SUPPORTING INFORMATION

Michael Acceptor-Based Peptidomimetic Inhibitor of Main Protease from Porcine Epidemic Diarrhea Virus

Fenghua Wang,[†,+] Cheng Chen,[†,‡,+] Kailin Yang,[#,+] Yang Xu,[‡] Xiaomei Liu,[‡] Fan Gao,[‡] He Liu,[‡] Xia Chen,[†] Qi Zhao,[§] Xiang Liu,[‡] Yan Cai,[‡,*] and Haitao Yang[†,‡,*]

[†]School of Life Sciences, Tianjin University, Tianjin 300072, China

[‡]Tianjin International Joint Academy of Biotechnology and Medicine, Tianjin 300457, China

[#]Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, Ohio 44120, United States

[§]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520, United States

[+]F.W., C.C., and K.Y. contributed equally and are considered as co-first authors.

Corresponding Authors

*H.Y.: phone, +86 22 27403906; fax, +86 22 27403902; e-mail, yanght@tju.edu.cn.

*C.Y.: phone, +86 22 65378009; fax, +86 22 65378036; e-mail, caiyan_86@163.com.

Table of Contents

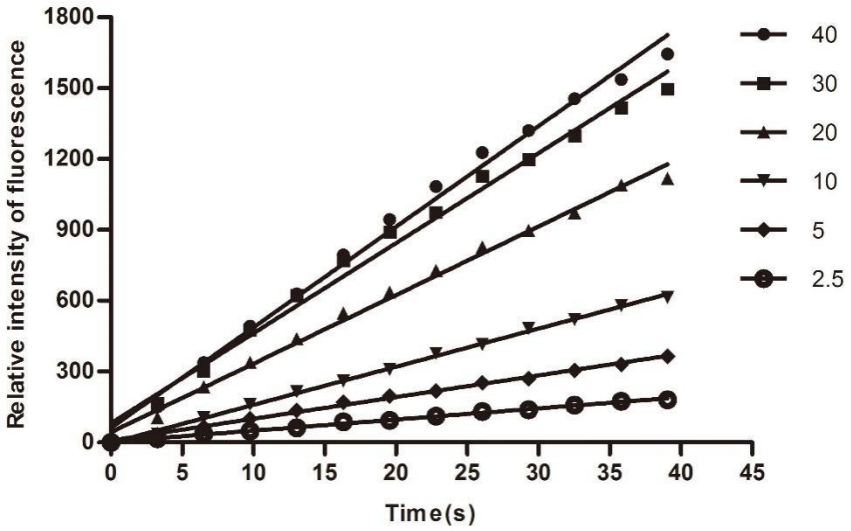


Figure S1. Enzyme activity of PEDV M^{pro}. Reaction progress curves in the presence of increasing fluorescence-labeled substrate concentration.

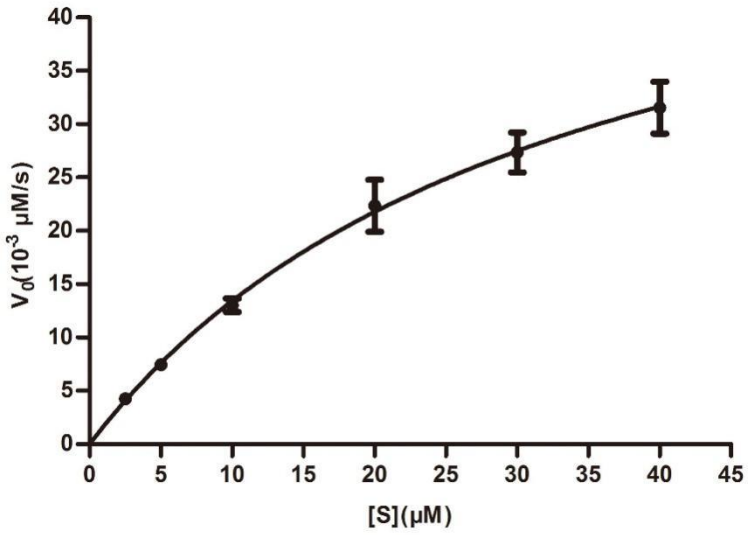


Figure S2. Initial rate constant of PEDV M^{pro}. Plot initial rate constant V_0 over the concentration of fluorescence-labeled substrate.

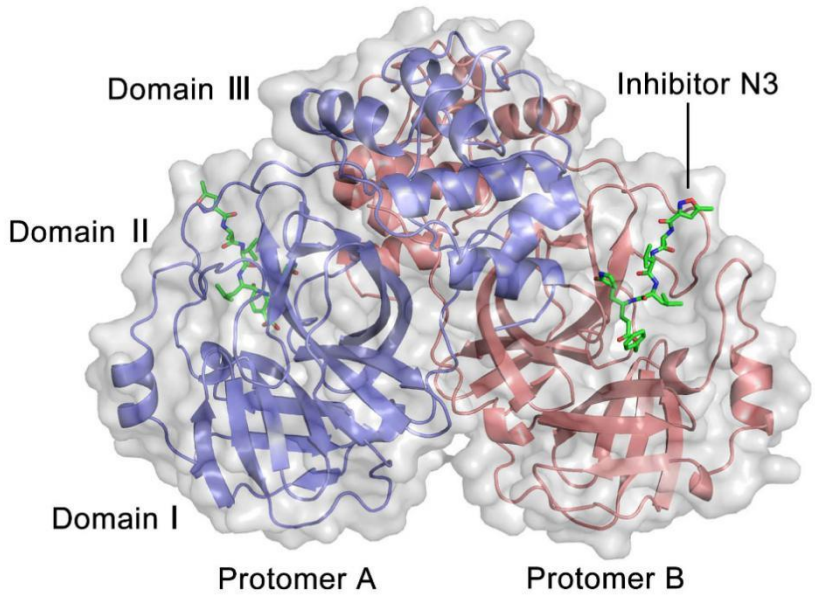


Figure S3. Overview of PEDV M^{pro} homodimer (PDB ID 5GWZ) in one asymmetric unit (A: slate and B: deep salmon). Protomers are shown in cartoons, and compound **1** (inhibitor N3) are shown as green sticks.

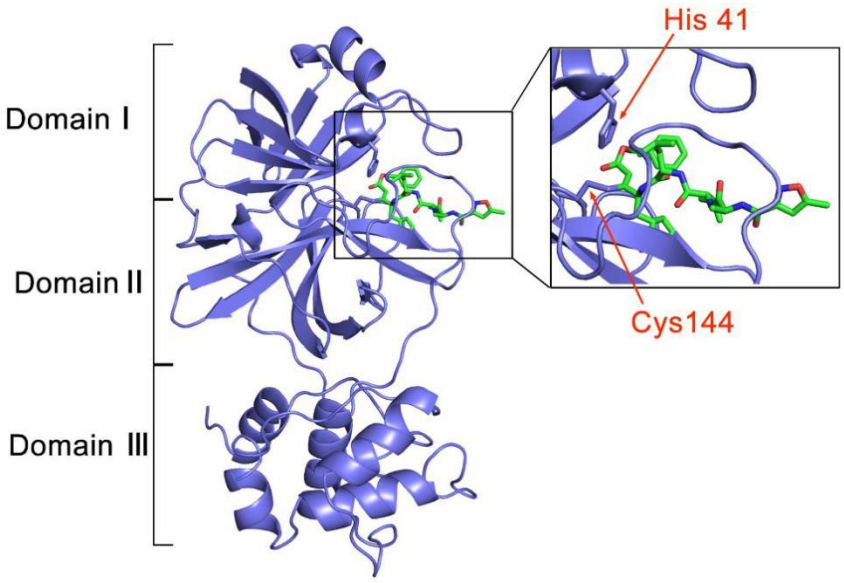


Figure S4. Structural overview of PEDV M^{pro} interaction with compound **1** (PDB ID 5GWZ). The backbone of PEDV M^{pro} is shown in cartoons, and compound **1** is presented as green sticks.

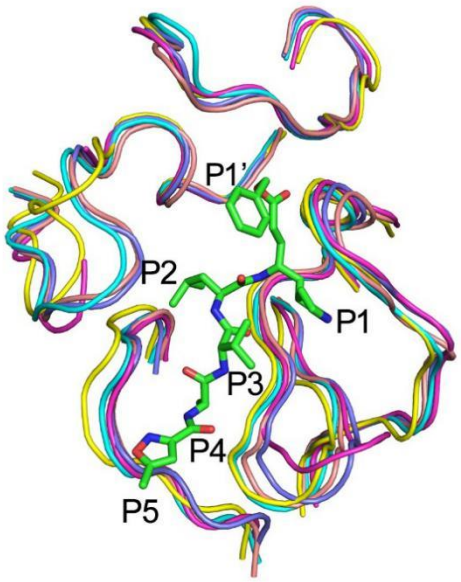


Figure S5. Comparison of the complex structure of compound **1**-PEDV M^{pro} with those from other coronavirus species: PEDV (PDB ID 5GWZ), slate; TGEV (PDB ID 2AMP),[1] salmon; SARS-CoV (PDB ID 2AMQ),[1] yellow; HCoV-NL63 (PDB ID 5GWY),[2] cyan; and HCoV-HKU1 (PDB ID 3D23),[3] light magentas.

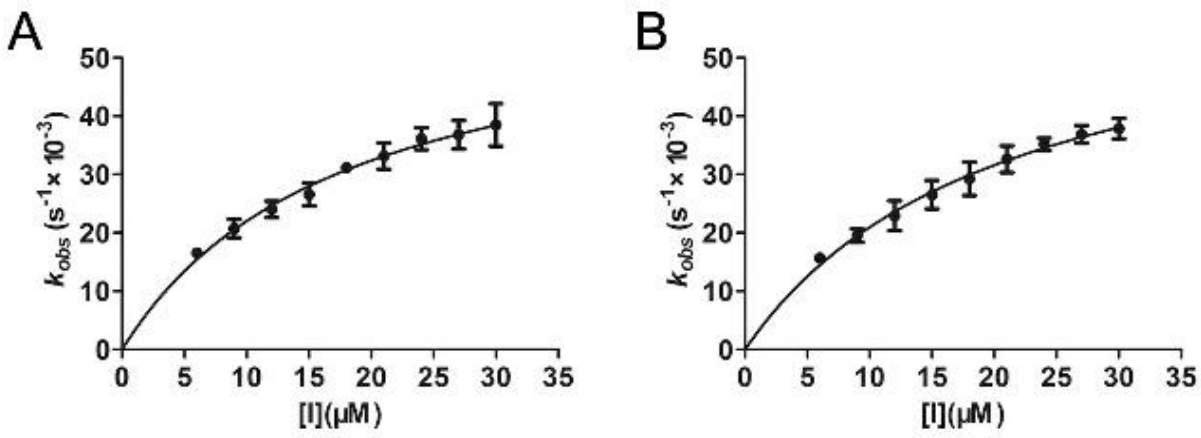


Figure S6. Enzymatic inhibition of compounds **3** and **15** against PEDV M^{pro}. A: k_{obs}-[I] for compound **3**. B: k_{obs}-[I] for compound **15**.

Table S1. X-ray data-processing and refinement statistics.

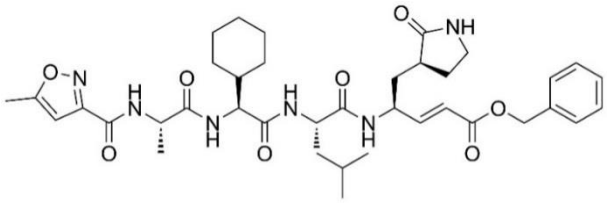
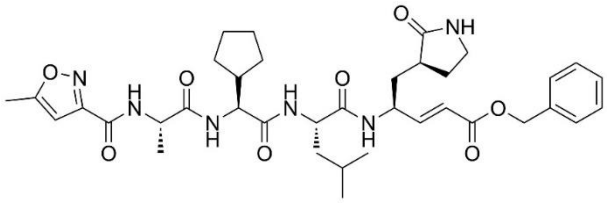
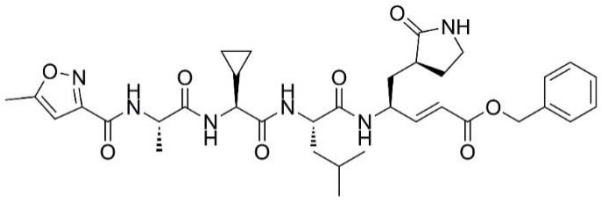
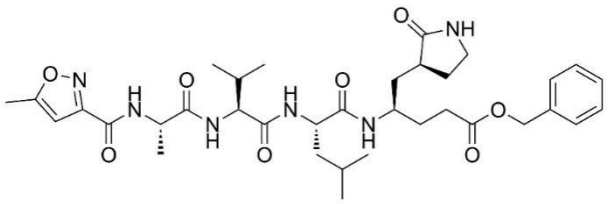
Statistics	**Value for the PEDV M^{pro}-compound 1 complex**
Data collection	
Wavelength (Å) ……	1.0000
Resolution limit (Å) ……	50.0-2.44 (2.90-2.85)
Space group ……	*R*3
Cell parameters	
a (Å) ……	175.3
b (Å) ……	175.3
c (Å) ……	58.7
α = β(°) …….	90
γ (°) ……	120
Total no. of reflections ……	144327
No. of unique reflections ……	24884
Completeness (%) ……	100.0 (99.2)
Redundancy……	5.8 (4.6)
R_{merge}(%)……	5.2 (29.9)
Sigma cutoff ……	0
I/σ(I) ……	32.6 (5.3)
Refinement	
Resolution range (Å) ……	50.0-2.44 (2.53-2.44)
R_{work}(%) ……	18.5
R_{free}(%)……	21.9
Rmsd from ideal geometry	
Bonds (Å) ……	0.005
Angles (°) ……	0.99
Avg B factor (Å^2) ……	46.3
Protein ……	46.2

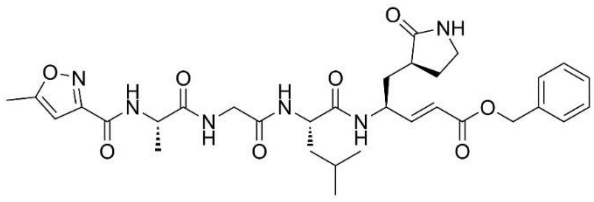
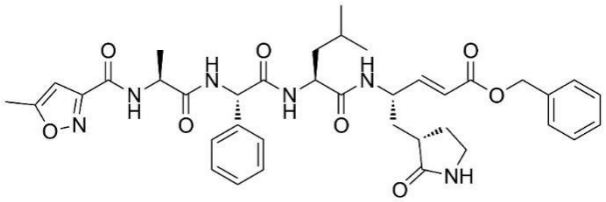
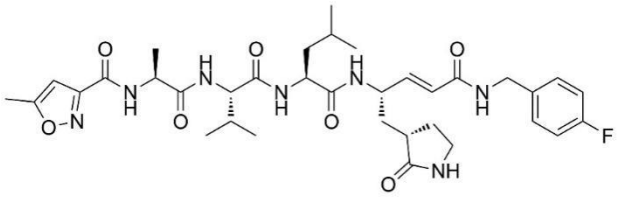
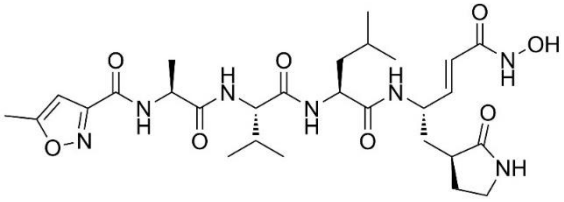
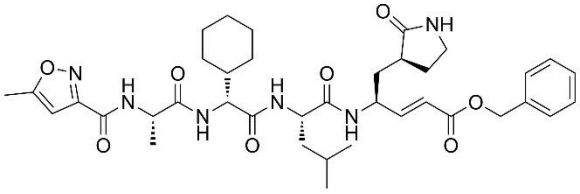
Ligand ……...	57.7
Solvent……	42.3
Ramachandran plot	
Favored (%) ……	96.0
Allowed (%) ……	4.0
Outliers (%) ……	0.0

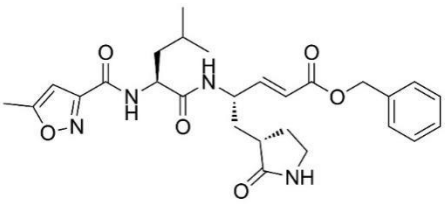
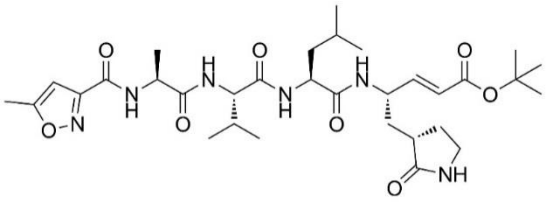
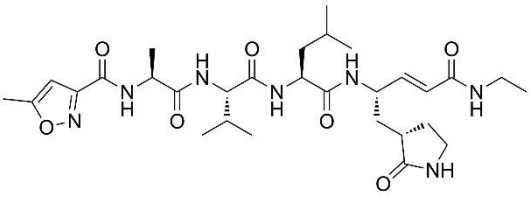
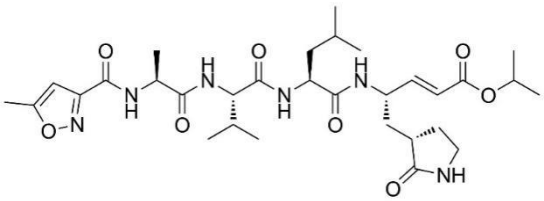
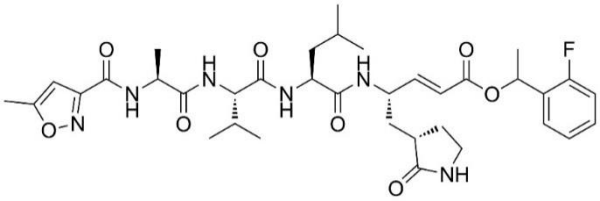
Table S2. Covalent and hydrogen bonds between PEDV M^{pro} and compound 1.

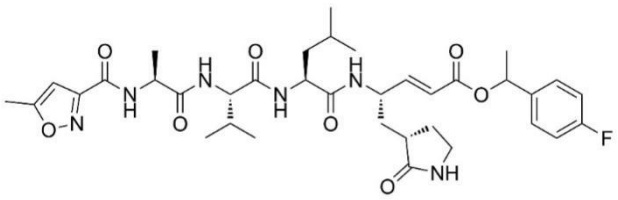
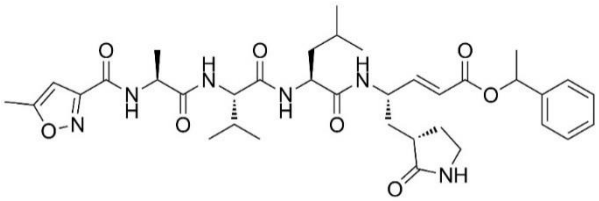
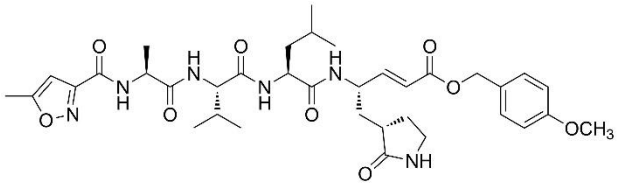
PEDV M^{pro}		Compound 1	Bond length(Å)
Residue	**Atom**	**Atom**	
Cys144	$S\gamma$	C20	1.83
Thr189	O	N2	3.07
Glu165	O	N3	2.88
Glu165	$O\varepsilon_2$	N6	3.01
Gln163	O	N5	3.10
Glu165	N	O4	3.05
Phe139	O	N6	3.59
Cys144	N	O6	3.48
His162	$N\varepsilon_2$	O8	2.38

Table S3. Design of 17 new Michael acceptor-based peptidomimetic inhibitors[4] for PEDV M^{pro}.

Compounds	Structure of compounds	Percentage purity (determined by analytical HPLC)	Exact mass (determined by ESI-MS)
2 (M1)	 Chemical Formula: $C_{38}H_{52}N_6O_8$ Exact Mass: 720.38	99.4%	720.54
3 (M2)	 Chemical Formula: $C_{37}H_{50}N_6O_8$ Exact Mass: 706.37	97.7%	706.49
4 (M3)	 Chemical Formula: $C_{35}H_{46}N_6O_8$ Exact Mass: 678.34	96.6%	678.49
5 (M5)	 Chemical Formula: $C_{35}H_{50}N_6O_8$ Exact Mass: 682.37	99.8%	682.51

6 (M6)	 Chemical Formula: $C_{32}H_{42}N_6O_8$ Exact Mass: 638.31	>99.9%	638.46
7 (M7)	 Chemical Formula: $C_{38}H_{46}N_6O_8$ Exact Mass: 714.34	97.2%	714.49
8 (M8)	 Chemical Formula: $C_{35}H_{48}FN_7O_7$ Exact Mass: 697.36	>99.9%	697.60
9 (M9)	 Chemical Formula: $C_{28}H_{43}N_7O_8$ Exact Mass: 605.32	99.5%	605.46
10 (M10)	 Chemical Formula: $C_{38}H_{52}N_6O_8$ Exact Mass: 720.38	99.1%	720.52

11 (M11)	 Chemical Formula: $C_{27}H_{34}N_4O_6$ Exact Mass: 510.25	98.5%	510.38
12 (M12)	 Chemical Formula: $C_{32}H_{50}N_6O_8$ Exact Mass: 646.37	99.4%	646.54
13 (M13)	 Chemical Formula: $C_{30}H_{47}N_7O_7$ Exact Mass: 617.35	98.8%	617.48
14 (M14)	 Chemical Formula: $C_{31}H_{48}N_6O_8$ Exact Mass: 632.35	98.6%	632.51
15 (M17)	 Chemical Formula: $C_{36}H_{49}FN_6O_8$ Exact Mass: 712.36	98.5%	712.51

16 (M18)	 Chemical Formula: $C_{36}H_{49}FN_6O_8$ Exact Mass: 712.36	96.1%	712.52
17 (M19)	 Chemical Formula: $C_{36}H_{50}N_6O_8$ Exact Mass: 694.37	>99.9%	694.50
18 (M25)	 Chemical Formula: $C_{36}H_{50}N_6O_9$ Exact Mass: 710.36	98.6%	710.51

Supplementary Experimental Methods

Protein Expression and Purification

Protein expression and purification of PEDV main protease has been described previously.[5] The coding sequence was synthesized and cloned into pGEX-6P-1 vector, and transformed into *E. coli* strain BL21 (DE3). Cell culture was grown in LB medium containing 100 μg ml^{-1} ampicillin at 37 °C until optical density (OD_{600}) reached 0.6. Protein expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM and further cultured at 16 °C for 16 hours. Cell pellets were harvested by centrifugation, and resuspended in phosphate-buffered saline solution supplemented with 1 mM dithiothreitol (DTT) and 10% glycerol. Cell lysate was prepared with sonication and centrifugation (12,000 g, 50 min, 4 °C). GST-tagged PEDV M^{pro} fusion protein was bound to glutathione Sepharose 4B affinity resin, and GST tag was removed through on-column cleavage using commercial PreScission protease (GE Healthcare) at 4 °C for 18 hours. Recombinant PEDV M^{pro} protein was subject to an additional step of anion-exchange chromatography using HiTrap Q column (GE Healthcare), with a linear gradient of 25 to 250 mM NaCl (20 mM Tris-HCl pH=8.0, 10% glycerol, 1 mM DTT).

Crystallization and Data Collection

Purified protein was supplemented with 10% DMSO and concentrated to 1mg ml^{-1}. The complex of PEDV M^{pro} with compound **1** was prepared by adding compound **1** to PEDV M^{pro} protein at a molar ratio between 3:1 and 5:1, and incubating at 4 °C for 4 hours. The complex was then centrifuged at 12,000 g for 10 min, and concentrated to 10 mg ml^{-1} in a buffer containing 10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT. Using hanging-drop vapor diffusion method at 16 °C,

best crystals were obtained after 1 day using a reservoir solution containing 25-29% (v/v) 2-propanol, 0.1 M Tris pH 7.5-7.9, 5%(w/v) polyethylene glycol 8000.[5]

Data for PEDV M^{pro}-compound **1** complex was collected to a 2.44-Å resolution at 100K on beamlineBL-5A of the Photon Factory, KEK, Japan, using an ADSC Q315r detector. The cryoprotectant solution contained 25% (v/v) 2-propanol, 0.1 M Tris pH 7.7, 5% (w/v) polyethylene glycol 8000, and 20% glycerol. All data integration and scaling were performed using HKL2000.[6] The Matthews coefficient of the crystal suggested two molecules per asymmetric unit, and the solvent content was 53.1%.

Enzymatic Activity and Inhibition Assays

Enzymatic assay was performed using a fluorogenic substrate with consensus sequence of CoV M^{pro}, MCA-AVLQ↓SGFR-Lys(Dnp)-Lys-NH2 (>95% purity, GL Biochem Shanghai Ltd., Shanghai, China), as previously reported.[1, 3, 7] Fluorescence intensity was monitored using a Fluoroskan Ascent instrument (Thermo Scientific, USA) with excitation and emission wavelengths of 320 nm and 405 nm, respectively. The assay was performed in a buffer solution consisted of 50 mM Tris-HCl (pH 7.3) and 1 mM EDTA at 30 °C. Kinetic parameters, including K_m and k_{cat} of PEDV M^{pro} and K_i and k_3 of compound **1**, were determined using methods described in detail in our previous work.[1, 3] Briefly, the strict kinetic parameters were determined for compound **1**. First-order inhibition rate constant (k_{obs}) was obtained by fitting time-dependent inhibition progress curves to a first-order exponential equation (1).[8] P is the product fluorescence; v_0 is the initial velocity; t is time; and D is a displacement term to account for the fact that the emission is nonzero at the start of data collection. The values of K_i and k_3 were calculated from plots of $1/k_{obs}$ obtained from equation (1) versus 1/[I] according to equation (2). [I] is inhibitor

concentration; [S] is substrate concentration; K_m is the Michaelis-Menten constant for the substrate; K_i is the equilibrium constant; and k_3 is the rate constant of inactivation.

(1)

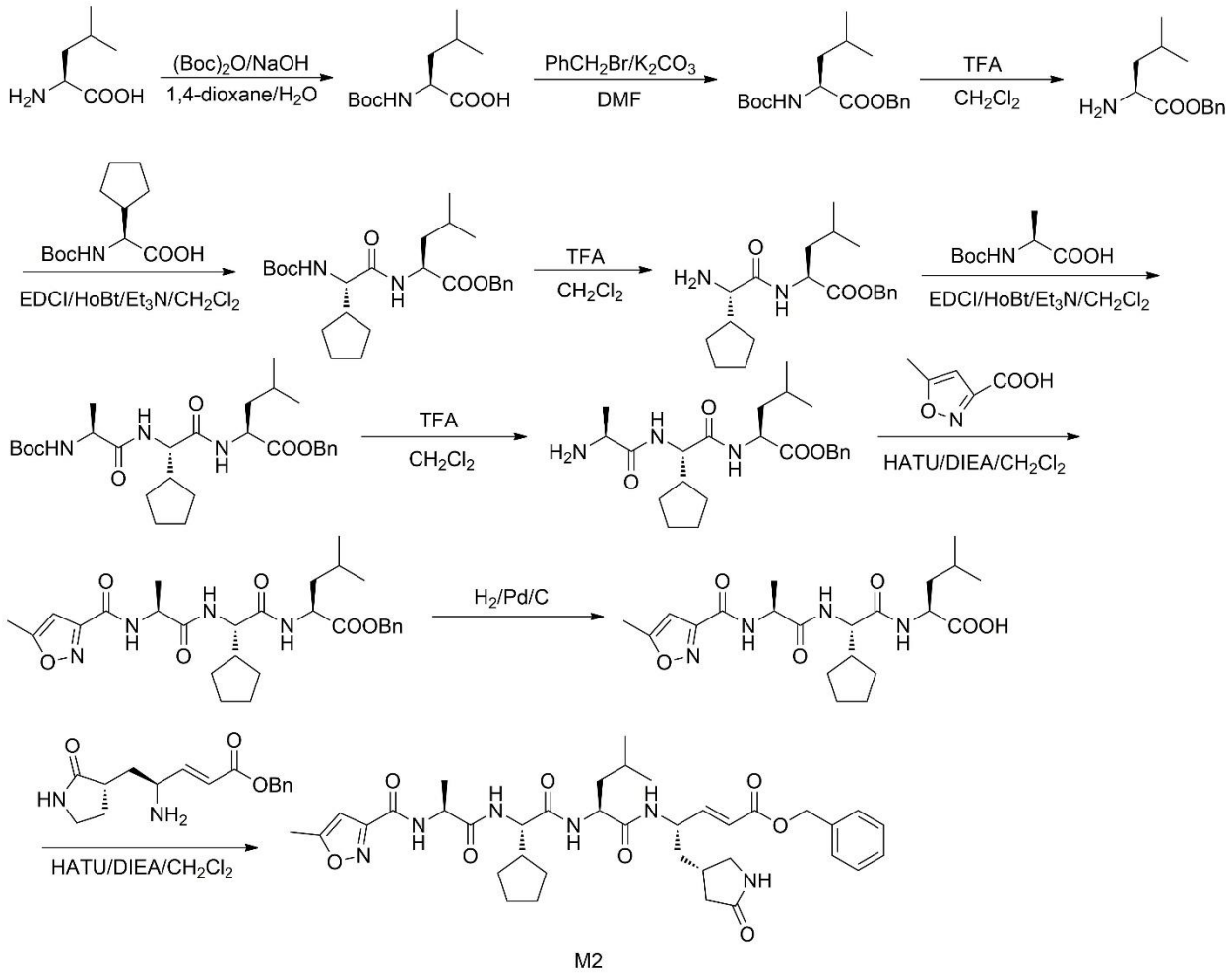
(2)

The values of K_i and k_3 parameters for the irreversible inhibitors were obtained from reactions initiated by addition of PEDV M^{pro}. The substrate concentration was set at 40μM, depending on the enzymatic activity. The inhibitors vary from 6 different concentrations (10-60 μM). Data from the continuous assays were analyzed with the nonlinear regression analysis program Origin. The enzymatic assay of compound **3** and compound **15** is similar to compound **1**.

Synthesis and Characterization of Compounds 3 and 15

Compound 3 (inhibitor M2)

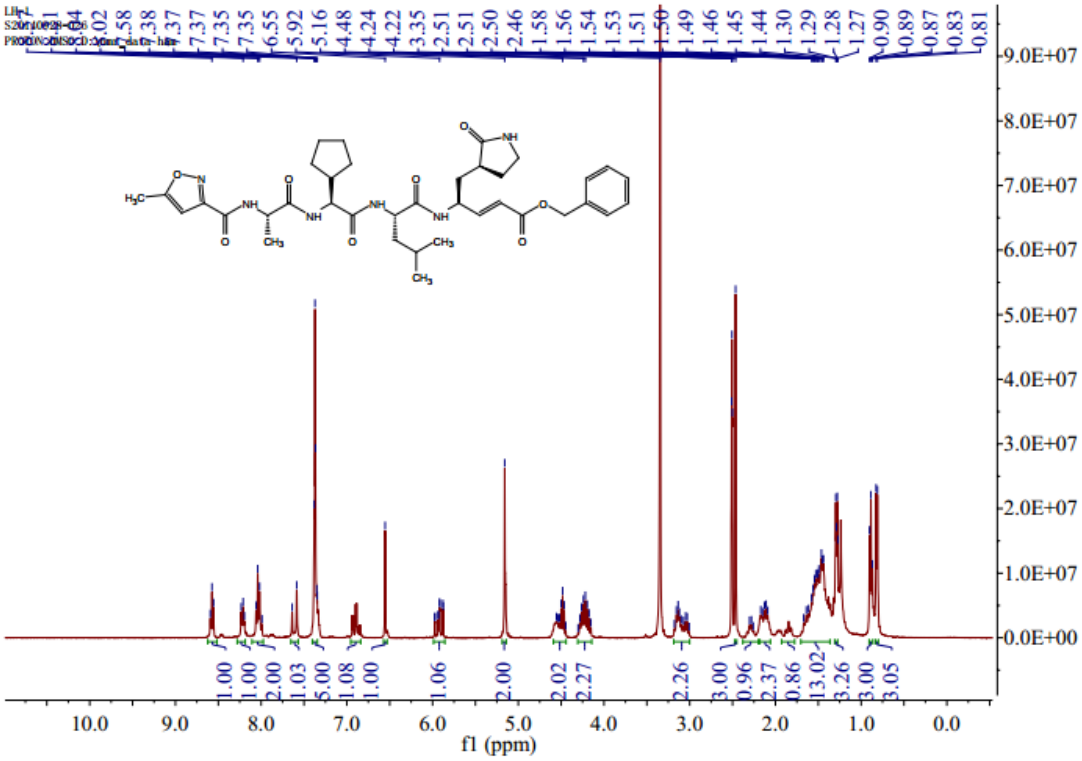
Synthetic Pathway:



Compound **3**

^{1}H NMR (400 MHz, DMSO-d_6):

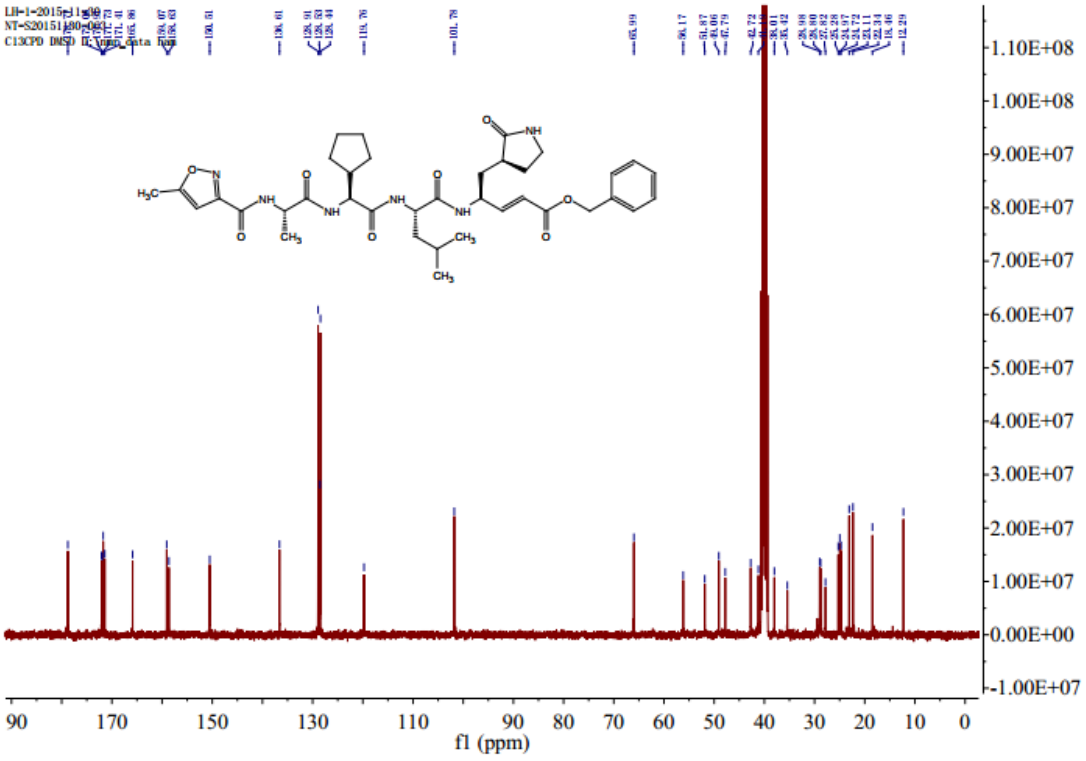
δ 8.56 (d, J = 7.6 Hz, 1H), 8.22 (d, J = 8.5 Hz, 1H), 7.97 -8.07 (m, 2H), 7.61 (d, J = 21.5 Hz, 1H), 7.37 (s, 5H), 6.91 (m, 1H), 6.55 (s, 1H), 5.86 -5.99 (m, 1H), 5.16 (s, 2H), 4.44 -4.59 (m, 2H), 4.14 -4.31 (m, 2H), 3.00 -3.18 (m, 2H), 2.46 (s, 3H), 2.29 (m, 1H), 2.05 -2.18 (m, 2H), 1.78 -1.93 (m, 1H), 1.36 -1.70 (m, 13H), 1.29 (d, J = 7.1 Hz, 3H), 0.89 (d, J = 6.7 Hz, 3H), 0.82 (d, J = 6.4 Hz,3H).



Compound **3**

^{13}C NMR (101 MHz, DMSO-d_6):

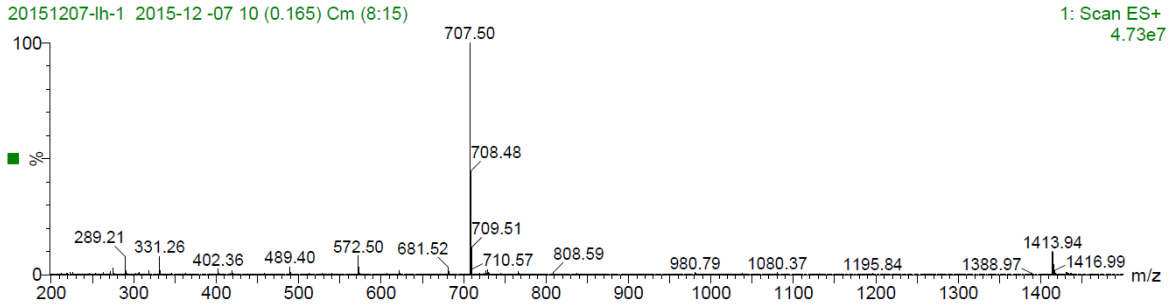
δ 178.77, 172.06, 171.91, 171.73, 171.41, 165.86, 159.07, 158.63, 150.51, 136.61, 128.91(2), 128.53, 128.44(2), 119.76, 101.78, 65.99, 56.17, 51.87, 49.06, 47.79, 42.72, 41.19, 38.01, 35.42, 29.47, 28.98 , 28.80, 27.82, 25.28, 24.97, 24.72, 23.11, 22.34, 18.46,12.29.



Compound **3**

ESI-MS:

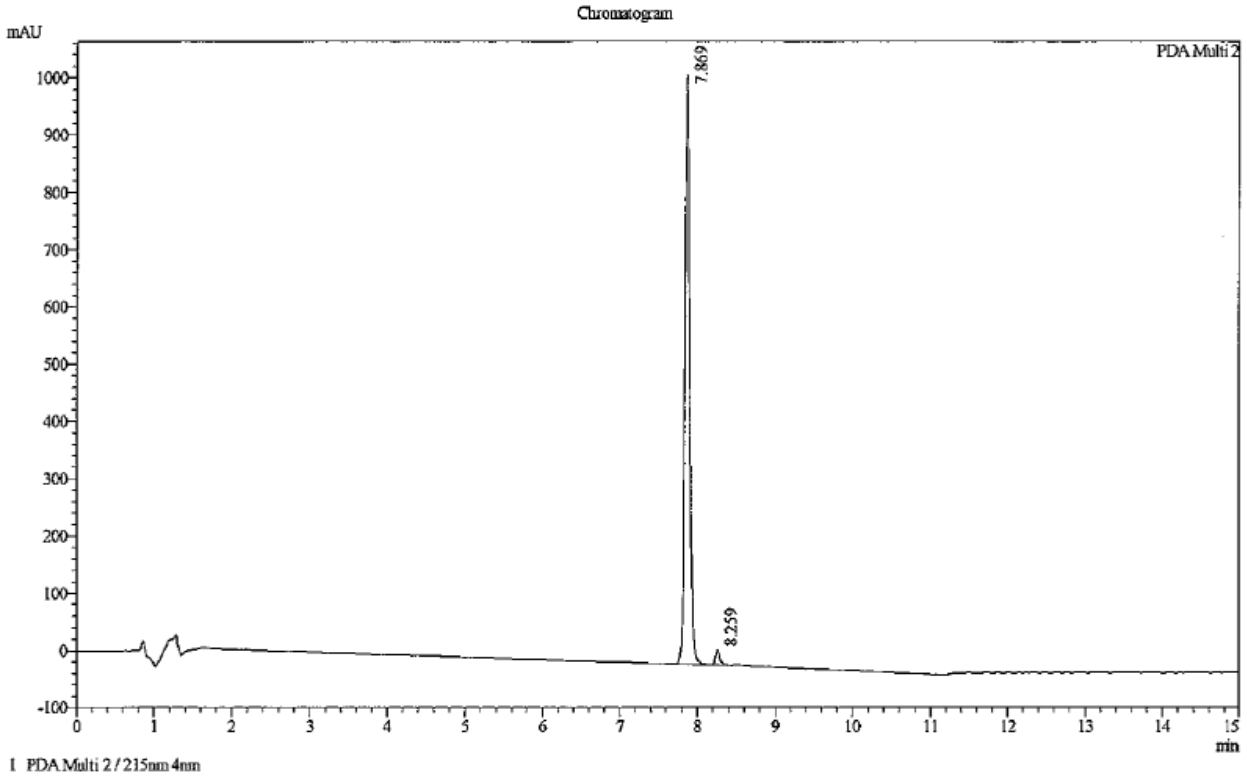
Calculated mass for $[C_{37}H_{51}N_6O_8, M+H]^+$: 707.38 Found: 707.50.



Compound **3**

Analytic HPLC:

Purity: 97.7%



Integration Result

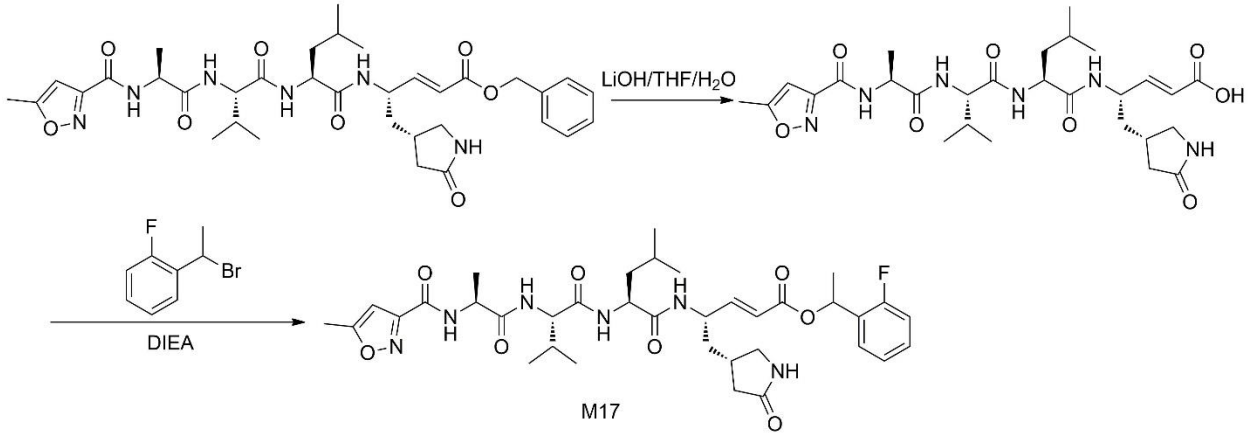
PDA Ch2 215nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	7.869	1025103	97.514	0.111	4469147	97.725
2	8.259	26136	2.486	0.102	104026	2.275

Compound **3**

Compound 15 (inhibitor M17)

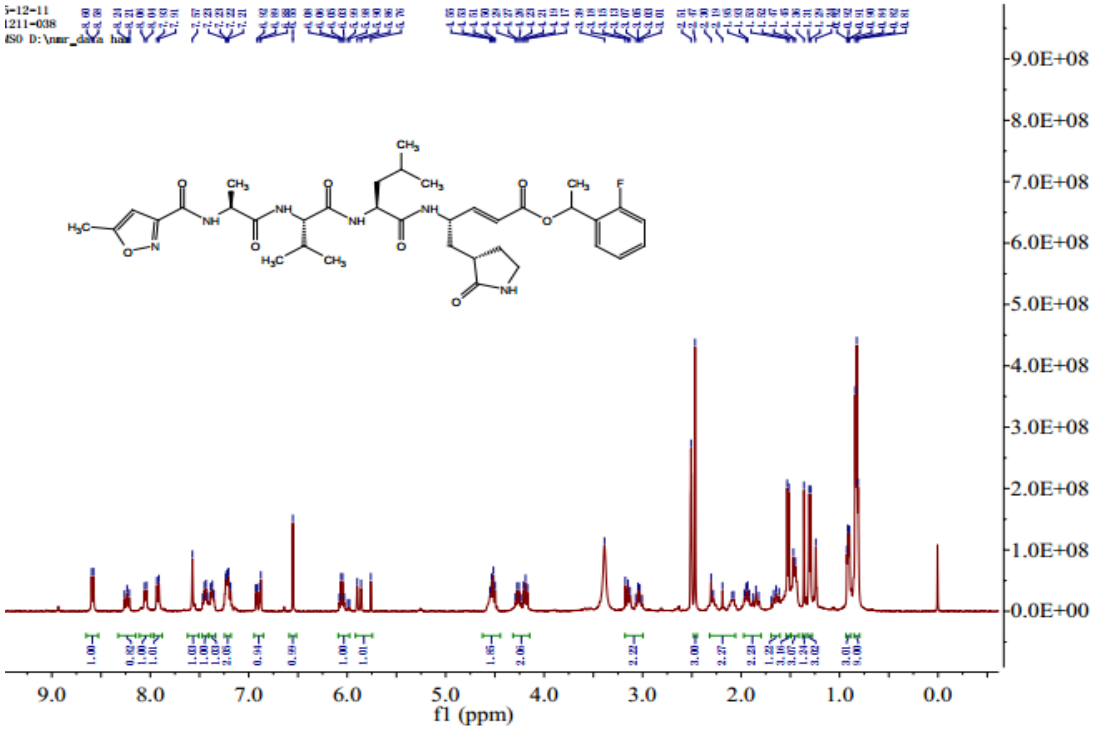
Synthetic Pathway:



Compound **15**

^{1}H NMR (400 MHz, DMSO-d_6):

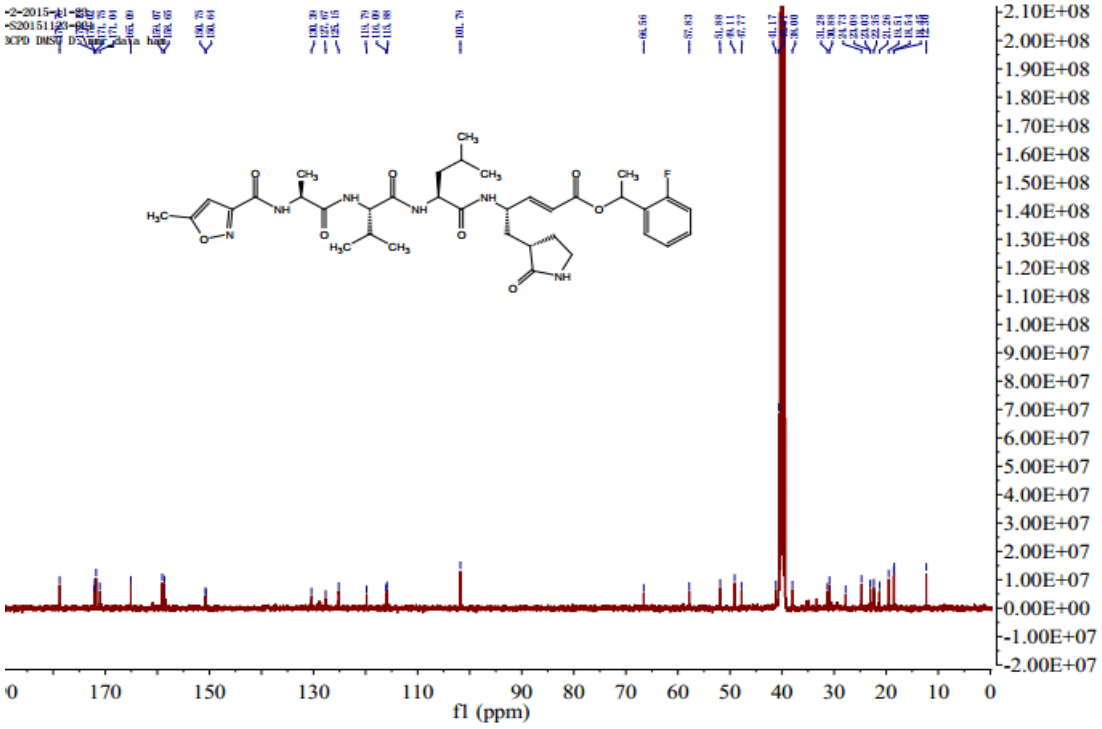
δ 8.59 (d, *J* = 7.4 Hz, 1H), 8.15 -8.33 (m, 1H), 8.05 (d, *J* = 7.5 Hz, 1H), 7.92 (d, *J* = 8.7 Hz, 1H), 7.57 (s, 1H), 7.44 (m, 1H), 7.33 -7.40 (m, 1H), 7.18 -7.25 (m, 2H), 6.90 (m, 1H), 6.55 (s, 1H), 6.05 (d, *J* = 6.6 Hz, 1H), 5.88 (d, *J* = 15.8 Hz, 1H), 4.44 -4.63 (m, 2H), 4.14 -4.32 (m, 2H), 2.99 -3.17 (m, 2H), 2.47 (s, 3H), 2.06 -2.32 (m, 2H), 1.79 -1.97 (m, 2H), 1.60-1.70 (m, 1H), 1.52 (d, *J* = 6.6 Hz, 3H), 1.41 -1.49 (m, 3H), 1.28 -1.37 (m, 4H), 0.89 -0.93 (m, 3H), 0.79 -0.85 (m, 9H).



Compound **15**

^{13}C NMR (101 MHz, DMSO-d_6):

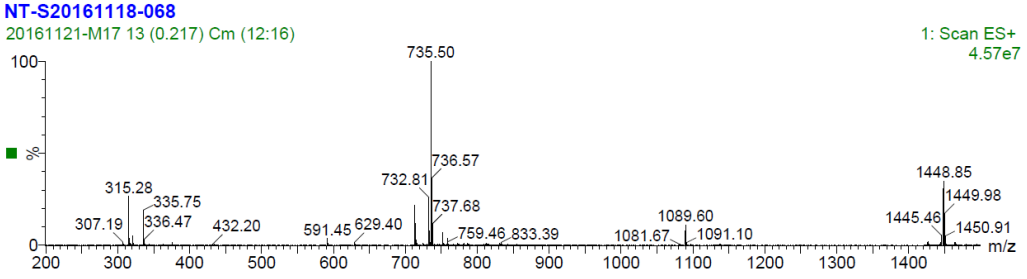
δ178.76, 172.13, 172.02, 171.75, 171.04, 165.09, 159.07, 158.65, 150.75, 150.64, 130.39 (d,C-F), 127.67, 125.15, 119.79, 116.09, 115.88, 101.79, 66.56, 57.83, 51.88, 49.11, 47.77, 41.17, 38.00, 31.28, 30.88, 27.78, 24.73, 23.09, 23.03, 22.43, 22.35, 19.51, 18.54, 18.45, 12.30.



Compound **15**

ESI-MS:

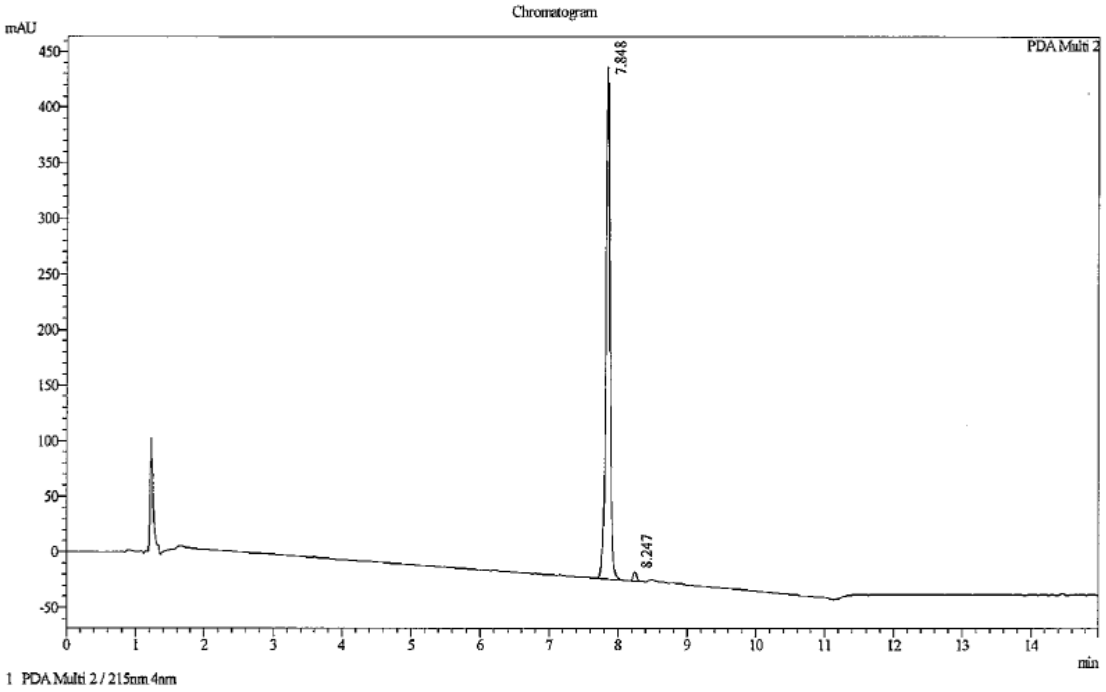
Calculated mass for $[C_{36}H_{49}FN_6O_8Na, M+Na]^+$: 735.35 Found: 735.50.



Compound **15**

Analytic HPLC:

Purity: 98.5%



Integration Result

PDA Ch2 215nm 4nm

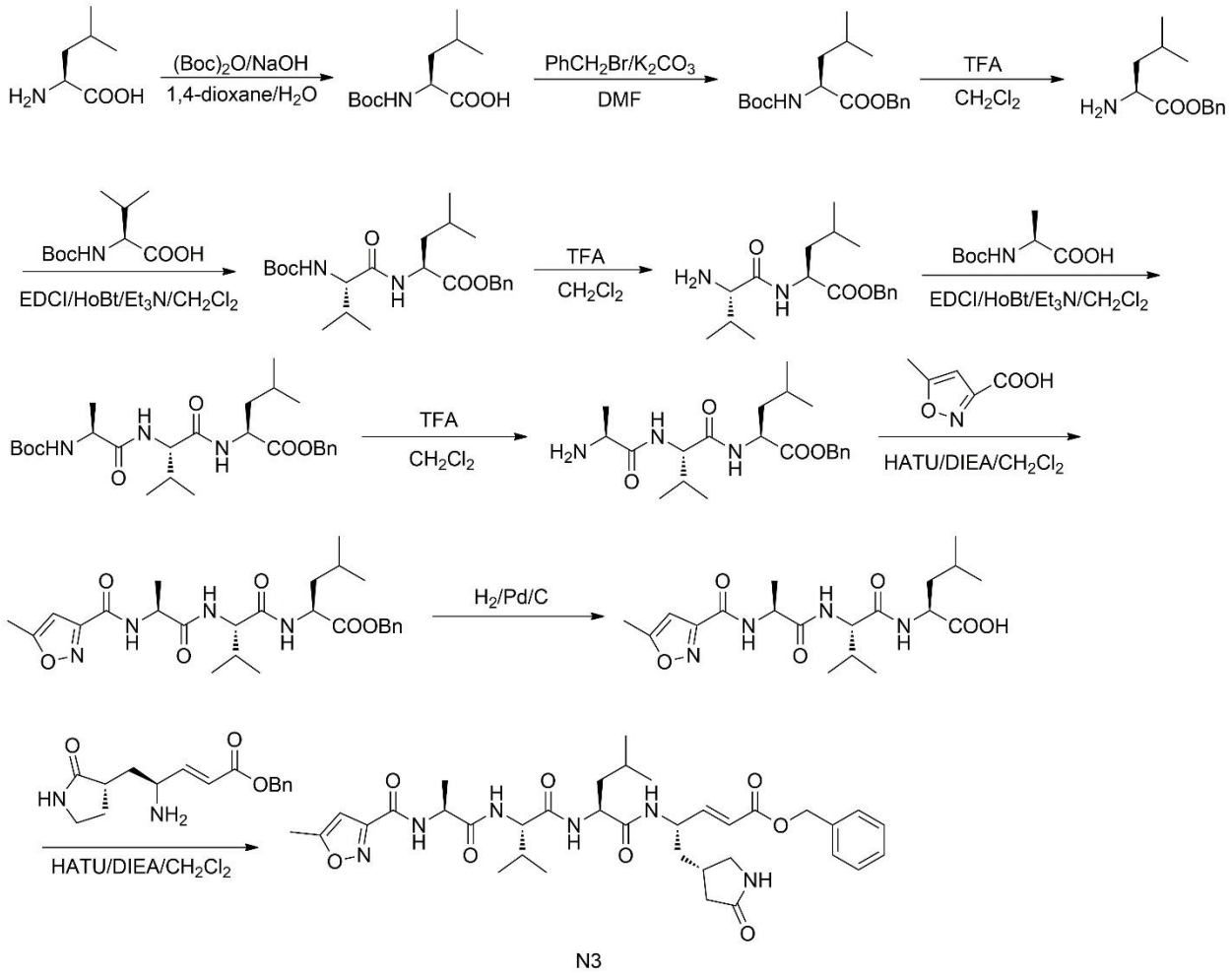
Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	7.848	454010	98.284	0.109	1971857	98.527
2	8.247	7927	1.716	0.098	29474	1.473

Compound **15**

Synthesis and Characterization of Compounds 1−2, 4−14, and 16−18

Compound 1 (Inhibitor N3)

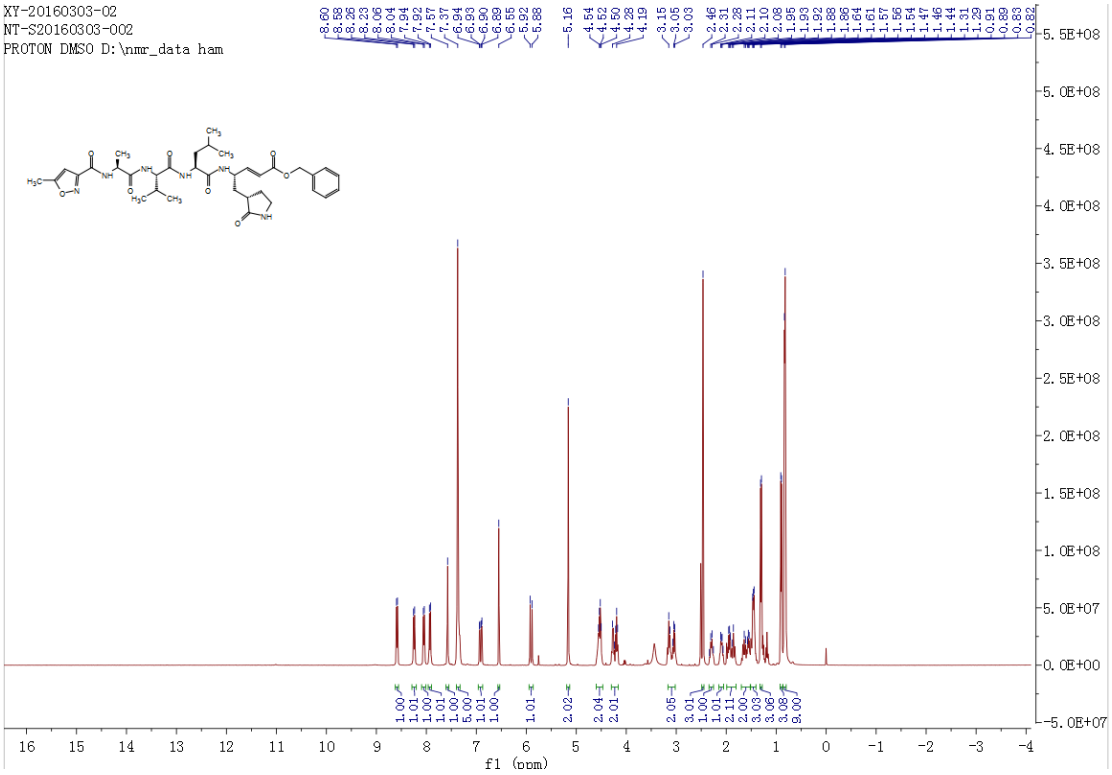
Synthetic Pathway:



Compound **1**

^{1}H NMR (400 MHz, DMSO-d_6):

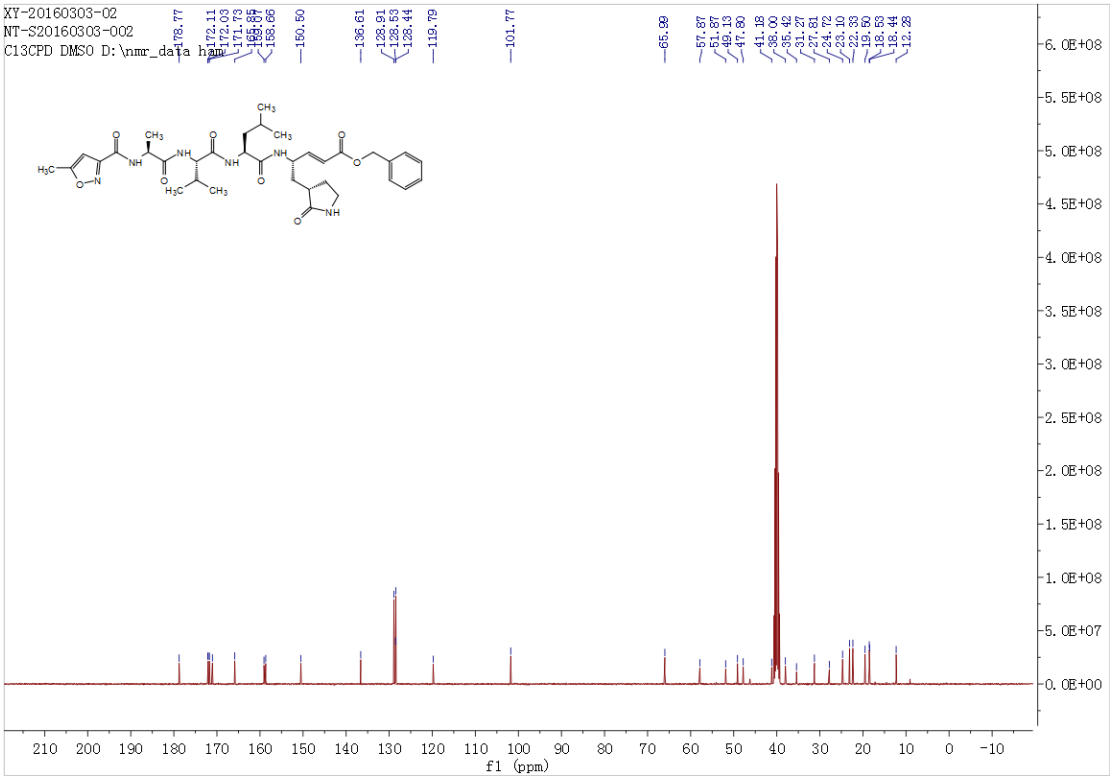
δ 8.59 (d, J = 7.5 Hz, 1H), 8.25 (d, J = 8.7 Hz, 1H), 8.05 (d, J = 7.6 Hz, 1H), 7.93 (d, J = 8.8 Hz, 1H), 7.57 (s, 1H), 7.40 – 7.33 (m, 5H), 6.96 – 6.87 (m, 1H), 6.55 (s, 1H), 5.90 (d, J = 15.7 Hz, 1H), 5.16 (s, 2H), 4.60 – 4.47 (m, 2H), 4.30 – 4.16 (m, 2H), 3.17 – 3.02 (m, 2H), 2.46 (s, 3H), 2.30 (m, 1H), 2.15 – 2.05 (m, 1H), 1.99 – 1.80 (m, 2H), 1.70 – 1.52 (m, 2H), 1.51 – 1.41 (m, 3H), 1.30 (d, J = 7.1 Hz, 3H), 0.90 (d, J = 6.4 Hz, 3H), 0.83 (d, J = 6.0 Hz, 9H).



Compound **1**

^{13}C NMR (101 MHz, DMSO-d_6):

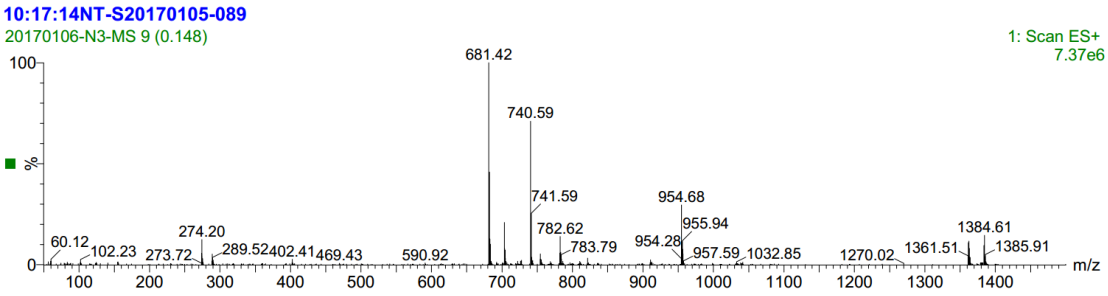
δ 178.77 , 172.11, 172.03 , 171.73 , 171.05 , 165.85 , 159.07 , 158.66 , 150.50 , 136.61 , 128.91(2) , 128.53, 128.44 (2), 119.79 , 101.77 , 65.99 , 57.87 , 51.87 , 49.13 , 47.80 , 41.18 , 38.00 , 35.42 , 31.27 , 27.81 , 24.72 , 23.10 , 22.33(2) , 19.50 , 18.53, 18.44, 12.28.



Compound **1**

ESI-MS:

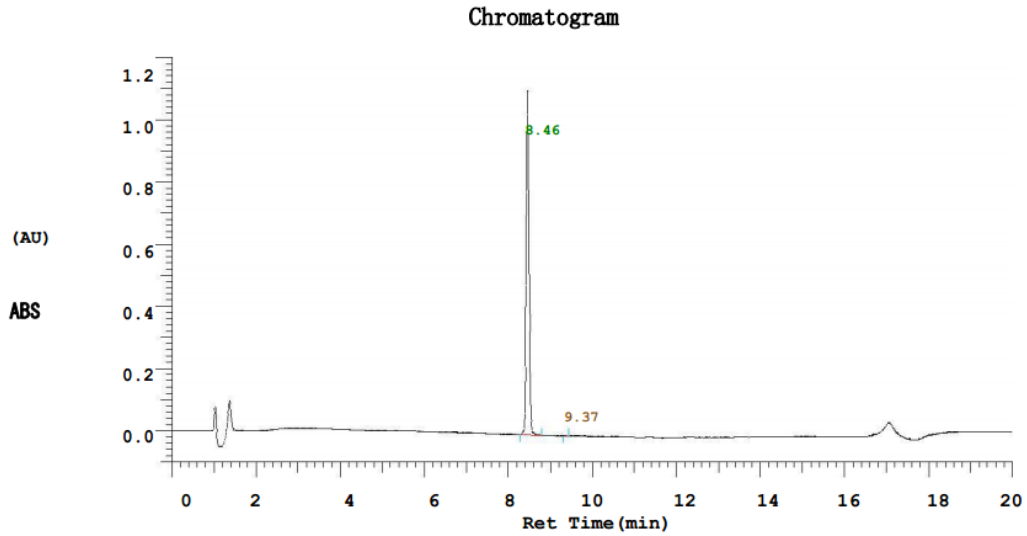
Calculated mass for $[C_{35}H_{49}N_6O_8, M+H]^+$: 681.36 Found: 681.42.



Compound **1**

Analytic HPLC:

Purity: 99.9%



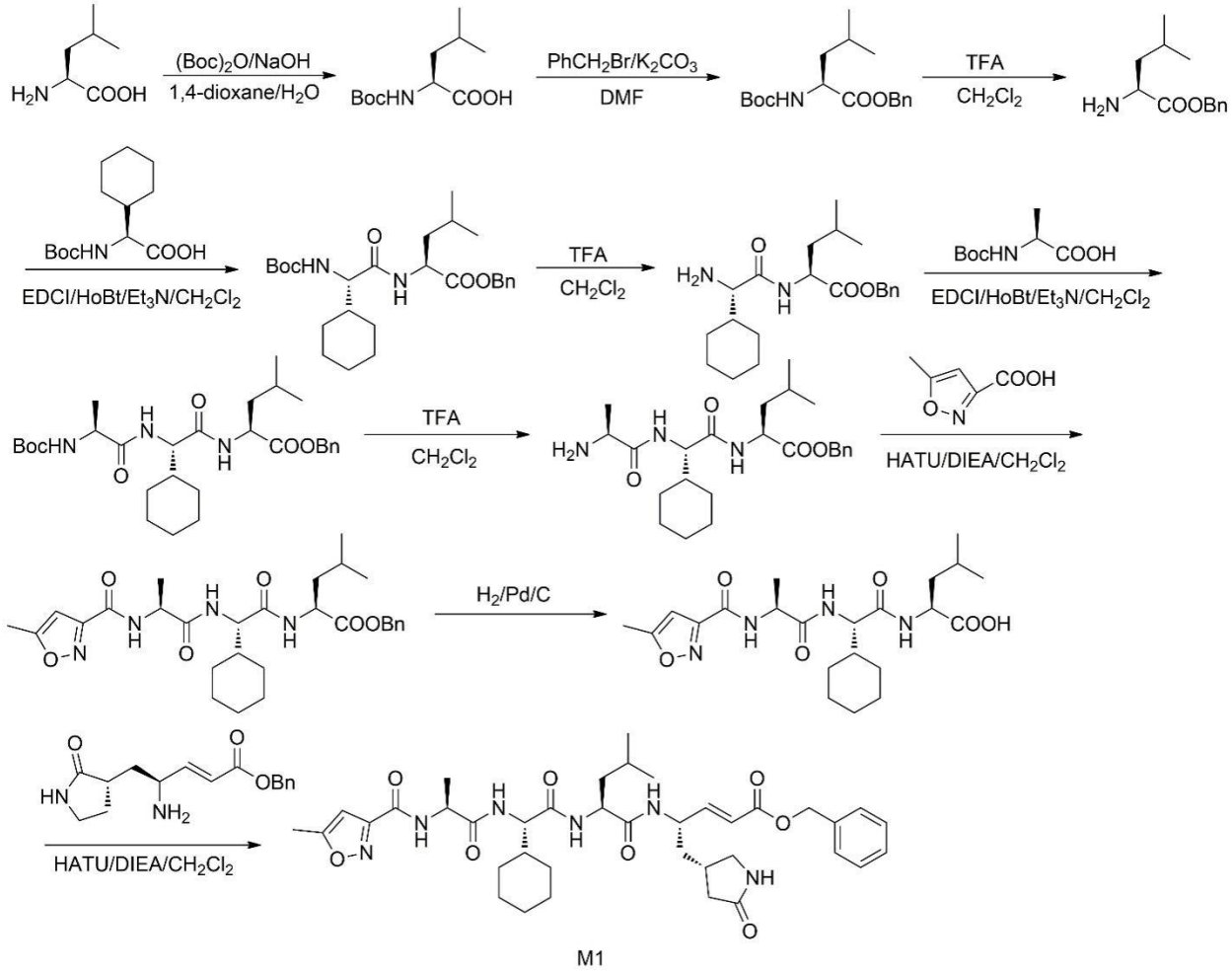
254 nm Integration Result

Peak No.	RT	Height	Area	Area%
1	8.46	552743	2547526	99.954
2	9.37	351	1182	0.046
		553094	2548708	100.000

Compound **1**

Compound 2 (Inhibitor M1)

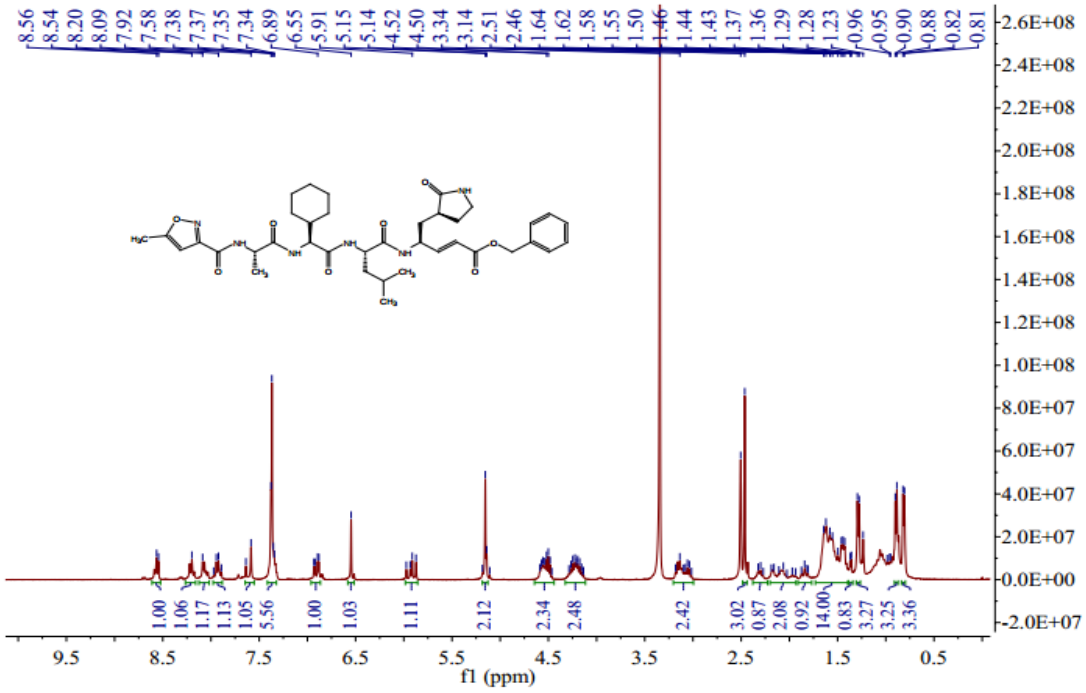
Synthetic Pathway:



Compound **2**

^{1}H NMR (400 MHz, DMSO-d_6):

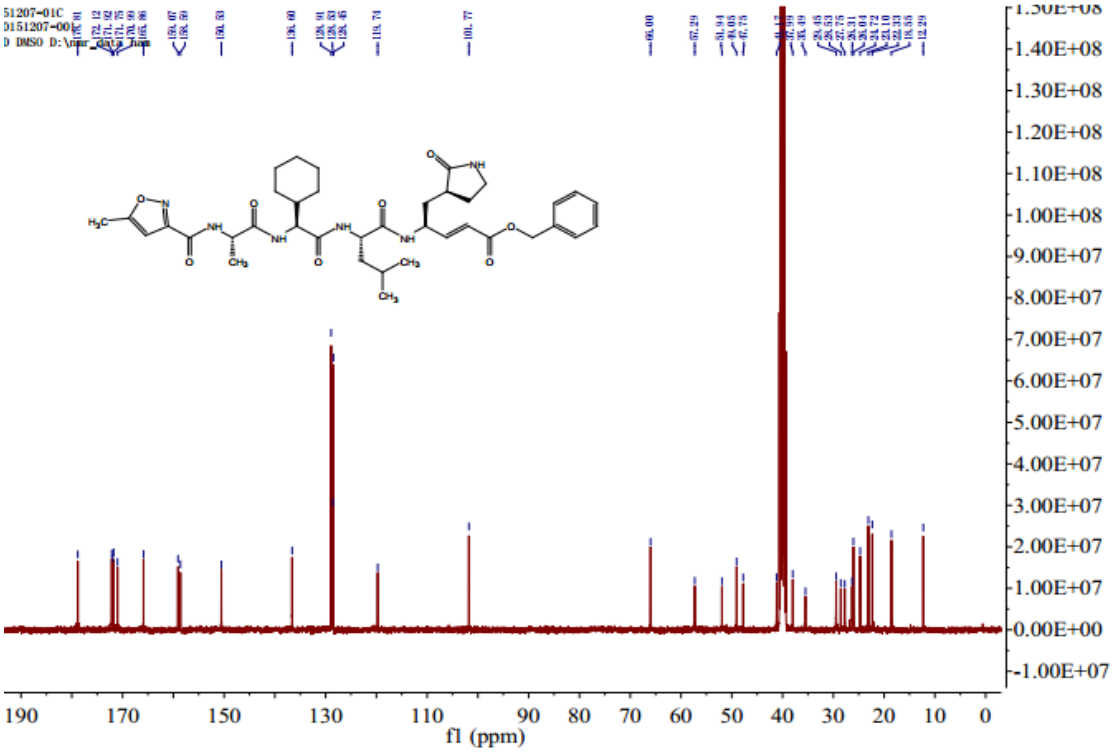
δ 8.55 (d, J = 7.7 Hz, 1H), 8.21 (d, J = 9.1 Hz, 1H), 8.08 (d, J = 7.5 Hz, 1H), 7.93 (d, J = 8.9 Hz, 1H), 7.58 (s, 1H), 7.37 (s, 5H), 6.91 (m, 1H), 6.55 (s, 1H), 5.89 (d, J = 15.7 Hz, 1H), 5.15 (s, 2H), 4.44 -4.64 (m, 2H), 4.11 -4.33 (m, 2H), 3.00 -3.20 (m, 2H), 2.46 (s, 3H), 2.23 -2.37 (m, 1H), 1.93 -2.20 (m, 2H), 1.77 -1.91 (m, 1H), 1.34 -1.73 (m, 15H), 1.29 (d, J = 7.0 Hz, 3H), 0.89 (d, J = 6.7 Hz, 3H), 0.81 (d, J = 6.3 Hz, 3H).



Compound **2**

^{13}C NMR (101 MHz, DMSO-d_6):

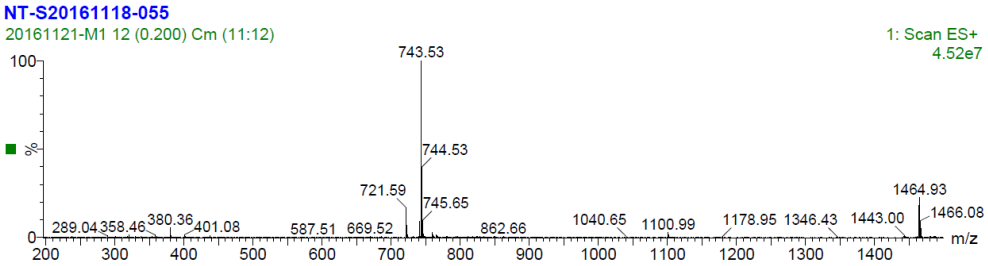
δ178.81, 172.12, 171.92, 171.75, 170.99, 165.86, 159.07, 158.59, 150.53, 136.60, 128.91(2), 128.53, 128.45(2), 119.74, 101.77, 66.00, 57.29, 51.94, 49.05, 47.75, 41.17, 37.99, 35.49, 29.45, 28.53, 27.75, 26.31(2), 26.04(2), 24.72, 23.10(2), 22.33, 18.55, 12.29.



Compound **2**

ESI-MS:

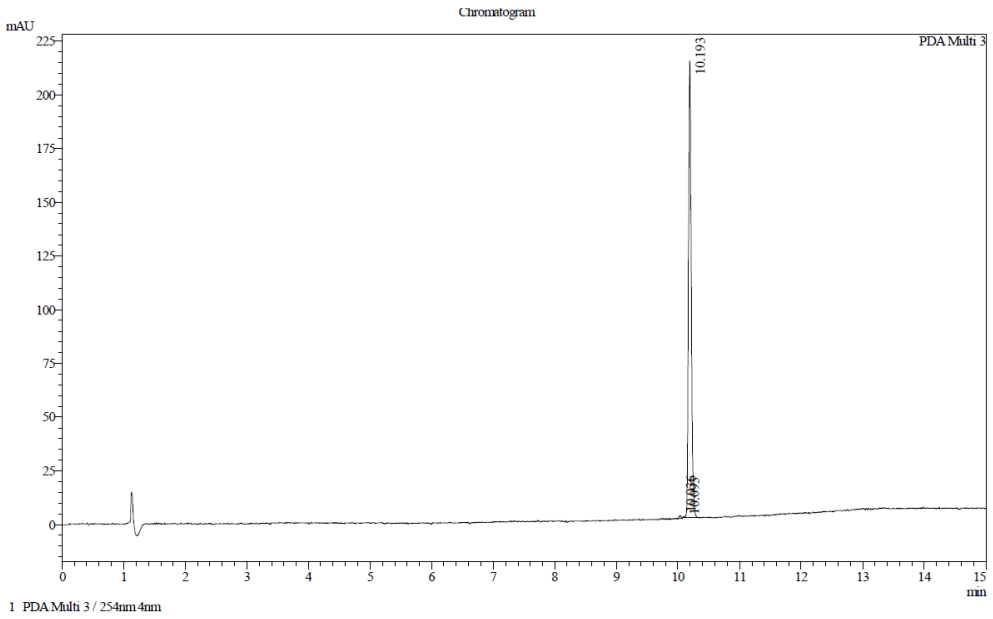
Calculated mass for $[C_{38}H_{52}N_6O_8Na, M+Na]^+$: 743.36 Found: 743.53.



Compound **2**

Analytic HPLC:

Purity: 99.4%



Integration Result

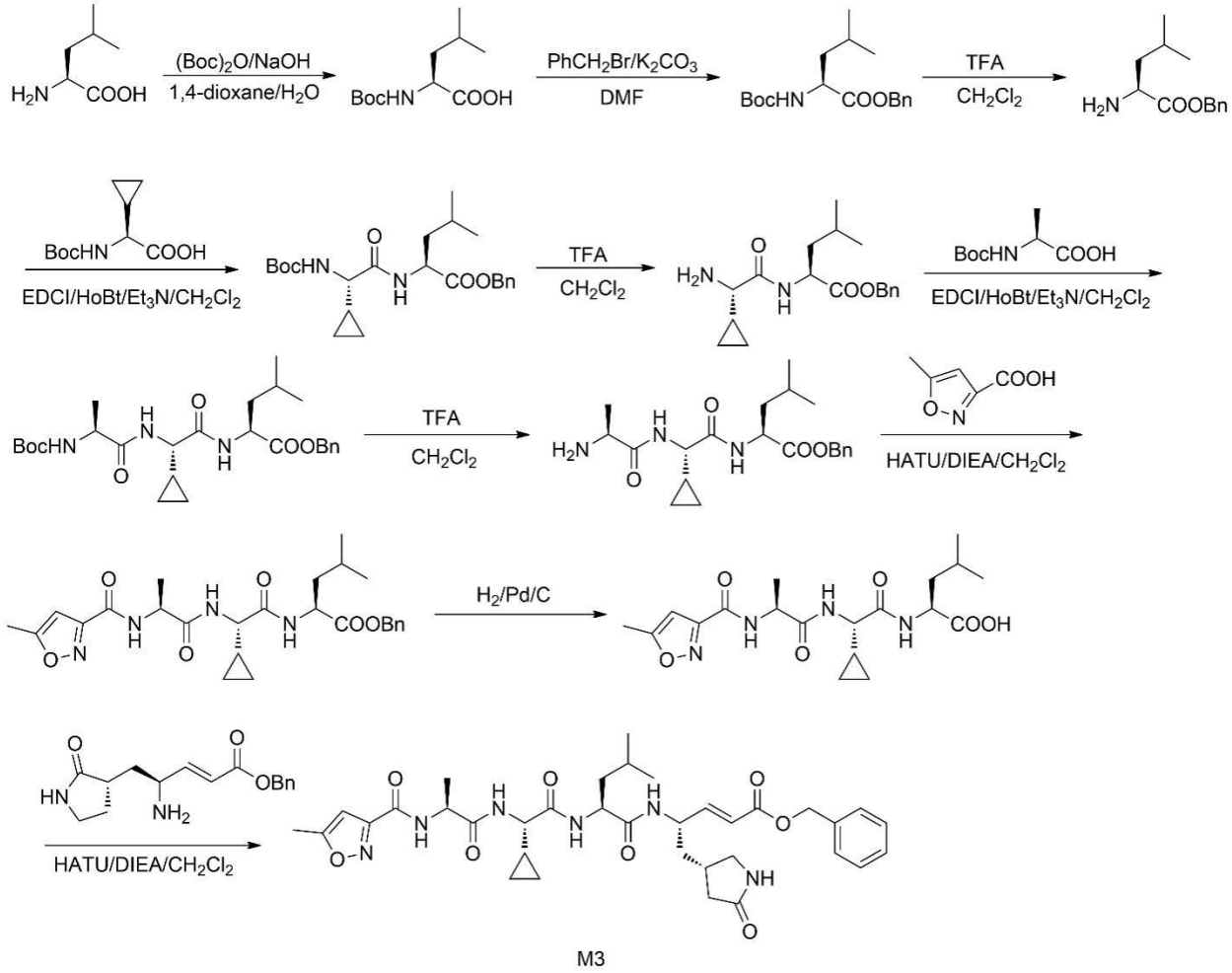
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	10.036	1196	0.568	0.065	2715	0.406
2	10.093	471	0.224	1.397	1287	0.192
3	10.193	208969	99.209	0.084	664648	99.401

Compound **2**

Compound 4 (Inhibitor M3)

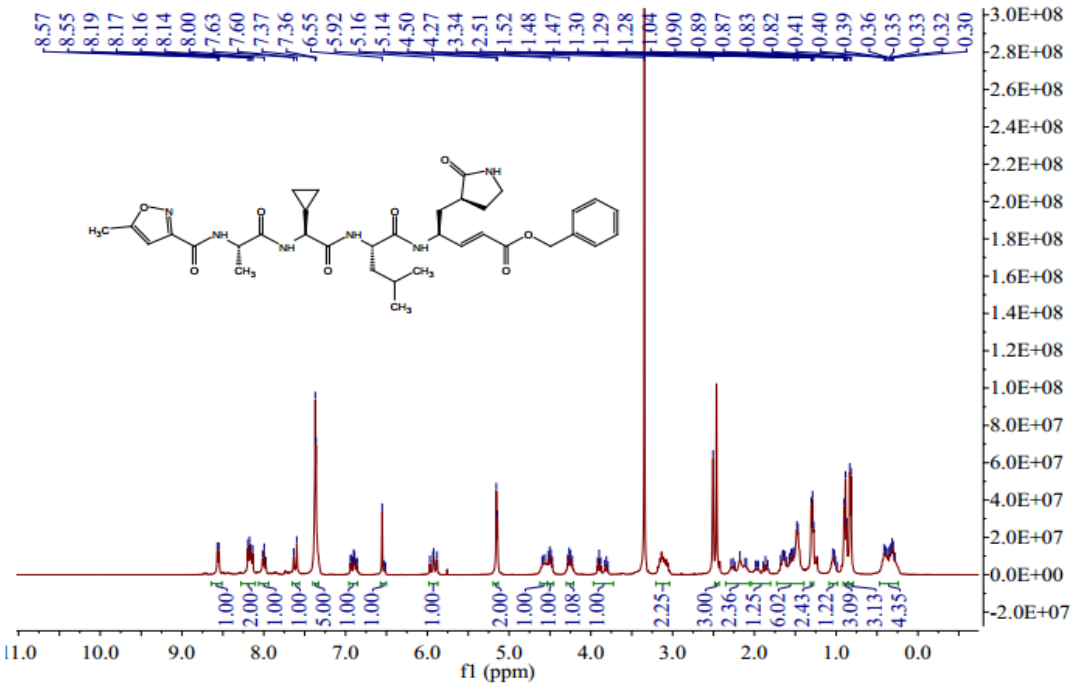
Synthetic Pathway:



Compound **4**

^{1}H NMR (400 MHz, DMSO-d_6):

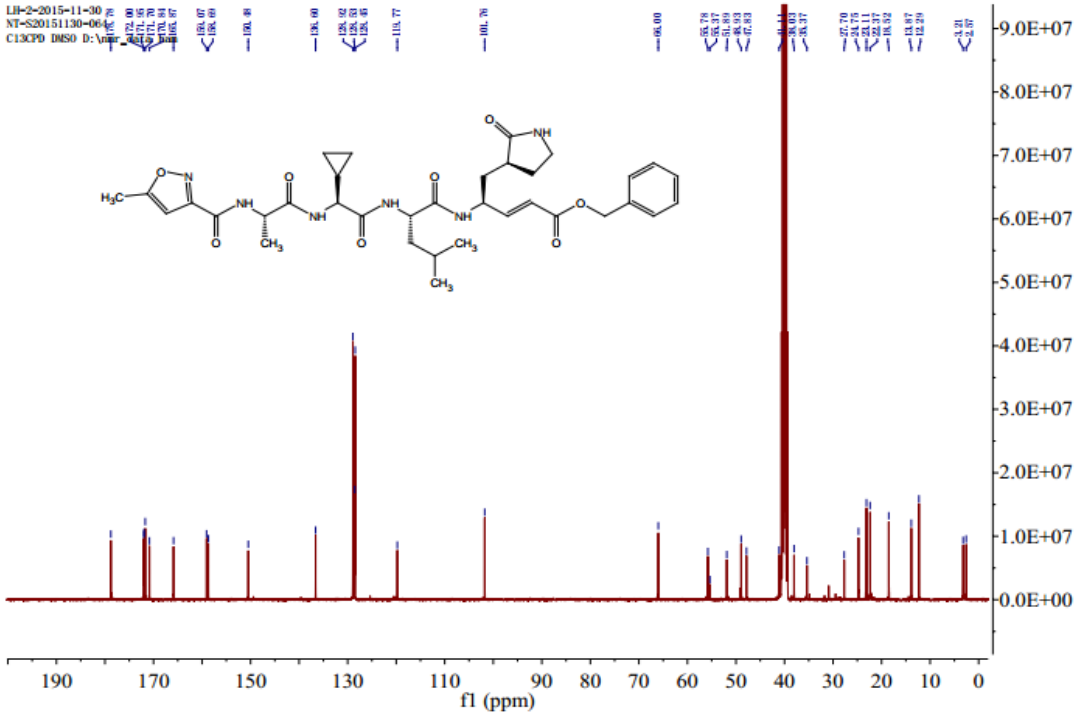
δ8.56 (d, J = 7.4 Hz, 1H), 8.17 (m, 2H), 7.99 (m, 1H), 7.62 (d, J = 15.0 Hz, 1H), 7.37 (d, J = 3.4 Hz, 5H), 6.85-6.94 (m, 1H), 6.50-6.56 (m, 1H), 5.93 (d, J = 19.4 Hz, 1H), 5.15 (d, J = 5.1 Hz, 2H), 4.57 (d, J = 6.9 Hz, 1H), 4.44- 4.54 (m, 1H), 4.20-4.31 (m, 1H), 3.77-3.93 (m, 1H), 3.03 -3.20 (m, 2H), 2.46 (s, 3H), 2.06- 2.34 (m, 2H), 1.81-2.03 (m, 1H), 1.39 -1.72 (m, 6H), 1.26 -1.35 (m, 2H) 1.03 (d, J = 7.6 Hz, 1H)0.89 (m, 3H), 0.83 (d, J = 6.2 Hz, 3H), 0.25-0.46 (m, 4H).



Compound **4**

^{13}C NMR (101 MHz, DMSO-d_6):

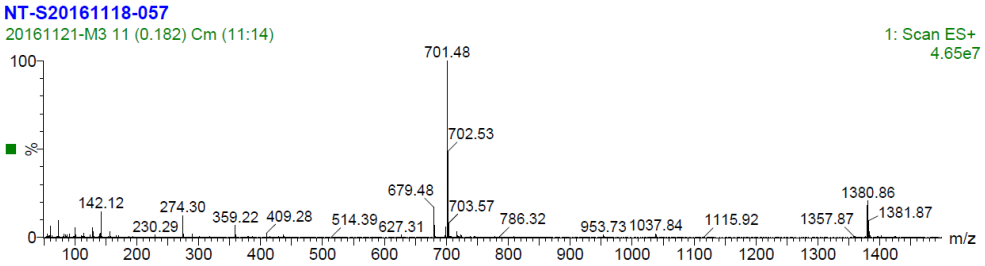
δ178.78, 172.00, 171.95, 171.70, 170.84, 165.87, 159.07, 158.69, 150.48, 136.60, 128.92(2), 128.53, 128.45(2), 119.77, 101.76, 66.00, 55.78, 55.37, 51.89, 48.93, 47.83, 41.11, 38.03, 35.37, 27.70, 24.75, 23.11, 22.37, 18.52, 13.87, 12.29, 3.21, 2.57.



Compound **4**

ESI-MS:

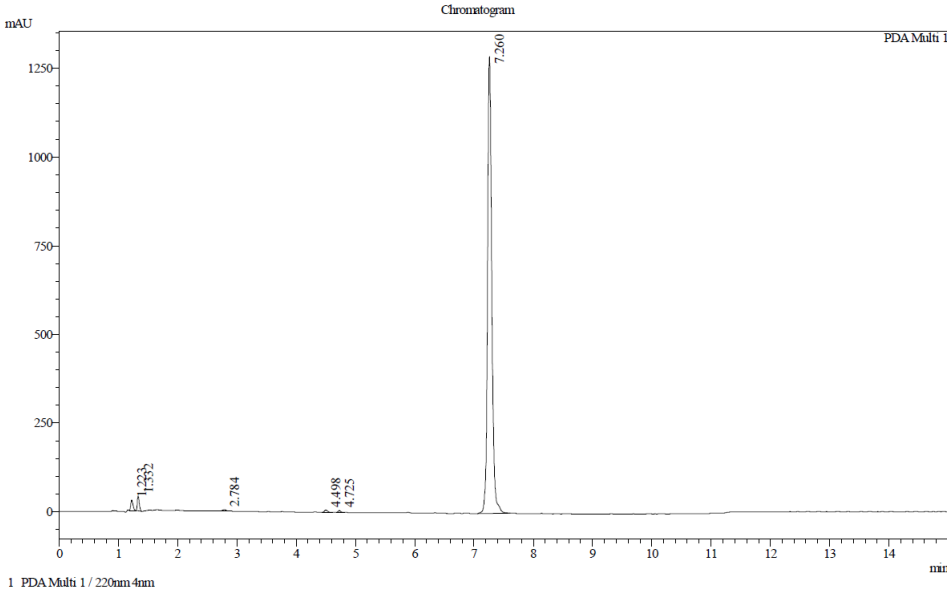
Calculated mass for $[C_{35}H_{46}N_6O_8Na, M+Na]^+$: 701.33 Found: 701.48.



Compound **4**

Analytic HPLC:

Purity: 96.6%



Integration Result

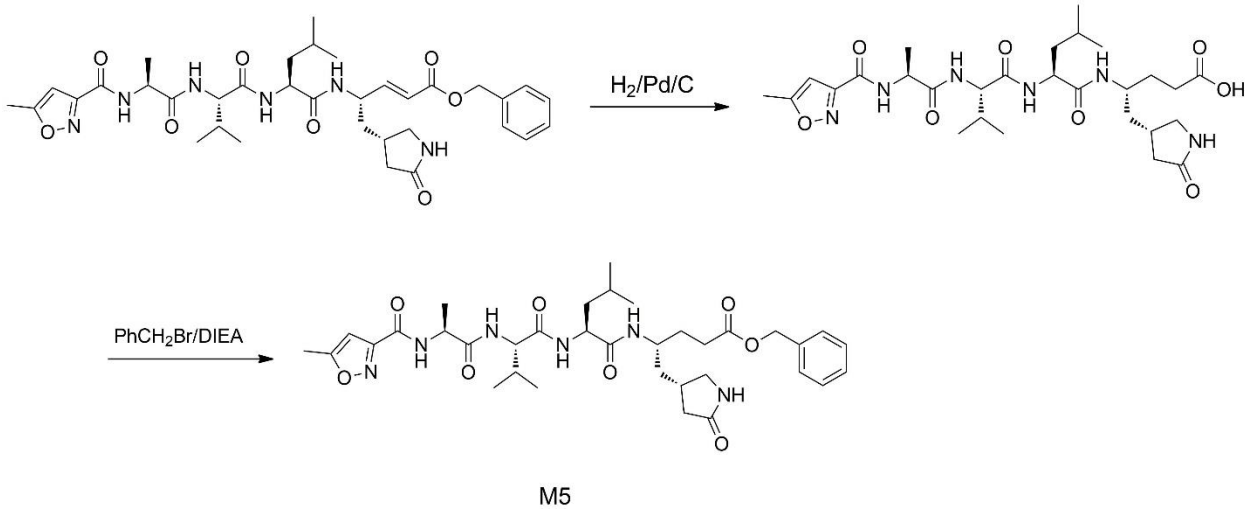
PDA Ch1 220nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	1.223	29595	2.157	0.072	77592	1.173
2	1.332	41911	3.055	0.063	93410	1.413
3	2.784	3688	0.269	0.111	15582	0.236
4	4.498	5934	0.433	0.097	21210	0.321
5	4.725	4961	0.362	0.083	15253	0.231
6	7.260	1285878	93.725	0.122	6390019	96.627

Compound **4**

Compound 5 (Inhibitor M5)

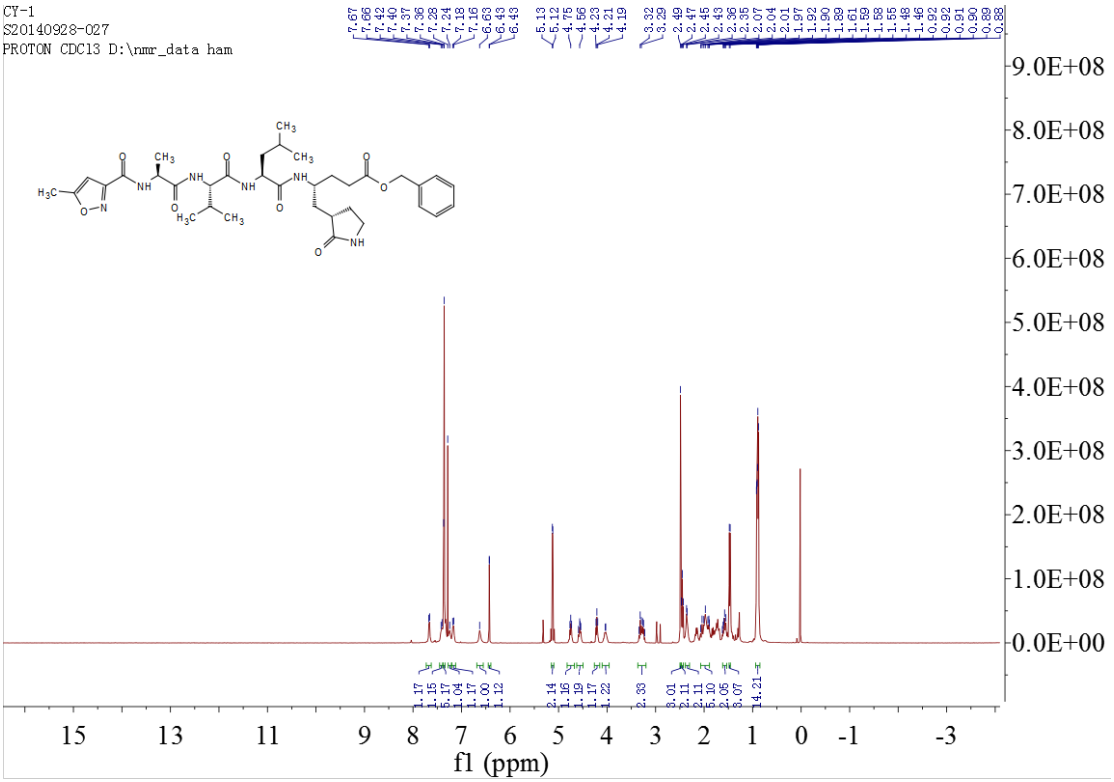
Synthetic Pathway:



Compound **5**

^{1}H NMR (400 MHz, $CDCl_3$):

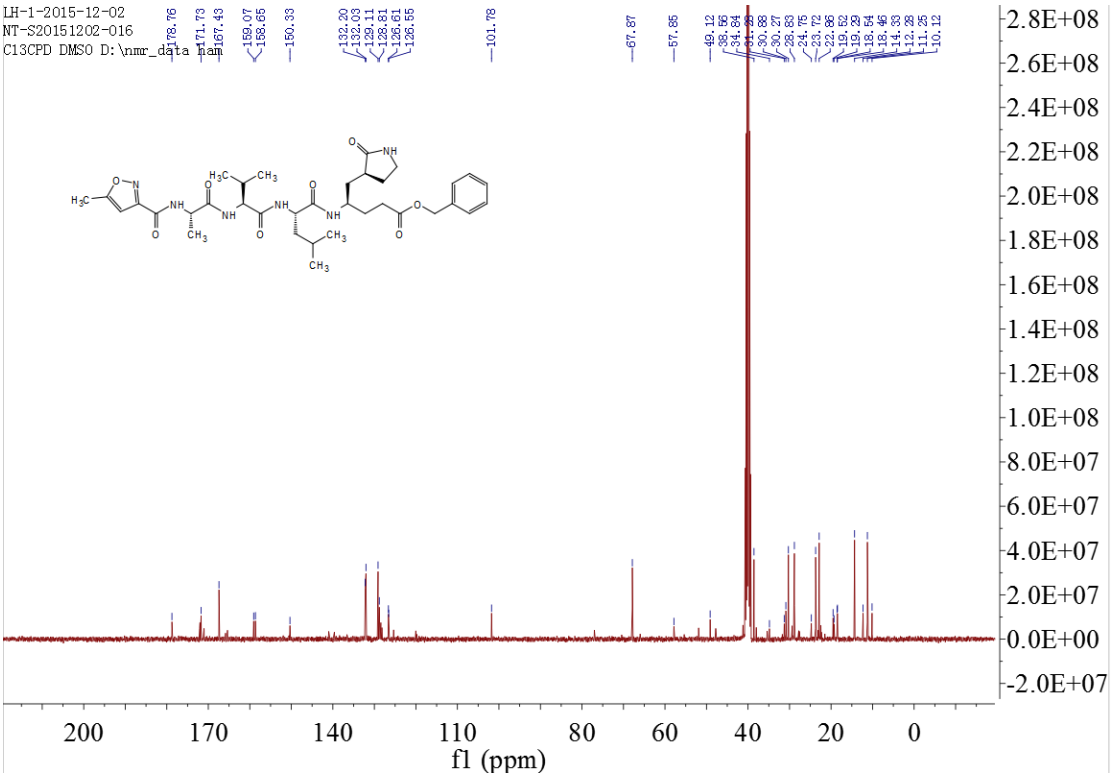
δ7.67 (d, J = 6.4 Hz, 1H), 7.41 (d, J = 8.3 Hz, 1H), 7.36 (s, 5H), 7.24 (s, 1H), 7.17 (d, J = 8.8 Hz, 1H), 6.63 (s, 1H), 6.43 (d, J = 0.7 Hz, 1H), 5.12 (s, 2H), 4.67 -4.82 (m, 1H), 4.50 -4.63 (m, 1H), 4.21 (m, 1H), 4.03 (d, J = 4.3 Hz, 1H), 3.20 -3.36 (m, 2H), 2.49 (s, 3H), 2.45 (m, 2H), 2.30 -2.38 (m, 2H), 1.89 - 2.07 (m, 5H), 1.53 -1.62 (m, 2H), 1.47 (d, J = 7.0 Hz, 3H), 0.85 -0.94 (m, 14H).



Compound **5**

^{13}C NMR (101 MHz, DMSO-d_6):

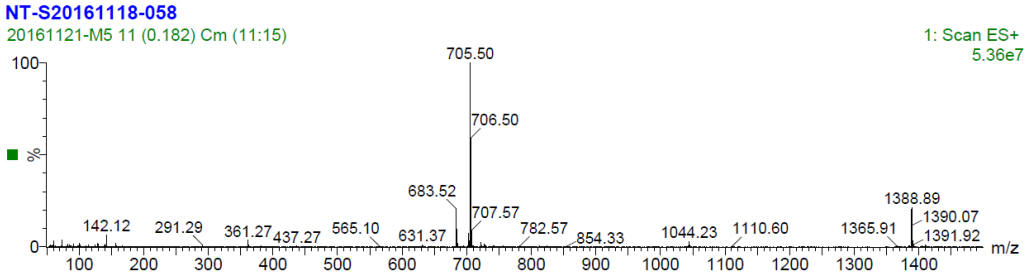
δ 178.76 , 171.73 , 167.43 , 159.07 , 158.65 , 150.33 , 132.20 , 132.03 , 129.11(2) , 128.81(2) , 126.61 , 126.55 , 101.78 , 67.87 , 57.85, 49.12 , 38.56 , 34.84 , 31.28 , 30.88 , 30.27 , 28.83 , 24.75 , 23.72 , 22.86 , 19.52 , 19.29 , 18.54 , 18.46 , 14.33 , 12.28, 11.25 , 10.12.



Compound **5**

ESI-MS:

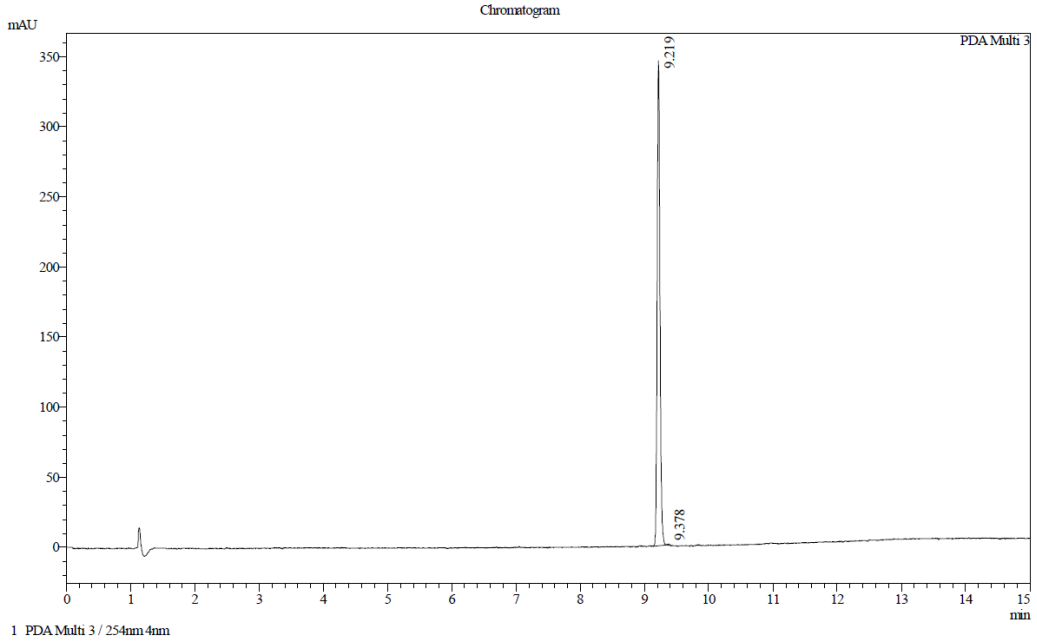
Calculated mass for $[C_{35}H_{50}N_6O_8Na, M+Na]^+$: 705.36 Found: 705.50.



Compound **5**

Analytic HPLC:

Purity: 99.8%



Integration Result

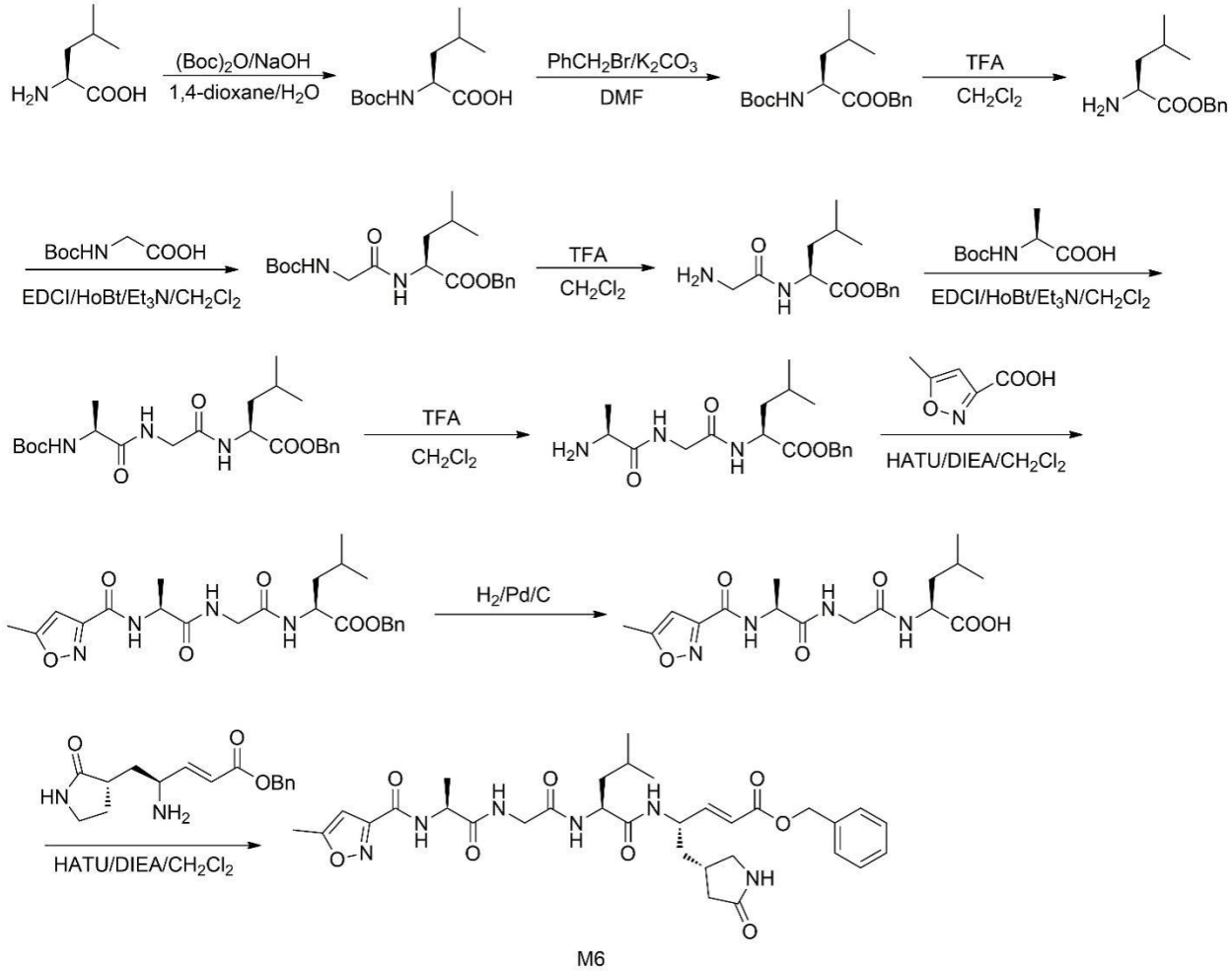
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	9.219	337902	99.772	0.081	1042437	99.823
2	9.378	772	0.228	0.067	1851	0.177

Compound **5**

Compound 6 (Inhibitor M6)

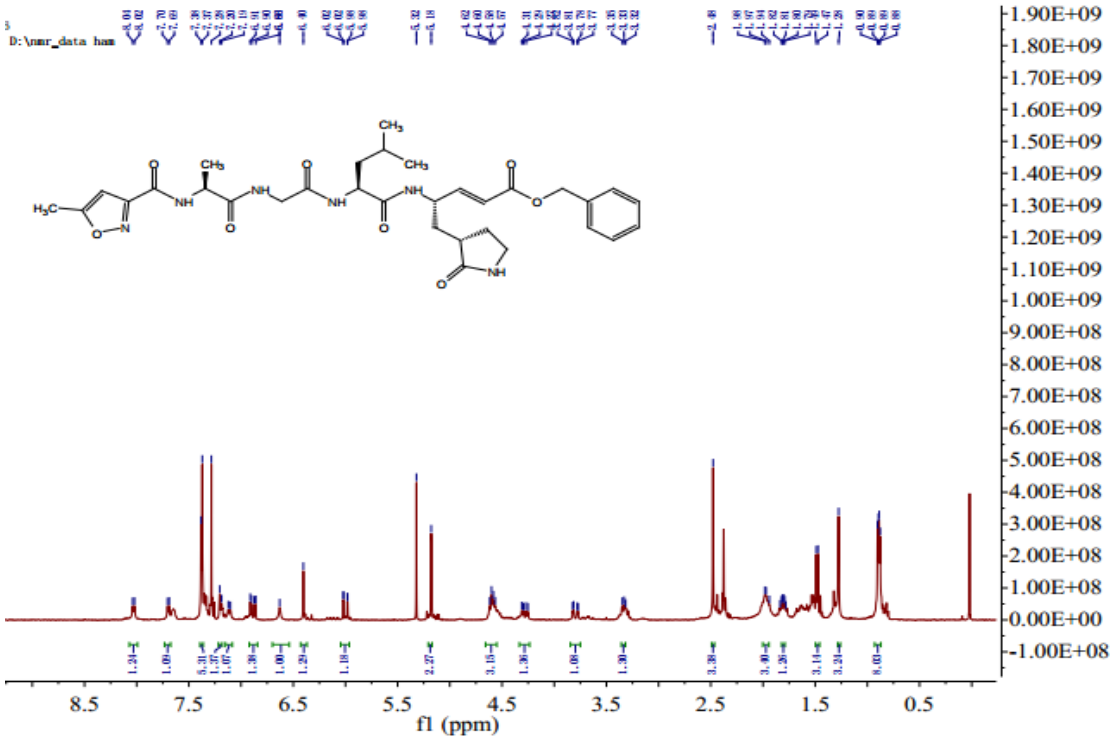
Synthetic Pathway:



Compound **6**

^{1}H NMR (400 MHz, $CDCl_3$):

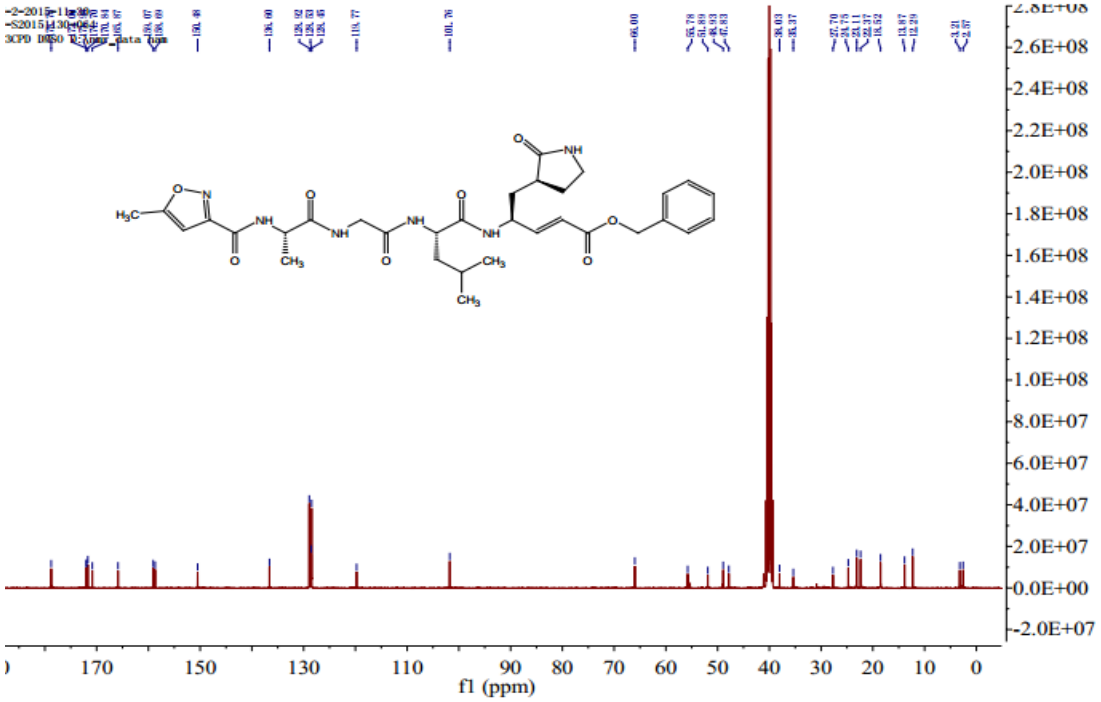
δ8.03 (d, J = 7.0 Hz, 1H), 7.70 (d, J = 6.6 Hz, 1H), 7.36 -7.39 (m, 5H), 7.19 (d, J = 7.5 Hz, 1H), 7.11 (d, J = 8.6 Hz, 1H), 6.85-6.92 (m, 1H), 6.63 (s, 1H), 6.40 (s, 1H), 5.96 -6.04 (m, 1H), 5.18 (s, 2H), 4.55 -4.66 (m, 3H), 4.24 -4.34 (m, 1H), 3.75-3.84 (m, 1H), 3.31 -3.36 (m, 1H), 2.48 (s, 3H), 1.95-2.00 (m, 3H), 1.79 -1.81 (m, 1H), 1.48 (d, J = 7.0 Hz, 3H), 1.26 -1.29 (m, 3H), 0.87-0.93 (m, 8H).



Compound **6**

^{13}C NMR (101 MHz, DMSO-d_6):

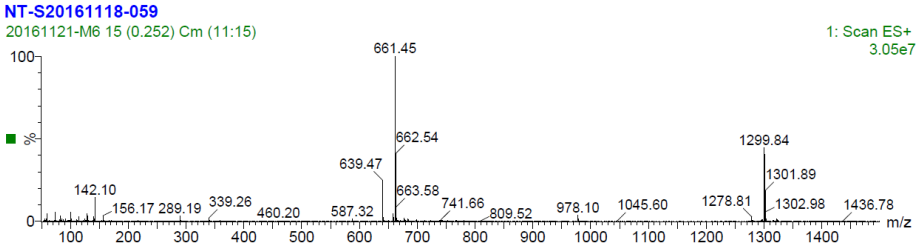
δ178.78, 172.00, 171.95, 171.70, 170.84, 165.87, 159.07, 158.69, 150.48, 136.60, 128.92 (2), 128.53, 128.45 (2), 119.77, 101.76, 66.00, 55.78, 51.89, 48.93, 47.83, 38.03, 35.37, 27.70, 24.75, 23.11, 22.37, 18.52, 13.87, 12.29, 3.21.



Compound **6**

ESI-MS:

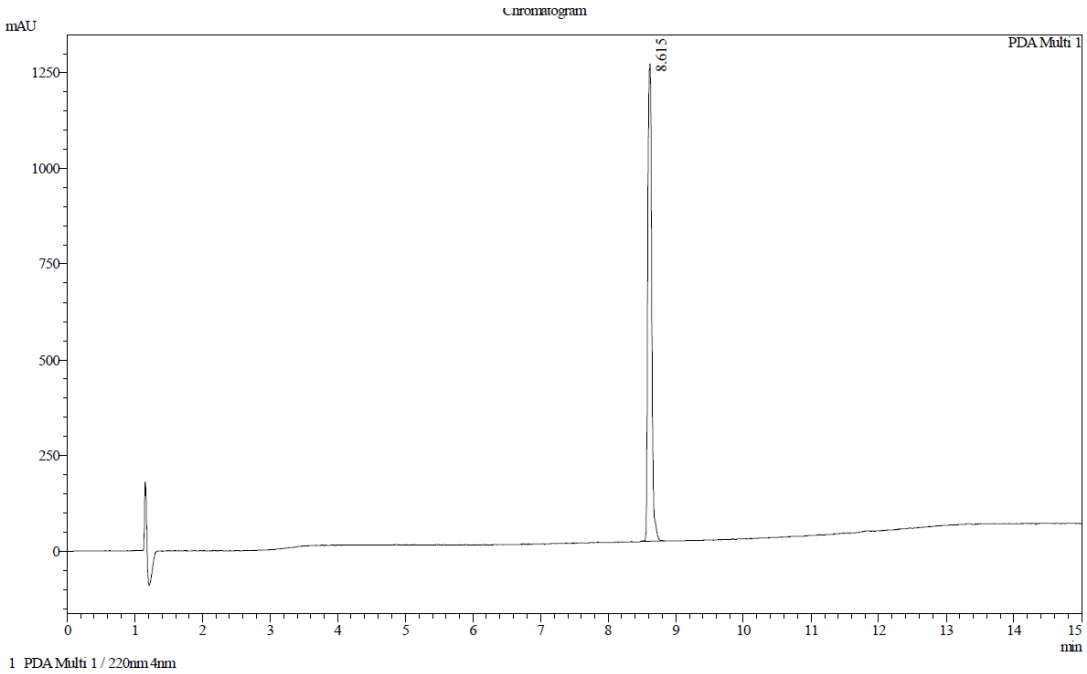
Calculated mass for $[C_{32}H_{42}N_6O_8Na, M+Na]^+$: 661.30 Found: 661.45.



Compound **6**

Analytic HPLC:

Purity: >99.9%



Integration Result

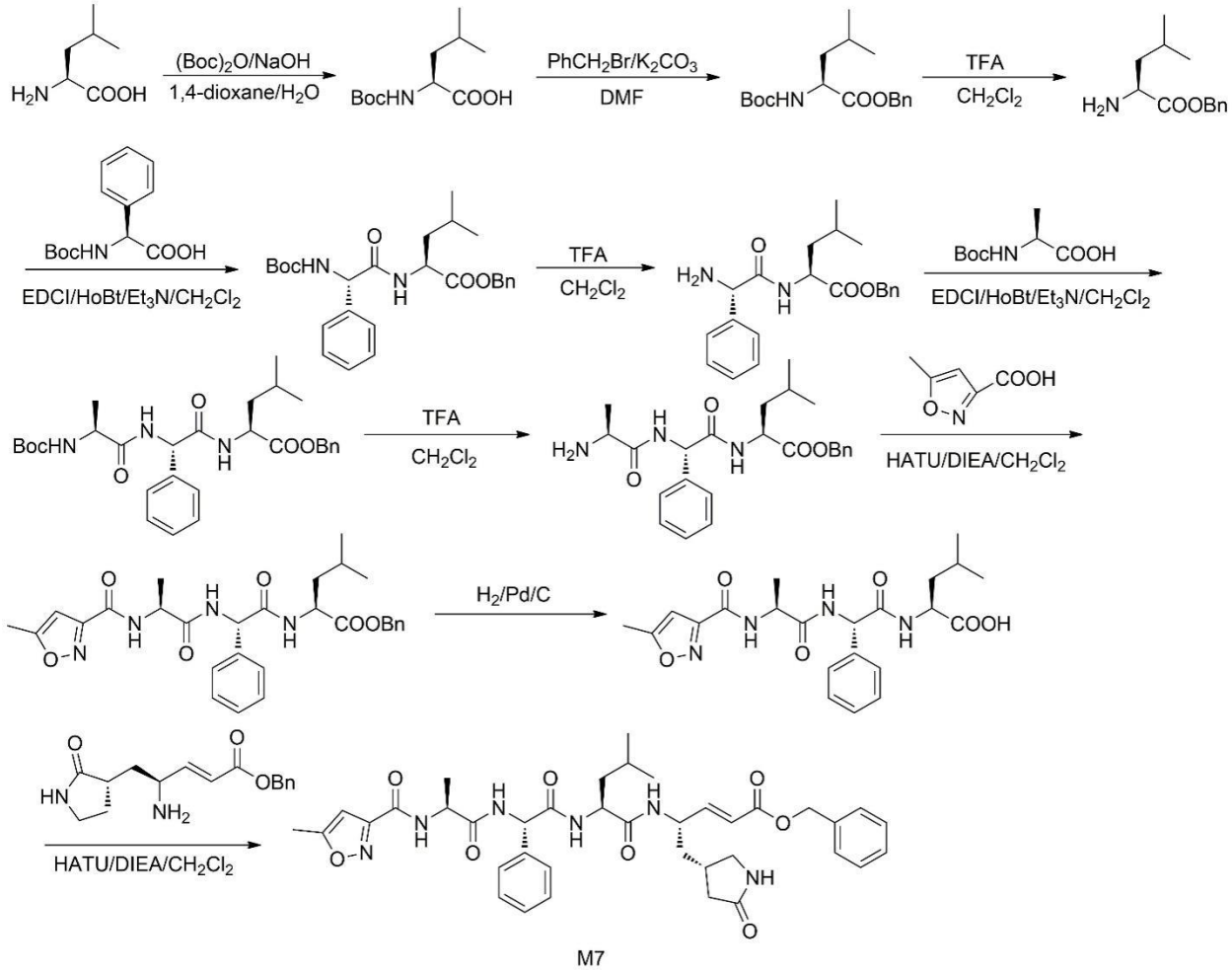
PDA Ch1 220nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	8.615	1238748	100.000	0.098	5088810	100.000

Compound **6**

Compound 7 (Inhibitor M7)

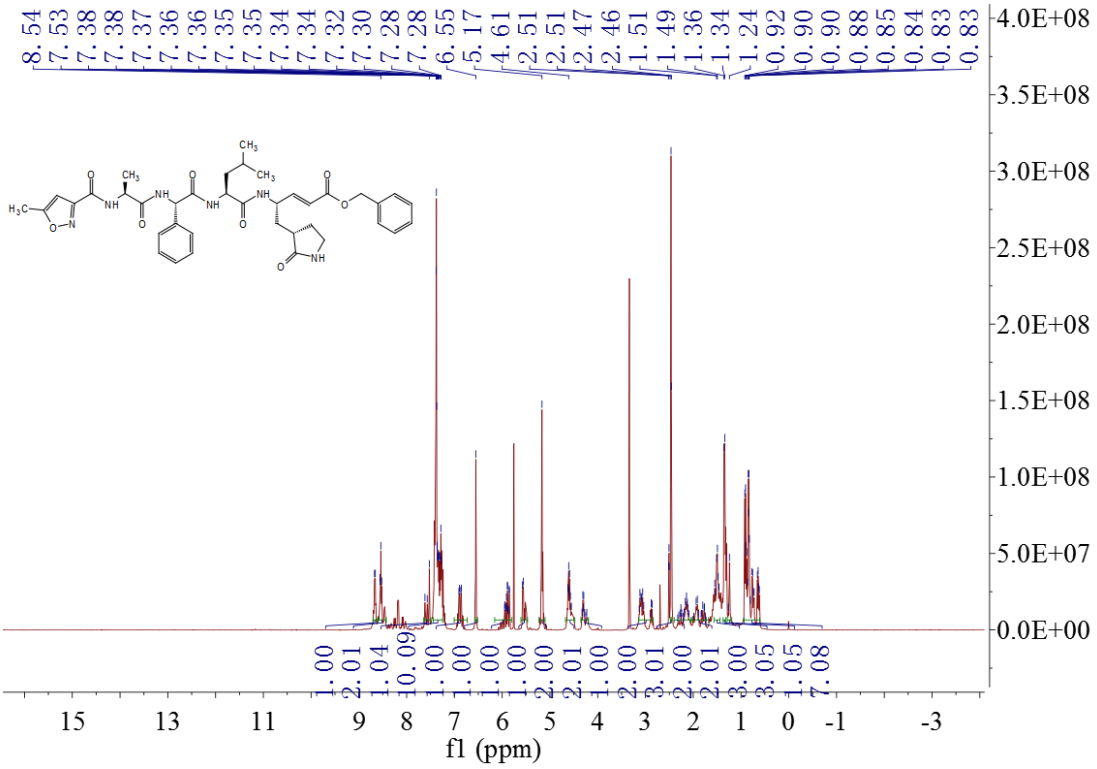
Synthetic Pathway:



Compound **7**

^{1}H NMR (400 MHz, DMSO-d_6):

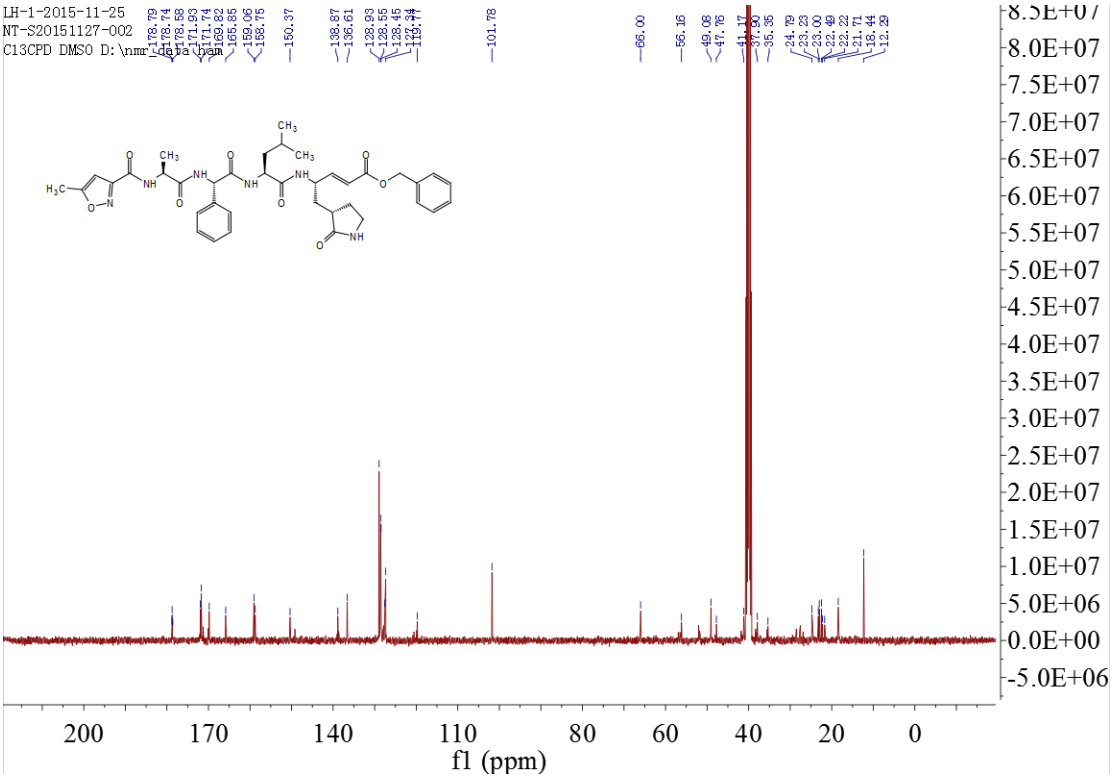
δ 8.66 (d, *J* = 7.6 Hz, 1H), 8.54 (m, 2H), 7.50-7.64 (m, 1H), 7.25 -7.44 (m, 10H), 6.88 (m, 1H), 6.55 (s, 1H), 5.80 - 6.15 (m, 1H), 5.56 (d, *J* = 8.1 Hz, 1H), 5.17 (s, 2H), 4.47 -4.67 (m, 2H), 4.18 - 4.34 (m, 1H), 2.84 -3.14 (m, 2H), 2.46 (s, 3H), 2.02 -2.40 (m, 2H), 1.68 -2.05 (m, 2H), 1.44 - 1.56 (m, 3H), 1.35 (d, *J* = 7.1 Hz, 3H), 1.19 -1.29 (m, 1H), 0.61 -0.95 (m, 7H).



Compound **7**

^{13}C NMR (101 MHz, DMSO-d_6):

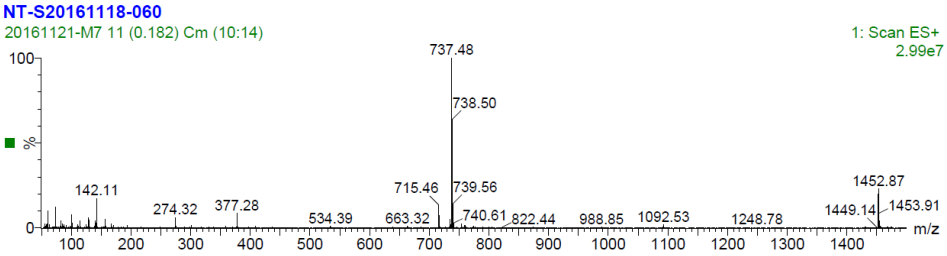
δ 178.79, 178.74, 178.58, 171.93, 171.74 , 169.82, 165.85, 159.06, 158.75, 150.37, 138.87, 136.61, 128.93, 128.55 (2), 128.45 (2), 127.56 (2), 127.34 (2), 119.77, 101.78, 66.00, 56.16, 49.08, 47.76, 41.17, 37.90, 35.35, 24.79, 23.23, 23.00, 22.49, 22.22, 21.71, 18.44, 12.29.



Compound **7**

ESI-MS:

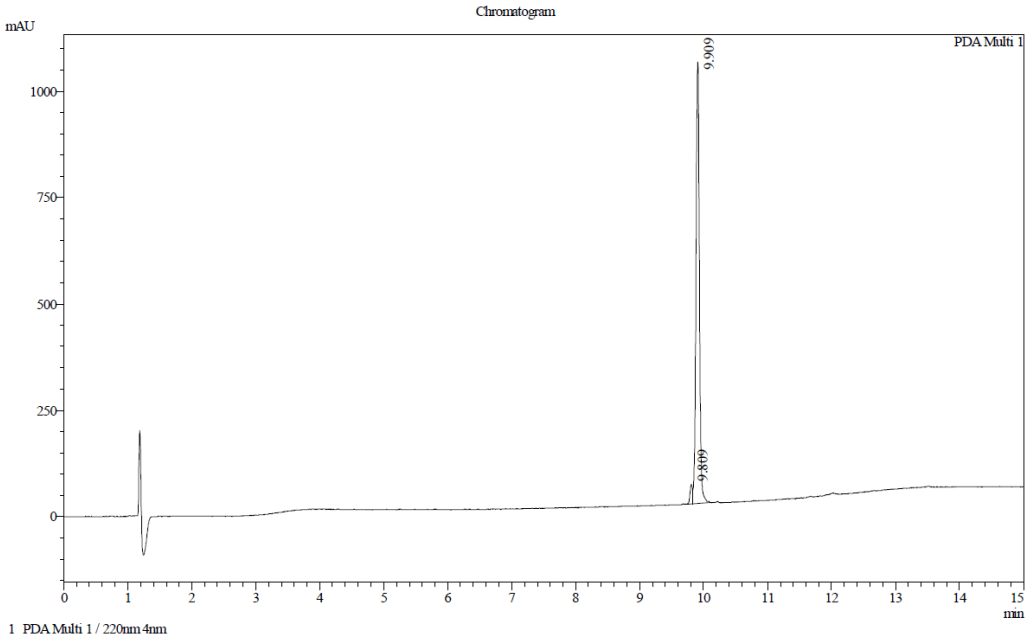
Calculated mass for $[C_{38}H_{46}N_6O_8Na, M+Na]^+$: 737.33 Found: 737.48.



Compound **7**

Analytic HPLC:

Purity: 97.2%



Integration Result

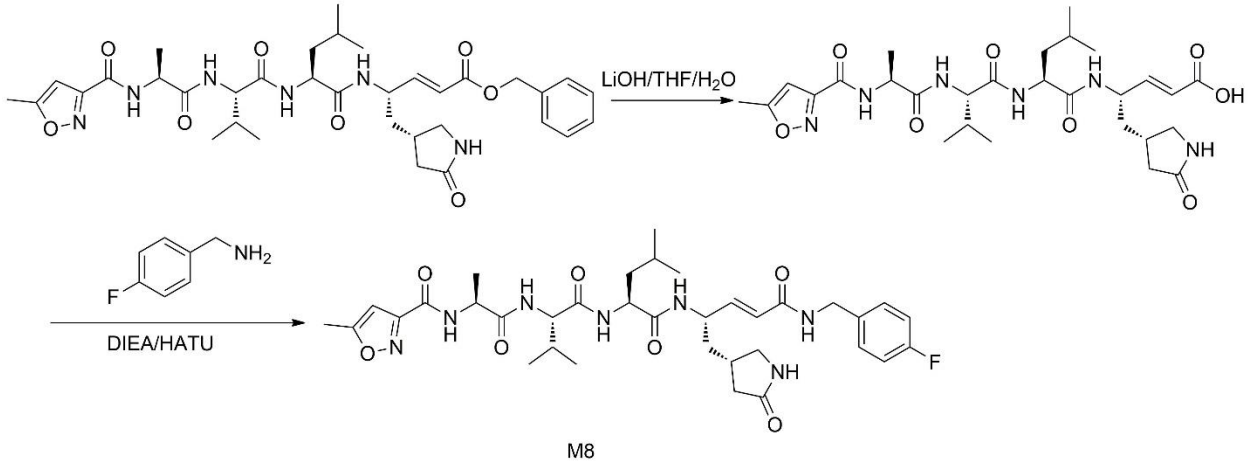
PDA Ch1 220nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	9.809	44786	4.243	0.098	109264	2.745
2	9.909	1010792	95.757	0.095	3870848	97.255

Compound **7**

Compound 8 (Inhibitor M8)

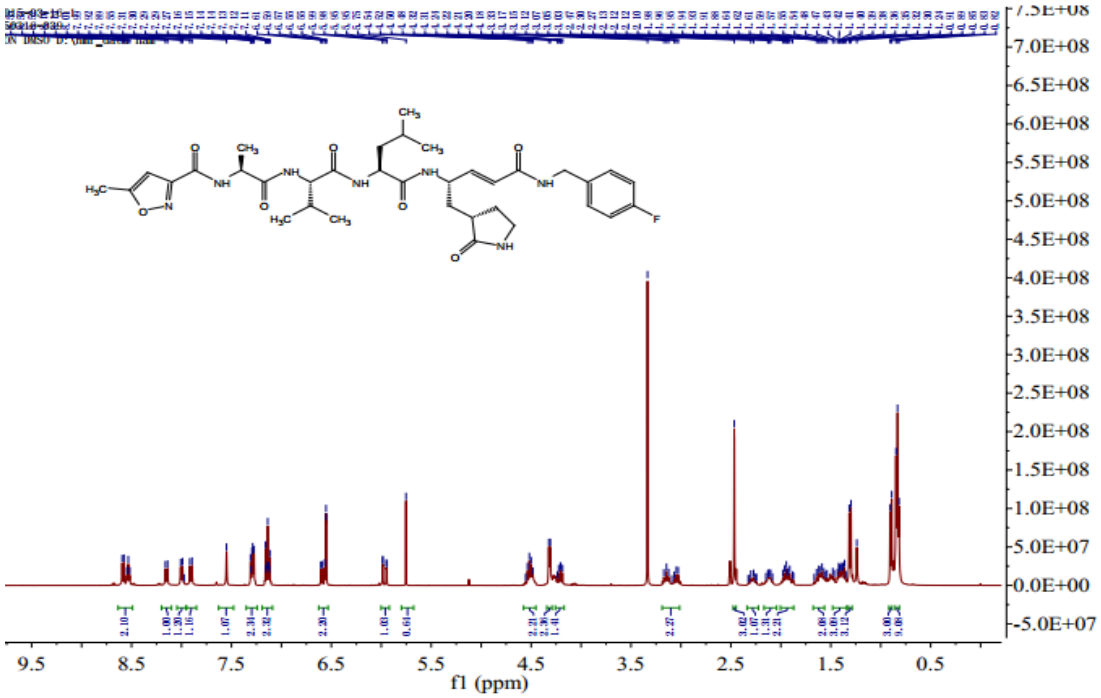
Synthetic Pathway:



Compound **8**

^{1}H NMR (400 MHz, DMSO-d_6):

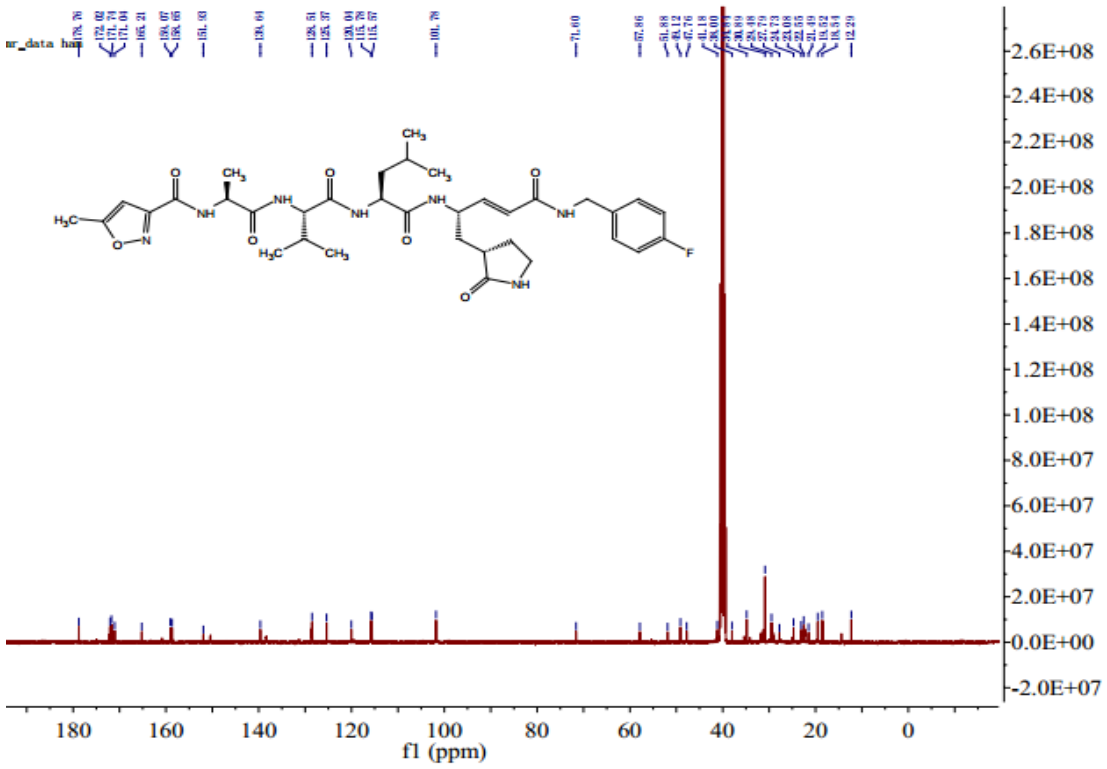
δ8.49 -8.64 (m, 2H), 8.15 (d, J = 8.8 Hz, 1H), 7.95-8.04 (m, 1H), 7.91 (d, J = 8.9 Hz, 1H), 7.55 (s, 1H), 7.24 -7.35 (m, 2H), 7.09-7.19 (m, 2H), 6.53 -6.63 (m, 2H), 5.92 -6.00 (m, 1H) 5.75 (s, 1H), 4.46-4.58 (m, 2H), 4.29 -4.34 (m, 2H) ,4.17 4.26 (m, 1H), 3.01 -3.18 (m, 2H), 2.47 (s, 3H), 2.23 -2.34 (m, 1H), 2.04 -2.17 (m, 1H), 1.87 -2.00 (m, 2H), 1.56-1.67 (m, 2H), 1.34-1.48 (m, 3H), 1.31 (d, J = 7.1 Hz, 3H), 0.89-0.92 (m, 3H), 0.81 - 0.85 (m, 9H).



Compound **8**

^{13}C NMR (101 MHz, DMSO-d_6):

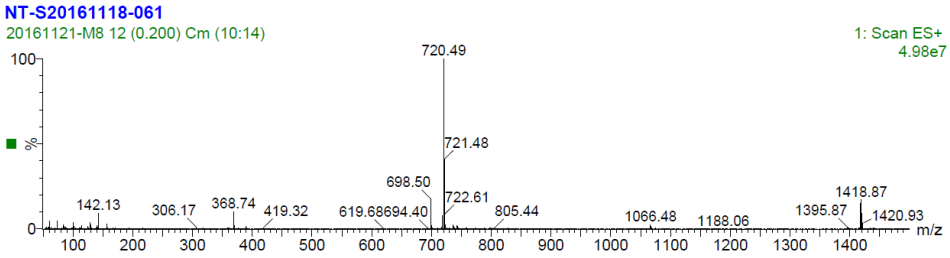
δ178.76, 172.02, 171.74, 171.04, 165.21, 159.07, 158.65, 151.93, 139.64, 128.51(t,C-F), 125.37, 120.04, 115.78(2), 115.57(2), 101.78, 71.60, 57.86, 51.88, 49.12, 47.76, 38.00, 34.84, 30.89, 29.48, 27.79, 24.73, 23.08, 22.55, 22.39, 19.52, 18.54, 14.41, 12.29.



Compound **8**

ESI-MS:

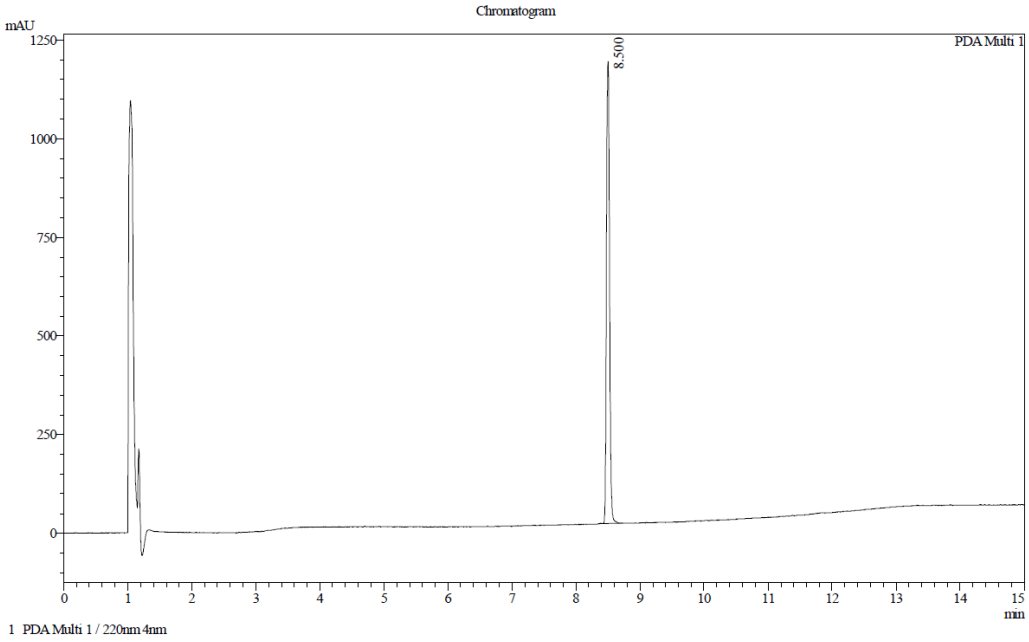
Calculated mass for $[C_{35}H_{48}N_7O_7Na, M+Na]^+$: 720.35 Found: 720.49.



Compound **8**

Analytic HPLC:

Purity: >99.9%



Integration Result

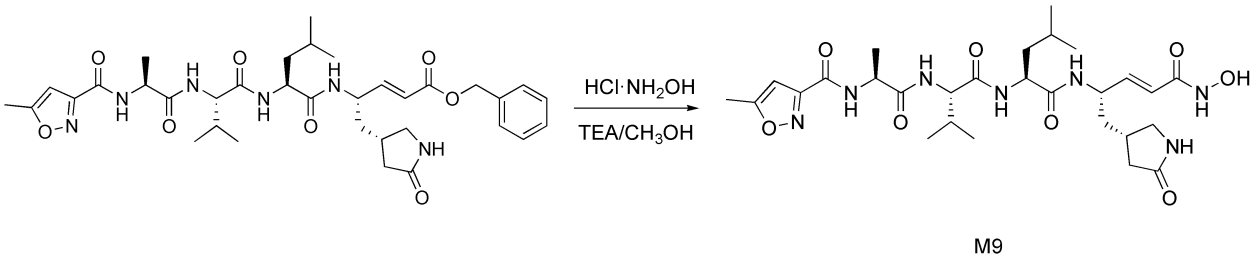
PDA Ch1 220nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	8.500	1160024	100.000	0.087	3943231	100.000

Compound **8**

Compound 9 (Inhibitor M9)

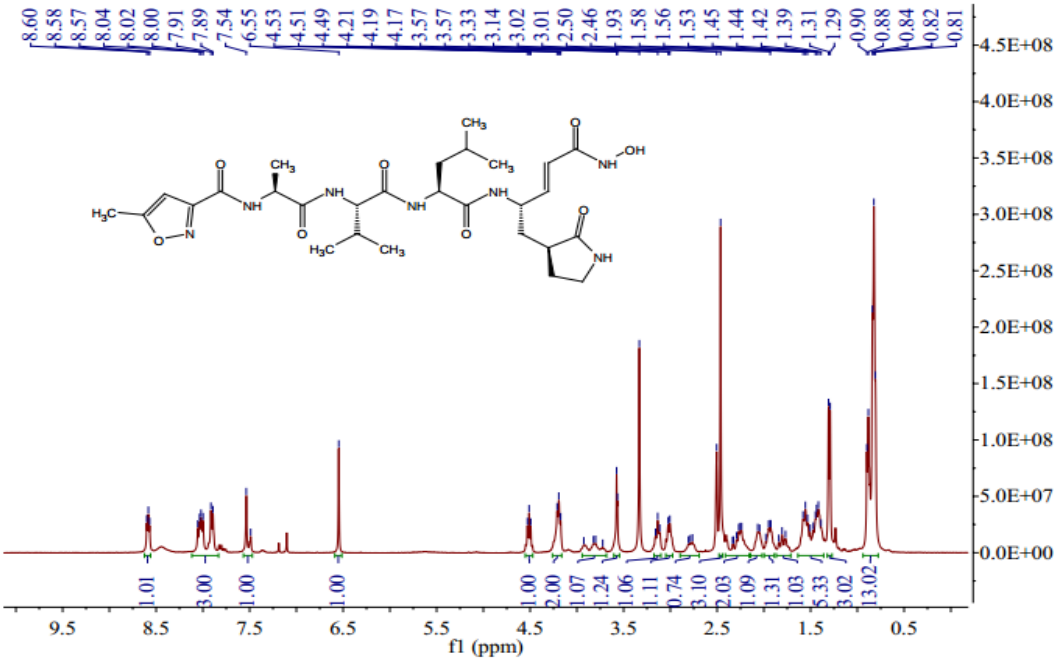
Synthetic Pathway:



Compound **9**

^{1}H NMR (400 MHz, DMSO-d_6):

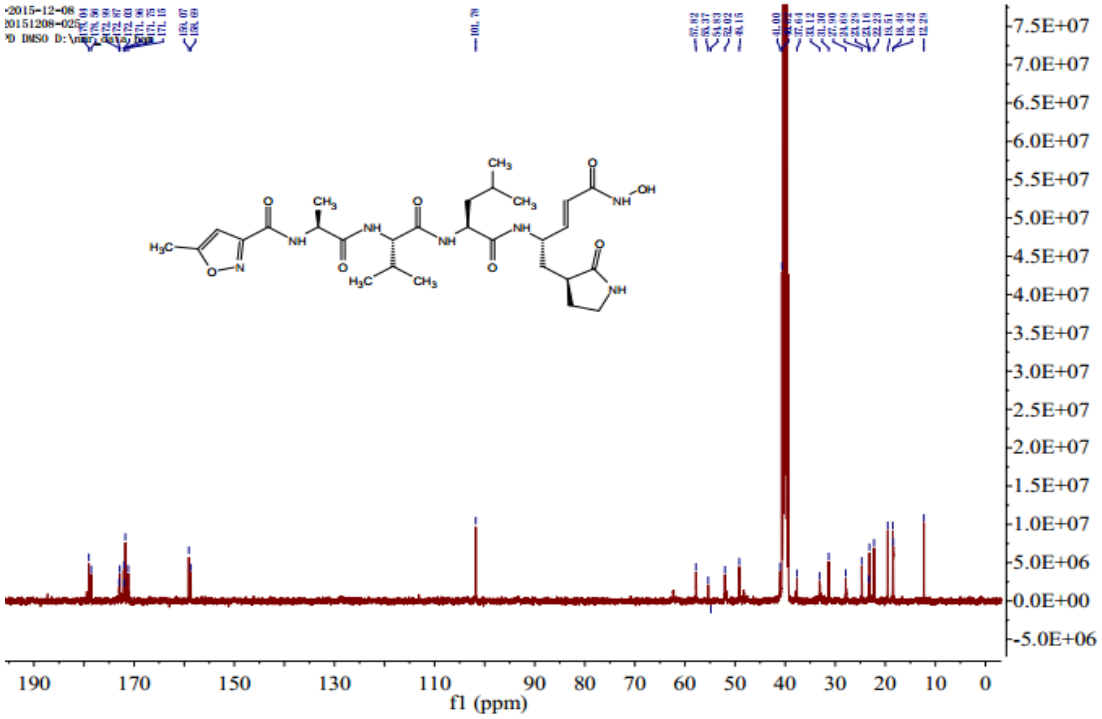
δ 8.56 -8.63 (m, 1H), 8.03 (m, 2H), 7.90 (d, J = 8.7 Hz, 1H), 7.47 -7.57 (m, 1H), 6.55 (s, 1H), 4.47 -4.55 (m, 1H), 4.16 -4.27 (m, 2H), 3.68 -3.94 (m, 1H), 3.57 (d, J = 3.4 Hz, 1H), 3.14 (m, 1H), 2.98 -3.05 (m, 1H), 2.69 -2.89 (m, 1H), 2.46 (s, 3H), 2.16 -2.41 (m, 2H), 2.01 -2.13 (m, 1H), 1.89 -1.99 (m, 1H), 1.71 -1.87 (m, 1H), 1.37 -1.61 (m, 5H), 1.30 (d, J = 7.0 Hz, 3H), 0.78 -0.94 (m, 13H).



Compound **9**

^{13}C NMR (101 MHz, DMSO-d_6):

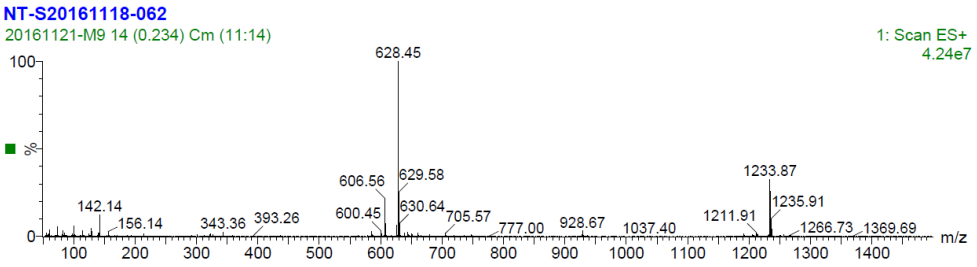
δ179.04, 178.56, 172.99, 172.87, 172.03, 171.75, 171.15, 159.07, 158.69, 158.64, 101.78, 62.25, 57.82, 55.37, 52.02, 49.15, 48.28, 41.00, 37.64, 33.12, 31.30, 27.90, 24.69, 23.16, 22.23, 19.51, 18.49, 12.29.



Compound **9**

ESI-MS:

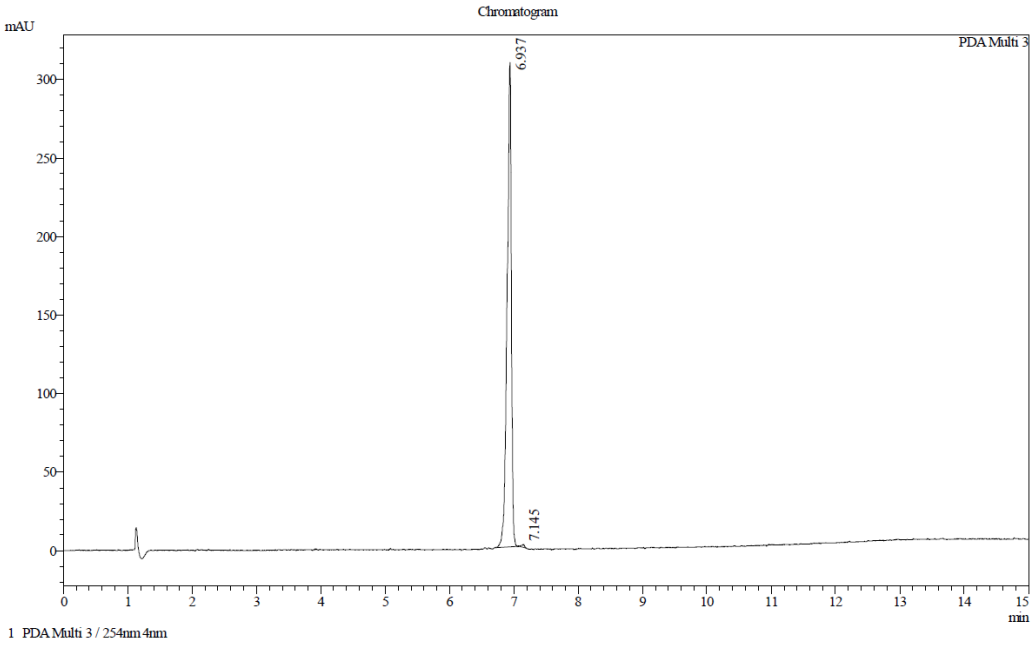
Calculated mass for $[C_{28}H_{43}N_7O_8Na, M+Na]^+$: 628.31 Found: 628.45.



Compound **9**

Analytic HPLC:

Purity: 99.5%



Integration Result

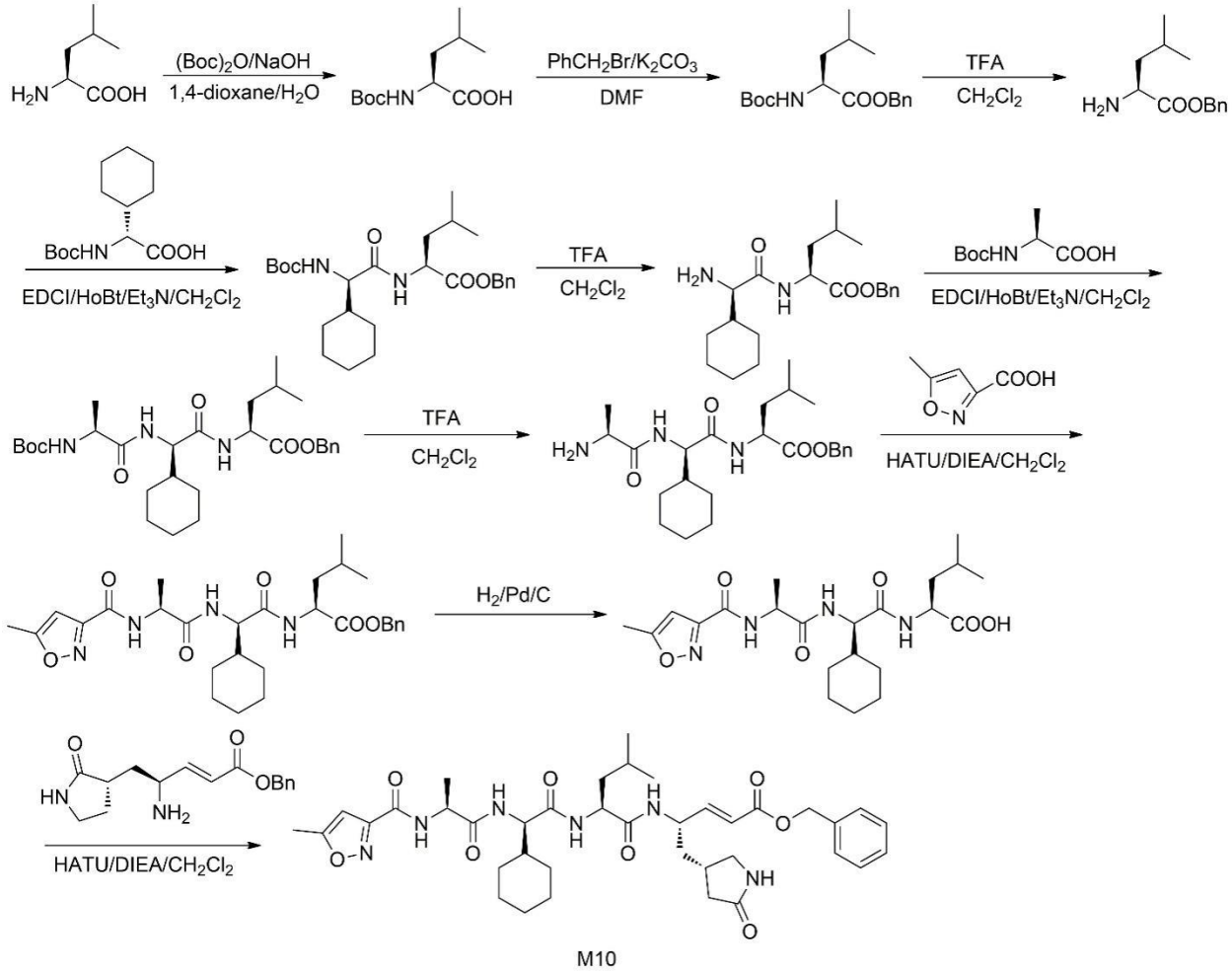
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	6.937	303138	99.413	0.116	1402278	99.572
2	7.145	1791	0.587	0.082	6026	0.428

Compound **9**

Compound 10 (Inhibitor M10)

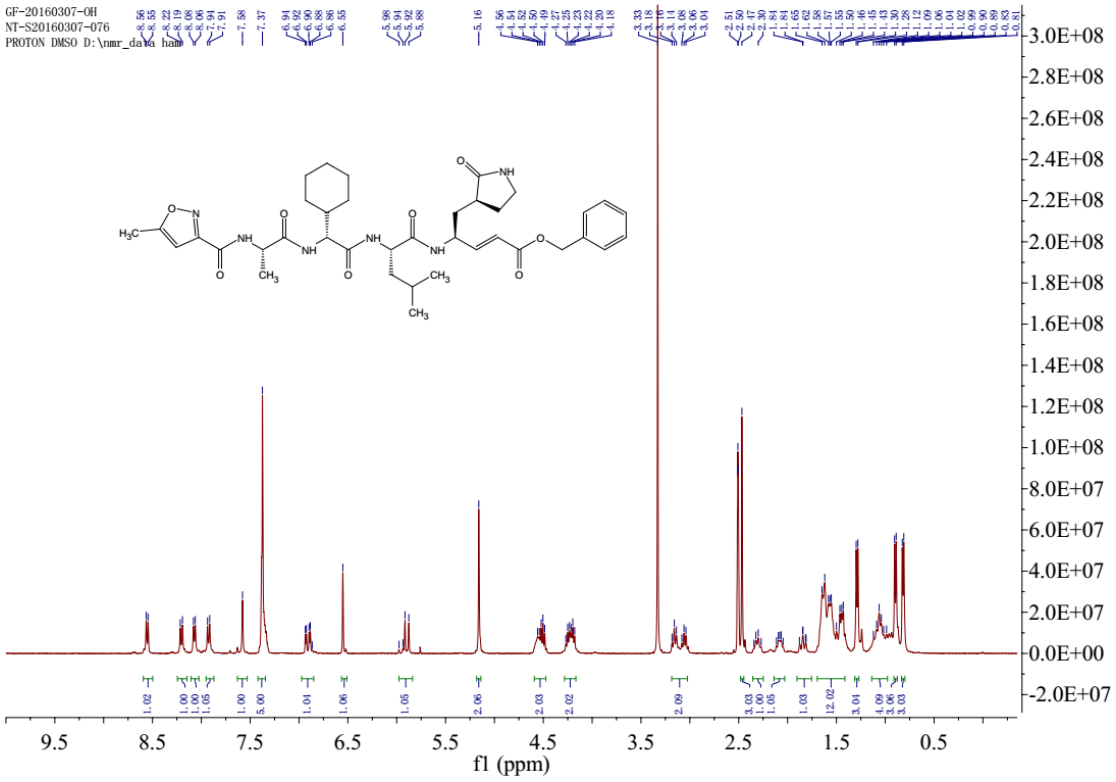
Synthetic Pathway:



Compound **10**

^{1}H NMR (400 MHz, DMSO-d_6):

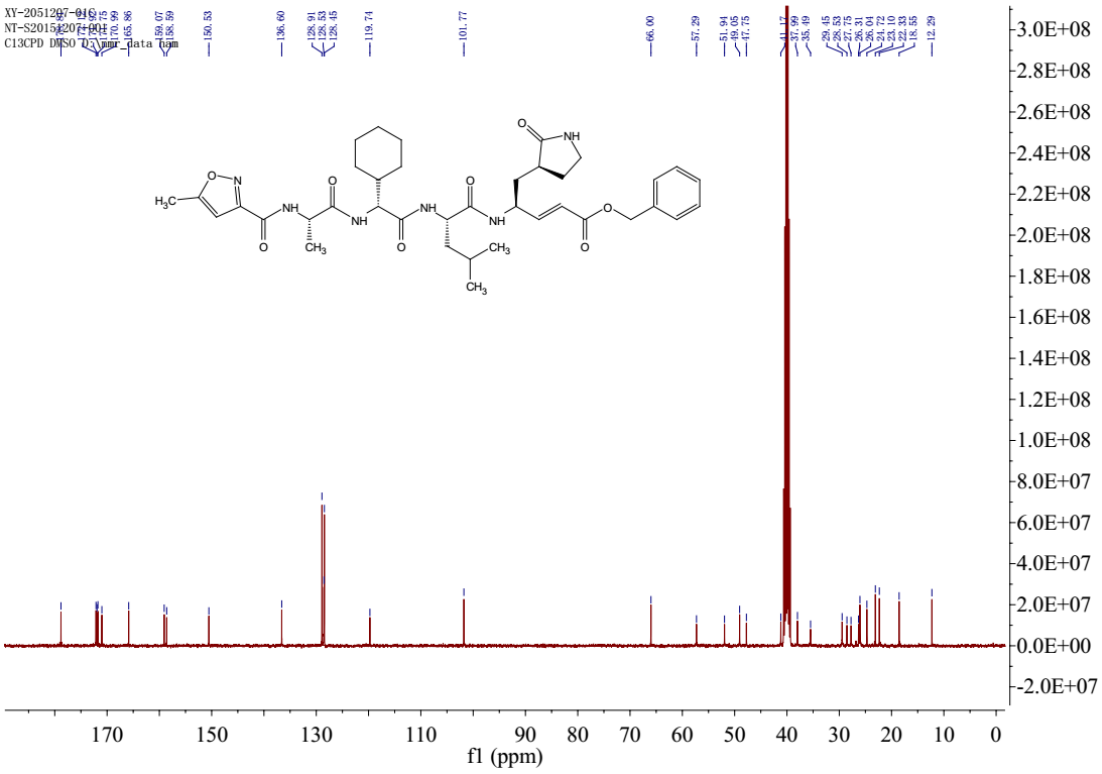
δ8.51-8.62(m, 1H), 8.20-8.28 (m, 1H), 8.02-8.11 (m, 1H), 7.88-7.99 (m, 1H), 7.61 (d, J = 21.3 Hz, 1H), 7.34-7.42 (m, 5H), 6.92 (m, 1H), 6.55 (s, 1H), 5.92 (m,1H), 5.10-5.18 (m, 2H), 4.44 -4.60 (m, 2H,), 4.10-4.32 (m, 2H), 2.99 -3.19 (m, 2H), 2.47 (s, 3H), 2.20-2.37 (m, 1H), 1.93 -2.18 (m, 2H), 1.76 -1.93 (m, 1H), 1.39 -1.70 (m, 14H), 1.36 (d, J = 5.3 Hz, 1H), 1.29 (d, J = 7.0 Hz, 3H), 0.89 (d, J = 6.7 Hz, 3H), 0.81 (d, J = 6.3 Hz, 3H).



Compound **10**

^{13}C NMR (101 MHz, DMSO-d_6):

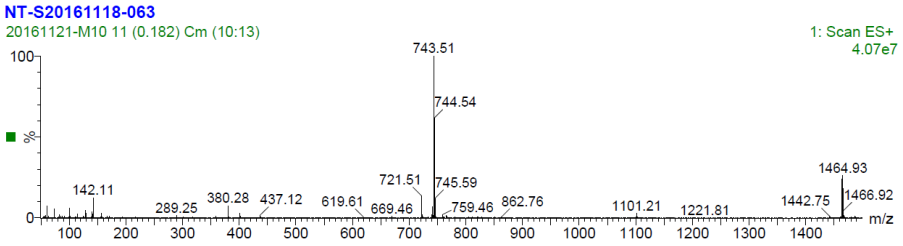
δ178.81, 172.12, 171.92, 171.75, 170.99, 165.86, 159.07, 158.59, 150.53, 136.60, 128.91(2), 128.53, 128.45(2), 119.74, 101.77, 66.00, 57.29, 51.94, 49.05, 47.75, 41.17, 37.99, 35.49, 29.45, 28.53, 27.75, 26.31(2), 26.04(2), 24.72, 23.10(2), 22.33, 18.55, 12.29.



Compound **10**

ESI-MS:

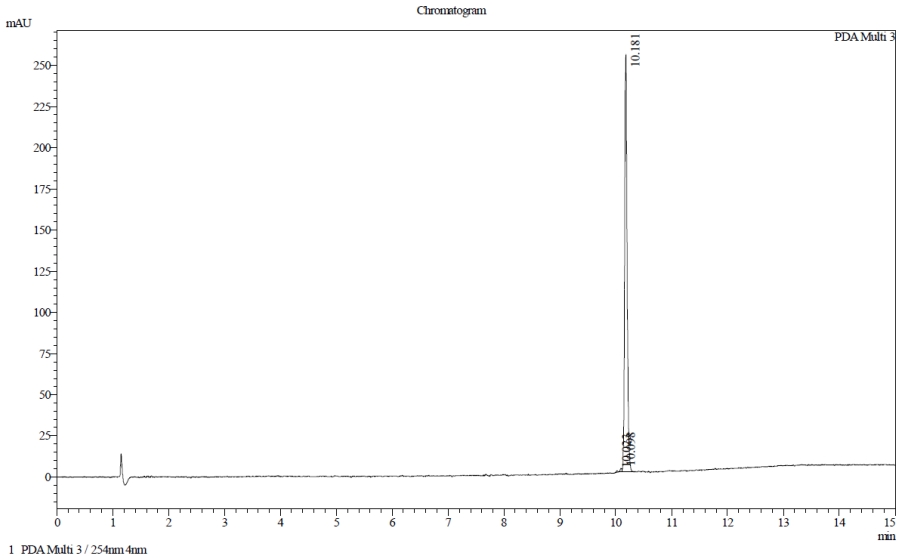
Calculated mass for $[C_{38}H_{52}N_6O_8Na, M+Na]^+$: 743.36 Found: 743.51.



Compound **10**

Analytic HPLC:

Purity: 99.1%



Integration Result

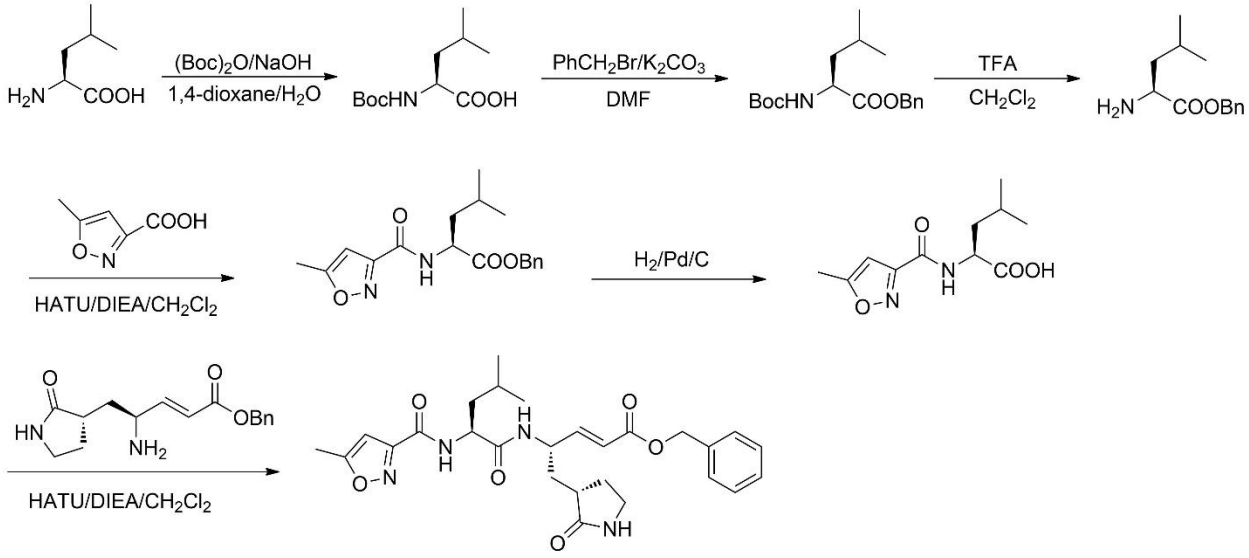
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	10.023	832	0.330	0.055	1955	0.247
2	10.098	1992	0.791	0.099	4827	0.609
3	10.181	248949	98.878	0.083	785731	99.144

Compound **10**

Compound 11 (Inhibitor M11)

Synthetic Pathway:

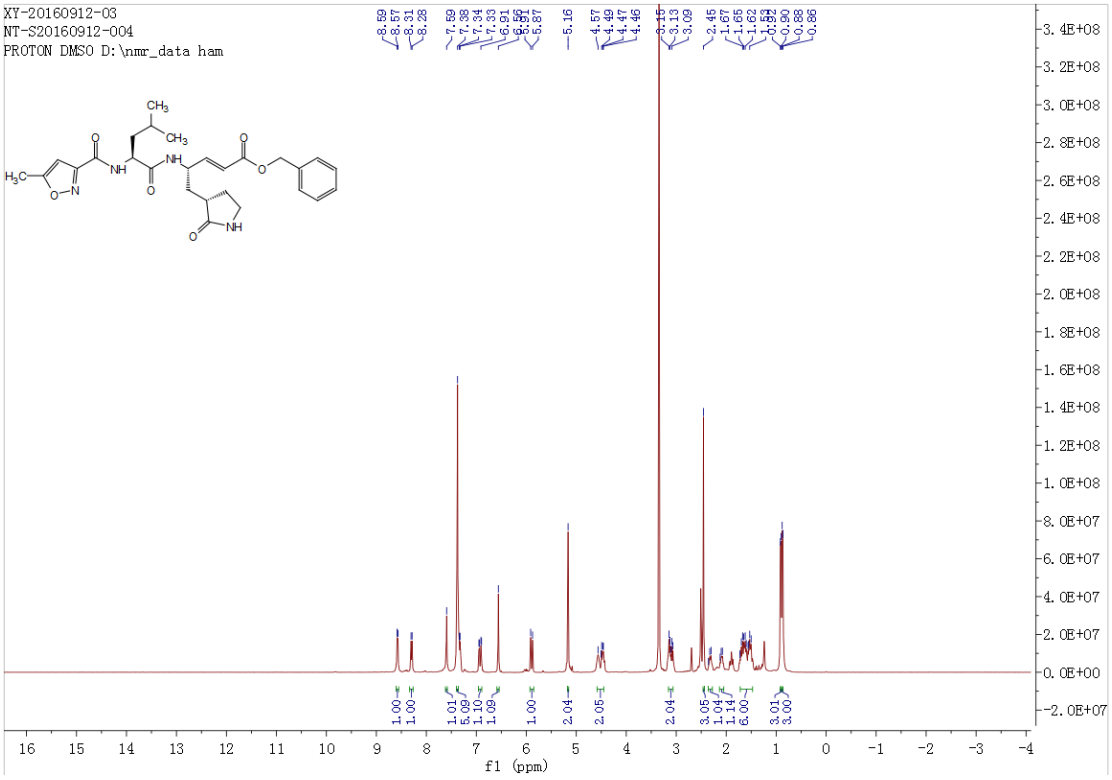


M11

Compound **11**

^{1}H NMR (400 MHz, DMSO-d_6):

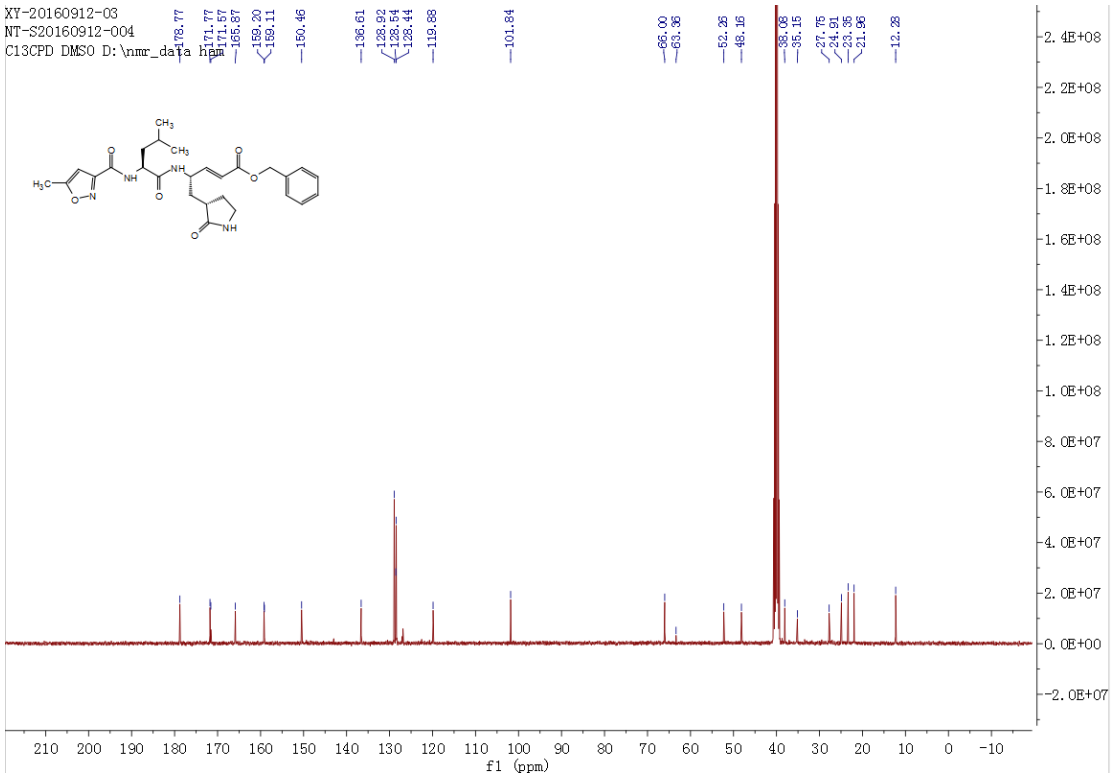
δ 8.58 (d, J = 8.0 Hz, 1H), 8.30 (d, J = 8.4 Hz, 1H), 7.59 (s, 1H), 7.38 (s, 5H), 6.96 – 6.89 (m, 1H), 6.56 (s, 1H), 5.89 (d, J = 15.2 Hz, 1H), 5.16 (s, 2H), 4.58 – 4.45 (m, 2H), 3.15 – 3.06 (m, 2H), 2.45 (s, 3H), 2.36 – 2.28 (m, 1H), 2.14 – 2.05 (m, 1H), 1.72 – 1.47 (m, 6H), 0.91 (d, J = 6.3 Hz, 3H), 0.87 (d, J = 6.2 Hz, 3H).



Compound **11**

^{13}C NMR (101 MHz, DMSO-d_6):

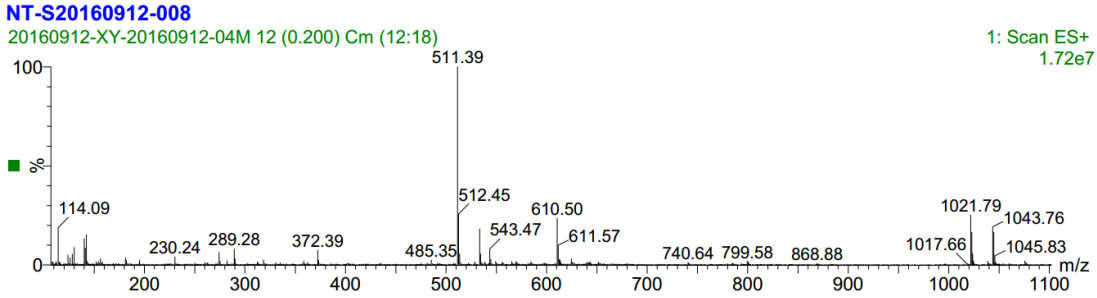
δ 178.77 , 171.77 ,171.57, 165.87 , 159.20, 159.11, 150.46 , 136.61 , 128.92(2) , 128.54(2),128.44 , 119.88 , 101.84, 66.00 , 63.36 , 52.26 , 48.16 , 38.08 , 35.15 , 27.75 , 24.91 , 23.35(2), 21.96, 12.28.



Compound **11**

ESI-MS:

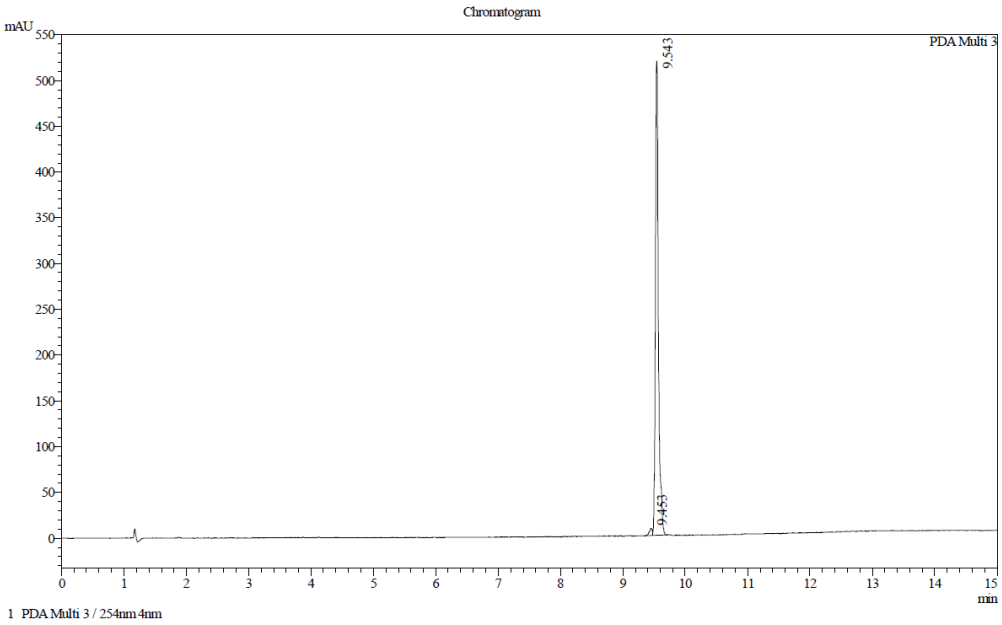
Calculated mass for $[C_{27}H_{35}N_4O_6, M+H]^+$: 511.26 Found: 511.39.



Compound **11**

Analytic HPLC:

Purity: 98.5%



Integration Result

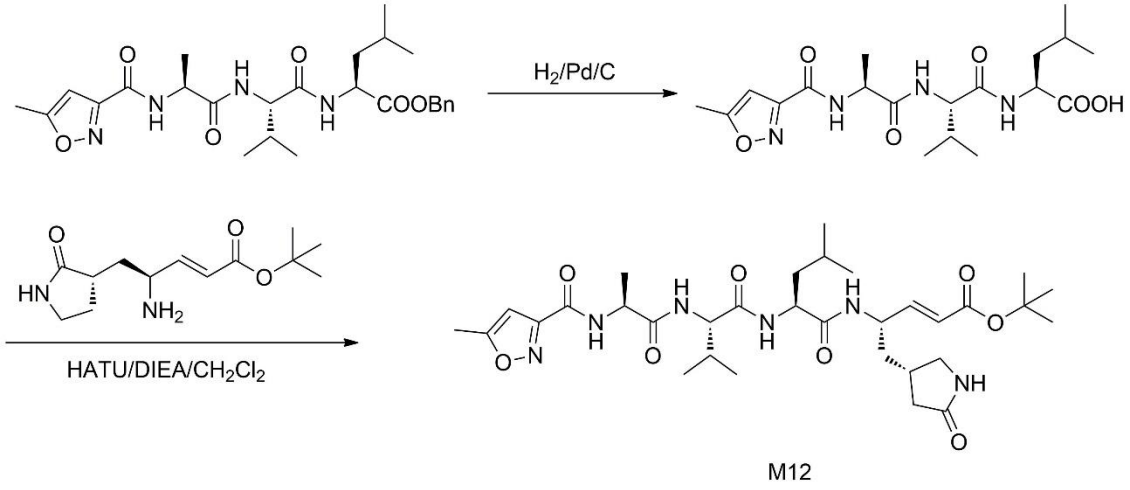
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	9.453	7440	1.458	0.096	25704	1.471
2	9.543	502739	98.542	0.084	1721695	98.529

Compound **11**

Compound 12 (Inhibitor M12)

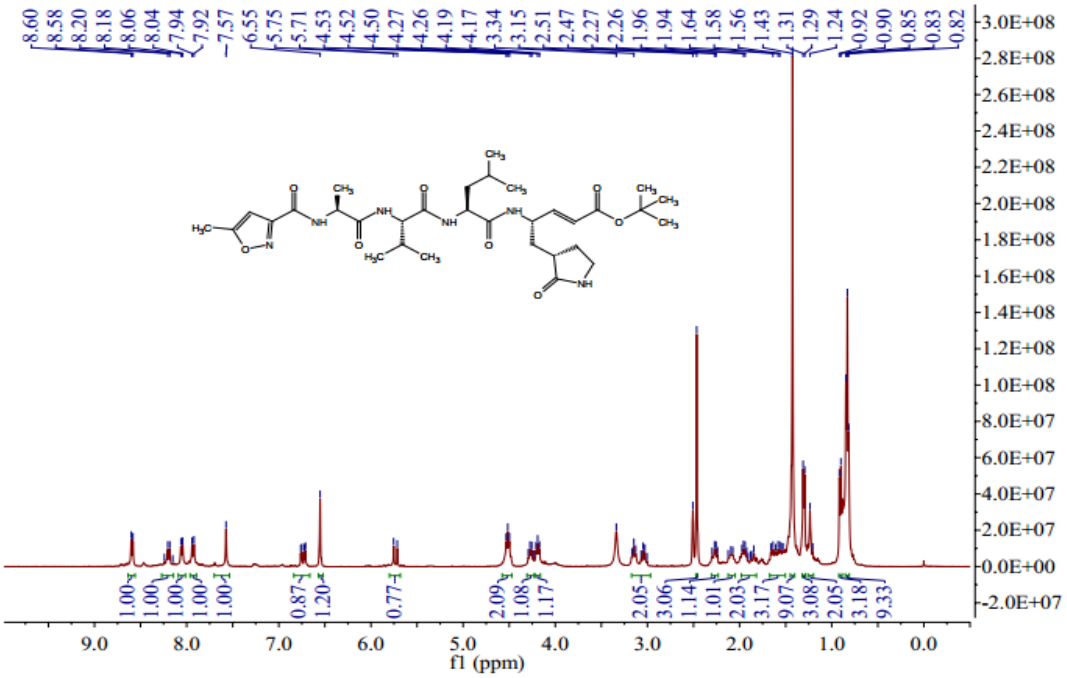
Synthetic Pathway:



Compound **12**

^{1}H NMR (400 MHz, DMSO-d_6):

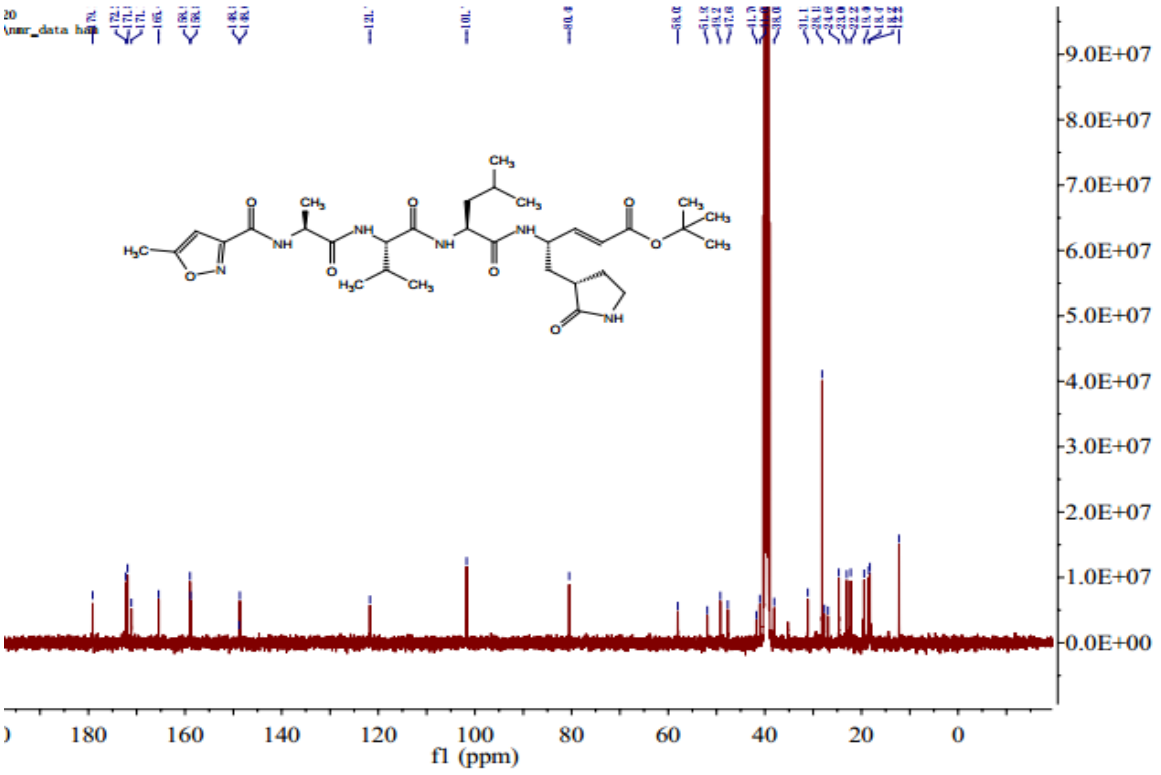
δ 8.59 (d, *J* = 7.5 Hz, 1H), 8.19 (d, *J* = 8.7 Hz, 1H), 8.05 (d, *J* = 7.8 Hz, 1H), 7.93 (d, *J* = 8.8 Hz, 1H), 7.57 (s, 1H), 6.74 (m, 1H), 6.55 (s, 1H), 5.73 (d, *J* = 15.8 Hz, 1H), 4.47 -4.58 (m, 2H), 4.26 (m, 1H), 4.16 -4.22 (m, 1H), 2.97 -3.19 (m, 2H), 2.47 (s, 3H), 2.23 -2.31 (m, 1H), 2.05 -2.13 (m, 1H), 1.81-1.99 (m, 2H), 1.50 -1.67 (m, 3H), 1.43 (s, 9H), 1.30 (d, *J* = 6.9 Hz, 3H), 1.20 -1.26 (m, 2H), 0.89 -0.92 (m, 3H), 0.81 -0.85 (m, 9H).



Compound **12**

^{13}C NMR (101 MHz, DMSO-d_6):

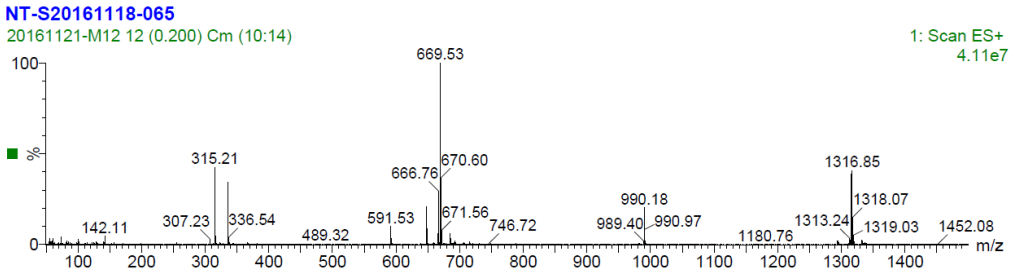
δ179.11, 172.26, 171.89, 171.12, 165.46, 158.96, 158.86, 148.81, 148.66, 121.73, 101.72, 80.48, 58.02, 51.92, 49.21, 47.68, 41.76, 41.03, 38.05, 31.11, 28.15, 27.68, 26.92, 24.69, 23.06, 22.23, 19.46, 18.47, 18.27, 12.23(3).



Compound **12**

ESI-MS:

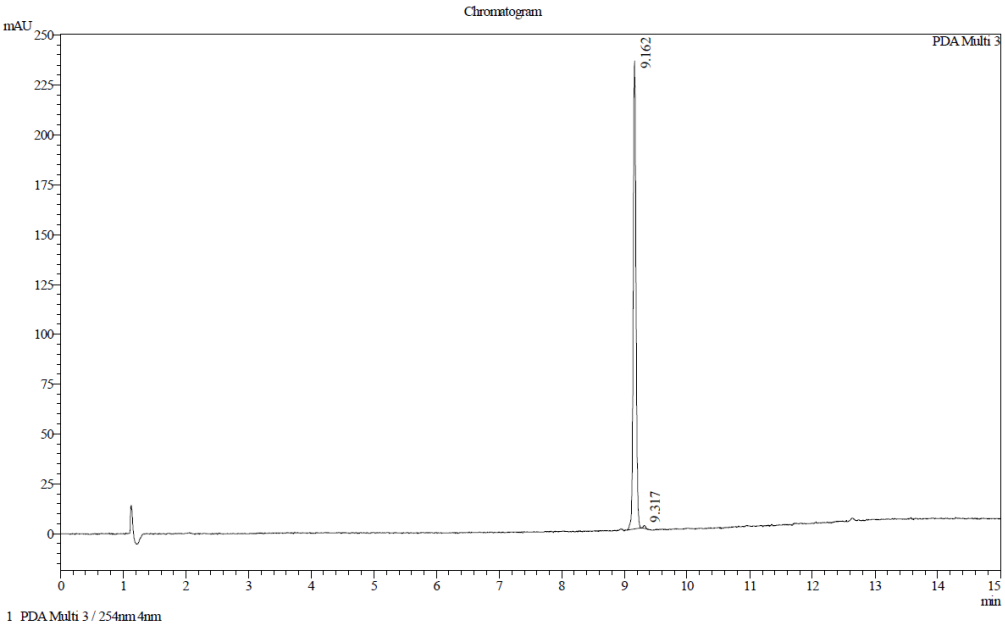
Calculated mass for $[C_{32}H_{50}N_6O_8Na, M+Na]^+$: 669.36 Found: 669.53.



Compound **12**

Analytic HPLC:

Purity: 99.4%



Integration Result

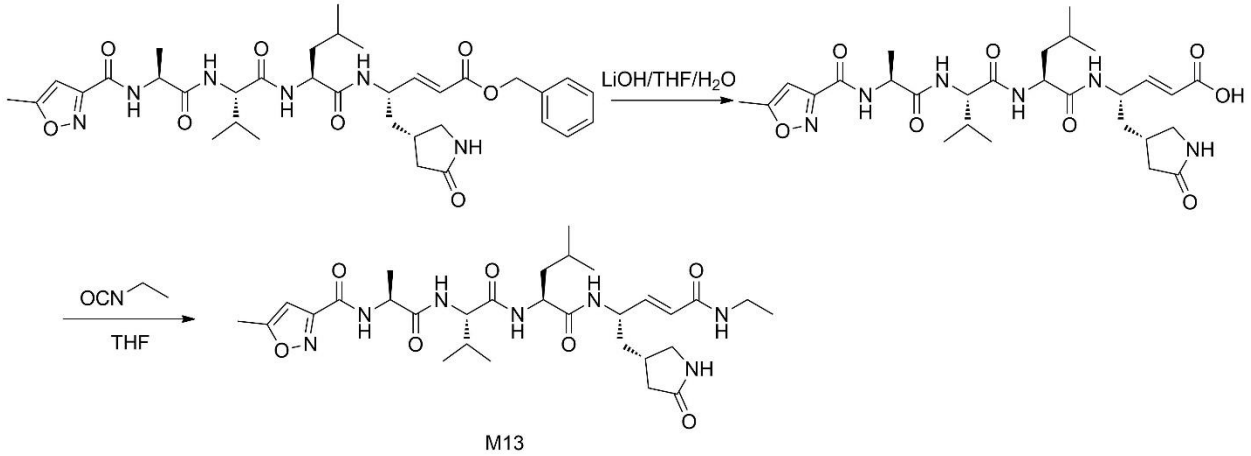
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	9.162	223298	99.373	0.082	719333	99.466
2	9.317	1410	0.627	0.075	3863	0.534

Compound **12**

Compound 13 (Inhibitor M13)

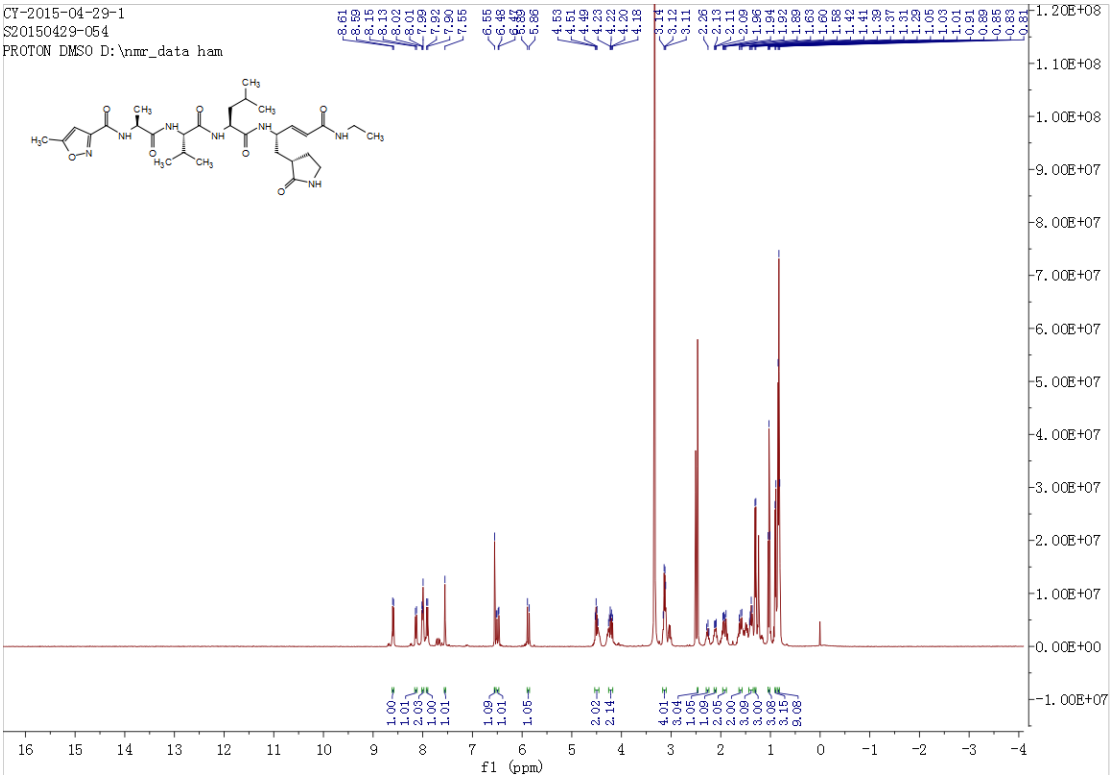
Synthetic Pathway:



Compound **13**

^{1}H NMR (400 MHz, DMSO-d_6):

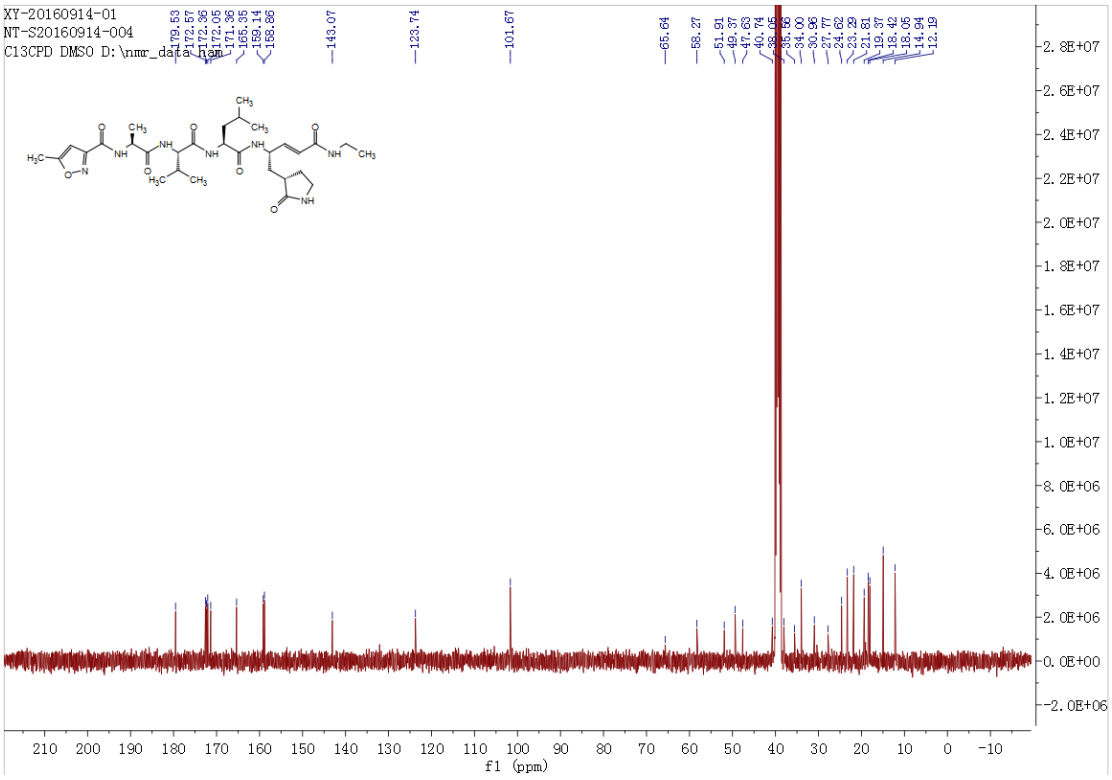
δ 8.60 (d, J = 7.5 Hz, 1H), 8.14 (d, J = 8.8 Hz, 1H), 7.98 – 8.02 (m, 2H), 7.91 (d, J = 8.9 Hz, 1H), 7.55 (s, 1H), 6.55 (s, 1H), 6.47 – 6.52 (m, 1H), 5.87 (d, J = 14.3 Hz, 1H), 4.45 – 4.54 (m, 2H), 4.17 – 4.26 (m, 2H), 3.10 – 3.17 (m, 4H), 2.47 (s, 3H), 2.24 – 2.29 (m, 1H), 2.09 – 2.14 (m, 1H), 1.89 – 1.96 (m, 2H), 1.57 – 1.63 (m, 2H), 1.35 – 1.43 (m, 3H), 1.30 (d, J = 7.1 Hz, 3H), 1.01 – 1.05 (m, 3H), 0.90 (d, J = 6.5 Hz, 3H), 0.82 – 0.85 (m, 9H).



Compound **13**

^{13}C NMR (101 MHz, DMSO-d_6):

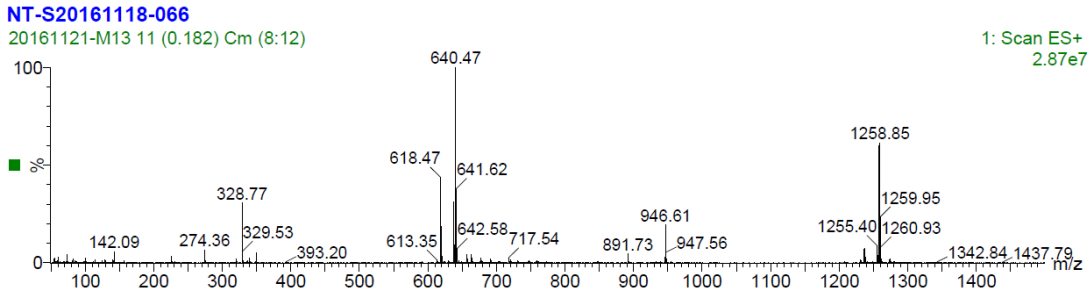
δ 179.53 , 172.57 , 172.36 , 172.05 , 171.36 , 165.35 , 159.14 , 158.86 , 143.07 , 123.74 , 101.67 , 65.64 , 58.27 , 51.91 , 49.37 , 47.63 , 40.74 , 38.05 , 35.56 , 34.00 , 30.96 , 27.77 , 24.62 , 23.29 , 21.81 , 19.37 , 18.42 , 18.05 , 14.94 , 12.19.



Compound **13**

ESI-MS:

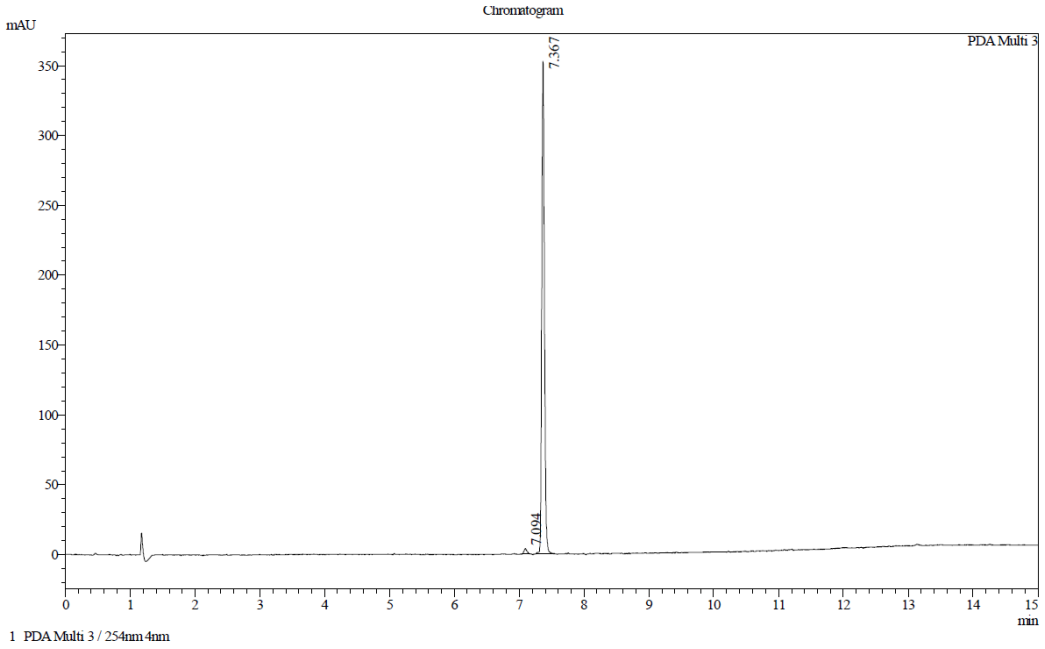
Calculated mass for $[C_{30}H_{47}N_7O_7Na, M+Na]^+$: 640.34 Found: 640.47.



Compound **13**

Analytic HPLC:

Purity: 98.8%



Integration Result

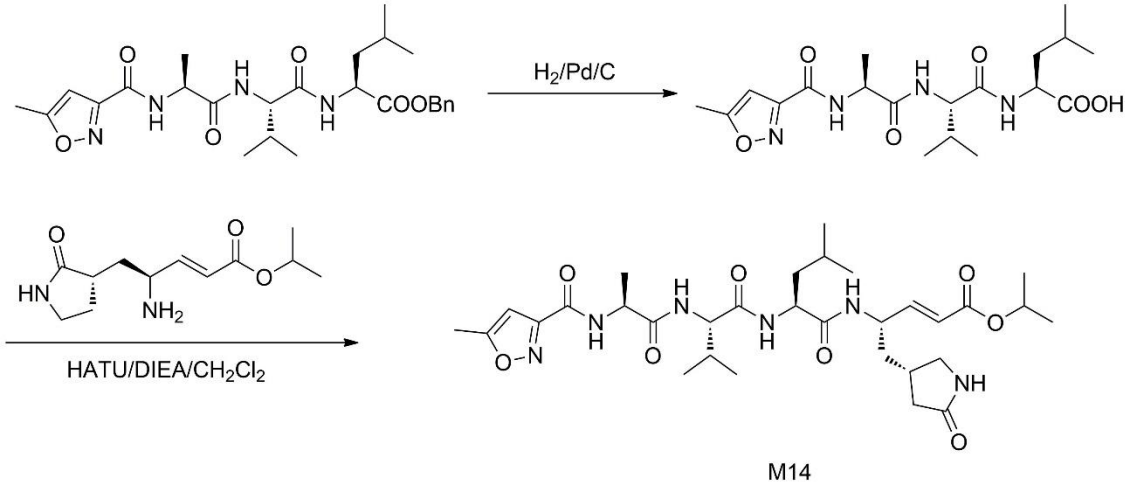
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	7.094	3604	1.034	0.089	11968	1.181
2	7.367	345117	98.966	0.077	1001430	98.819

Compound **13**

Compound 14 (Inhibitor M14)

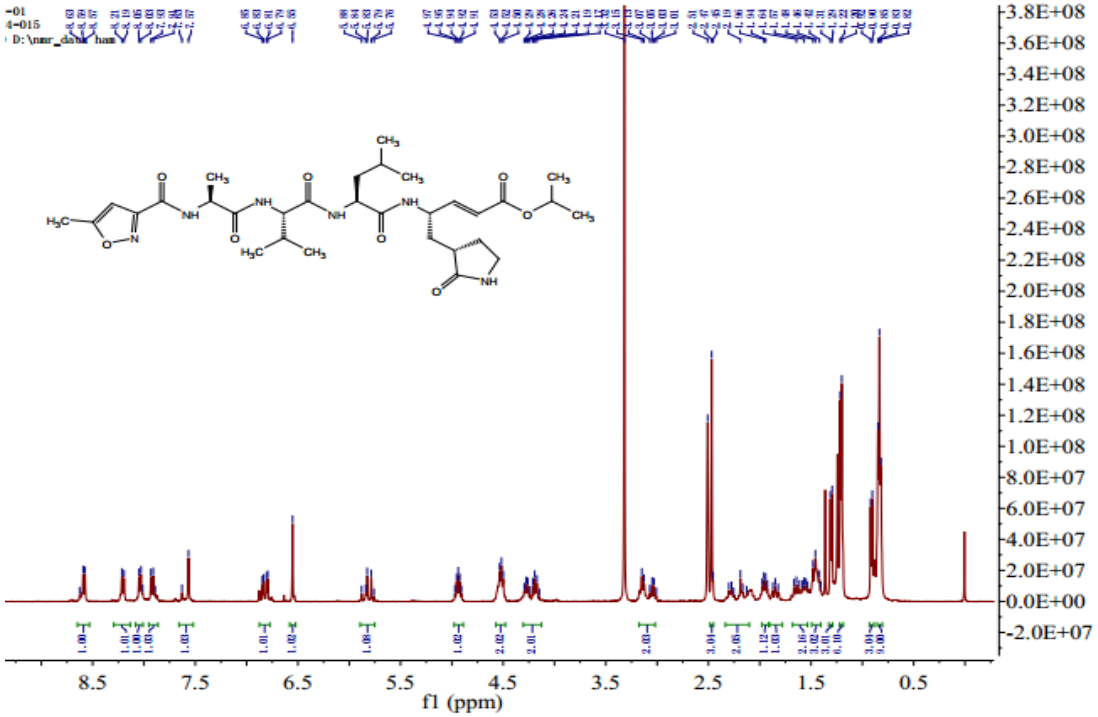
Synthetic Pathway:



Compound **14**

^{1}H NMR (400 MHz, DMSO-d_6):

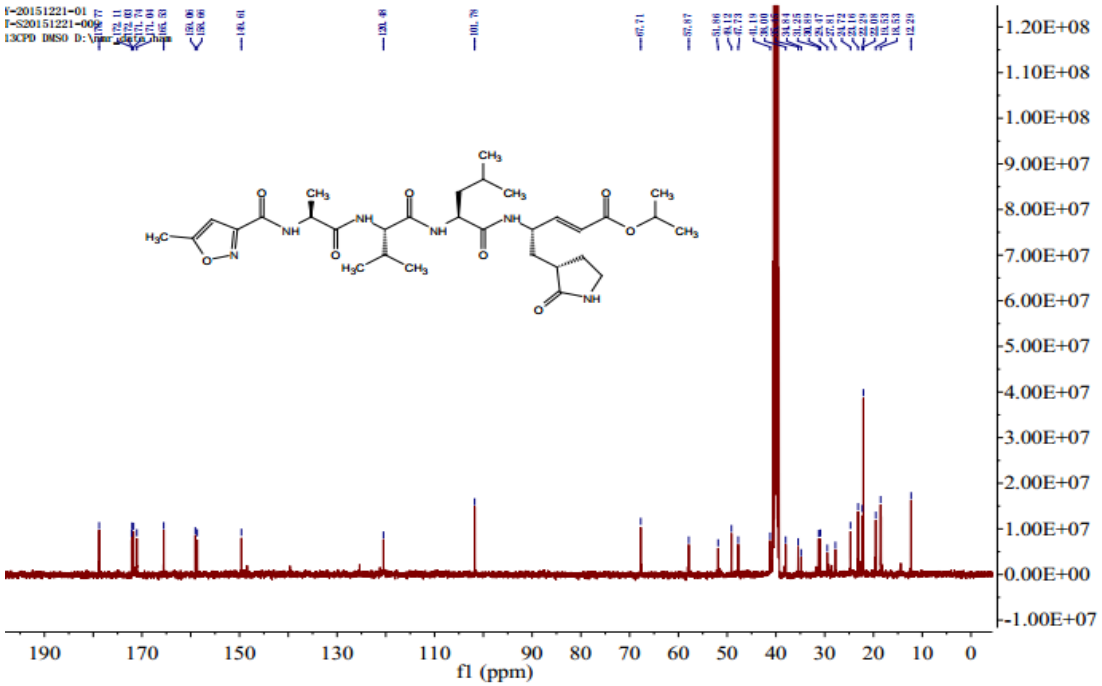
δ 8.58 (d, *J* = 7.5 Hz, 1H), 8.20 (d, *J* = 8.7 Hz, 1H), 8.04 (d, *J* = 7.5 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.57 (s, 1H), 6.82 (m, 1H), 6.55 (s, 1H), 5.81 (d, *J* = 15.8 Hz, 1H), 4.94 (m, 1H), 4.48 -4.58 (m, 2H), 4.12 -4.31 (m, 2H), 3.02 -3.18 (m, 2H), 2.47 (s, 3H), 2.10 -2.33 (m, 2H), 1.92 -1.98 (m, 1H), 1.78 -1.90 (m, 1H), 1.53 -1.68 (m, 2H), 1.40 -1.50 (m, 3H), 1.30 (d, *J* = 7.1 Hz, 3H), 1.21 (d, *J* = 6.0 Hz, 6H), 0.89 -0.93 (m, 3H), 0.81 -0.86 (m, 9H).



Compound **14**

^{13}C NMR (101 MHz, DMSO-d_6):

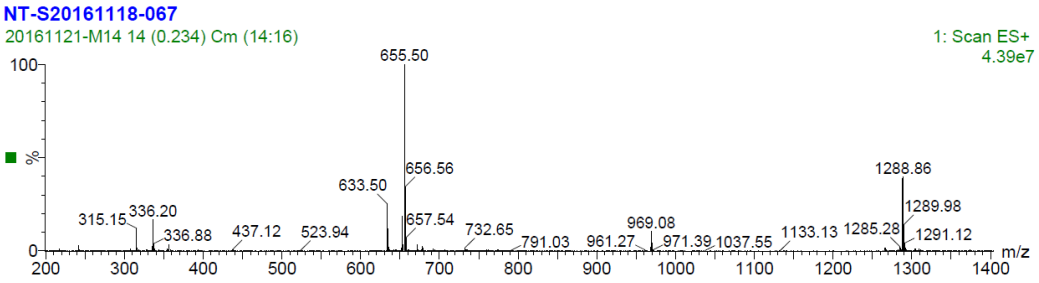
δ178.77, 172.11, 172.03, 171.74, 171.04, 165.53, 159.06, 158.66, 149.61, 120.48, 101.78, 67.71, 57.87, 51.86, 49.12, 47.73, 41.19, 38.00, 35.45, 31.25, 30.89, 29.47, 27.81, 24.72, 23.16, 22.29, 22.08, 19.53, 18.53, 18.44, 12.29.



Compound **14**

ESI-MS:

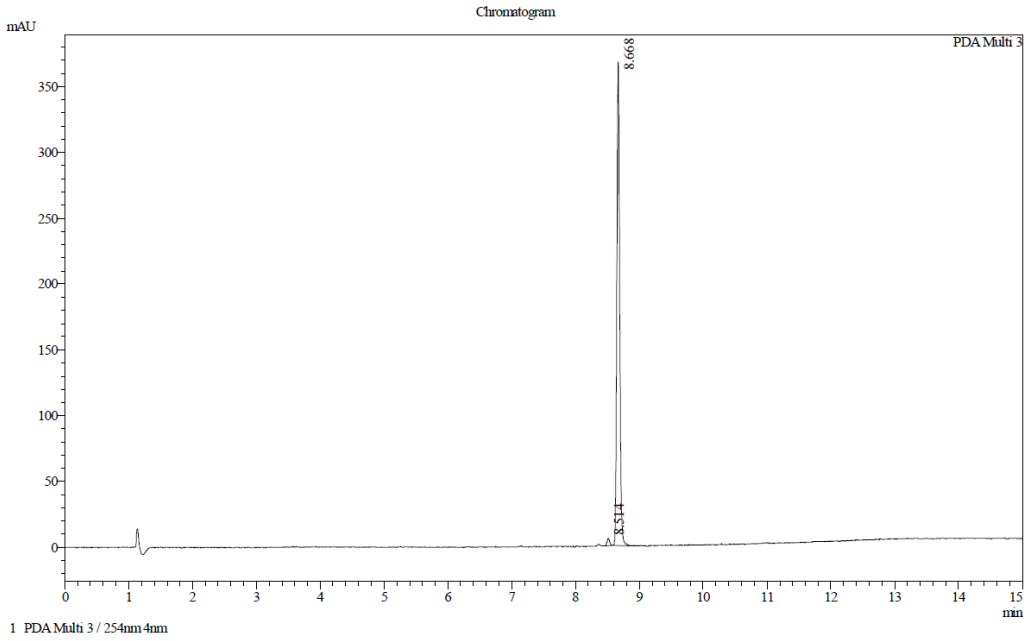
Calculated mass for $[C_{31}H_{48}N_6O_8Na, M+Na]^+$: 655.34 Found: 655.50.



Compound **14**

Analytic HPLC:

Purity: 98.6%



Integration Result

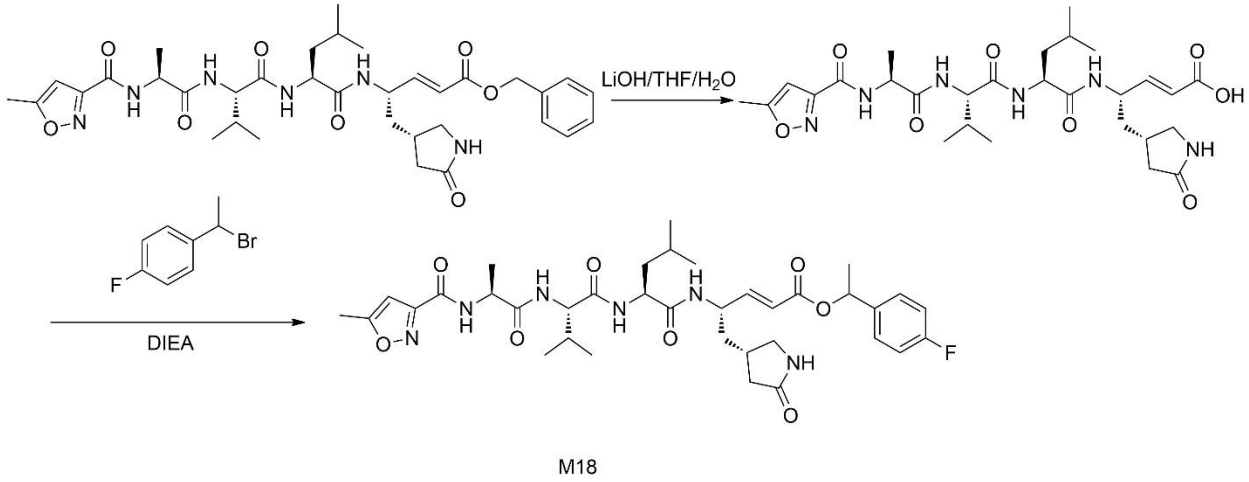
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	8.514	5404	1.528	0.079	15447	1.344
2	8.668	348344	98.472	0.083	1133682	98.656

Compound **14**

Compound 16 (Inhibitor M18)

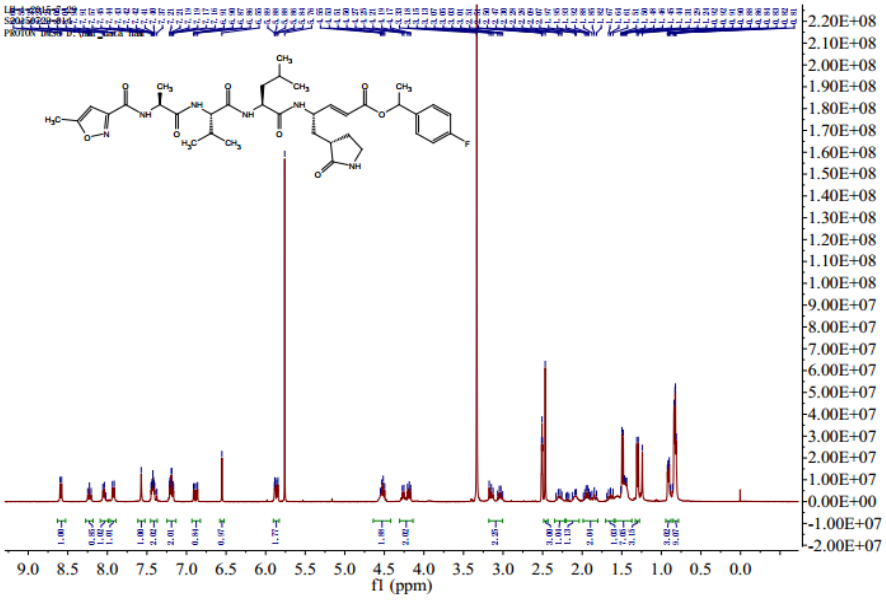
Synthetic Pathway:



Compound **16**

^{1}H NMR (400 MHz, DMSO-d_6):

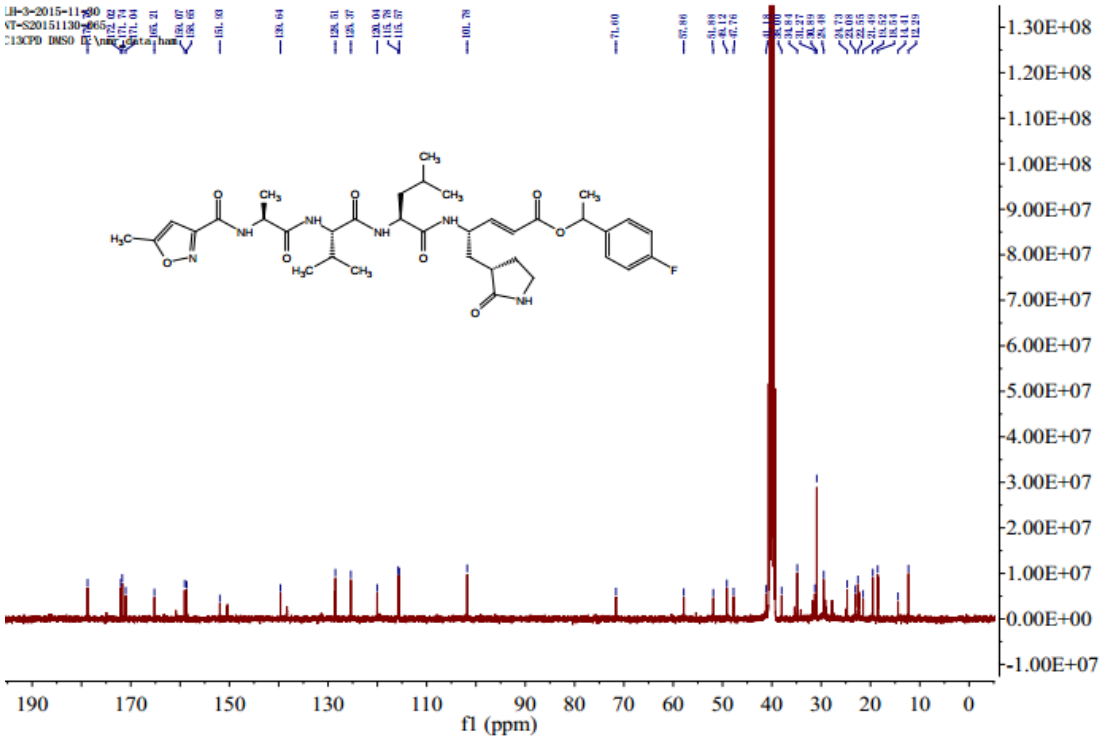
δ 8.59 (d, *J* = 7.5 Hz, 1H), 8.23 (m, 1H), 8.05 (d, *J* = 7.4 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.57 (s, 1H), 7.37 -7.45 (m, 2H), 7.13 -7.25 (m, 2H), 6.89 (m, 1H), 6.55 (s, 1H), 5.87 -5.90 (m, 1H), 5.85 (d, *J* = 5.7 Hz, 1H), 4.41 -4.64 (m, 2H), 4.14 -4.31 (m, 2H), 3.00 -3.19 (m, 2H), 2.47 (s, 3H), 2.22 -2.34 (m, 1H), 2.03 -2.20 (m, 1H), 1.80 -1.99 (m, 2H), 1.60 -1.70 (m, 1H), 1.37 -1.58 (m, 7H), 1.30 (d, *J* = 7.1 Hz, 3H), 0.87 -0.94 (m, 3H), 0.78 -0.85 (m, 9H).



Compound **16**

^{13}C NMR (101 MHz, DMSO-d_6):

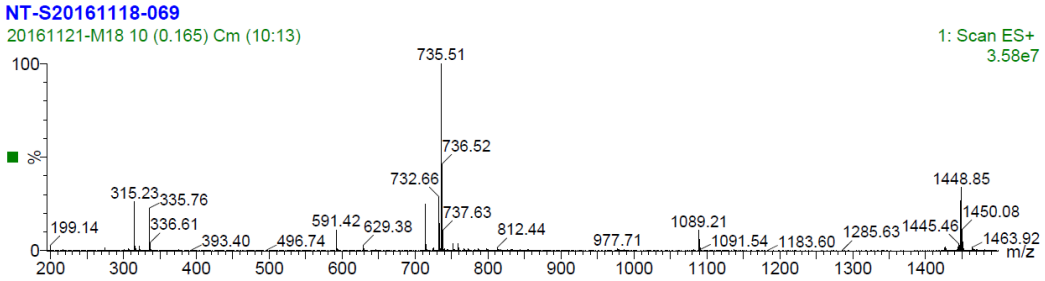
δ178.76, 172.09, 171.74, 171.04, 165.21, 159.07, 158.65, 151.93, 139.64, 128.51 (d,C-F), 125.37, 120.04, 115.78 (2), 115.57 (2), 101.78, 71.60, 57.86, 51.88, 49.12, 47.76, 41.18, 38.00, 34.84, 31.27, 30.89, 29.48, 24.73, 23.08, 22.55, 22.39, 21.49, 19.52, 18.45, 12.29.



Compound **16**

ESI-MS:

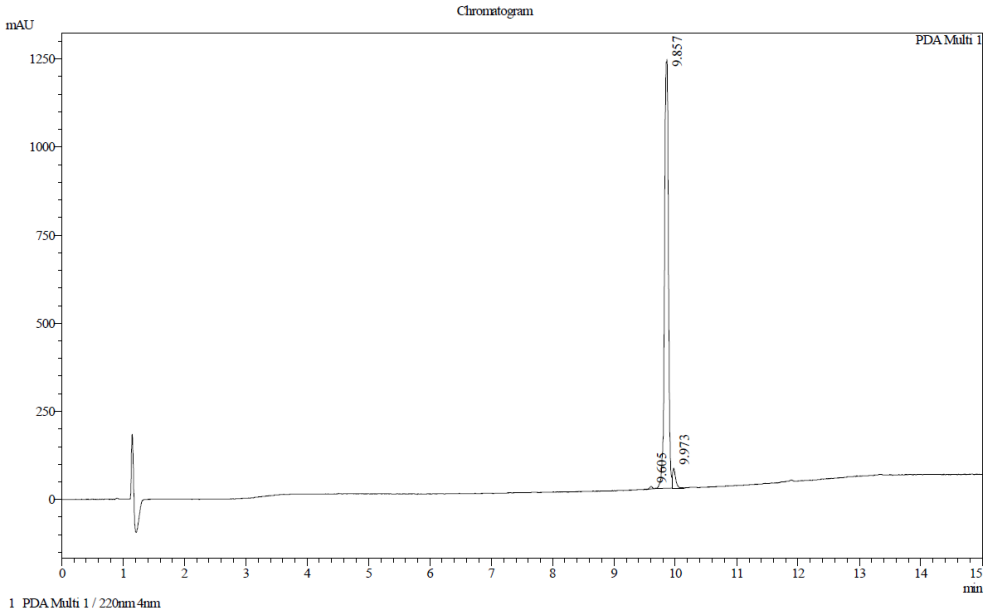
Calculated mass for $[C_{36}H_{49}FN_6O_8Na, M+Na]^+$: 735.35 Found: 735.51.



Compound **16**

Analytic HPLC:

Purity: 96.1%



Integration Result

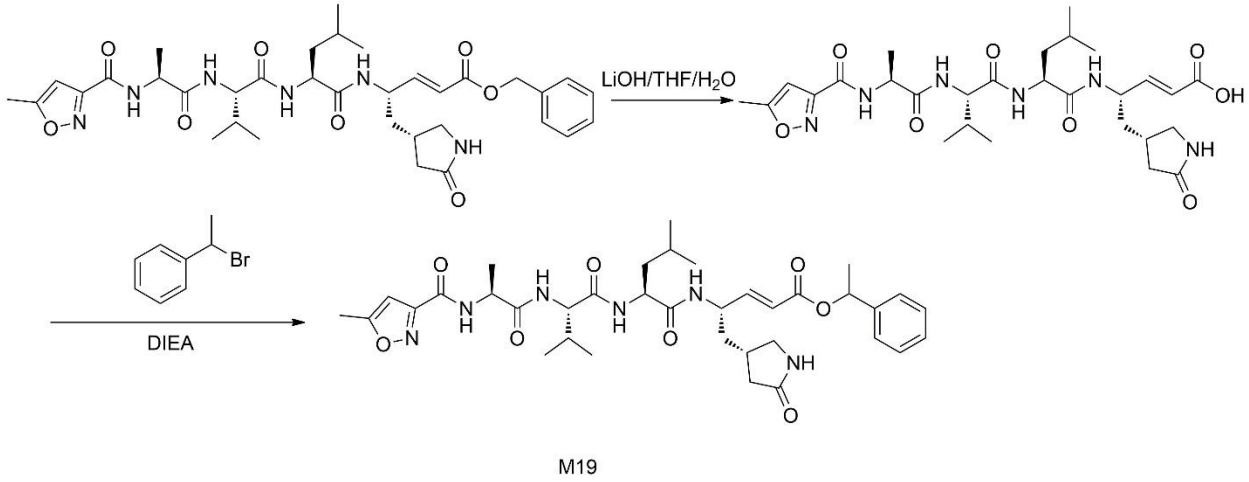
PDA Ch1 220nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	9.605	6792	0.534	0.080	19875	0.352
2	9.857	1211930	95.268	0.106	5434092	96.159
3	9.973	53411	4.199	0.111	197185	3.489

Compound **16**

Compound 17 (Inhibitor M19)

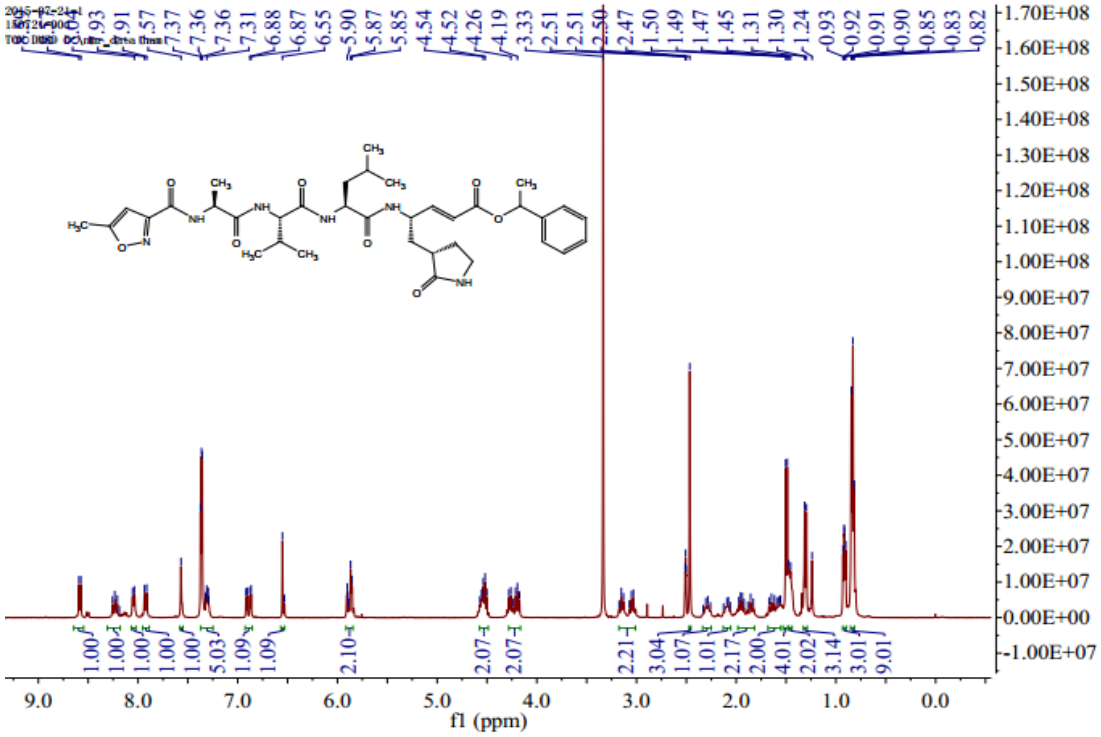
Synthetic Pathway:



Compound **17**

^{1}H NMR (400 MHz, DMSO-d_6):

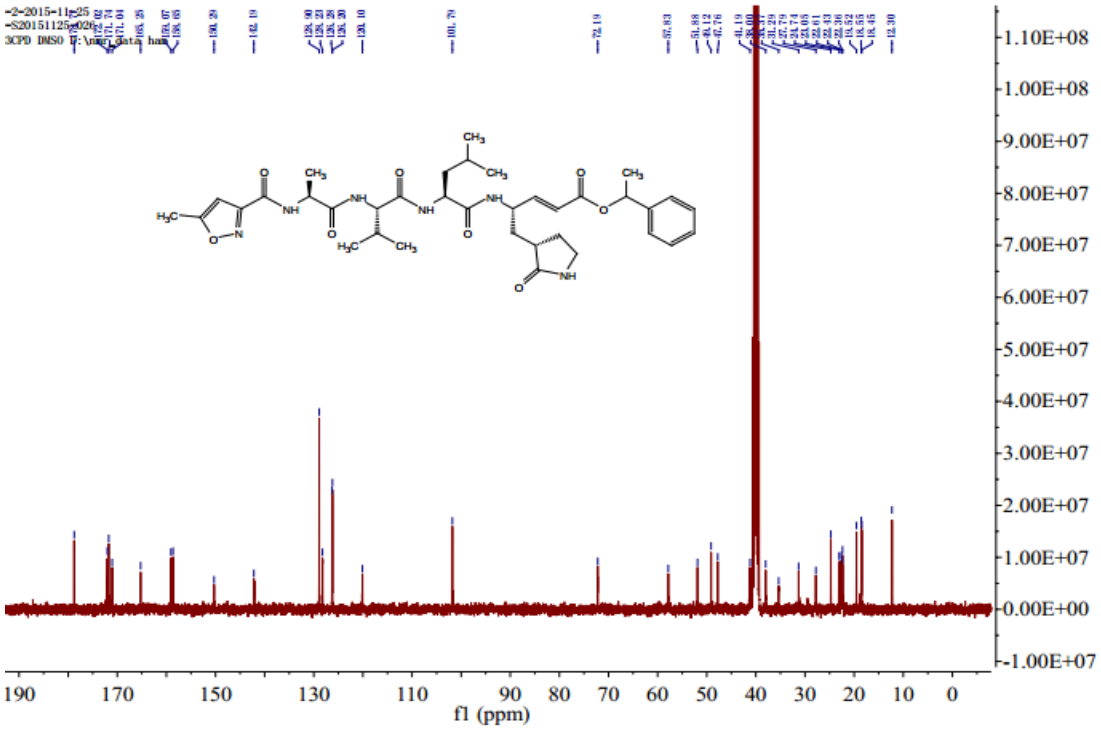
δ 8.58 (d, *J* = 7.5 Hz, 1H), 8.18 -8.31 (m, 1H), 8.05 (d, *J* = 6.8 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.57 (s, 1H), 7.32 -7.39 (m, 5H), 6.89 (m, 1H), 6.55 (s, 1H), 5.84 -5.91 (m, 2H), 4.48 -4.59 (m, 2H), 4.16 -4.28 (m, 2H), 3.01 -3.18 (m, 2H), 2.47 (s, 3H), 2.25 -2.34 (m, 1H), 2.06 -2.13 (m, 1H), 1.82 -1.98 (m, 2H), 1.55 -1.68 (m, 2H), 1.45 -1.51 (m, 6H), 1.30 (d, *J* = 7.1 Hz, 3H), 0.92 (d, *J* = 6.2, 4.7 Hz, 3H), 0.81 -0.85 (m, 9H).



Compound **17**

^{13}C NMR (101 MHz, DMSO-d_6):

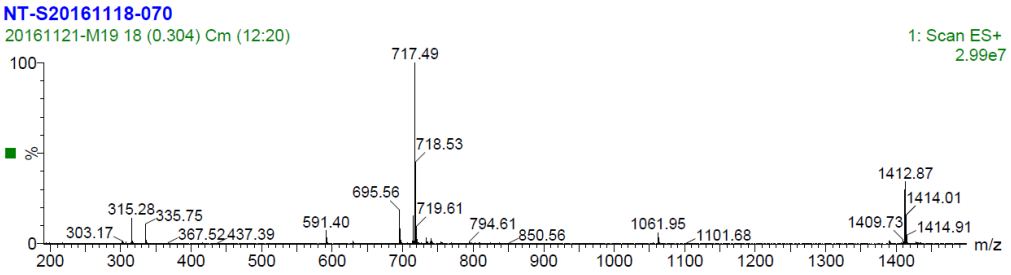
δ178.77, 172.02, 171.74, 171.04, 165.25, 159.07, 158.65, 150.29, 142.19, 128.90(2), 128.23(2), 126.28, 126.20, 120.10, 101.79, 72.19, 57.83, 51.88, 49.12, 47.76, 41.19, 38.00, 31.29, 27.79, 24.74, 23.05, 22.68, 22.61, 22.43, 22.36, 19.52, 18.55, 18.45, 12.30.



Compound **17**

ESI-MS:

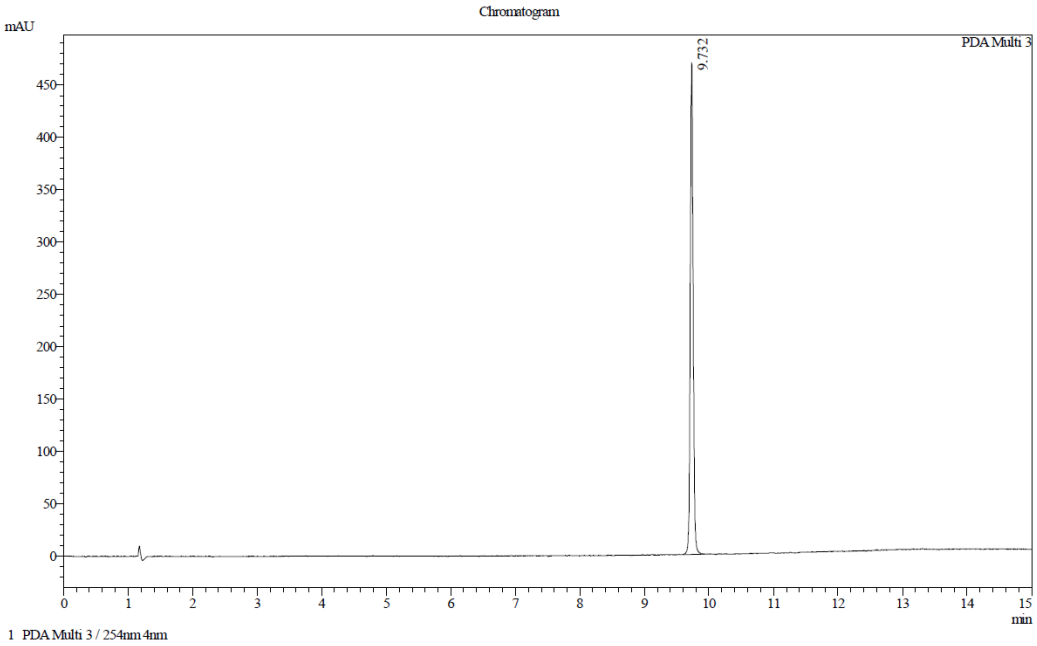
Calculated mass for $[C_{36}H_{50}N_6O_8Na, M+Na]^+$: 717.36 Found: 717.49.



Compound **17**

Analytic HPLC:

Purity: >99.9%



Integration Result

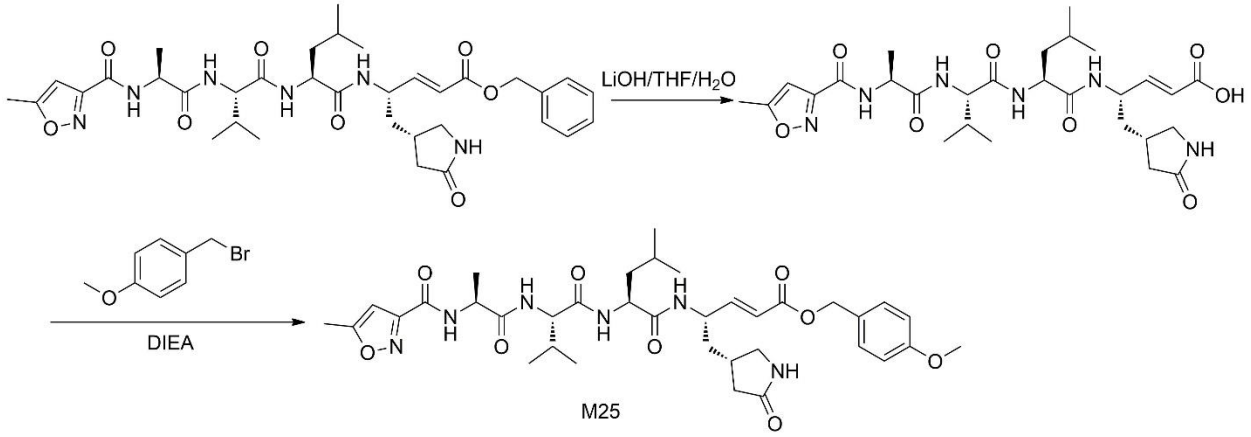
PDA Ch3 254nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	9.732	442076	100.000	0.083	1468938	100.000

Compound **17**

Compound 18 (Inhibitor M25)

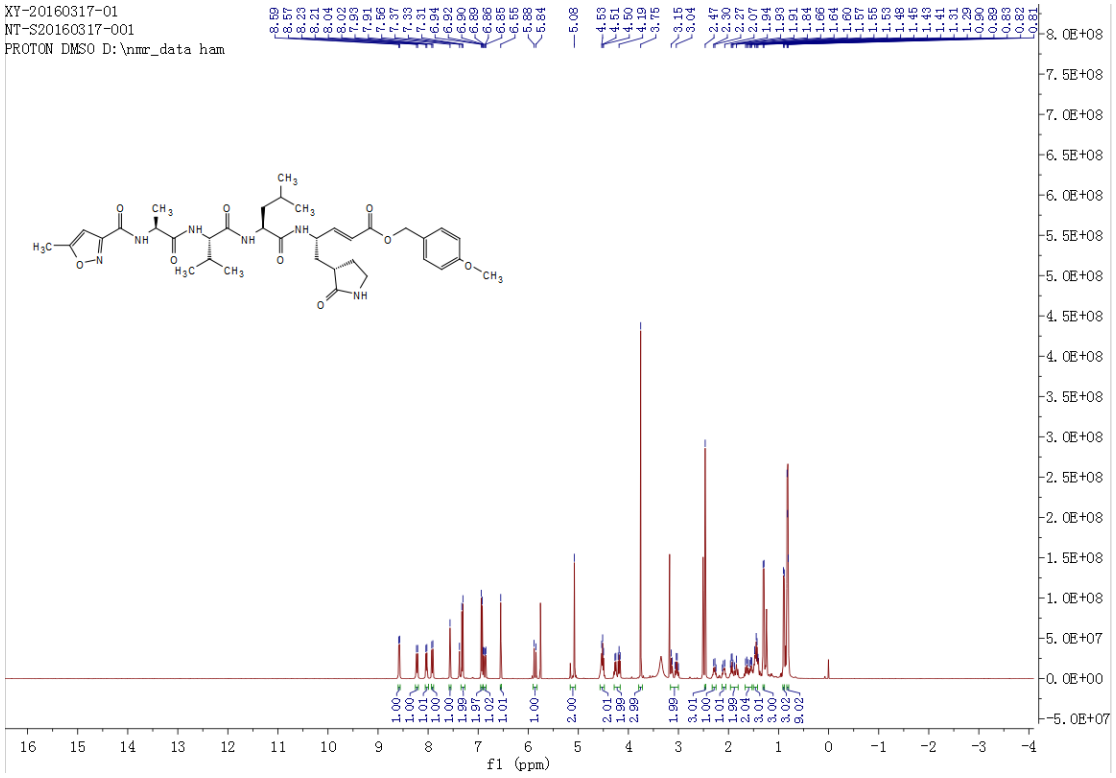
Synthetic Pathway:



Compound **18**

^{1}H NMR (400 MHz, DMSO-d_6):

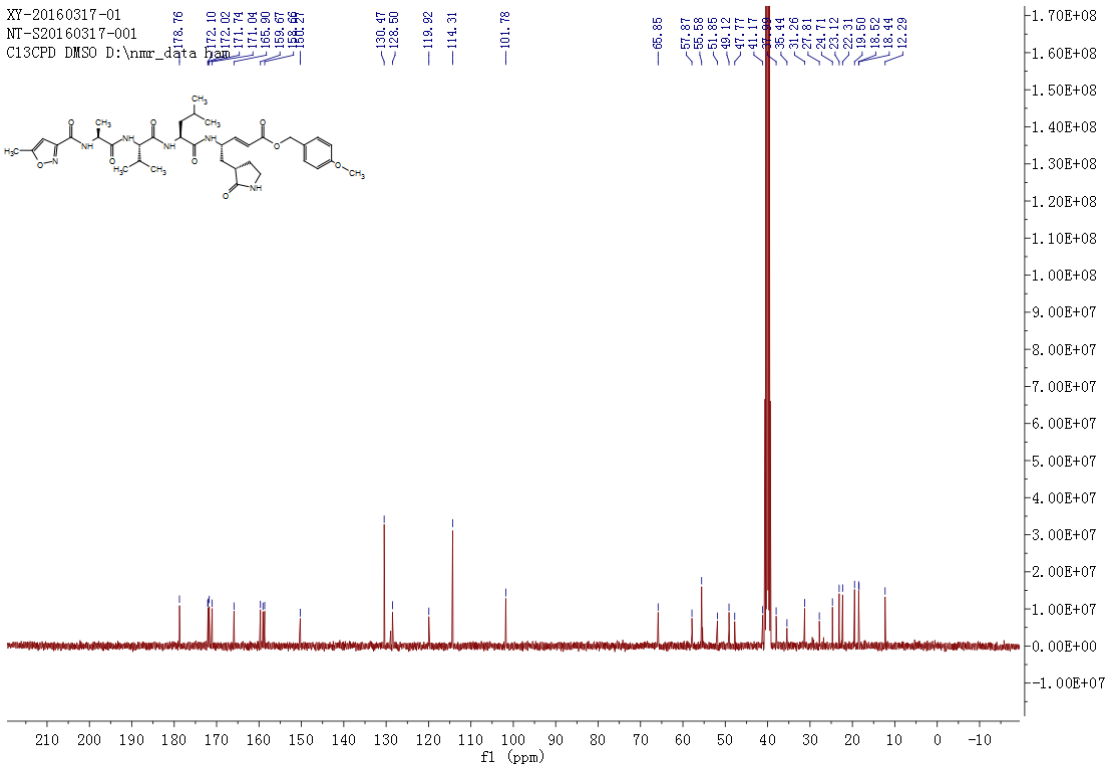
δ 8.58 (d, J = 7.5 Hz, 1H), 8.22 (d, J = 8.7 Hz, 1H), 8.03 (d, J = 7.7 Hz, 1H), 7.92 (d, J = 8.8 Hz, 1H), 7.56 (s, 1H), 7.32 (d, J = 8.6 Hz, 2H), 6.93 (d, J = 8.6 Hz, 2H,), 6.84 – 6.91 (m, 1H), 6.55 (s, 1H), 5.86 (d, J = 15.7 Hz, 1H), 5.08 (s, 2H), 4.48 – 4.57 (m, 2H), 4.16 – 4.28 (m, 2H), 3.75 (s, 3H), 3.00 – 3.16 (m, 2H), 2.47 (s, 3H), 2.25 – 2.33 (m, 1H), 2.05 – 2.13 (m, 1H), 1.81 – 1.96 (m, 2H), 1.53 – 1.67 (m, 2H), 1.42 – 1.50 (m, 3H), 1.30 (d, J = 7.1 Hz, 3H), 0.89 (d, J = 6.5 Hz, 3H), 0.80 – 0.84 (m, 9H).



Compound **18**

^{13}C NMR (101 MHz, DMSO-d_6):

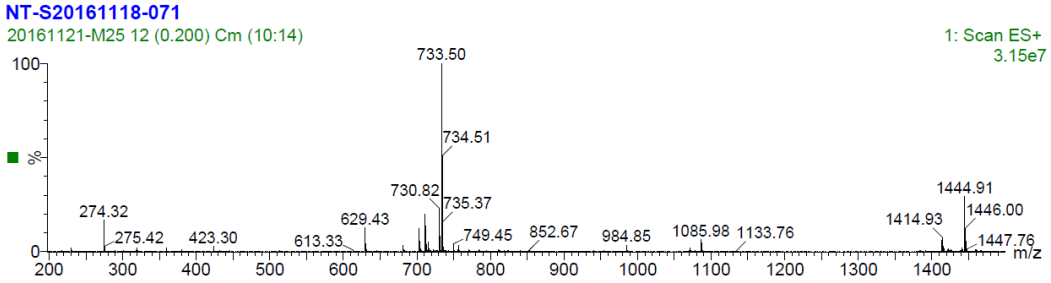
δ 178.76 , 172.10 ,172.02,171.74, 171.04 , 165.90 , 159.67 , 159.07 , 158.66 , 150.27 , 130.47 , 128.50(2) , 119.92(2), 114.31 , 101.78 , 65.85 , 57.87 , 55.58 , 55.37 , 51.85 , 49.12 , 47.77 , 41.17 , 37.99 , 35.44 , 31.26 , 27.81 , 24.71 , 23.12 , 22.31 , 19.50 , 18.52 , 18.44 , 12.29.



Compound **18**

ESI-MS:

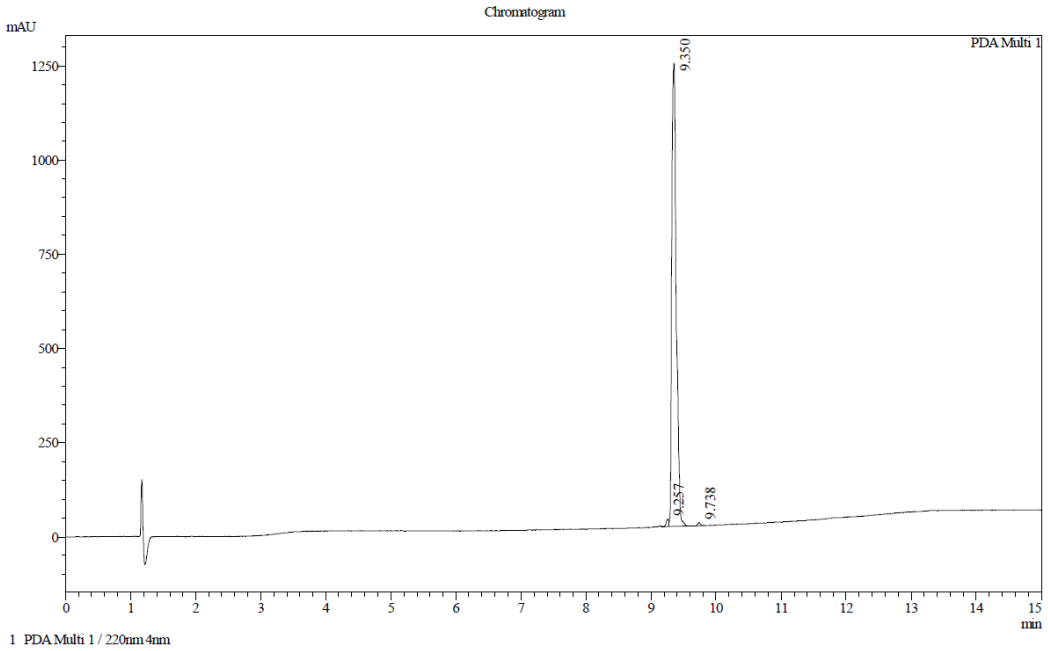
Calculated mass for $[C_{36}H_{50}N_6O_9Na, M+Na]^+$: 733.35 Found: 733.50.



Compound **18**

Analytic HPLC:

Purity: 98.6%



Integration Result

PDA Ch1 220nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	9.257	19005	1.520	0.102	54776	0.916
2	9.350	1223330	97.821	0.107	5898721	98.665
3	9.738	8248	0.660	0.078	25029	0.419

Compound **18**

Supplementary References:

1. Yang, H.; Xie, W.; Xue, X.; Yang, K.; Ma, J.; Liang, W.; Zhao, Q.; Zhou, Z.; Pei, D.; Ziebuhr, J.; Hilgenfeld, R.; Yuen, K. Y.; Wong, L.; Gao, G.; Chen, S.; Chen, Z.; Ma, D.; Bartlam, M.; Rao, Z. Design of wide-spectrum inhibitors targeting coronavirus main proteases. *PLoS Biol.* **2005,** *3*, e324.
2. Wang, F.; Chen, C.; Tan, W.; Yang, K.; Yang, H. Structure of main protease from human coronavirus NL63: insights for wide spectrum anti-coronavirus drug design. *Sci. Rep.* **2016,** *6*, 22677.
3. Zhao, Q.; Li, S.; Xue, F.; Zou, Y.; Chen, C.; Bartlam, M.; Rao, Z. Structure of the main protease from a global infectious human coronavirus, HCoV-HKU1. *J. Virol.* **2008,** *82*, 8647-8655.
4. Rao, Z.; Yang, H.; Fu, S.; Yang, C.; Wang, Z.; Cai, Y.; Li, S.; Guo, Y.; Liu, H. Small molecule inhibitors of coronavirus main protease: their preparation and application. China Patent 201510079787, Feb 15, 2015.
5. Tan, Y.; Wang, F.; Chen, X.; Wang, J.; Zhao, Q.; Li, S.; Wang, Z.; Fu, S.; Chen, C.; Yang, H. Crystallization and preliminary crystallographic study of Porcine epidemic diarrhea virus main protease in complex with an inhibitor. *Acta Crystallogr. Sect. F Struct. Biol. Commun.* **2014,** *70*, 1608-1611.
6. Otwinowski, Z.; Minor, W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. In *Macromolecular Crystallography, part A*, Carter, C. W., Jr.; Sweet, R. M., Eds. Academic Press: New York, NY, 1997; Vol. 276, pp 307-326.
7. Xue, X.; Yu, H.; Yang, H.; Xue, F.; Wu, Z.; Shen, W.; Li, J.; Zhou, Z.; Ding, Y.; Zhao, Q.; Zhang, X. C.; Liao, M.; Bartlam, M.; Rao, Z. Structures of two coronavirus main proteases: implications for substrate binding and antiviral drug design. *J. Virol.* **2008,** *82*, 2515-2527.
8. Tian, W. X.; Tsou, C. L. Determination of the rate constant of enzyme modification by measuring the substrate reaction in the presence of the modifier. *Biochemistry* **1982,** *21*, 1028-1032.