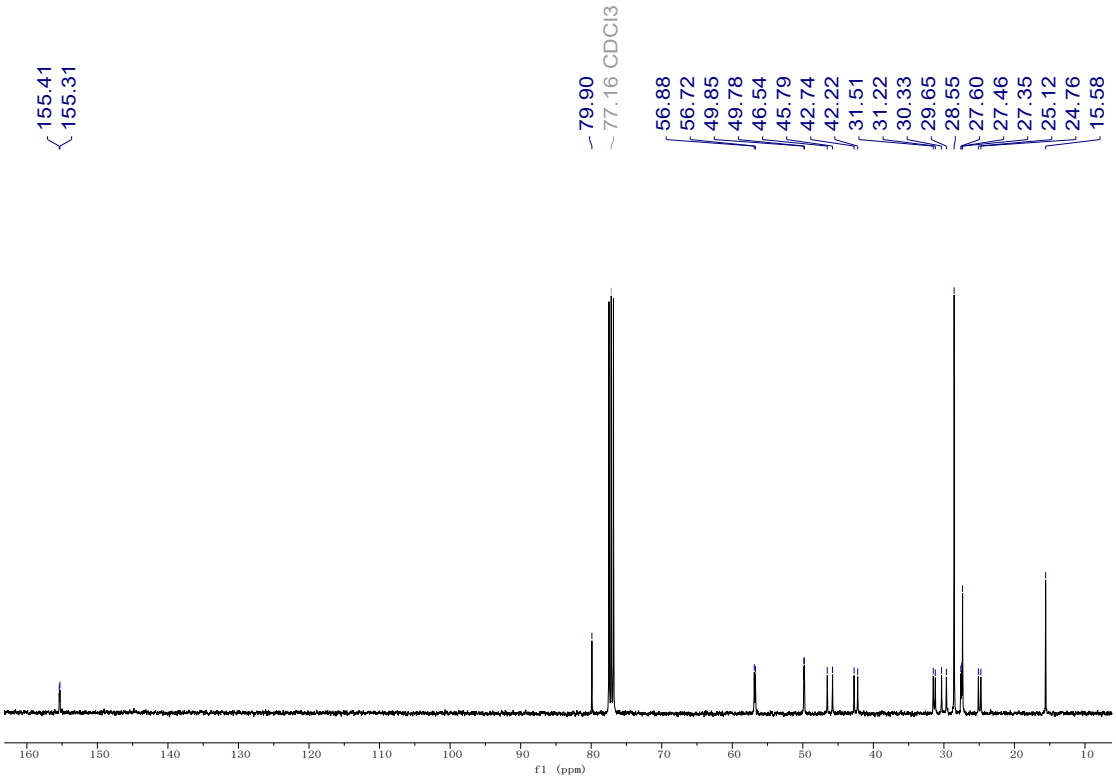

Figure 17. The ^{13}C NMR spectrum of **1e** in chloroform-*d* (100 MHz)

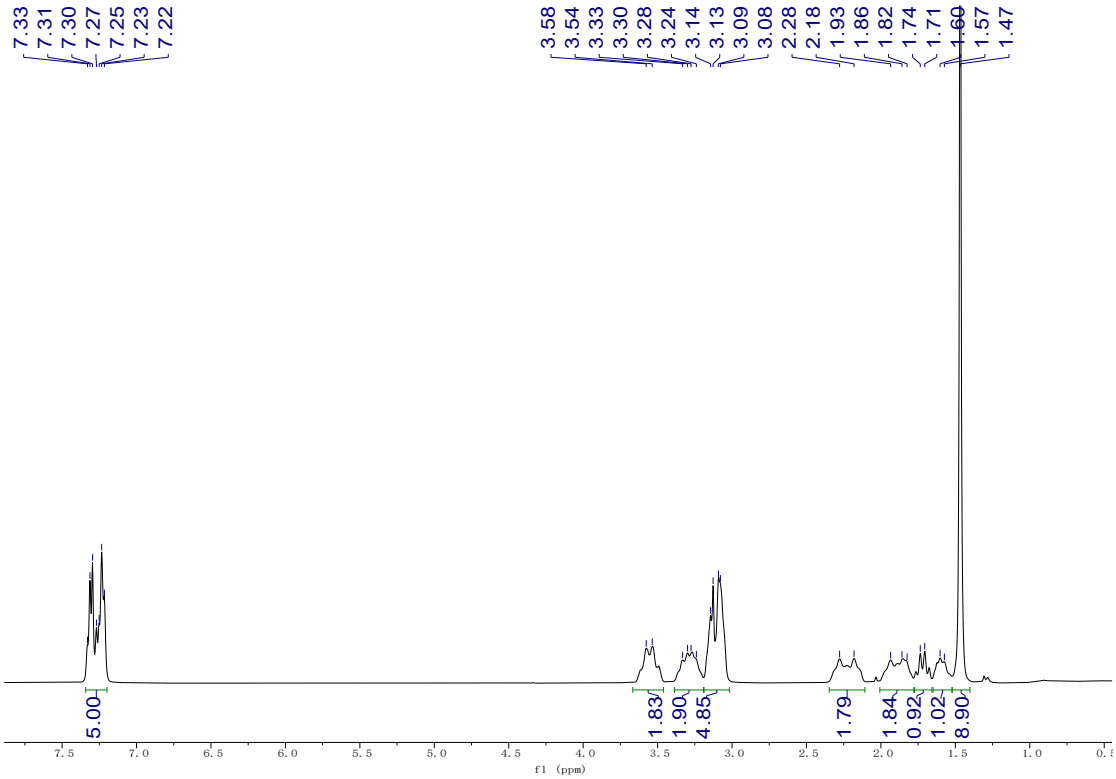

Figure 18. The ^{1}H NMR spectrum of **1f** in chloroform-*d* (400 MHz)

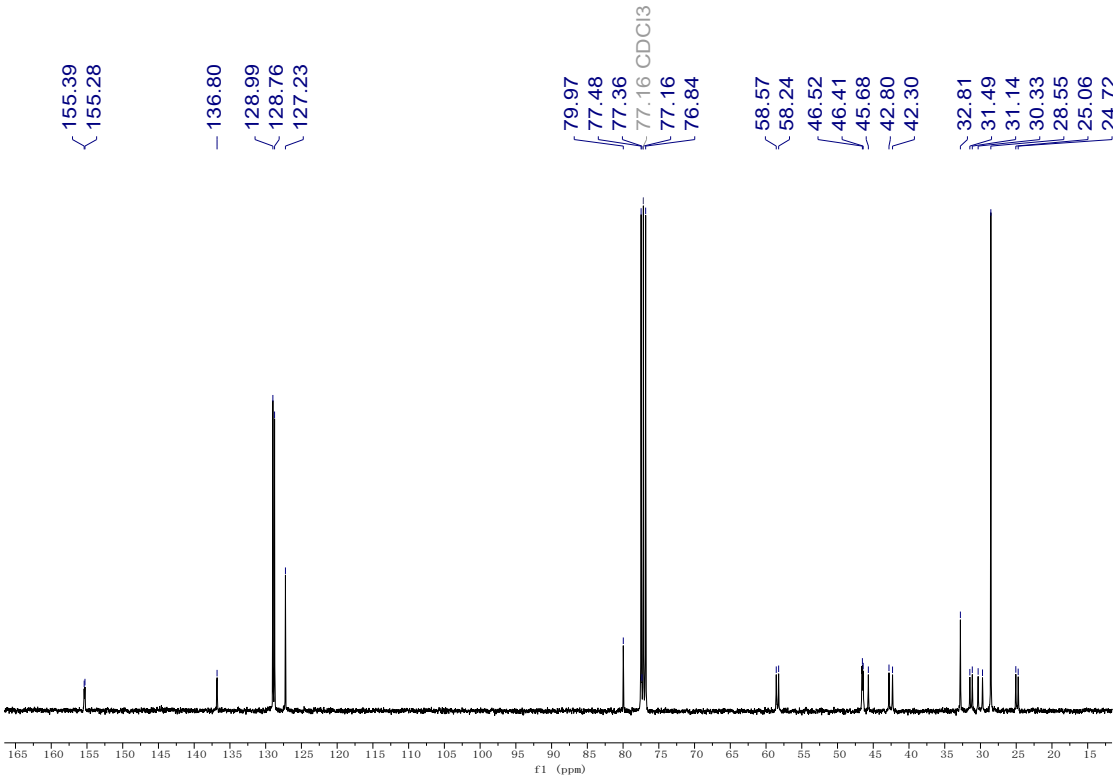

Figure 19. The ^{13}C NMR spectrum of **1f** in chloroform-*d* (100 MHz)

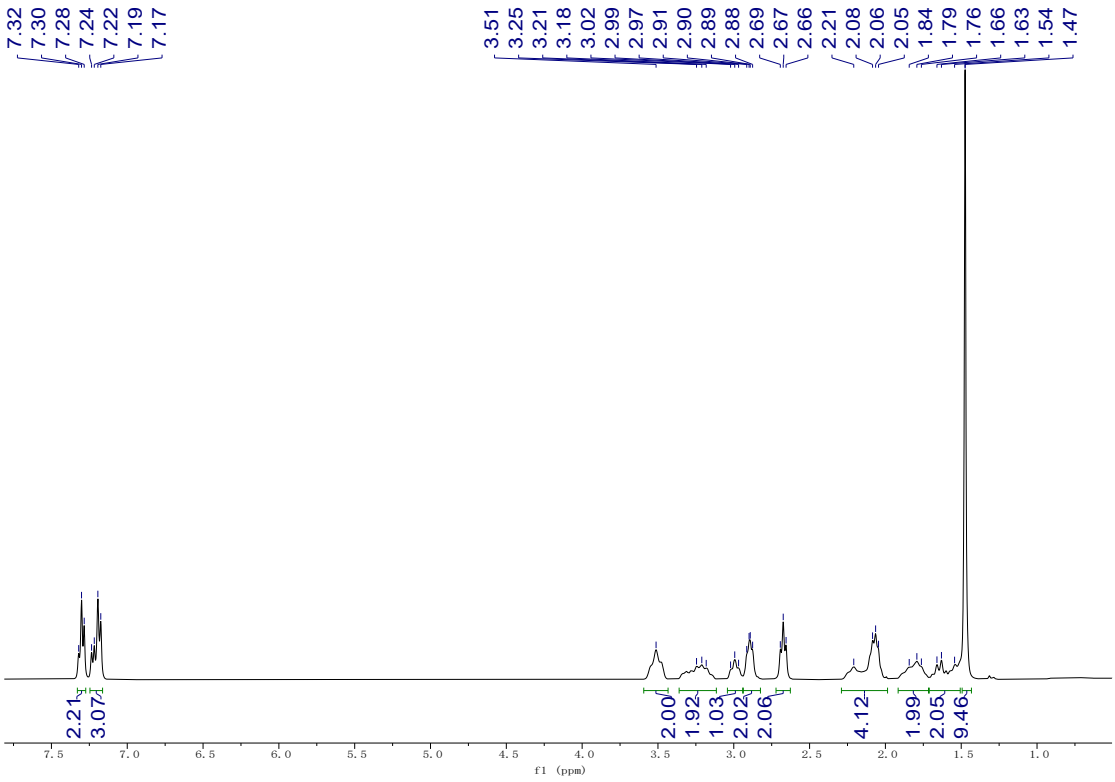

Figure 20. The ^{1}H NMR spectrum of **1g** in chloroform-*d* (400 MHz)

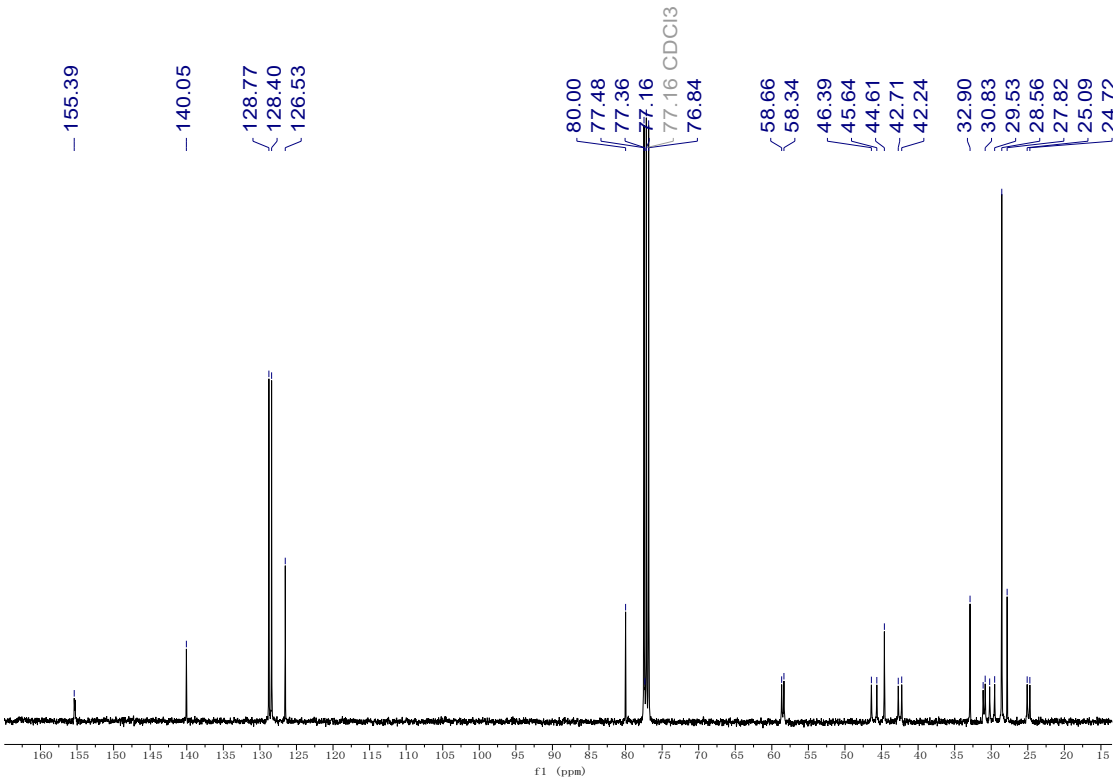

Figure 21. The ^{13}C NMR spectrum of **1g** in chloroform-*d* (100 MHz)

HR-ESI-MS spectra of amine products

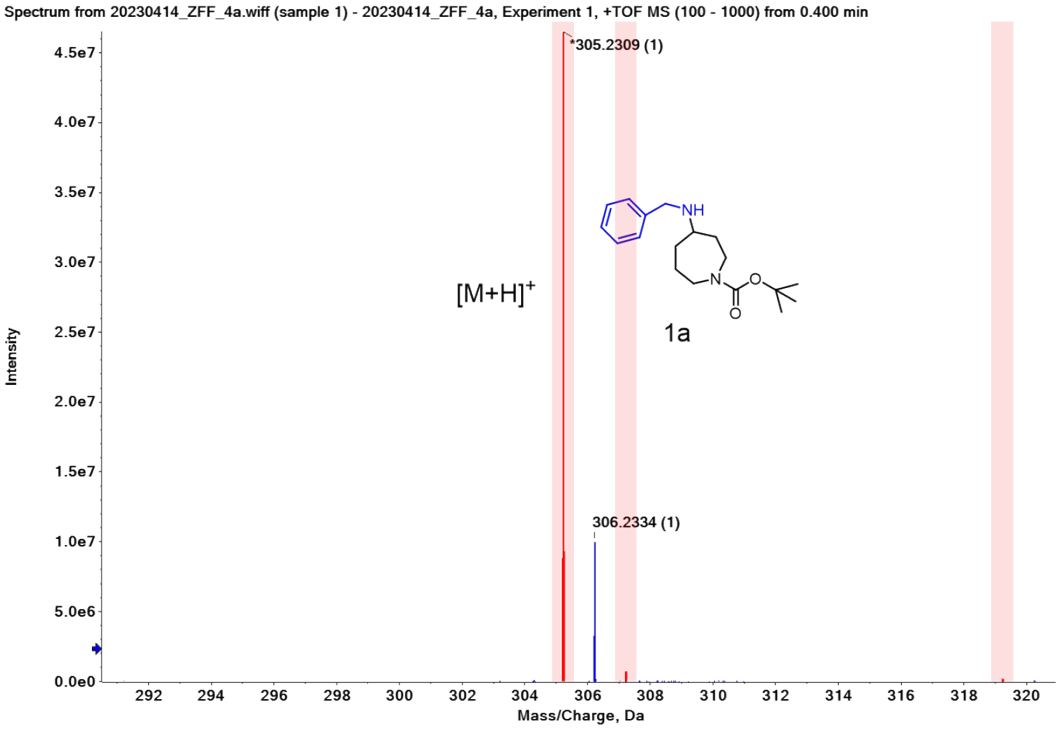

Figure 22. The HR-ESI-MS spectrum of **1a**.

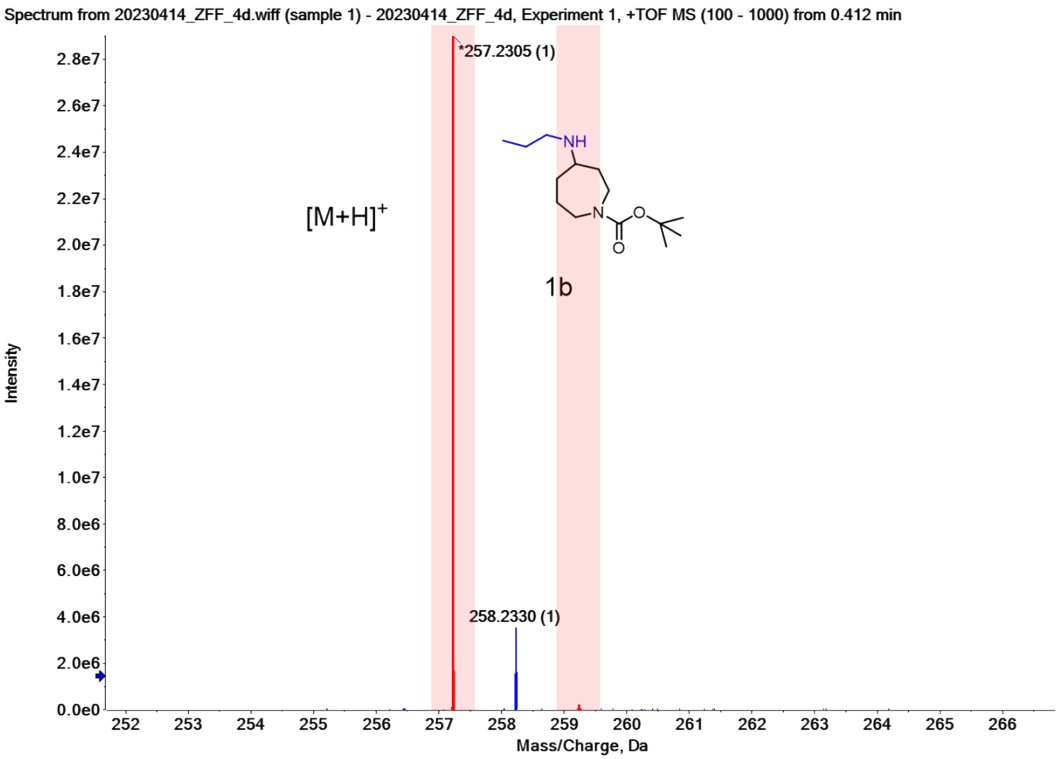

Figure 23. The HR-ESI-MS spectrum of **1b**.

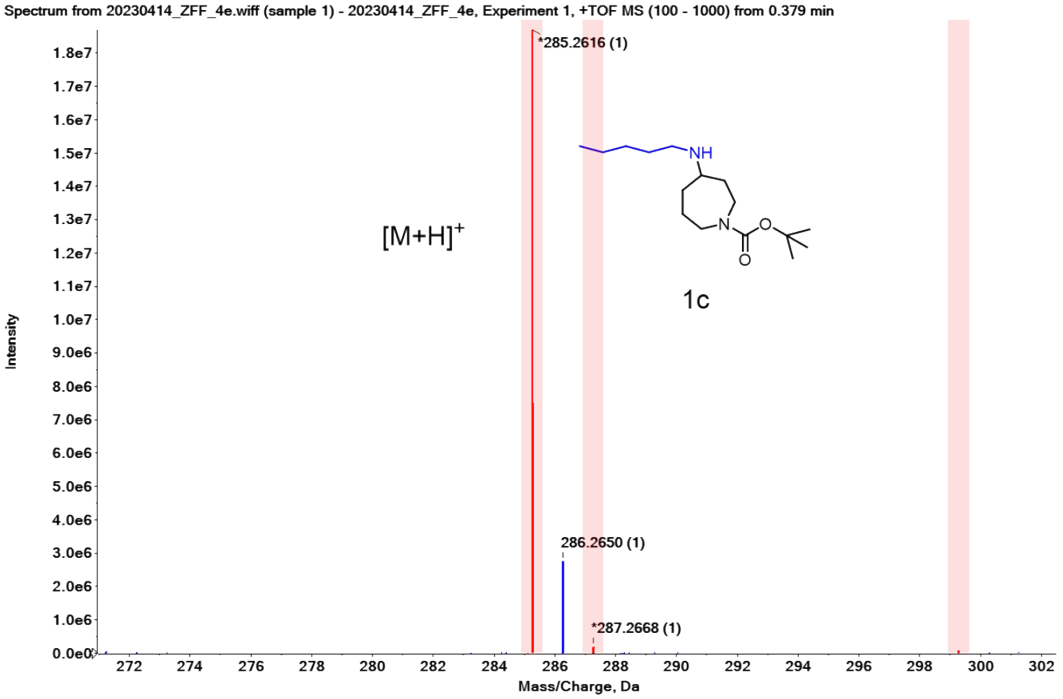

Figure 24. The HR-ESI-MS spectrum of **1c**.

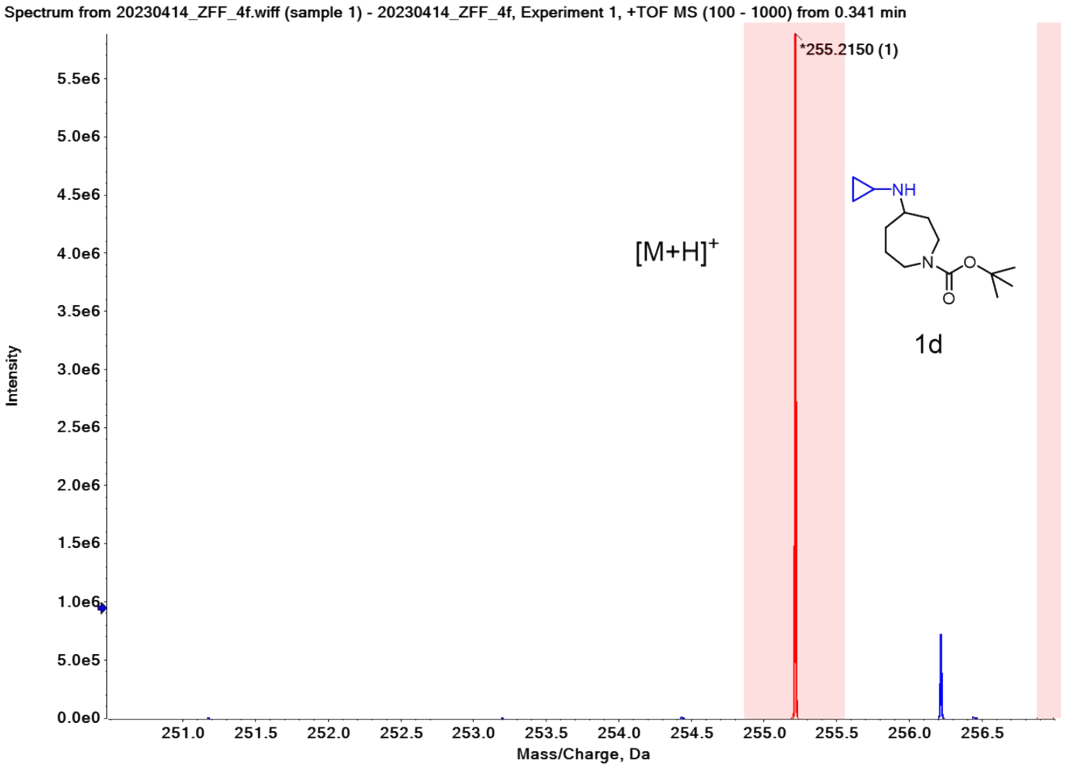

Figure 25. The HR-ESI-MS spectrum of **1d**.

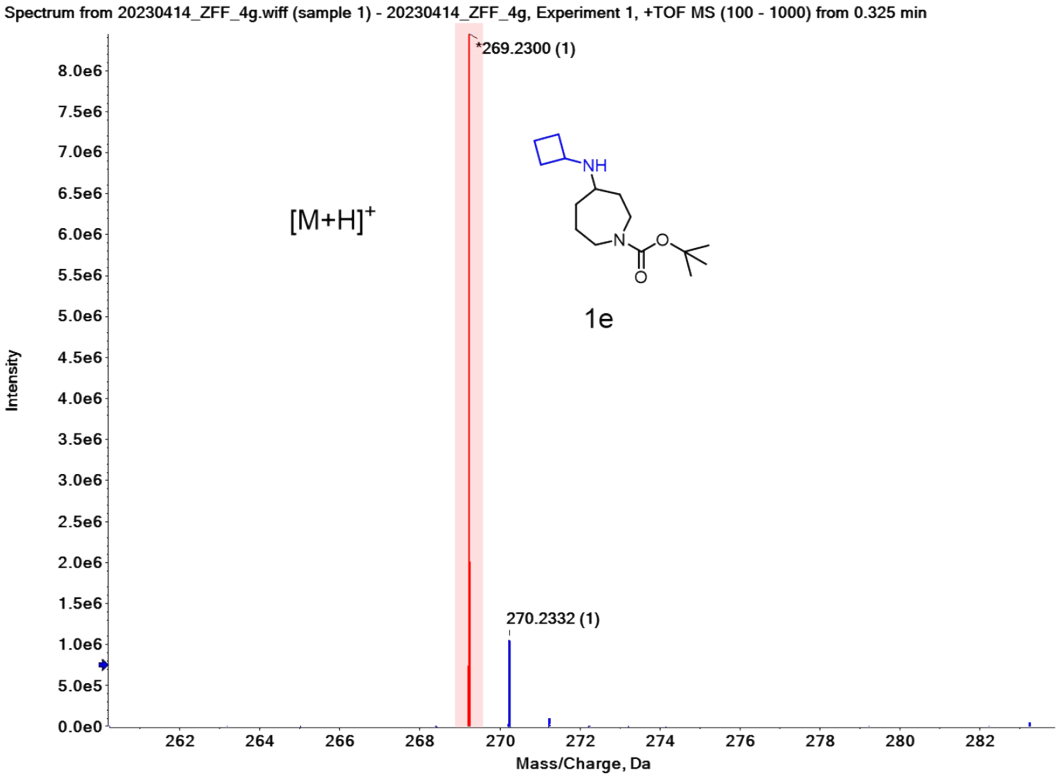

Figure 26. The HR-ESI-MS spectrum of **1e**.

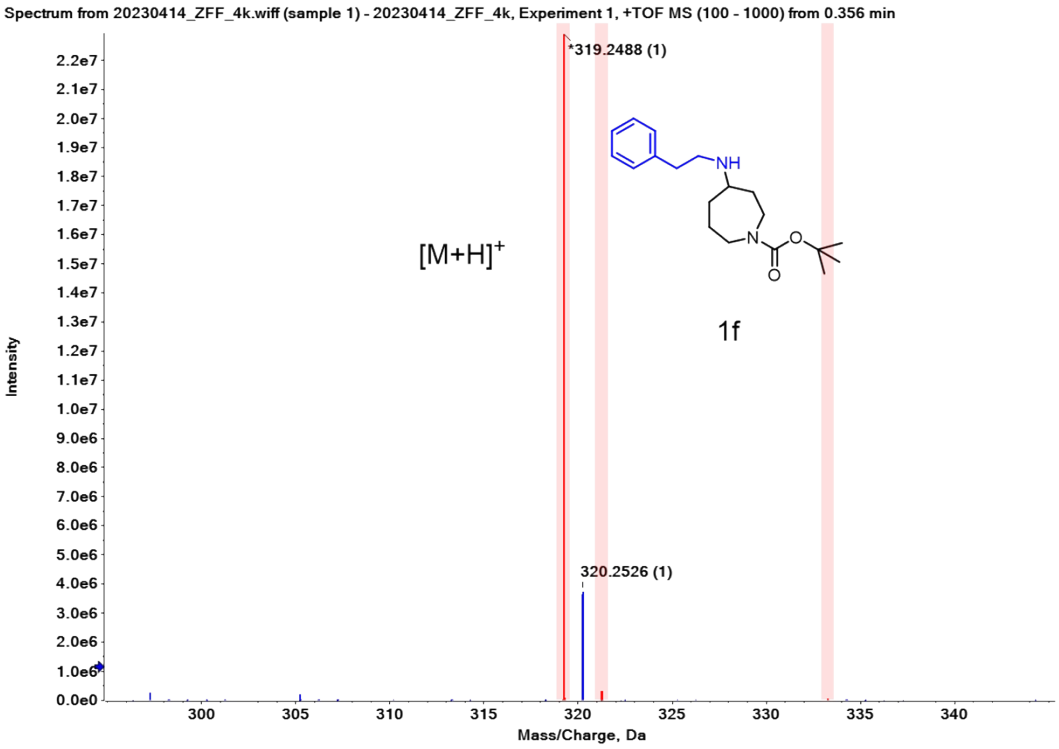

Figure 27. The HR-ESI-MS spectrum of **1f**.

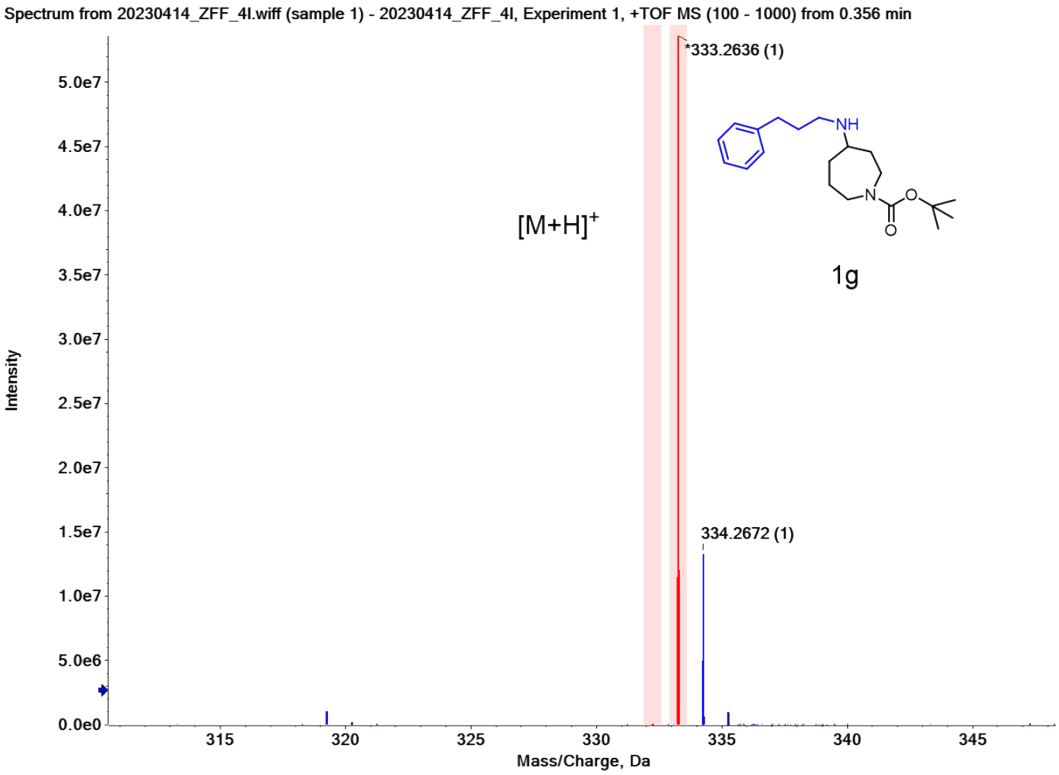

Figure 28. The HR-ESI-MS spectrum of **1g**.