Supporting Information

High Selective Azadipeptide Nitrile Inhibitors for Cathepsin K: Design, Synthesis and Activity Assay

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1. Inhibition Assays

1.1. Inhibition Assay of Cathepsin B

Human cathepsin B (Merck 219362, Human Liver, Germany) was assayed at FLx800 Fluorescence Microplate Reader (BioTek) using 96-well plates. The wavelength for excitation was at 360 nm and that for emission was 440 nm. Assay buffer was 100 mM MES-NaOH solution (pH 6.0) containing 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 0.001% Tween20 and 9% DMSO. A stock solution of 50 μg enzyme in 50 mM sodium acetate buffer, 1 mM EDTA, pH 5.0 was diluted to 50 nM with assay buffer containing 2.5 mM DTT. Stock solutions of inhibitor were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-AMC (ALX-260-131, Alexis, ≥ 98% (HPLC)) was prepared with DMSO and diluted with assay buffer. The final concentration of DMSO in enzymatic assay was 10%, and those of the substrate and cathepsin B was 20 μM and 5 nM, respectively. Into a well containing 39 μL assay buffer, 1 μL inhibitor solution (or DMSO) and 10 μL diluted solution of cathepsin B were added and mixed thoroughly, and then incubated at 37 ºC for 30 min. The reaction was initiated by adding 50 μL diluted solution of substrate and the fluorescence intensity (I) at 440 nm was measured (**Figure S1**). Experiments were performed in triplicate with at least five different concentrational inhibitor and three controls.

1.2. Inhibition Assay of Cathepsin K

Human cathepsin K (Merck 219461, His•Tag, Human, Recombinant, E. coli., Germany) was assayed at FLx800 Fluorescence Microplate Reader (BioTek) using 96-well plates. The wavelength for excitation was 360 nm and that for emission was 440 nm. Assay buffer was 100 mM MES-NaOH solution (pH 5.5) containing 2.5 mmol/L EDTA, 2.5 mmol/L DTT and 9% DMSO. A stock solution of 25 μg enzyme in 500 mM NaCl, 200 mM sodium acetate, 25 mM Tris-HCl, 10 mM trehalose, 5 mM DTT, 5 mM EDTA, 50% glycerol, pH 4.0 was diluted to 10 nM with assay buffer containing 2.5 mM DTT. Stock solutions of inhibitor were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-AMC (ALX-260-131, Alexis, ≥98% (HPLC)) was prepared with DMSO and diluted with assay buffer. The final concentration in enzymatic assay of DMSO was 10%, and those of the substrate and cathepsin K was 20 μM and 1 nM, respectively. Into a well containing 39 μL assay buffer, 1 μL inhibitor solution (or DMSO) and 10 μL diluted solution of cathepsin K were added and mixed thoroughly, and then incubated at 37 ºC for 30 min. The reaction was initiated by adding 50 μL diluted solution of substrate and the fluorescence intensity (I) at 440 nm was measured. Experiments were performed in triplicate with at least five different concentrational inhibitor and three controls.

1.3. Inhibition Assay of Cathepsin L

Human cathepsin L (Merck 219402, Human Liver, Germany) was assayed at FLx800 Fluorescence Microplate Reader (BioTek) using 96-well plates. The wavelength for excitation was 360 nm and that for emission was 440 nm. Assay

buffer was 100 mM MES-NaOH solution (pH 5.5) containing 2.5 mmol/L EDTA, 2.5 mmol/L DTT and 9% DMSO. A stock solution of 25 μg enzyme in 400 mM NaCl, 20 mM malonate buffer, 1 mM EDTA, pH 5.5 was diluted to 20 nM with assay buffer containing 2.5 mM DTT. Stock solutions of inhibitor were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-AMC (ALX-260-131, Alexis, ≥98% (HPLC)) was prepared with DMSO and diluted with assay buffer. The final concentration in enzymatic assay of DMSO was 10%, and those of the substrate and cathepsin L was 20 μM and 2 nM, respectively. Into a well containing 39 μL assay buffer, 1 μL inhibitor solution (or DMSO) and 10 μL diluted solution of cathepsin L were added and mixed thoroughly, and then incubated at 37 ºC for 30 min. The reaction was initiated by adding 50 μL diluted solution of substrate and the fluorescence intensity (I) at 440 nm was measured. Experiments were performed in triplicate with at least five different concentrational inhibitor and three controls.

1.4. Inhibition Assay of Cathepsin S

Human cathepsin S (Merck 219343, Human, Recombinant, E. coli., Germany) was assayed at FLx800 Fluorescence Microplate Reader (BioTek) using 96-well plates. The wavelength for excitation was 360 nm and that for emission was 440 nm. Assay buffer was 100 mM MES-NaOH solution (pH 6.5) containing 2.5mmol/L EDTA, 2.5 mmol/L DTT , 0.001%BSA and 9% DMSO. A stock solution of 50 μg enzyme in 35 mM potassium phosphate, 35 mM Sodium acetate, 2 mM DTT, 2 mM EDTA, 50% ethylene glycol, pH 6.5 was diluted to 50 nM with assay buffer containing 2.5 mM DTT. Stock solutions of inhibitor were prepared in DMSO. A 10 mM stock solution of the chromogenic substrate Z-VVR-AMC (Biomol BML-P199-0010, 99%) was prepared with DMSO and diluted with assay buffer. The final concentration in enzymatic assay of DMSO was 10%, and those of the substrate and cathepsin S was 20 μM and 5 nM, respectively. Into a well containing 39 μL assay buffer, 1 μL inhibitor solution (or DMSO) and 10 μL diluted solution of cathepsin S were added and mixed thoroughly, and then incubated at 37 ºC for 30 min. The reaction was initiated by adding 50 μL diluted solution of substrate and the fluorescence intensity (I) at 440 nm was measured. Experiments were performed in triplicate with at least five different concentrational inhibitor and three controls.

2. Representative Plots and Kinetic Parameters

2.1 Determination of IC_{50} for Cathepsin K, B, L and S

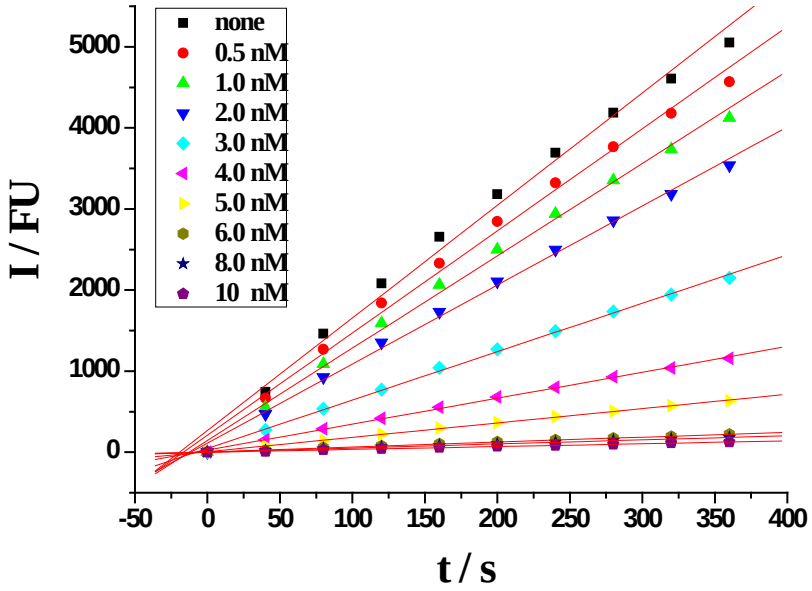


Figure S1. Monitoring of the human cathepsin B-catalyzed hydrolysis of Z-Phe-Arg-AMC (20 μM) in the presence of increasing concentrations of the azadipeptide nitrile inhibitor **1** (■, 0;●, 0.5 nM; ▲, 1 nM; ▼, 2 nM; ◆, 3 nM; ◂, 4 nM; ▸, 5 nM; +, 6 nM; ★, 8 nM; ╳, 10 nM). The reaction was initiated by the addition of substrate and the fluorescence intensity (I) at 440 nm was measured under the excitation of 360 nm. Fluorescence units (FU) were corrected for background fluorescence.

The apparent inhibition constants K_i' (IC_{50}) were determined by fitting

$$v = v_0/(1+[I]/ K_i') \quad (1)$$

to the experimental data, where v and v_0 is respectively the rate in the presence and absence of inhibitor, [I] is the inhibitor concentration and K_i' is the apparent inhibition constant.[1]

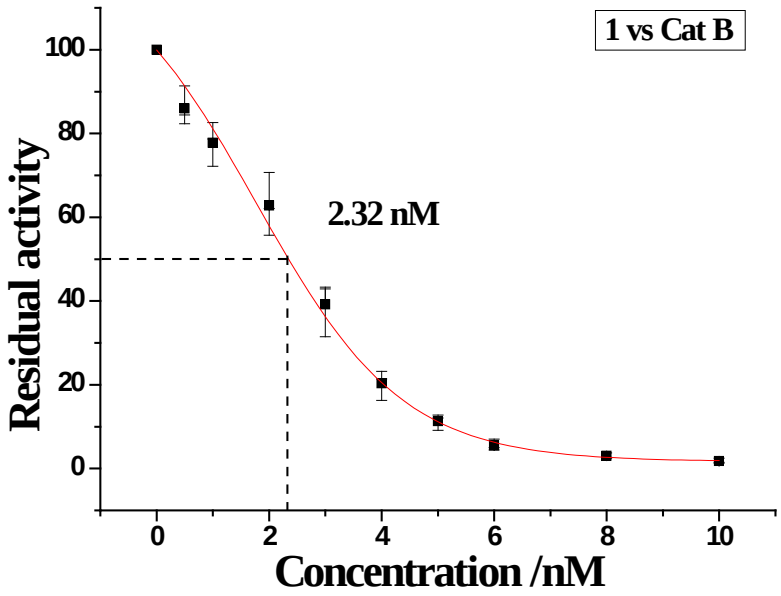


Figure S2. Plot of residual enzyme activity *versus* different concentrations of inhibitor **1**. Non-linear regression gave IC_{50} =2.32 nM.

Table S1. Cathepsin inhibition by azadipeptide dipeptide nitriles

No. of Compounds	IC_{50} (nM)			
	Cat K	Cat B	Cat L	Cat S
1	0.057	2.32	0.101	3.06
2	0.066	4.20	0.405	6.66
3	0.046	5.26	0.312	4.96
4	0.261	3.81	0.201	2.82
5	0.056	3.39	0.325	5.20
6	0.074	5.27	0.346	3.55
7	0.062	2.71	0.525	1.87
8	0.150	5.62	0.084	3.92
9	0.220	3.55	0.301	4.02
10	0.113	5.74	0.122	3.81
11	0.289	4.58	0.113	1.58
12	0.263	0.78	1.73	1.84
13	0.0065	3.55	0.208	3.94
14	0.256	3.65	0.244	2.89
15	0.459	1.81	1.404	2.35

2.2 Determination of K_m for Cathepsin K, B, L and S

To calculate the inhibition constant (K_i) of each inhibitor against enzyme, the Michaelis constants (K_m) for cathepsin K, B, L and S should be determined firstly**.** Using the similar approaches for enzymatic assay for cathepsins in **1.1**-**1.4**, in the absence of inhibitor and at the different concentrations of substrates, the reaction was initiated by adding 50 μL of the substrate solution. Experiments were performed in

triplicate for each group at 11 different substrate concentrations, and two parallel groups were measured.

The typical method for determining the K_m involves running a series of enzyme assays at varying substrate concentrations [S], and measuring the initial reaction rate. Before computing facilities to perform nonlinear regression became available, graphical methods involving linearization of the equation were used. Herein, by plotting reaction rate against concentration, and use SigmaPlot fit nonlinear regression of the Michaelis-Menten equation, the K_m and V_{max} were obtained.

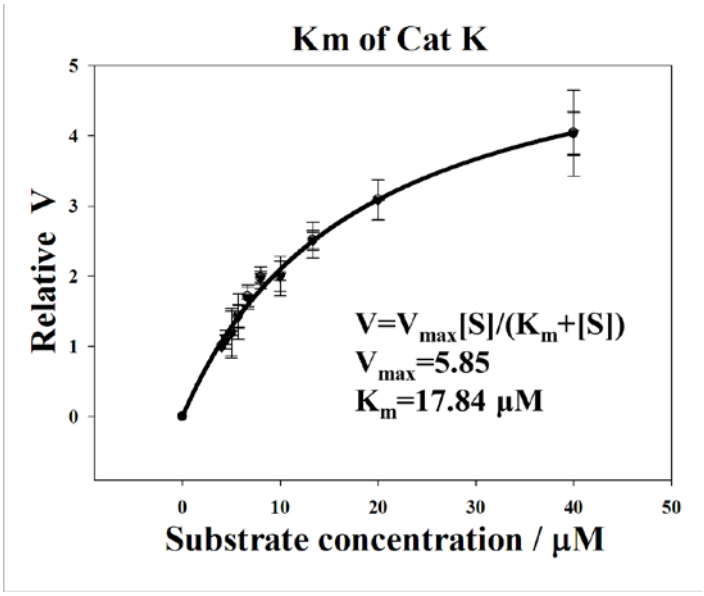


Figure S3 Representative plot of relative enzyme activities versus different concentrations of substrate at 3.64, 4.00, 4.44, 5.00, 5.71, 6.67, 8.00, 10.0, 13.3, 20.0 and 40.0 μM. Non-linear regression gave K_m=18.06 ± 0.22 μM for cathepsin K.

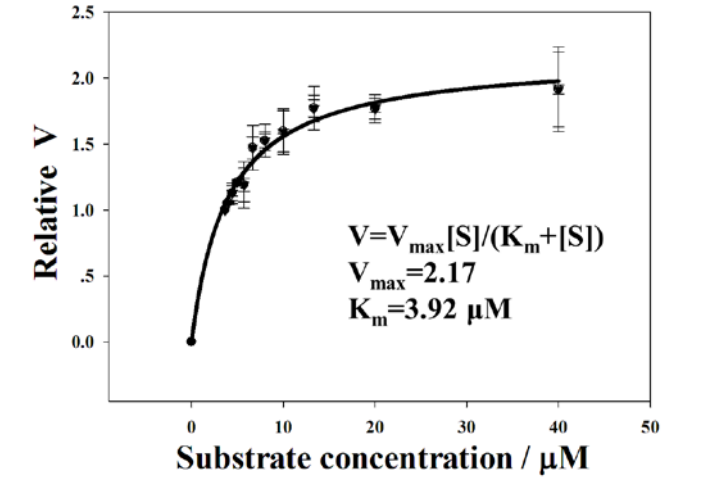


Figure S4 Representative plot of relative enzyme activities versus different concentrations of substrate at 3.64, 4.00, 4.44, 5.00, 5.71, 6.67, 8.00, 10.0, 13.3, 20.0 and 40.0 μM. Non-linear regression gave K_m=3.525 ± 0.405 μM for cathepsin L.

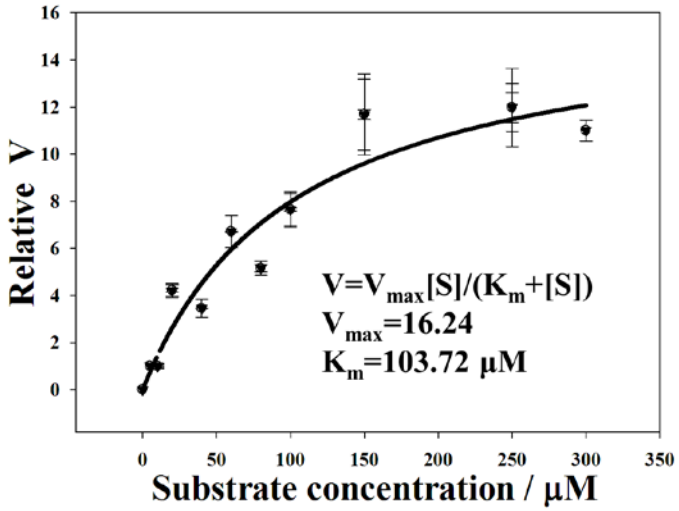


Figure S5 Representative plot of relative enzyme activities versus different concentrations of substrate at 5, 10, 20, 40, 60, 80, 100, 150, 200, 250 and 300 μM. Non-linear regression gave K_m=102.2 ± 1.52 μM for cathepsin S.

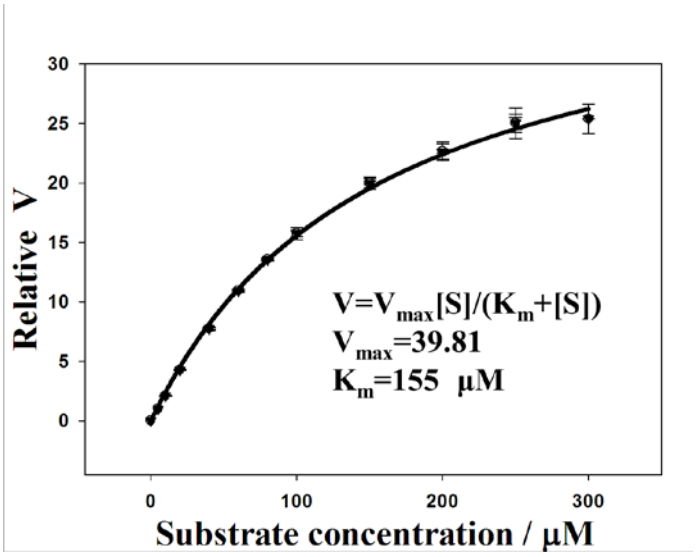


Figure S6 Representative plot of relative enzyme activities versus different concentrations of substrate at 5, 10, 20, 40, 60, 80, 100, 150, 200, 250 and 300 μM. Non-linear regression gave K_m=157.5 ± 2.5 μM for cathepsin B.

2.3 Determination of K_i for Cathepsin K, B, L and S

The true inhibition constants K_i were calculated by correction of K_i' according to

$$K_i = K_i'/(1+[S]/K_m) \quad (2)$$

where [S] is the substrate concentration and K_m is the Michaelis constant. The calculated K_i values of the inhibitor were illustrated in Table 2.

In order to validate the calculated K_i value in above, we also determined the K_i value of compound **1** against cathepsin K as an example. By changing the concentrations of inhibitor and substrate at the same time, we performed the enzymatic assay by using

the similar approaches in **1.2** for cathepsin K. Firstly, the reciprocal of the initial reaction rate *versus* the reciprocal of the substrate concentration at various concentrational inhibitors gave a straight-line plot as Figure S7. Then, the slope of the linear plot to inhibitor concentration supplied another linear relationship between them. Further extension of it would intersect with the X-axis and then supplied a value for $-K_i$ (Figure S8). The determined value (K_i = 0.026 nM) was comparable with the calculated K_i value (0.027 nM) based on K_m and IC_{50}, validating the approaches we used for K_i availability.

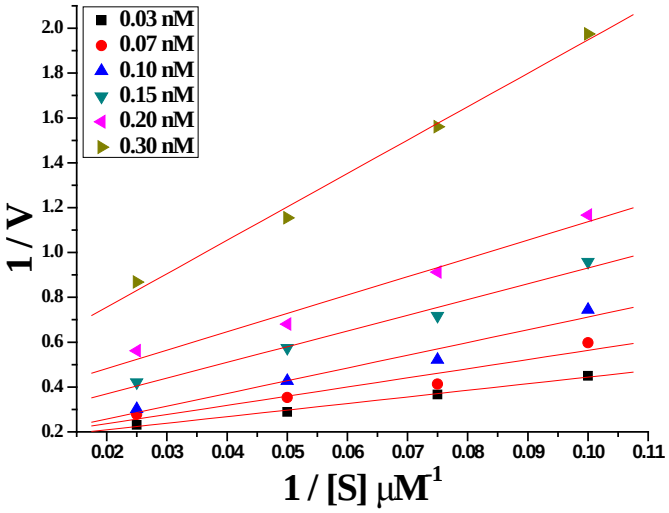


Figure S7. The double reciprocal plot of different compound 1 concentration against cathepsin K(■, 0.03 nM;●, 0.07 nM; ▲, 0.10 nM; ▼, 0.15 nM; ◂, 0.20 nM; ▸, 0.30 nM).

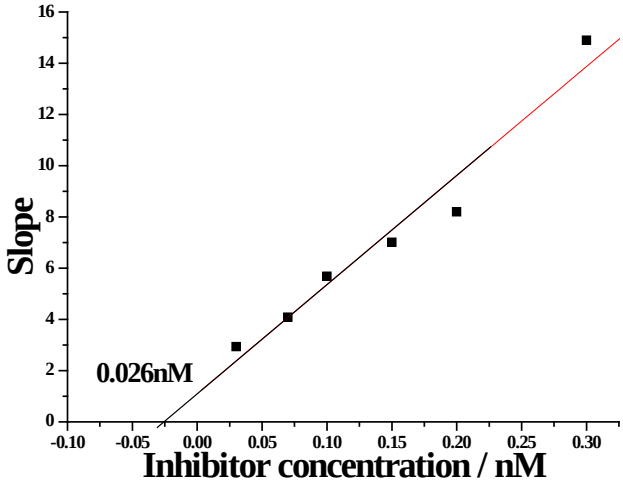


Figure S8. The slope of the line plot in Figure S7 to the concentration of inhibitor supplied a linear relationship between them, and a value for $-K_i$ was achieved by further extension of the line to the X-axis.

3. Preparation of Compounds 1-15

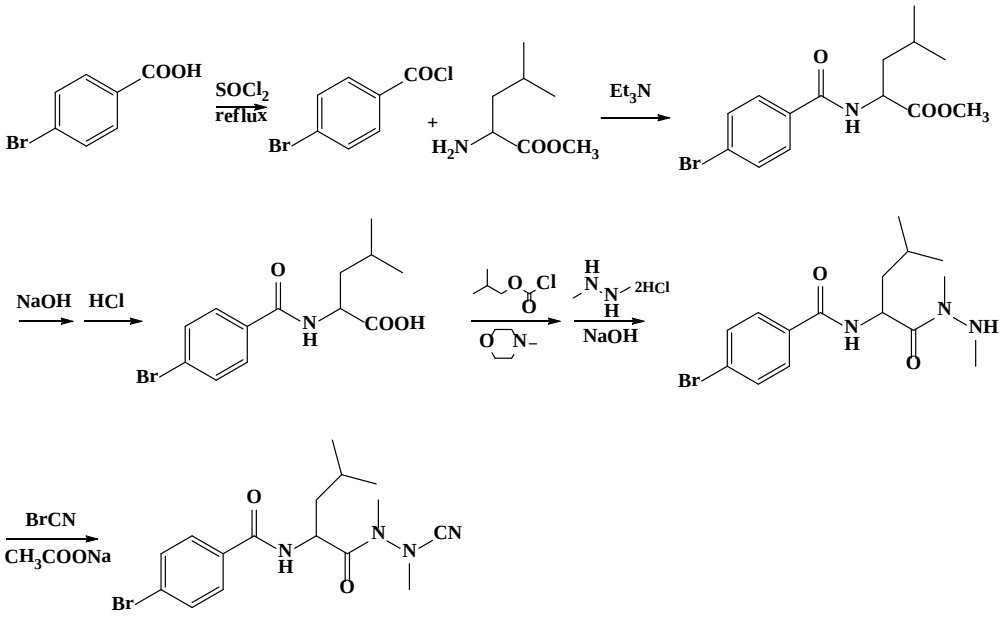
3.1. General Methods and Materials

Thin layer chromatography was performed on Branch of Qingdao Haiyang Chemical Co., Ltd aluminum sheets. Preparative column chromatography was performed on silica gel 60, 54-74μm. ^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra were recorded on a Bruker Avance 500 spectrometer. ESI-MS spectra were recorded on an Bruker HCT mass spectrometer. IR spectra were recorded on a Bruker Vertex 80V FT-IR spectrometer.

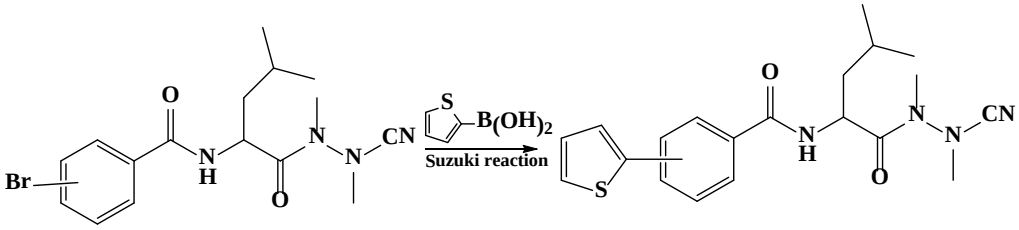
Amino acid was obtained from Beijing Dingguo Biotechnology Co., Ltd.. 3-bromobenzoic acid, 4-bromobenzoic acid, 4-methoxybenzoic acid, 4-nitrobenzoic acid, 4-(trifluoromethyl)benzoic acid were bought from Alfa Aesar China (Tianjin) Co., Ltd.. 1,2-dimethylhydrazine dihydrochloride was obtained from TCI Co., Ltd. Cyanogen bromide was bought from ACROS Co., Ltd. 2-thienylboronic acid, pyridin-4-ylboronic acid, phenylboronic acid and biphenyl-4-ylboronic acid were obtained from RED Chemical.

6-(4-(tert-butoxycarbonyl)piperazin-1-yl)pyridin-3-ylboronic acid were obtained from Frontier Scientific.

3.2. Schemes of synthesis

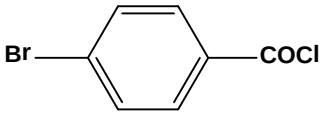


Scheme S1. Synthesis routes for compounds 1-4.



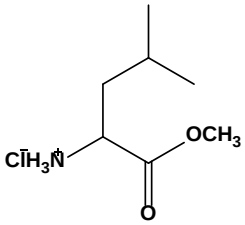
Scheme S2. Synthesis routes for compounds 5-7 and 9-15.

3.3. *4*-bromobenzoyl chloride



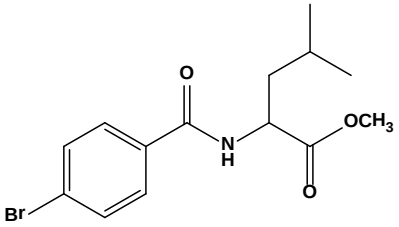
2.01 g 4-bromobenzoic acid (10 mmol) was added to a 50 mL round-bottomed flask equipped with a magnetic stir bar, 20 mL thionyl was then added. The mixture was stirred for 30 min, then it was heated at reflux for 60 min. After evaporation of the excessive thionyl, the solid residue was suspended in 30 mL cyclohexane for recrystallization. The mixture was heated at reflux for 30 min, then cooled slowly to room temperature and next to 0 °C in an ice bath. It was filtered through a fritted-glass funnel, the solid was washed with 15 mL cold cyclohexane, and then dried under vacuum to afford 4-bromobenzoyl chloride as a white solid.

3.4. *L*-leucine methyl ester hydrochloride



In an ice-bath, 5 mL thionyl was added dropwise under stirring to a 50 mL round-bottomed flask containing 30 mL anhydrous methanol. 10 min later 5 g L-leucine was added. After the mixture was stirred for 15 min in ice-bath, it was allowed to warm to room temperature and stirred for additional 30 min, then the mixture was heated at reflux for 60 min. The heated mixture was concentrated in a rotary evaporator to leave an oily residue, which was then suspended in 40 mL anhydrous aether. A white solid precipitated out and then was cooled to –20 °C for 2 h, the mixture was filtered through a fritted-glass funnel and the solid was washed with 15 mL cold anhydrous aether, and finally dried under vacuum to afford L-leucine methyl ester hydrochloride as a white solid.

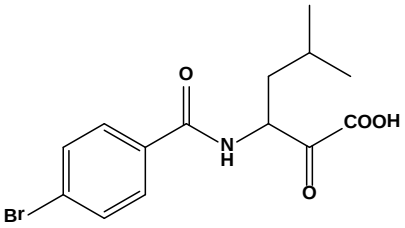
3.5. Methyl 2-(4-bromobenzamido)-4-methylpentanoate



0.5 g sodium bicarbonate was dissolved in 20 mL water, and 1.82 g L-leucine methyl ester hydrochloride (10 mmol) was added under stirring. After 15 min, the solution was extracted with dichloromethane for four times (15 mL×4). The combined organic layers were washed with H_2O (2×15 mL), dried with Na_2SO_4 and then concentrated in a rotary evaporator. 2.19 g 4-bromobenzoyl chloride (10 mmol) was added to the solution under ice-cooling and 1.40 mL anhydrous triethylamine was added dropwise.

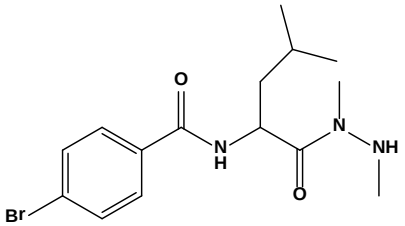
The mixture was stirred at 0 °C for 30 min, then it was allowed to warm to room temperature and stirred for additional 3 h. The mixture was washed with HCl (1×15 mL), sat.$NaHCO_3$ (1×15 mL), H_2O (1×15 mL) and brine (1×15 mL) in turn. The solvent was dried by Na_2SO_4 and then evaporated to obtain a white solid, then it was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (1:1) as eluent to obtain methyl 2-(4-bromobenzamido)-4-methylpentanoate as white solid.

3.6. *3*-(4-bromobenzamido)-5-methyl-2-oxohexanoic acid



3.42 g methyl 2-(4-bromobenzamido)-4-methylpentanoate (10 mmol) was dissolved in 15 mL THF, then 20 mL 1M NaOH was added under ice-cooling, then stirring was last for 3h. THF was removed in a rotary evaporator. The pH of the mixture was adjusted to 1-2, inducing a white solid precipitates. Then ethyl acetate was added to dissolve the precipitates, the organic layer was separated and the aqueous layer was extracted with ethyl acetate (15 mL×3). The combined organic phases were washed with brine twice (2×15 mL), then dried with Na_2SO_4 and evaporated to obtain 3-(4-bromobenzamido)-5-methyl-2-oxohexanoic acid as a white solid.

3.7. *4*-bromo-N-(1-(1, 2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)benzamide

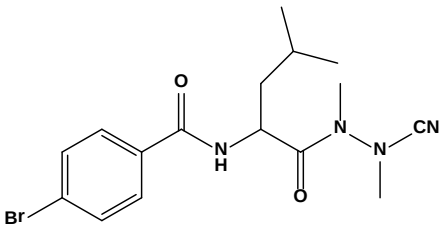


3.56 g 3-(4-bromobenzamido)-5-methyl-2-oxohexanoic acid (10 mmol) was dissolved in THF (15 mL) and cooled to –25 °C. To the stirred solution, 1.11 mL N-methylmorpholine (10 mmol) and 1.32 mL isobutylchloroformate (10 mmol) were added consecutively. 2.66 g 1,2-dimethylhydrazine dihydrochloride (20 mmol) was dissolved in 5 mL H_2O, and 5 M NaOH (8 mL) was added under ice-cooling. When the precipitation of N-methylmorpholine hydrochloride occurred this solution was added to the reaction mixture. It was allowed to warm to room temperature within 30 min and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (1×40 mL, 3×10 mL). The combined organic layers were washed with H_2O (1×15 mL), sat.$NaHCO_3$ twice (2×15 mL), H_2O

(1×15 mL) and brine (1×15 mL) in turn. The solvent was dried with Na_2SO_4 and evaporated to obtain a colorless oil. It was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (1:1) as eluent to obtain 4-bromo-N-(1-(1, 2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)benzamide as a colorless oil, which was then slowly solidified at room temperature.

3.8.

***4*-bromo-N-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)benzamide (1)**

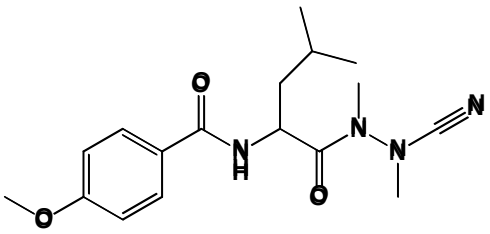


1.23 g sodium acetate (15 mmol) and 1.58 g cyanogen bromide (15 mmol) were added to a MeOH solution (20 mL) containing 1.78g (5 mmol) 4-bromo-N-(1-(1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)benzamide. The mixture was stirred at room temperature for 5 h and then the solvent was removed under reduced pressure. The residue was suspended in 10 mL H_2O, a pH of 1-2 was adjusted by using 10% $KHSO_4$, and then it was extracted with ethyl acetate for three times (3×20 mL). The combined organic layers were washed with H_2O (1×10 mL), sat. $NaHCO_3$ (2×10 mL) and brine (1×10 mL) in turn. The solvent was dried by Na_2SO_4 and removed in vacuo. The oily residue was purified by column chromatography on silica gel using petroleum ether / ethyl acetate (2:1) as eluent. The obtained oil was dried in a desiccator at room temperature for several days to obtain 4-bromo-N-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)benzamide as a colorless solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.62 – 7.57 (m, 2H), 7.52 – 7.48 (m, 2H), 7.10 (d, J = 8.2 Hz, 1H), 5.30 – 5.23 (m, 1H), 3.37 (s, 3H), 3.26 (s, 3H), 1.88 – 1.80 (m, 1H), 1.78 – 1.58 (m, 2H), 1.04 (dd, J = 25.0, 6.6 Hz, 6H).
^{13}C NMR (126 MHz, $CDCl_3$): δ 175.06 (s), 166.75 (s), 131.70 (s), 131.59 (s), 128.73 (s), 126.52 (s), 113.51 (s), 48.79 (s), 40.75 (d, *J* = 52.6 Hz), 30.58 (s), 25.16 (s), 23.26 (s), 21.25 (s).
FT-IR (KBr, cm^{-1}): 3268, 2960, 2936, 2872, 2222, 1708, 1660, 1590, 1542, 1482.
MS (ESI) m/z: $[M + H]^+$ 381.1, $[M+Na]^+$ 402.9, $[M+K]^+$ 418.9.
ee: 0.994.

3.9.

***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-4-methoxybenzamide (2)**



This compound was produced by using the similar procedures as for compound **1 (Scheme S1)** but it staring with 4-methoxybenzoic acid instead of 4-bromobenzoic acid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.77 (d, J = 8.7 Hz,2H), 6.94 (dd, J = 8.9, 2.1 Hz, 2H), 6.50 (d, J = 8.3 Hz, 1H), 5.32 (td, J = 10.1, 3.8 Hz, 1H), 3.87 (s, 3H), 3.36 (s, 3H), 3.23 (s, 3H), 1.87 – 1.78 (m, 1H), 1.75 – 1.55 (m, 2H), 1.09 – 1.02 (dd, J =25.8,6.6 Hz ,6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ 174.82 (s), 167.30 (s), 162.49 (s), 128.96 (s), 125.78 (s), 113.76 (s), 55.42 (s), 48.19 (s), 41.32 (s), 40.96 (s), 30.51 (s), 25.11 (s), 23.30 (s), 21.45 (s).

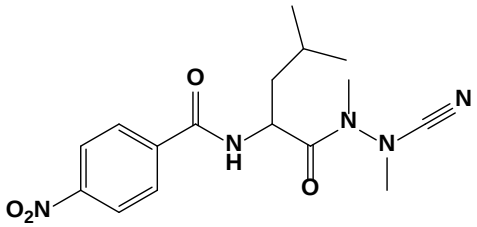
FT-IR (KBr, cm^{-1}): 3297, 2960, 2870, 2222, 1694, 1626, 1540, 1503, 1264.

MS (ESI) m/z: $[M + H]^+$ 332.9, $[M+Na]^+$ 354.8, $[M+K]^+$ 370.8.

ee: 0.992.

3.10.

***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-4-nitrobenzamide (3)**



This compound was produced by using the similar procedures as for compound **1** (**Scheme S1**) but it staring with 4-nitrobenzoic acid instead of 4-bromobenzoic acid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.24 (d, *J* = 8.7 Hz, 2H), 7.91 (d, *J* = 8.7 Hz, 2H), 6.98 (d, *J* = 8.1 Hz, 1H), 5.32 – 5.23 (m, 1H), 3.35 (s, 3H), 3.25 (s, 3H), 1.87 – 1.78 (m, 1H), 1.77 – 1.61 (m, 2H), 1.10 – 0.99 (m, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ 175.14 (s), 165.08 (d, *J* = 42.0 Hz), 149.36 (s), 139.25 – 137.16 (m), 128.30 (s), 123.22 (s), 114.39 – 112.02 (m), 49.31 (s), 40.86 (s), 39.82 (s), 30.63 (s), 25.11 (s), 23.12 (s), 20.98 (s).

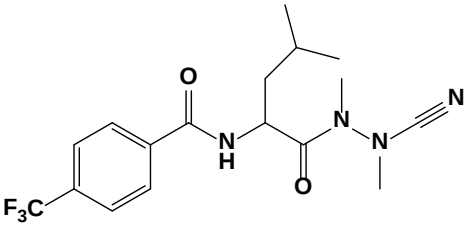
FT-IR (KBr, cm^{-1}): 3320, 3106, 3073, 2960, 2870, 2222, 1660, 1602, 1529, 1344.

MS (ESI) m/z: $[M + H]^+$ 347.9, $[M+Na]^+$ 369.7, $[M+K]^+$ 385.6.

ee: 1.

3.11.

***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-4-(trifluoromethyl)benzamide (4)**



This compound was produced by using the similar procedures as for compound **1** (**Scheme S1**) but it staring with 4-(trifluoromethyl)benzoic acid instead of 4-bromobenzoic acid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.86 (d, *J* = 8.1 Hz, 2H), 7.66 (d, *J* = 8.2 Hz, 2H), 6.86 (d, *J* = 7.7 Hz, 1H), 5.33 – 5.25 (m, 1H), 3.35 (s, 3H), 3.25 (s, 3H), 1.86 – 1.77 (m, 1H), 1.69 (dddd, *J* = 40.1, 13.7, 9.9, 3.8 Hz, 2H), 1.10 – 0.99 (m, 6H).

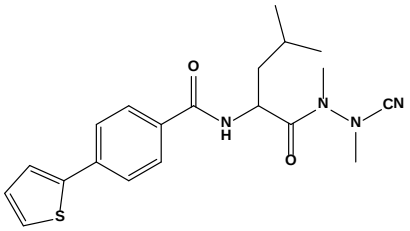
^{13}C NMR (126 MHz, $CDCl_3$): δ 175.41 (s), 165.90 (s), 135.75 (s), 132.57 (q, *J* = 32.4 Hz), 127.49 (s), 124.98 (s), 113.40 (s), 49.27 (s), 40.68 (s), 39.53 (s), 30.34 (s), 25.08 (s), 22.97 (s), 20.78 (s).

FT-IR (KBr, cm^{-1}): 3332, 2960, 2870, 2222, 1659, 1541, 1507, 1326.

MS (ESI) m/z: $[M + H]^+$ 370.7, $[M+Na]^+$ 392.7, $[M+K]^+$ 408.5.

ee: 0.85.

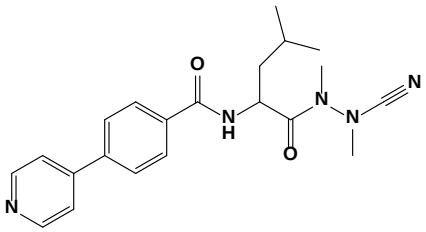
3.12. *N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-4-(thiophen-2-yl)benzamide (5)



In **Scheme S2**, a 100 mL, round-bottomed Schlenk flask equipped with a magnetic stir bar was charged with 0.256 g maternal compound **1**, 2-thienylboronic acid (2 mmol), 0.38 g 4-bromo-N-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)benzamide (1 mmol), 0.058 g tetrakis(triphenylphosphine)palladium (0.05 mmol), 2 mL 1M aqueous solution of Na_2CO_3, and 30 mL of THF. The mixture was degassed using five cycles of vacuum/nitrogen back-fill, and then was heated to 70 °C and kept there for 2.5 h with vigorous stirring. The mixture was cooled to room temperature, and the solvent was removed under reduced pressure. The residue was then suspended in H_2O (10 mL) and ethyl acetate (30 mL), the organic layer was separated while the aqueous layer was extracted with ethyl acetate (3×15 mL). The combined organic phases were washed with brine (2×15 mL), then dried with Na_2SO_4 and evaporated to obtain the crude product of *N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-4-(thiophen-2-yl)benzamide, which was purified by column chromatography on silica gel using

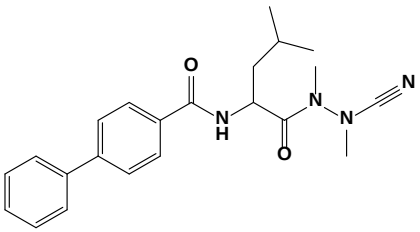
petroleum ether / ethyl acetate (2:1) as eluent.
^{1}H NMR (500 MHz, DMSO-d_6): δ 8.89 (d, J = 7.1 Hz, 1H), 8.13 (s, 1H), 7.82 (d, J = 7.6 Hz, 2H), 7.59 (d, J = 4.9 Hz, 2H), 7.51 (t, J = 7.7 Hz, 1H), 7.17 (dd, J = 5.0, 3.7 Hz, 1H), 5.03 (s, 1H),3.30 (s, 3H), 3.12 (s, 3H), 1.92 – 1.66 (m, 2H), 1.40 (d, J = 10.6 Hz, 1H), 0.96 (d, J = 6.1 Hz, 6H).
^{13}C NMR (126 MHz, $CDCl_3$): δ 175.29 (s), 166.67 (s), 142.94 (s), 137.27 (s), 131.63 (s), 131.34 (s), 128.80 (s), 128.26 (s), 127.86 (s), 126.30 (s), 125.34 (s), 124.16 (s), 113.56 (s), 49.05 (s), 40.82 (s), 39.90 (s), 30.57 (s), 25.13 (s), 23.22 (s), 21.09 (s).
FT-IR (KBr, cm^{-1}): 3478, 3332, 2960, 2870, 2222, 1653, 1602, 1586, 1535, 1467.
MS (ESI) m/z: $[M + H]^+$ 384.9, $[M+Na]^+$ 406.9.
ee: 0.33.

3.13.
***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-4-(pyridin-4-yl)benzamide (6)**



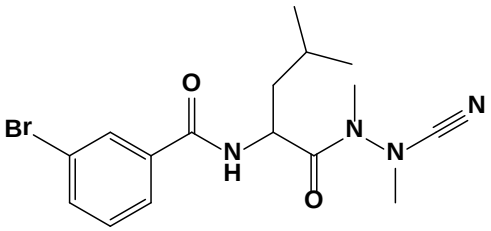
This compound was produced by using similar procedures as for compounds **5** (**Scheme S2**) but using pyridin-4-ylboronic acid instead of 2-thienylboronic acid.
^{1}H NMR (500 MHz, $CDCl_3$): δ 8.72 (d, *J* = 5.7 Hz, 2H), 7.91 (d, *J* = 8.1 Hz, 2H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.53 (d, *J* = 5.2 Hz, 2H), 6.89 (d, *J* = 6.9 Hz, 1H), 5.39 – 5.31 (m, 1H), 3.38 (s,3H), 3.27 (s, 3H), 1.86 (dd, *J* = 9.9, 6.7 Hz, 1H), 1.72 (tdd, *J* = 17.3, 14.0, 3.8 Hz, 2H), 1.08 (dd, *J* = 29.3, 6.5 Hz, 2H).
^{13}C NMR (126 MHz, $CDCl_3$): δ 174.88 (d, *J* = 37.8 Hz), 167.71 – 166.18 (m), 149.93 (s), 133.50 (s), 128.12 (d, *J* = 25.0 Hz), 126.71 (d, *J* = 19.9 Hz), 121.45 (s), 113.53 (s), 49.48 – 48.04 (m), 40.73 (s), 30.46 (s), 25.02 (s), 23.11 (s), 21.03 (s).
FT-IR (KBr, cm^{-1}): 3332, 2960, 2870, 2222, 1698, 1602, 1534, 1505, 1237.
MS (ESI) m/z: $[M+H]^+$ 380.2, $[M+Na]^+$ 402.1.
ee: 0.648.

3.14.
***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)biphenyl-4-carboxamide (7)**



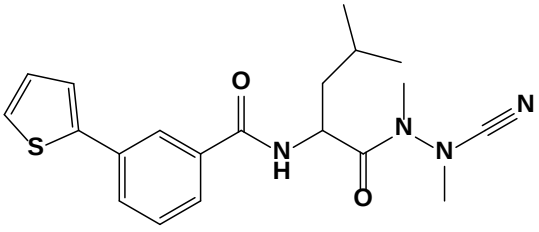
This compound was produced by using similar procedures as for compounds **5** (**Scheme S2**) but using phenylboronic acid instead of 2-thienylboronic acid.
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.86 (d, *J* = 5.6 Hz, 2H), 7.64 (dt, *J* = 8.6, 6.1 Hz, 4H), 7.46 (t, *J* = 7.6 Hz, 2H), 7.39 (t, *J* = 7.3 Hz, 1H), 6.64 (d, *J* = 8.3 Hz, 1H), 5.34 (td, *J* = 10.1, 3.7 Hz, 1H), 3.36 (s, 3H), 3.24 (s, 3H), 1.82 (dd, *J* = 12.1, 5.4 Hz, 1H), 1.77 – 1.63 (m, 2H), 1.12 – 1.00 (m, 6H).
^{13}C NMR (126 MHz, $CDCl_3$): δ 174.89 (s), 167.44 (s), 144.92 – 144.38 (m), 139.94 (s), 132.00 (s), 128.92 (s), 128.05 (s), 127.65 (s), 127.18 (d, *J* = 8.3 Hz), 113.63 (s), 48.48 (s), 41.58 – 41.24 (m), 41.01 (s), 30.55 (s), 25.17 (s), 23.32 (s), 21.42 (s).
FT-IR(KBr, cm^{-1}): 3461, 3347, 2973, 2870, 2222, 1652, 1606, 1530, 1362.
MS (ESI) m/z: $[M+H]^+$ 378.9.
ee: 0.977.

3.15. *3*-bromo-N-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)benzamide (8)



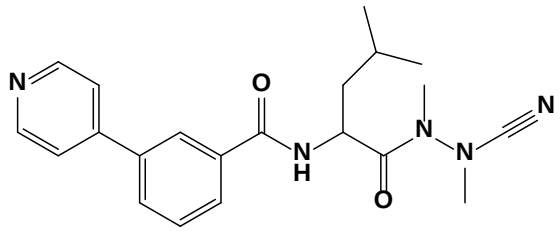
This compound was produced by using the similar procedures as for compound **1** (**Scheme S1**) but staring with 3-bromobenzoic acid instead of 4-bromobenzoic acid.
^{1}H NMR (500 MHz, DMSO-d_6): δ 8.89 (d, J = 7.0 Hz, 1H), 8.10 (s, 1H), 7.89 (d, J = 7.8 Hz, 1H), 7.76 (d, J = 7.7 Hz, 1H), 7.45 (t, J = 7.8 Hz, 1H), 5.00 (s, 1H), 3.32 (d, J = 13.7 Hz, 3H), 3.22 – 3.03 (m, 3H), 1.90 – 1.62 (m, 2H), 1.46 – 1.31 (m, 1H), 1.00 – 0.87 (m, 6H).
^{13}C NMR (126 MHz, $CDCl_3$): δ 175.63 (s), 165.79 (s), 134.56 (s), 134.22 (s), 130.18 (s), 129.76 (s), 125.71 (s), 122.23 (s), 113.50 (d, *J* = 10.1 Hz), 48.75 (d, *J* = 15.5 Hz), 40.78 (d, *J* = 14.2 Hz), 40.00 (s), 30.68 (d, *J* = 16.0 Hz), 25.07 (s), 23.21 (s), 21.22 (d, *J* = 8.6 Hz).
FT-IR (KBr, cm^{-1}): 3268, 2960, 2936, 2872, 2222, 1708, 1660, 1590, 1542, 1482.
MS (ESI) m/z: $[M+H]^+$ 381.1, $[M+Na]^+$ 402.9, $[M+K]^+$ 418.9.
ee: 1.

3.16. *N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-3-(thiophen-2-yl)benzamide (9)



This compound was produced by using the similar procedures as for compound **5** (**Scheme S2**) but it using compound **8** instead of **1** as maternal compound.
^{1}H NMR (500 MHz, DMSO-d_6): δ 8.89 (d, J = 7.1 Hz, 1H), 8.13 (s, 1H), 7.82 (d, J = 7.6 Hz, 2H), 7.59 (d, J = 4.9 Hz, 2H), 7.51 (t, J = 7.7 Hz, 1H), 7.17 (dd, J = 5.0, 3.7 Hz, 1H), 5.03 (s, 1H),3.30 (s, 3H), 3.12 (s, 3H), 1.92 – 1.66 (m, 2H), 1.40 (d, J = 10.6 Hz, 1H), 0.96 (d, J = 6.1 Hz, 6H).
^{13}C NMR (126 MHz, $CDCl_3$): δ 175.57, 167.22, 143.38, 134.49, 133.55, 130.34, 128.87, 128.02, 125.95, 125.32, 124.70, 123.75, 113.68, 48.91, 40.87, 40.23, 30.63, 25.21, 23.31, 21.27.
FT-IR(KBr, cm^{-1}): 3332, 2960, 2870, 2222, 1653, 1602, 1580, 1535.
MS (ESI) m/z: $[M+H]^+$ 384.9, $[M+Na]^+$ 406.9.
ee: 0.996.

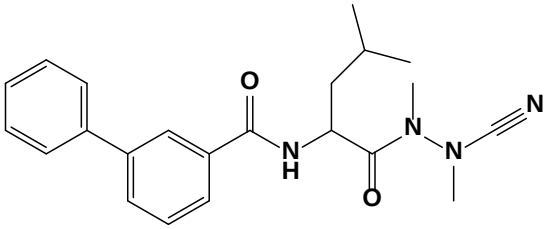
3.17.
***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-3-(pyridin-4-yl)benzamide (10)**



This compound was produced by using the similar procedures as for compound **5** (**Scheme S2**) but it using compound **8** instead of **1** as maternal compound, and using pyridin-4-ylboronic acid instead of 2-thienylboronic acid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.64 (d, J = 6.0 Hz, 2H), 7.96 (s, 1H), 7.91 (d, J = 7.8 Hz, 1H), 7.73 (d, J = 7.8 Hz, 1H), 7.62 (t, J = 8.5 Hz,1H), 7.44 (d, J = 6.0 Hz, 2H), 7.38 (t, J = 7.7 Hz, 1H), 5.33 – 5.25 (m, 1H), 3.39 (s, 3H), 3.25 (s, 3H), 1.94 – 1.85 (m,1H), 1.85 – 1.56 (m, 2H), 1.02 (dt, J = 13.8, 6.8 Hz, 6H).
^{13}C NMR (126 MHz, $CDCl_3$): δ 175.64 (s), 166.63 (d, *J* = 68.8 Hz), 149.86 (s), 147.43 (s), 137.78 (s), 133.58 (s), 129.68 (s), 128.96 (s), 127.81 (s), 125.69 (s), 121.57 (s), 113.46 (s), 49.11 (s), 40.76 (s), 39.73 (s), 30.53 (s), 25.08 (s), 24.73 (s), 23.16 (s), 20.99 (s).
FT-IR(KBr, cm^{-1}): 3484, 2960, 2870, 2222, 1653, 1602, 1535, 1473.
MS (ESI) m/z: $[M+H]^+$ 380.3.
Enantiomeric excess,ee: 0.33.

3.18.
***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)biphenyl-3-carboxamide (11)**



This compound was produced by using the similar procedures as for compound **5** (**Scheme S2**) but it using compound **8** instead of **1** as maternal compound, and using phenylboronic acid instead of 2-thienylboronic acid.

1H NMR (500 MHz, $CDCl_3$): δ 8.01 (d, J = 5.6 Hz, 1H), 7.74 (dd, J = 12.6, 7.2 Hz, 2H), 7.64 – 7.59 (m, 2H), 7.47 (dd, J = 10.8, 4.4 Hz, 3H), 7.42 – 7.37 (m, 1H), 6.94 (d, J = 26.9 Hz, 1H), 5.38 – 5.31 (m, 1H), 3.38 (s, 3H), 3.24 (s, 3H), 1.85 (ddd, J = 14.2, 10.3, 6.3 Hz, 1H), 1.80 – 1.58 (m, 2H), 1.11 – 1.02 (m, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ 174.61 (s), 167.40 (s), 144.18 (s), 141.00 (s), 140.46 (s), 138.73 (s), 132.21 (d, *J* = 1.5 Hz), 128.89 (s), 127.63 (d, *J* = 10.1 Hz), 127.08 (s), 113.60 (s), 48.27 (s), 41.79 – 40.79 (m), 30.54 (s), 25.17 (s), 23.33 (s), 21.50 (s).

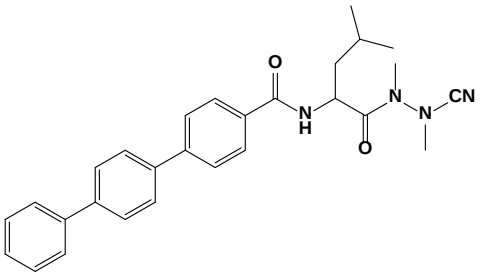
FT-IR (KBr, cm^{-1})： 3332, 2960, 2870, 2222, 1656, 1529, 1474.

MS (ESI) m/z: $[M+H]^+$ 379.4, $[M+Na]^+$ 401.1.

ee: 0.96.

3.19.

***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-4'-phenyl-biphenyl-4-carboxamide (12)**



This compound was produced by using similar procedures as for compound **5** (**Scheme S2**) in staring with compound **1** as maternal compound but using biphenyl-4-ylboronic acid instead of 2-thienylboronic acid.

^1H NMR (500 MHz, $CDCl_3$): δ 7.91 (d, *J* = 8.1 Hz, 2H), 7.76 – 7.71 (m, 6H), 7.67 (d, *J* = 7.5 Hz, 2H), 7.50 (t, *J* = 7.5 Hz, 2H), 7.41 (t, *J* = 7.3 Hz, 1H), 6.61 (d, *J* = 8.4 Hz, 1H), 5.38 (td, J = 10.0, 3.8 Hz, 1H), 3.39 (s, 3H), 3.27 (s, 3H), 1.87 (s, 1H), 1.73 (ddd, *J* = 23.4, 21.8, 12.1 Hz, 2H), 1.10 (dd, *J* = 28.6, 6.5 Hz, 6H).

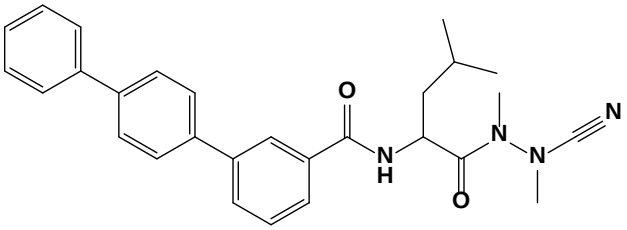
^{13}C NMR (126 MHz, $CDCl_3$): δ 174.62 (s), 167.40 (s), 144.19 (s), 141.00 (s), 140.45 (s), 138.73 (s), 132.20 (s), 128.89 (s), 127.63 (d, *J* = 10.1 Hz), 127.08 (s), 113.60 (s), 48.27 (s), 42.00 – 41.38 (m), 41.05 (s), 30.54 (s), 25.17 (s), 23.33 (s), 21.50 (s).

FT-IR (KBr, cm^{-1})： 3372, 2960, 2871, 2222, 1695, 1644, 1519, 1480, 1381.

MS (ESI) m/z: $[M+H]^+$ 455.5, $[M+Na]^+$ 476.9, $[M+K]^+$ 492.8.

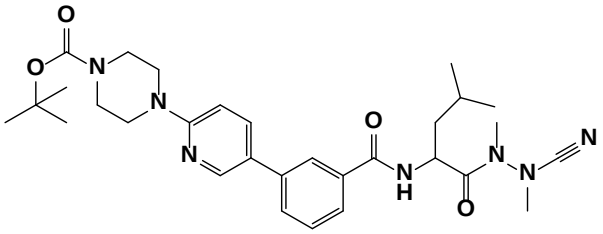
ee: 0.999.

3.20.
***N*-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-yl)-4'-phenyl-biphenyl-3-carboxamide (13)**



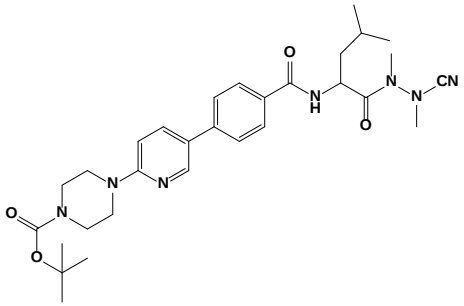
This compound was produced by using the same procedure as for compound **5** (**Scheme S2**) in staring with compound **8** as maternal compound but using biphenyl-4-ylboronic acid instead of 2-thienylboronic acid.
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.91 (d, J = 8.1 Hz, 2H), 7.76 – 7.71 (m, 6H), 7.67 (d, J = 7.5 Hz, 2H), 7.50 (t, J = 7.5 Hz, 2H), 7.41 (t, J = 7.3 Hz, 1H), 6.61 (d, J = 8.4 Hz, 1H), 5.39 (d, J = 6.5 Hz, 1H), 3.39 (s, 3H), 3.27 (s, 3H), 1.87 (s, 1H), 1.73 (ddd, J = 23.4, 21.8, 12.1 Hz, 2H), 1.10 (dd, J = 28.6, 6.5 Hz, 6H);
^{13}C NMR (126 MHz, $CDCl_3$): δ 174.68 (d, *J* = 2.9 Hz), 167.42 (s), 144.18 (d, *J* = 4.4 Hz), 140.98 (s), 140.45 (s), 138.74 (s), 132.18 (s), 128.89 (s), 127.59 (s), 127.08 (s), 113.61 (s), 48.32 (s), 41.41 (s), 41.04 (s), 30.55 (s), 25.17 (s), 23.34 (s), 21.49 (s).
FT-IR (KBr, cm^{-1})： 3337, 2960, 2870, 2222, 1687, 1653, 1608, 1518, 1484；
MS (ESI) m/z： $[M+H]^+$ 455.5, $[M+Na]^+$ 476.9, $[M+K]^+$ 492.8.
ee: 0.994.

3.21.*Tert*-butyl
4-(5-(3-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-ylcarbamoyl)phenyl)pyridin-2-yl)piperazine-1-carboxylate (14)



This compound was produced by using the same procedure as for **5** (**Scheme S2**) in staring with compound **8** as maternal compound but using *tert*-butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate instead of 2-thienylboronic acid.
^{1}H NMR (500 MHz, $CDCl_3$): δ 8.45 (d, J = 2.2 Hz, 1H), 7.92 (s, 1H), 7.76 (d, J = 7.5 Hz, 1H), 7.66 (t, J = 8.7 Hz,2H), 7.47 (t, J = 7.7 Hz,1H), 6.73 (d, J = 8.8 Hz, 1H), 6.70 (d, J = 8.3 Hz, 2H), 5.33 (dt, J = 15.7, 4.6 Hz, 1H), 3.59 (d, J = 9.0 Hz, 8H), 3.36 (s, 3H), 3.25 (s, 3H), 1.87 – 1.78 (m,1H), 1.77 – 1.62 (m, 2H), 1.50 (s, 9H), 1.06 (dd, J = 27.9, 6.6 Hz, 6H).
FT-IR (KBr, cm^{-1}): 3334, 2960, 2870, 2222, 1689, 1656, 1593, 1542, 1389, 1298.
MS (ESI) m/z: $[M+H]^+$ 564.4

3.22.*Tert*-butyl 4-(5-(4-(1-(2-cyano-1,2-dimethylhydrazinyl)-4-methyl-1-oxopentan-2-ylcarbamoyl)phenyl)pyridin-2-yl)piperazine-1-carboxylate (15)



This compound was produced by using the same procedure as for compound **5** (**Scheme S2**) in staring with compound **1** as maternal compound but using *tert*-butyl 4-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate instead of 2-thienylboronic acid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.46 (s, 1H), 7.82 (d, J = 8.2 Hz, 2H), 7.75 (d, J = 8.7 Hz, 1H), 7.56 – 7.50 (m, 2H), 6.98 (s, 1H), 6.73 (d, J = 8.8 Hz, 1H), 5.32 (t, J = 8.8 Hz, 1H), 3.60 (d, J = 14.6 Hz, 8H), 3.37 (s, 3H), 3.24 (s, 3H), 1.95 – 1.80 (m, 1H), 1.80 – 1.59 (m, 2H), 1.50 (s, 9H), 1.05 (dd, J = 26.9, 6.4 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ 174.73 (s), 167.36 (s), 158.63 (s), 154.81 (s), 146.25 (s), 141.63 (s), 136.22 (s), 131.56 (s), 127.83 (s), 125.98 (s), 125.03 (s), 113.59 (s), 106.96 (s), 80.06 (s), 48.34 (s), 45.03 (s), 41.28 (s), 41.02 (s), 30.55 (s), 28.44 (s), 25.15 (s), 23.30 (s), 21.44 (s).

FT-IR (KBr, cm^{-1}): 3340, 2960, 2866, 2223, 1700, 1656, 1601, 1540, 1463, 1386, 1238.

MS (ESI) m/z: $[M+H]^+$ 564.3, $[2M+H]^+$ 1127.6.

ee: 0.984.

4. Reference

[1] R. Löser, K. Schilling, E. Dimmig, M. Gütschow, *J. Med. Chem.*, 2005, **48**, 7688-7707.

5. Copies of ^{13}C-NMR spectra

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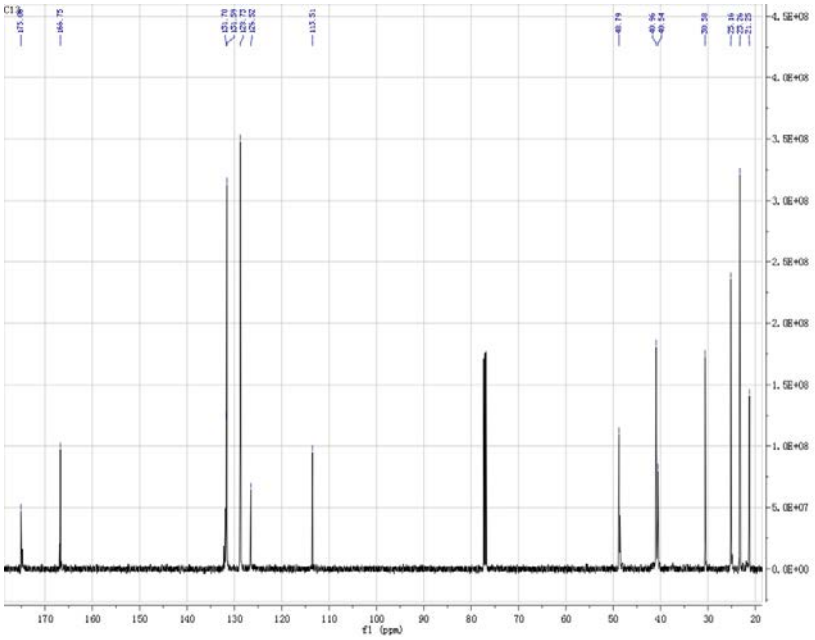

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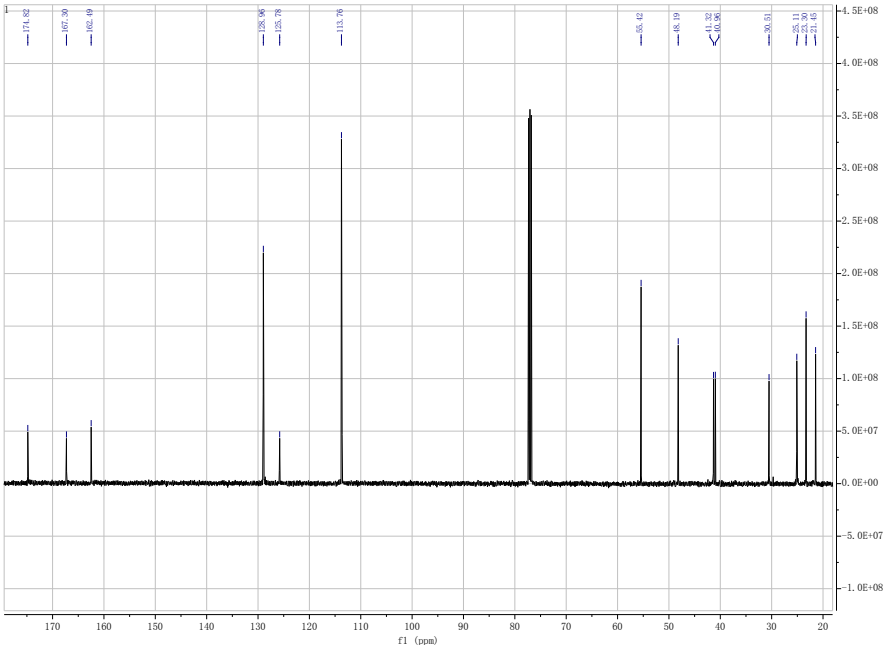

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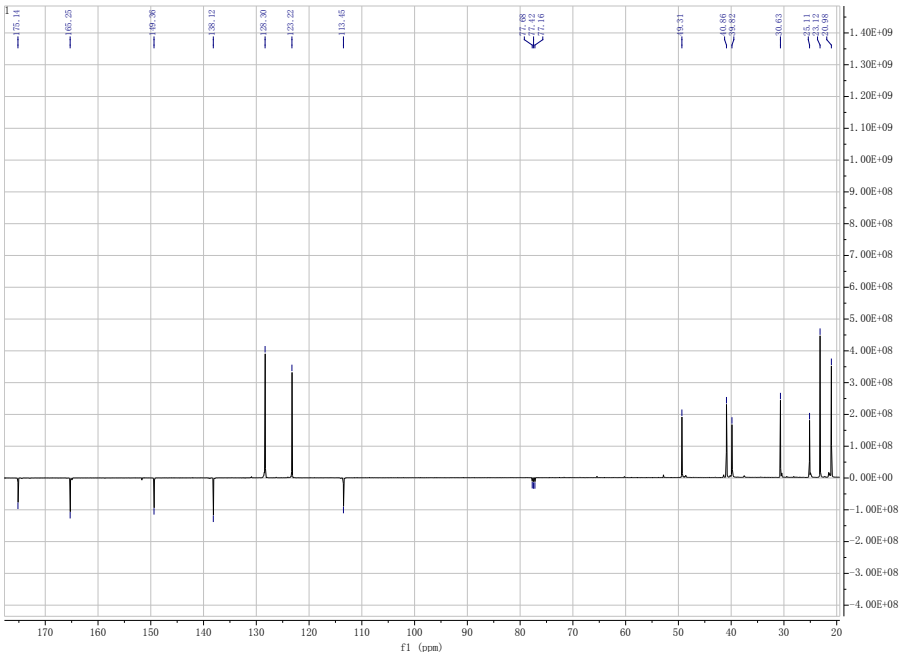
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138.12
128.30
123.22
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77.16
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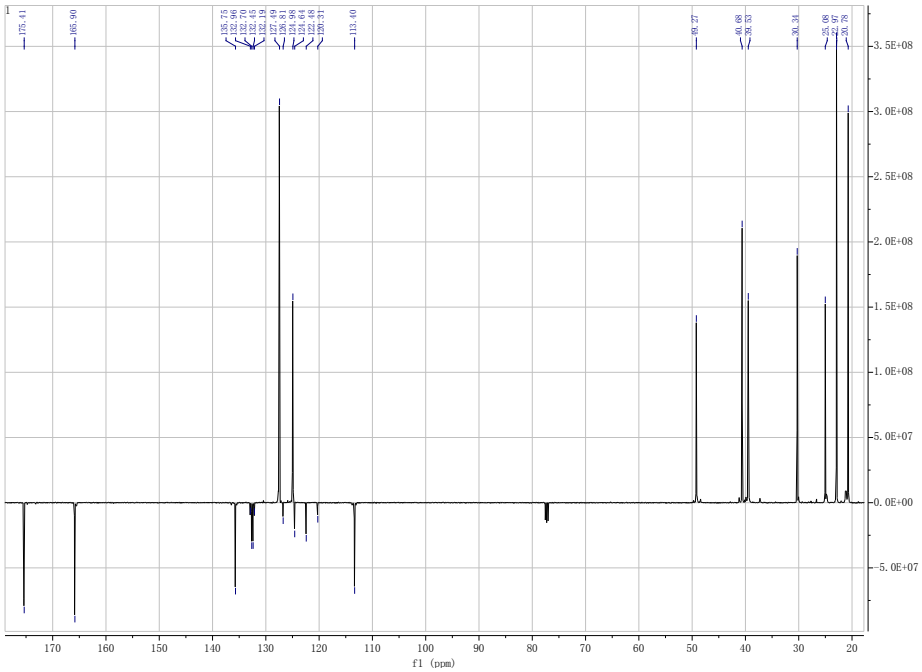
25.11
23.12
20.98
f1 (ppm)

4.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132.50
132.45
132.19
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126.81
124.98
123.46
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113.40
69.27
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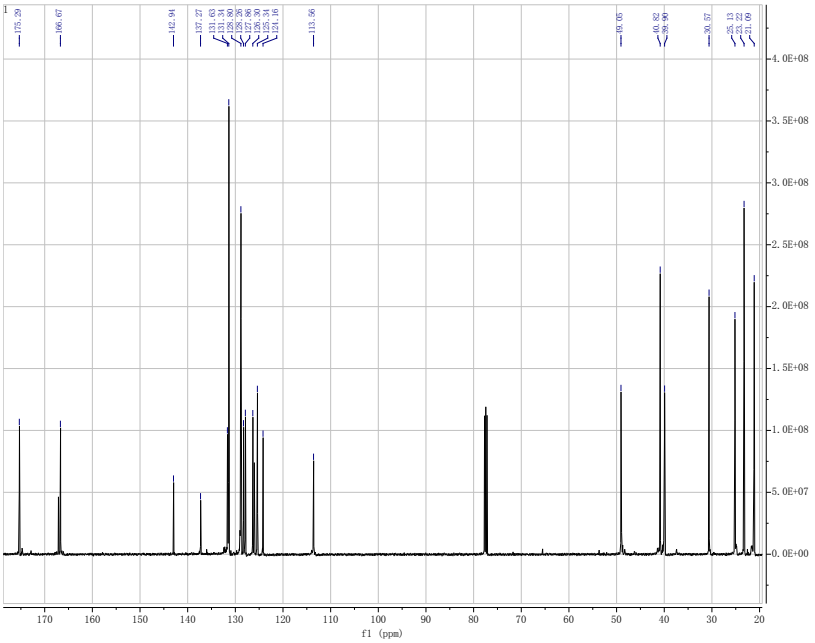
20.78
f1 (ppm)

5.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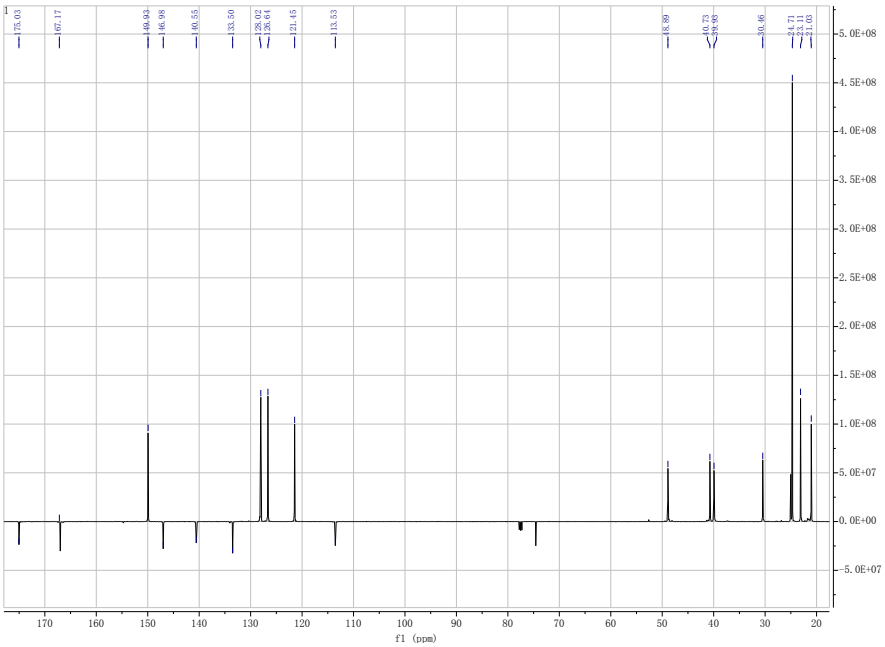
6.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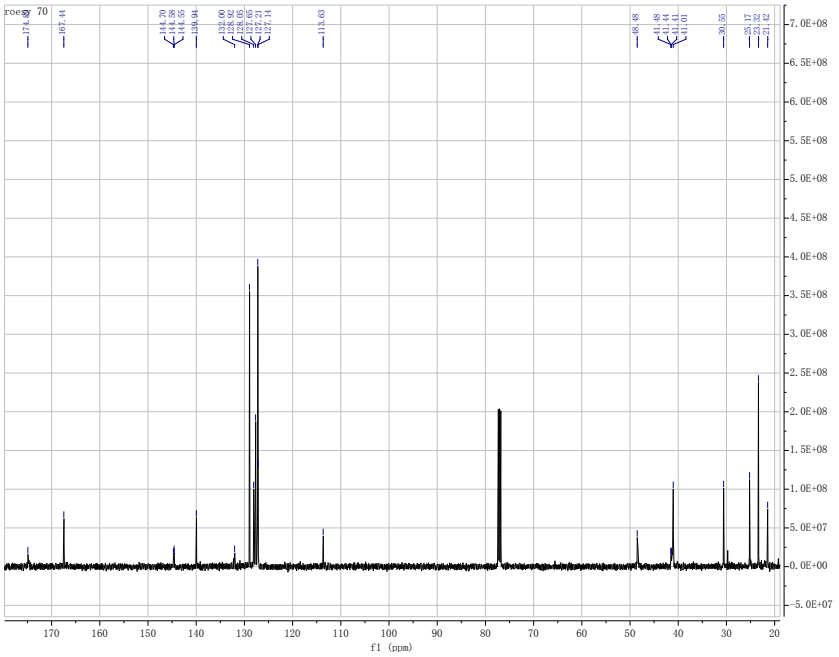
39.93
30.46
24.71
21.11
21.03
f1 (ppm)

7.


roesy 70
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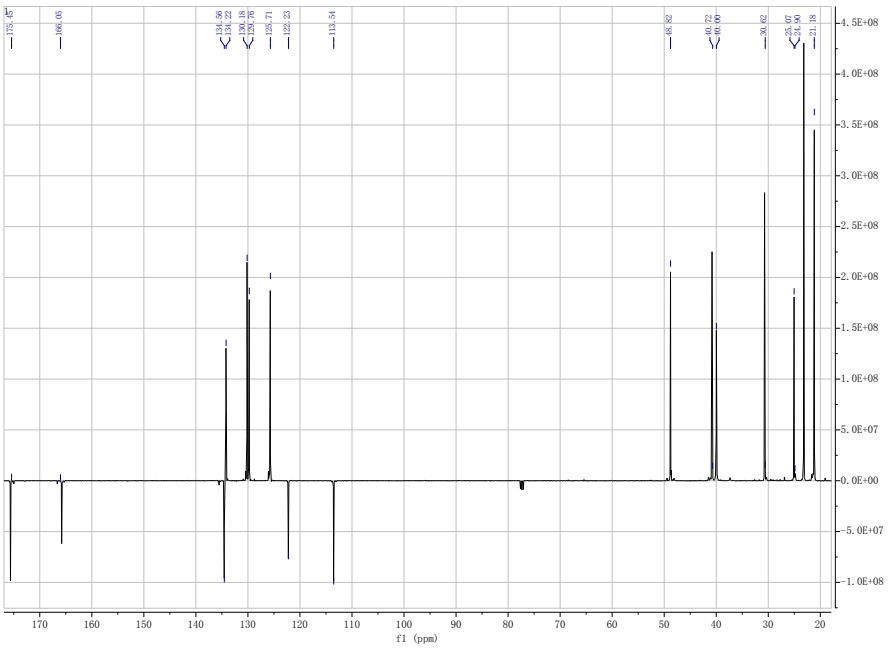
167.44
144.20
141.98
140.55
139.94
132.00
129.67
128.05
127.65
127.31
127.11
123.14
118.63
68.48
41.48
41.44
41.41
41.01
30.55
25.17
23.32
21.42
7.0E+08
6.5E+08
6.0E+08
5.5E+08
5.0E+08
4.5E+08
4.0E+08
3.5E+08
3.0E+08
2.5E+08
2.0E+08
1.5E+08
1.0E+08
5.0E+07
0.0E+00
-5.0E+07
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

8.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25.07
24.90
21.18
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3.5E+08
3.0E+08
2.5E+08
2.0E+08
1.5E+08
1.0E+08
5.0E+07
0.0E+00
-5.0E+07
-1.0E+08
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50
40
30
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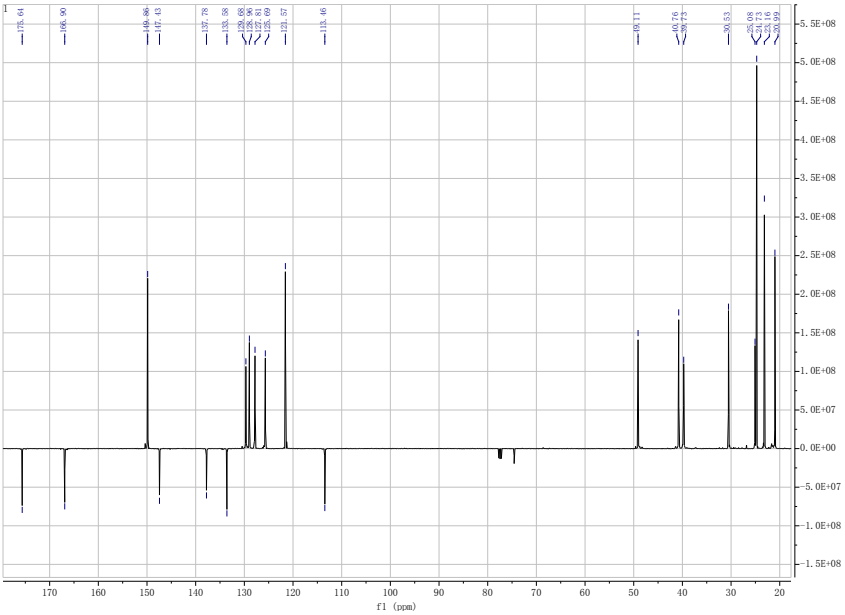
f1 (ppm)

10.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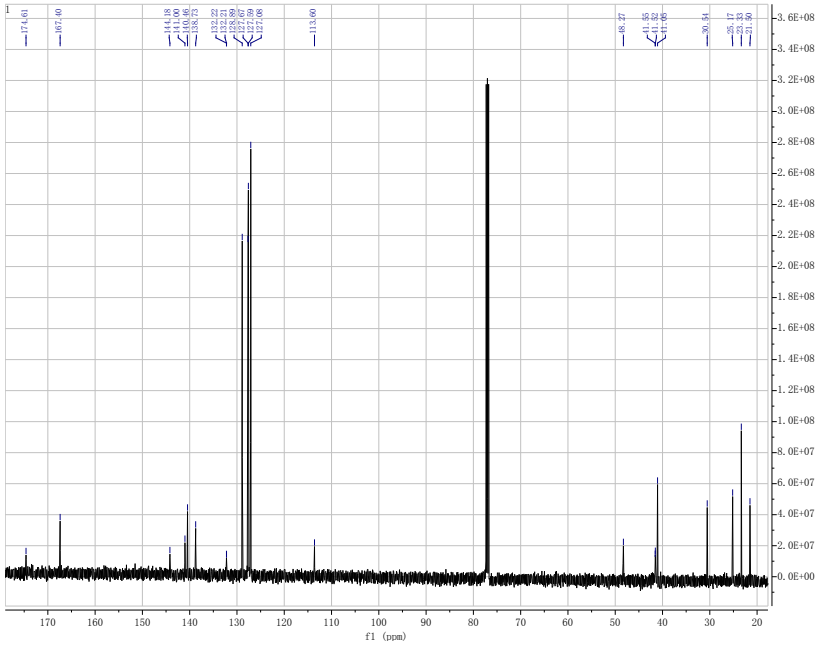
f1 (ppm)

11.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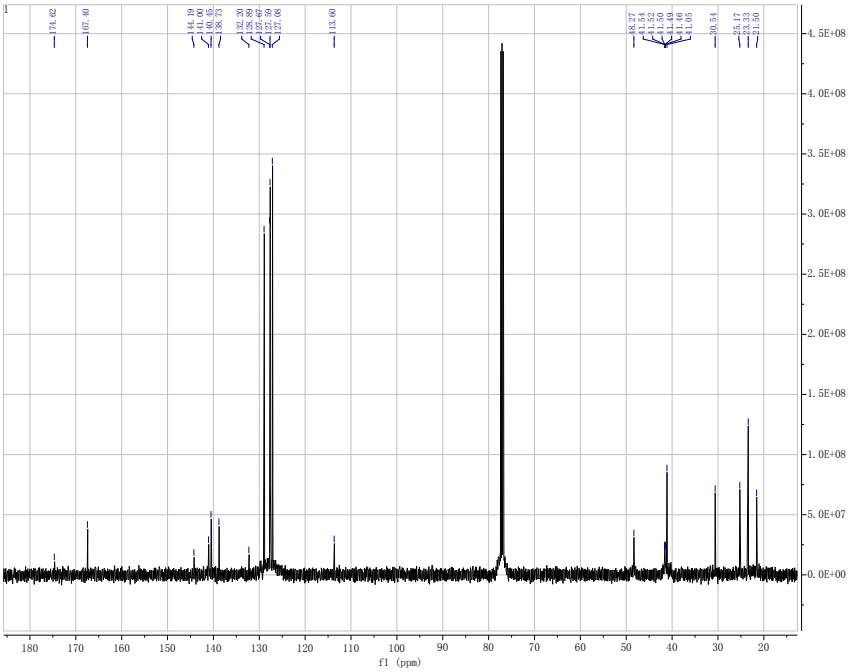
f1 (ppm)

12.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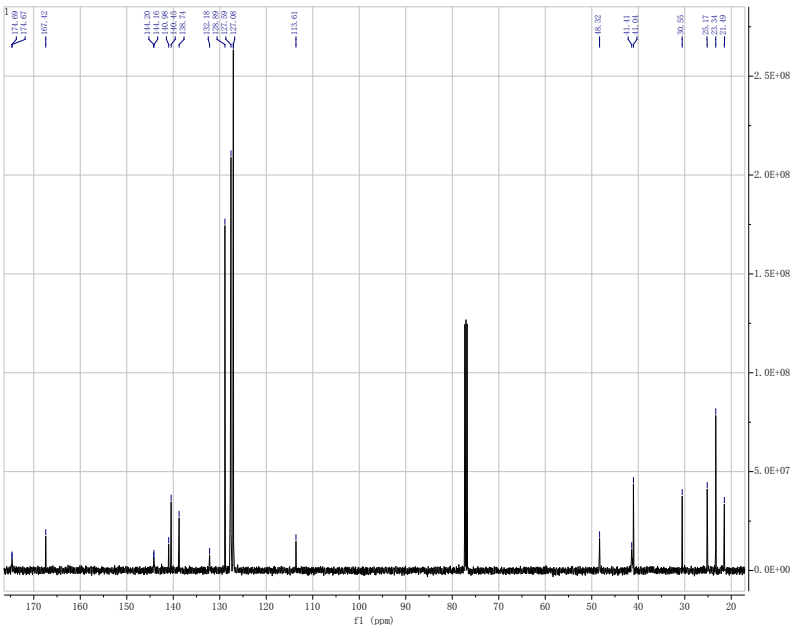
13.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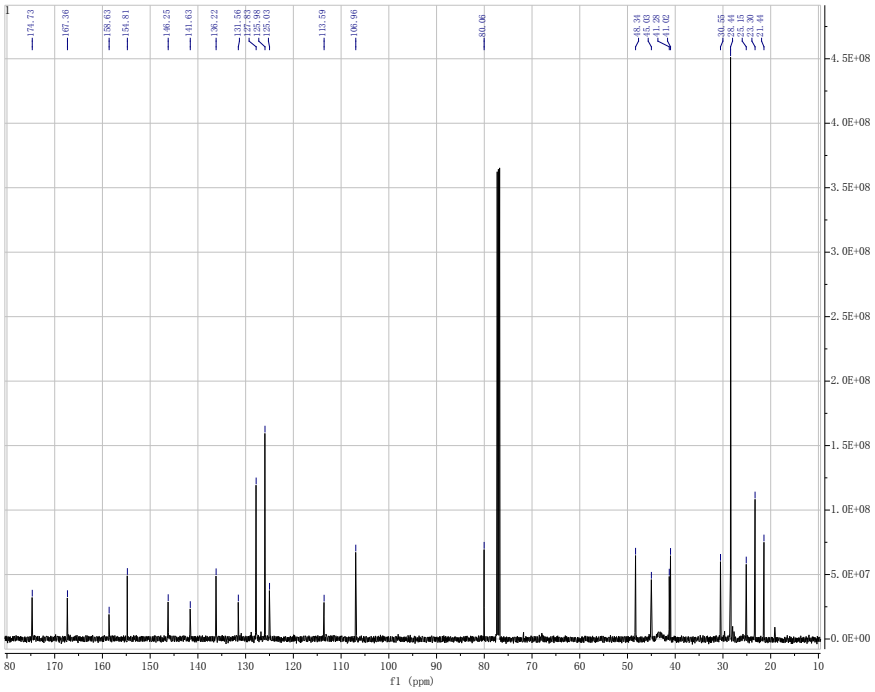
f1 (ppm)

15.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125.03
113.59
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3.5E+08
3.0E+08
2.5E+08
2.0E+08
1.5E+08
1.0E+08
5.0E+07
0.0E+00
80
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)