Design, synthesis, and antitumor activity of cyclic peptide-lenalidomide conjugated small molecules

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1．Experimental Section

1.1 General Experimental Procedures

Boc-L-isoleucine (13139-16-7), Boc-L-leucine（13139-15-6）, Boc-L-proline（15761-39-4）, Boc-L-isoleucine（13139-16-7）, BON-Ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinline (EEDQ, 16357-59-8), Lenalidomide(191732-72-6), Chloroacetyl chloride(79-04-9), N-[(Trimethylsilyl)methyl]-benzylamine (53215-95-5), Phthalylglycyl chloride (6780-38-7) and Trifluoroacetic acid (TFA, 76-05-1) were purchased from Energy Chemical. Dichloromethane, methanol, ethyl acetate, petroleum ether, acetone, N,N-Dimethylformamide(DMF), tetrahydrofuran(THF), 1, 4-Dioxane were analytical reagent. Roswell Park Memorial Institute (RPMI-1640), penicillin, fetal bovine serum (FBS), and streptomycin were purchased from Beijing Dingguo Biotechnology Co. Phosphate buffered saline (PBS) purchased from Invitrogen (10010) was used as a balanced salt solution in cell culture. Acridine orange acts as a fluorescent indicator of cellular autophagy. All the solvents were distilled and purified by standard procedures. All the above chemicals reagents were used without further purification. ^{1}H and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively, on an AMX400 spectrometer (Bruker, Bremen, Germany) with tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded on a JEOL JMS-700 spectrometer using the fast atom bombardment (FAB) or electron impact (EI) mode.

1.2 Preparation of 3-Hydroxy-isoindolinone-cyclo-(Gly-Ile-Ile) (1)

The carboxyl group on tert-butyl carbamate isoleucine (N-Boc-isoleucine) (1.0 g, 4.32mmol) was condensed with N-benzyl-1-(trimethylsilyl)-methylamine (BnTMSA) (0.83g, 4.32mmol) using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (1.28g, 5.18mmol) as the condensation agent on the amino group. The crude product obtained was treated directly with trifluoroacetic acid (TFA) (2~3ml) to deprotect the t-Boc group. The deprotected product (1.0g, 3.26mmol) was then condensed with N-Boc-Ile by using EEDQ (0.8g, 3.26mmol) as a condensation agent, after which the t-Boc group was deprotected by TFA (2~3ml) to obtain a linear peptide. The linear peptide was then acylated with phthalylglycyl chloride under dilute conditions to obtain

the photoactive precursor linear peptide. Finally, the cyclic peptides were generated by photoreaction in a JRS-7825-34 (Julabo, Germany) photoreactor using a 450 W medium-pressure mercury lamp (Hanovia) capable of delivering 200-400 nm UV radiation. The linear peptides were purified by column chromatography and identified by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry.

1.3 Preparation of 3-Hydroxy-isoindolinone-cyclo-(Gly-Ile-Pro-Pro-Leu) (2)

First, the carboxyl group on tert-butyl phenylalanine carbamate (N-Boc-leucine) (1.5g, 6.48mmol) was condensed with the amino group on N-benzyl-1-(trimethylsilyl)-methylamine (BnTMSA) (1.25g, 6.48mmol) using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (1.9g, 7.78mmol) as a condensation agent. The crude product obtained was treated directly with trifluoroacetic acid (TFA) (3~4.5ml) to deprotect the t-Boc group. The deprotected product was then successively condensed with N-Boc-amino acids by using EEDQ as a condensation agent, after which the t-Boc group was deprotected by TFA to obtain linear peptide. The linear peptide was then acylated with phthaloylglycyl under dilution conditions to obtain photoactive precursors. Finally, the photoreaction was performed in a JRS-7825-34 (Julabo, Germany) photoreactor using a 450 W medium-pressure mercury lamp (Hanovia) capable of delivering 200-400 nm UV radiation to generate cyclic peptides, which were identified by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry.

1.4 Preparation of 3-Lenalidomide-acetoxyl-isoindolinone-cyclo-(Gly-Ile-Ile) (5)

The cyclo-(Gly-Ile-Ile) (1.0g, 1.87 mmol) and triethylamine (0.78ml, 5.61mmol) were dissolved in 10ml of tetrahydrofuran solution, and cooled in ice bath. Chloroacetyl chloride (0.16ml, 2.0mmol) was added drop by drop, then the reaction was carried out at room temperature for 24 h under nitrogen protection. The product was dissolved in ethyl acetate (10ml), and the precipitated triethylamine hydrochloride was removed by filtration. The filtrate was concentrated to obtain the chloroacetyl-cyclo- (Gly-Ile-Ile), which was dissolved with acetone (10ml), and NaI (0.27g, 1.8mmol) was added, and the reaction was refluxed at 75℃ for 3h. The solvent was then removed by reduced pressure distillation. The obtained product was dissolved with DMF (10ml), and added lenalidomide (0.47g, 1.80 mmol) and potassium (0.50g, 3.60mmol) carbonate were.

After 36h stirring at 70℃ for, the mixture was concentrated, and the crude product was purified by column chromatography to obtain 3-Lenalidomide-acetoxyl-isoindolinone-cyclo-(Gly-Ile-Ile).

1.5 Preparation of 3-lenalidomide-acetoxyl-isoindolinone-cyclo-(Gly-Ile-Pro-Pro-Leu) (6)

Cyclo-(Gly-Ile-Pro-Pro-Leu) (1.0g, 1.37mmol) synthesized by photo-induce reaction previously was used as raw material. The cyclopeptide and triethylamine (0.57ml，4.11mmol) were dissolved in tetrahydrofuran solution (10ml) and cooled in an ice bath. Chloroacetyl chloride (0.16ml, 2.0mmol) was added drop by drop, then the reaction was carried out at room temperature for 24 h under nitrogen protection. After the reaction was finished, the product was dissolved in ethyl acetate (10ml), and the precipitated triethylamine hydrochloride was filtered. The filtrate was concentrated to obtain the chloroacetyl-cyclo-(Gly-Ile-Pro-Pro-Leu), which was dissolved with acetone (10ml), and NaI (0.24g, 1.5mmol) was added, and the reaction was refluxed at 75℃ for 3h. The solvent was then concentrated. The obtained product was and dissolved in DMF (10ml), and added lenalidomide (0.35g, 1.38 mmol) and potassium (0.51g, 3.72mmol l) carbonate. After 48h stirring at 70℃ for, the mixture was concentrated, and the crude product was purified by column chromatography to obtain 3-Lenalidomide-acetoxyl-isoindolinone-cyclo-(Gly-Ile-Ile). The spectroscopic data are shown below.

3-Hydroxy-isoindolinone-cyclo-(Gly-Ile-Ile)（1）

White solid (yield 60%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.70~0.97 (m,12H,$(CH_3)_4$), 1.12~1.28(m, 4H, CH_2,), 1.99~2.04 (m, 2H, CH_2(**CH**)$_2CH_3$), 3.74(m, 1H, NCH2), 3.89~4.01(m, 3H, NCH_2), 4.41(m, 1H, NHCH), 4.52(m, 1H, NH**CH**CH_2), 4.59~5.01(m, 2H, N**CH$_2$**Ph), 7.24~7.25(m, 2H, phH), 7.27~7.44(m, 3H, ph), 7.50~7.58(m, 3H, Phthaloyl), 8.04(d, 1H, Phthaloyl). ^{13}C NMR (CDCl3, 300 MHz) δ: 10.73, 11.89, 14.74, 16.68, 24.78, 25.71, 34.44, 35.56, 44.52, 52.03, 56.30, 57.51, 59.62, 77.00, 87.90, 123.62, 124.32, 127.08, 127.62, 128.02, 128.10, 128.60, 129.04, 129.65, 134.53, 136.25, 144.43, 168.82, 170.04, 171.64, 173.07. 170.6. MS (ESI) m/z calcd for $C_{30}H_{38}N_4O_5$ Na^+ $(M+Na)^+$ 557.3, found 557.3.

3-Hydroxy-isoindolinone-cyclo-(Gly-Ile-Pro-Pro-Leu)（2）

White solid (yield 35%). ^{1}H NMR ($CDCl_3$) δ: 0.86-1.00 (m, 12H, CH3), 1.27-1.83 (m, 13H, **CH_2**CH_3 and **C**H(CH_3)$_2$ and **CH_2**$CHCH_3$ and NCH_2**CH_2**CH_2CH and NCH_2CH_2**CH_2**CH), 2.77-2.95 (m, 1H, **C**HCH_2CH_3), 3.00-3.10 (m, 2H, N**CH_2**C(OH)), 3.13-4.22 (m, 6H, N**CH_2**CH_2CH_2CH and N**CH_2**CO), 4.39-4.46 (m, 1H, **C**H$CHCH_2CH_3$), 4.60-4.64 (m, 2H, $NCH_2CH_2CH_2$**CH**), 5.10-5.52 (m, 3H, **C**H$CH_2CH(CH_3)_2$ and **CH_2**Ph), 7.11-7.39 (m, 5H, ArH), 7.70-7.88 (m, 4H, Phthaloyl); ^{13}C NMR (CDCl3) δ: 14.2,16.6, 22.1, 23.4, 23.6, 24.9, 30.8, 40.2, 41.6, 42.6, 46.8, 47.1, 47.6, 53.2, 61.6, 96.8,123.4, 123.6, 126.8, 128.9, 132.3, 133.9, 134.1, 134.2, 136.6, 166.0, 166.3, 167.7, 167.9, 170.7, 173.2. MS (ESI) m/z calcd for $C_{40}H_{52}N_6O_7Na^+$ $(M+Na)^+$ 751.4, found 751.5.

N-phthalimido-Gly-Ile-Pro-Pro-Leu-Si(CH_3)$_3$ (2a)

White solid (yield 68%). ^{1}H NMR ($CDCl_3$) δ: 0.04-0.15 (m, 9H, $SiMe_3$), 0.78-1.06 (m, 12H, CH_3), 1.14-1.21 (m, 1H, **C**H(CH_3)$_2$), 1.29-1.36 (m, 2H, **CH_2**CH_3), 1.59-1.62 (m, 2H, **CH_2**CH(CH_3)$_2$), 1.87-2.19 (m, 9H, N**CH_2**CH_2CH_2CH and N**CH_2**CH_2CH_2CH and **C**HCH_2CH_3), 2.63-2.90 (m, 2H, **CH_2**$SiMe_3$), 3.59-3.83 (m, 4H, N**CH_2**CH_2CH_2CH), 4.12-4.45 (m, 3H, $NCH_2CH_2CH_2$**CH** and **C**H$CH_2CH(CH_3)_2$), 4.53-4.95 (m, 5H, **C**H$CHCH_2CH_3$ and **CH_2**Ph and N**CH_2**CO), 7.19-7.23 (m, 2H, ArH), 7.30-7.39 (m, 3H, ArH), 7.76-7.92 (m, 4H, Phthaloyl); ^{13}C NMR ($CDCl_3$) δ: -1.2, 10.9, 15.2, 21.7, 23.3, 24.4, 24.7, 27.8, 28.7, 37.8, 38.9, 40.4, 42.5, 47.1, 47.7, 53.2, 55.1, 58.0, 59.7, 61.4, 77.8 (solvent peak), 123.5, 126.8, 127.6, 128.8, 132.1, 134.1, 136.5, 166.0, 167.8, 170.7, 170.9, 171.0, 171.5. MS (ESI) m/z calcd for $C_{43}H_{60}N_6O_7SiNa^+$$(M+Na)^+$823.4, found 823.4.

3-Lenalidomide-acetoxyl-isoindolinone-cyclo-(Gly-Ile-Ile) (5)

Yellow solid (yield 18%). ^{1}H NMR (DMSO) δ:0.61~0.94 (m, 12H, $(CH_3)_4$), 1.31~1.34 (m, 4H, (**CH_2**)$_4$CH3), 1.67(m, 1H, NHCH**CH_2**CH2CO), 1.88~2.02 (m, 3H, CH_3**C**HCH, CH_2**CH_2**CONH), 2.66~2.72(m, 2H, **CH_2**CONH),4.05~4.18(m, 4H, NH**(CH_2)$_2$**), 4.51~4.61(m, 6H, N**(CH_2)$_2$**,NH**C**H, NH**C**HCO), 4.66~4.72(m, 3H, NCHCO, Ph**CH_2**), 6.81 (m, 1H, Phthaloyl), 7.14~7.32(m, 6H, PhH), 7.50~7.58(m, 3H, Phthaloyl), 7.77 (m, 1H, $PhCH_2$), 7.94 (m, 1H, Phthaloyl). ^{13}C NMR (DMSO) δ: 10.45,

11.79, 15.26, 15.99, 23.18, 24.51, 25.13, 30.01, 36.58, 37.58, 44.70, 46.42, 47.12, 51.60, 56.25, 57.92, 59.42, 59.80, 93.87, 116.39, 117.25, 123.27, 123.59, 125.16, 126.76, 126.92, 127.30, 127.62, 127.90, 129.13, 129.29, 129.45, 130.79, 133.48, 137.46, 140.63, 144.66, 167.55, 167.71, 168.20, 169.54, 169.86, 170.19, 171.53, 172.23, 172.55. MS (ESI) m/z calcd for $C_{45}H_{51}N_7O_9Na^+$ $(M+Na)^+$ 856.3, found 856.3.

3-lenalidomide-acetoxyl-isoindolinone-cyclo-(Gly-Ile-Pro-Pro-Leu) (6)

Yellow solid (yield 15%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ:0.87~0.93(m, 12H, $(CH_3)_4$), 1.22~1.40（m, 2H, $CH_3\mathbf{CH_2}$）, 1.65~1.67(m, 4H, $CH_3(\mathbf{CH})_2CH_2$, $NCH\mathbf{CH_2}CH_2$), 1.73~1.91(m, 7H, $NCH_2(\mathbf{CH_2})_3$, $CH_3\mathbf{CH}CHNH$), 1.97~2.23(m, 4H, $NCH(\mathbf{CH_2})_2$), 2.51~2.59(m, 2H, $CO\mathbf{CH_2}CH_2$), 3.32~3.58(m, 4H, $N(\mathbf{CH_2})_2CH_2$), 4.15~4.24(m, 7H, $N(\mathbf{CH_2})_3$, $N\mathbf{CH_2}C$), 4.39~4.45(m, 4H, $N(\mathbf{CH_2})_2CH$), 4.56~4.63(m, 4H, NCH, $CH_2NCH\mathbf{CH_2}$, NCHCO), 7.25~7.33(m, 6H, PhH), 7.54~7.66(m, 3H, Phthaloyl), 7.80(m, 1H, Phthaloyl), 8.04(m, 1H, ArH). ^{13}C NMR (DMSO, 300 MHz) δ: 10.21, 15.39, 22.74, 24.28, 25.24, 26.27, 28.45, 29.64, 30.80, 37.91, 39.50, 40.34, 43,52, 45.88, 46.72, 47.50, 48.12, 50.37, 52.50, 55.50, 56.80, 56.96, 60.45, 61.74, 95.69, 116.89, 117.19, 123.95, 125.20, 126.74, 127.13, 127.46, 128.07, 128.31, 128.38, 128.45, 128.60, 131.75, 133.44, 138.20, 139.72, 144.75, 164.61, 168.11, 168.82, 169.06, 170.77, 171.23, 172.00, 172.93. MS (ESI) m/z calcd for $C_{51}H_{56}N_9O_{11}Na^+$ $(M-C_4H_9+2H)^+$ 971.3, found 971.4.

The photo-induced cyclization process (Fig.S1) is shown below.

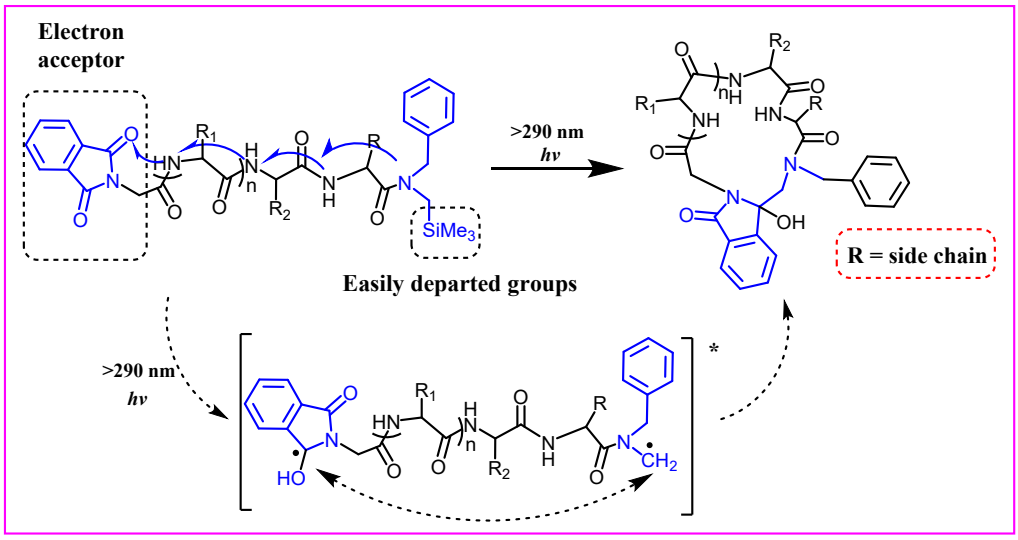


Fig. S1. Schematic diagram of photo-induced ring formation mechanism.

2. MTT experiment

The synthesized compounds were dissolved in DMSO to prepare different concentrations of samples (300, 200, 150, 100, 75, 50, 37.5, 25, 18.75 μg/mL), and stored at 4 °C for use. CT26 (Mouse colon cancer cells ATCC CRL-2638) provided by Harbin Institute of Technology, Harbin, China. The cells in logarithmic growth phase were digested with trypsin. The cell suspension with a cell concentration of 5000 cells/well was prepared with 10% bovine serum and inoculated in 96-well plates. The cells were divided into 9 experimental groups and 1 blank group. Different concentrations of drugs were added to the experimental group and no drugs were added to the blank control group. Each drug dose was repeated for six wells. After 48 h of incubation, MTT solution (100 μL, 0.5 mg/mL) was added to each well and incubated for further 4 h at 37 °C. Then the supernatant was discarded, 150 μL of DMSO was added to each well and the supernatant was shaken at low speed for 10 min until it was fully dissolved. The absorbance (OD) values were measured at 490 nm with a microplate reader. The data obtained were processed using SPSS19 statistical software to calculate the cell inhibition rate and to obtain IC50 values. Each set of data was analyzed by one-way ANOVA. Cell viability was calculated using equation (1):

$$\text{Cell survival rate}(\%) = A_s/A_b \times 100\% \quad (1)$$

A_s is the absorbance value of the experimental group, and Ab is the absorbance value of the blank group.

3. Computational details and the docking

Conformational analysis was performed by fixing the absolute configuration of C-3 with the MMFF94 molecular mechanics force field. The obtained conformers were optimized at the DFT/B3LYP/6-31G(d,p) level by Gaussian 09 (Gaussian 09, Revision C.01; Gaussian, Inc.: Wallingford, CT, 2010.) Time-dependent DFT calculations were performed on the lowest energy conformations (>5% population) to calculate excitation energy (denoted by wavelength in nm) and rotatory strength R at the level of TDDFT/ωB97XD/cc-PVDZ using polarizable continuum model (PCM) to consider the solvent. ECD curves were calculated based on rotatory strengths using half bandwidth of 0.30~0.40 eV by Specdis 1.71(T. Bruhn, et. al. Chirality, 2013, 25, 243). The

calculated spectra were treated by UV correction to facilitate comparison to the experimental data. Docking experiments were performed by AutoDock vina 1.1.2. Docking experiments were performed by AutoDockVina 1.1.2. The three-dimensional structure of the molecular docking target protein was downloaded from the RCSB protein database (RCSB PDB, http://www.pdb.org/), and the modified ligand and water were separated using PyMol software. The compound structure was based on the optimized structure at the level of B3LYP/6-311++G(d,p). The hydrogen was added and charge calculated by AutoDock Vina 1.1.2 software, and Discovery Studio 2019 Client was used to process docking results.

4. Cellular autophagy

CT26 cells at the growth stage were inoculated in a six-well plate (1×10^6/ml) with a cover glass in advance, and incubated in an incubator at 37℃ and 5% CO_2 for 24h. After that, the medium was discarded and washed once with PBS, one of the wells was added to serum-free 1640 medium, and 1 mL of sample solution (10μg/ml and 5μg/ml respectively) was added to each of the other five wells. The cells were incubated for 24h, washed three times with PBS, fixed with 4% paraformaldehyde for 10min, and washed again with PBS. Then 1ml of acridine orange solution (20μg/ml) was added to each well and maintained for 15min, washed once with PBS, then observed with confocal laser microscope.

5. Effects of cyclic peptides on MDM2 and P53 expression

Sample preparation:

4T1 cells in logarithmic growth phase were inoculated in 6-well culture plates, and 1 mL of different concentrations of drug (0, 6.25, 12.5, 25, 37.5, and 50 μg/mL) was added to each well and incubated in the incubator for 24 h. Each concentration was performed in triplicate. The adherent cells were gently washed with cold PBS and then separated by trypsin, collected by centrifugation at 1000r for 5 min, and the cells were washed three times in cold PBS. Cells were resuspended in fresh lysis buffer at a concentration of 10^7 cells/mL. If necessary, the cells were sonicated until the solution

was clarified. Centrifuge the cells at 4°C 12000g for 5 minutes at 2-8°C to remove cell debris and remove the supernatant for subsequent assays.

Experimental flow:

Prepare standard wells and sample wells. Add 50 μL of different concentrations of standards to each standard well. Add 10 μL of the sample to be tested, followed by adding 40 μL of the sample diluent to the sample wells (do not add anything to blank wells). To each standard and sample well, add 100μL horseradish peroxidase (HRP)-labeled detection antibody, seal with a film, incubate for 60min at 37°C in a water bath or thermostat. Discard liquid, pat dry on blotting paper, fill each well with washing solution for one minute before shaking off excess solution and repeating this process five times (or wash plates using a plate washer). Repeat this process five times. Add substrate A and B (50μL) to each well; incubate at 37°C for fifteen minutes before adding termination solution (50μL) to each well. Measure OD value at wavelength of 450nm within fifteen minutes.

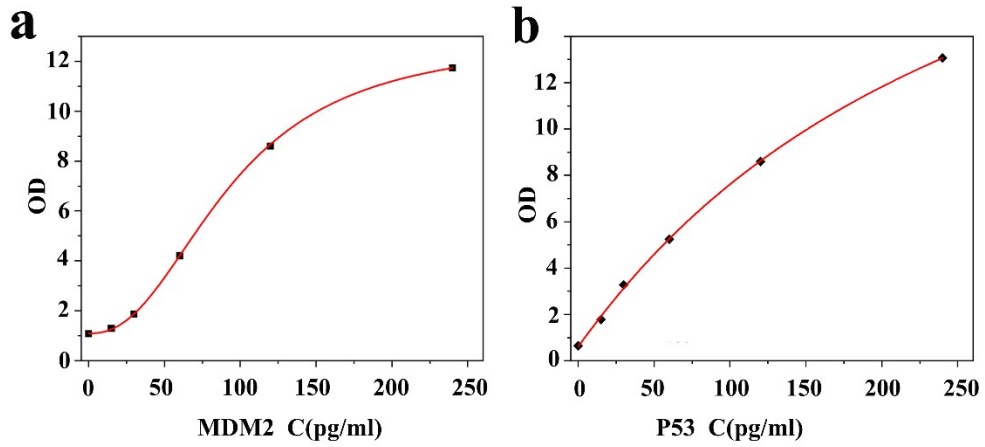
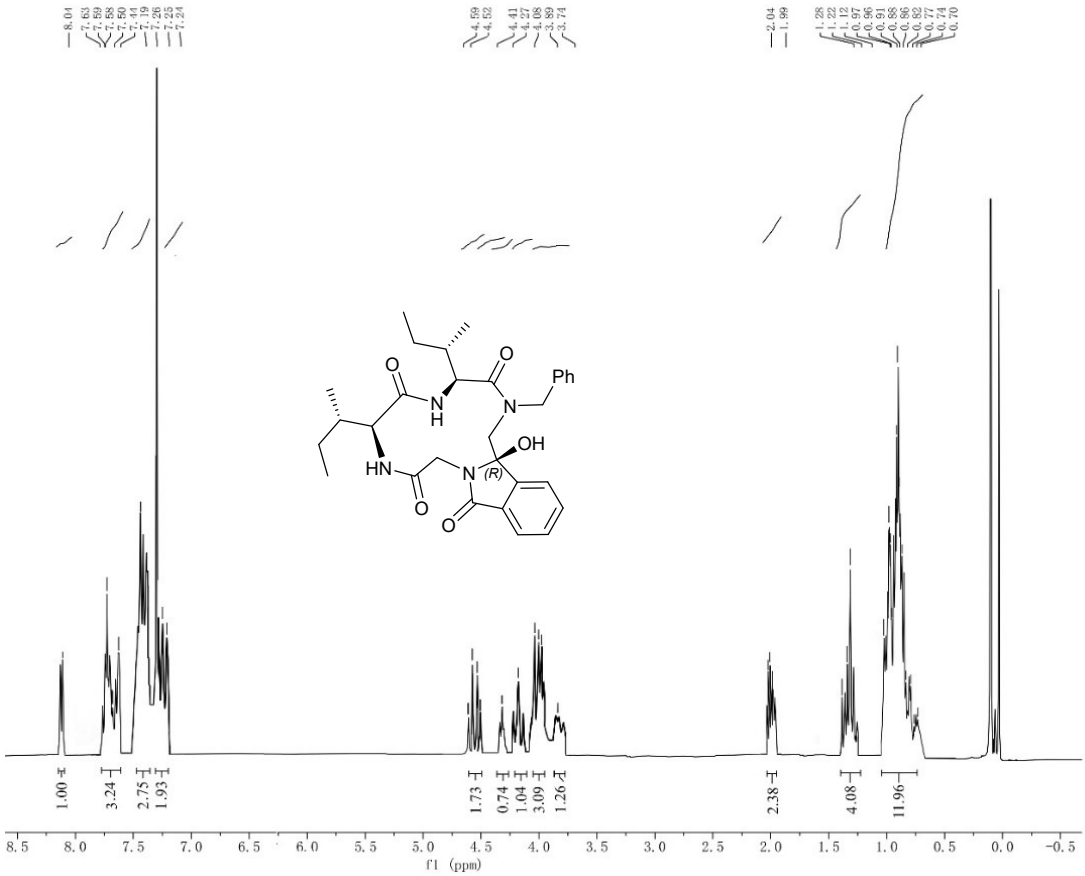
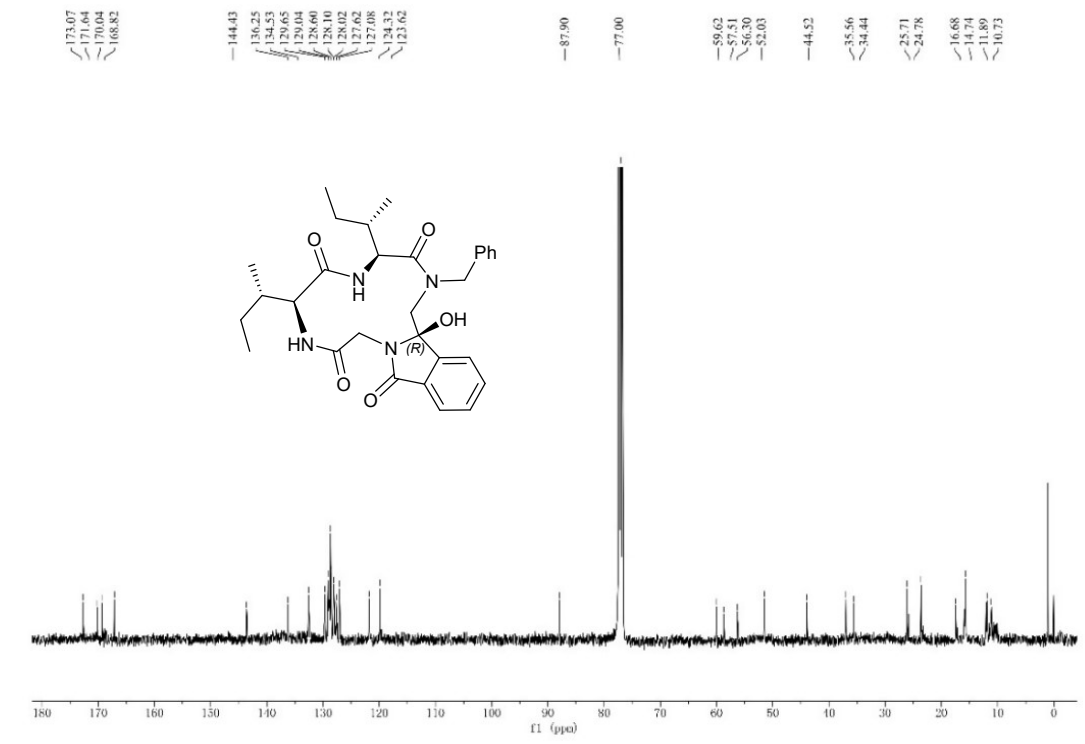


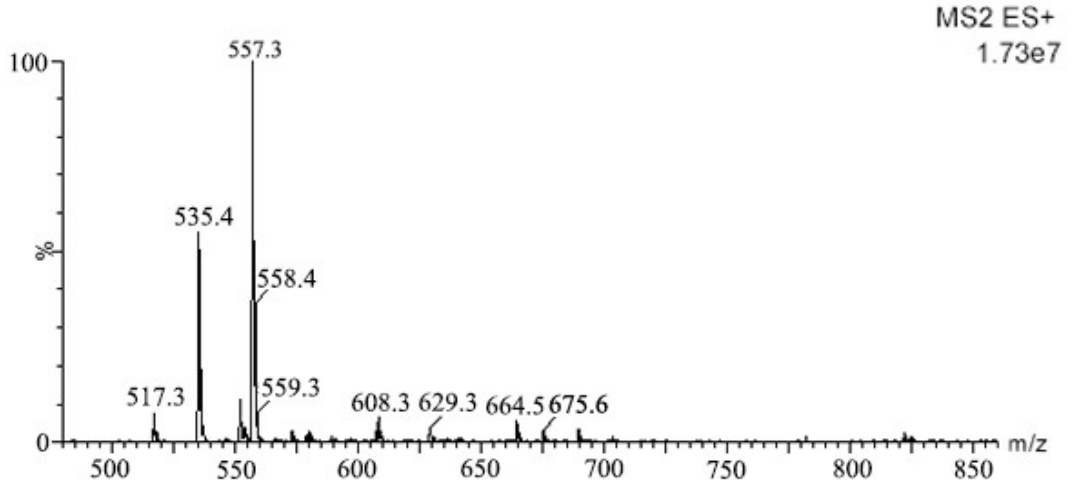
Fig. S2. Standard curve of changes of MDM2 and P53 content

6. NMR spectra and mass spectrometry

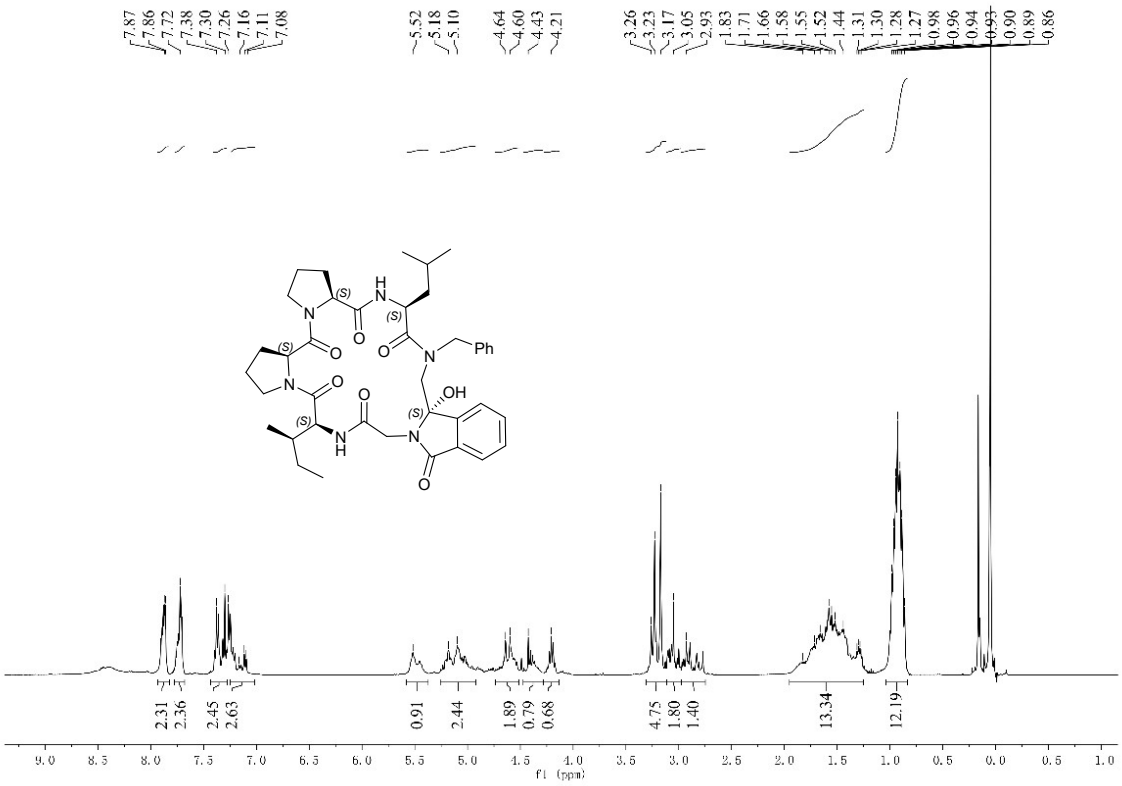
6.1 ^{1}H-NMR, ^{13}C-NMR and MS of compound **1**

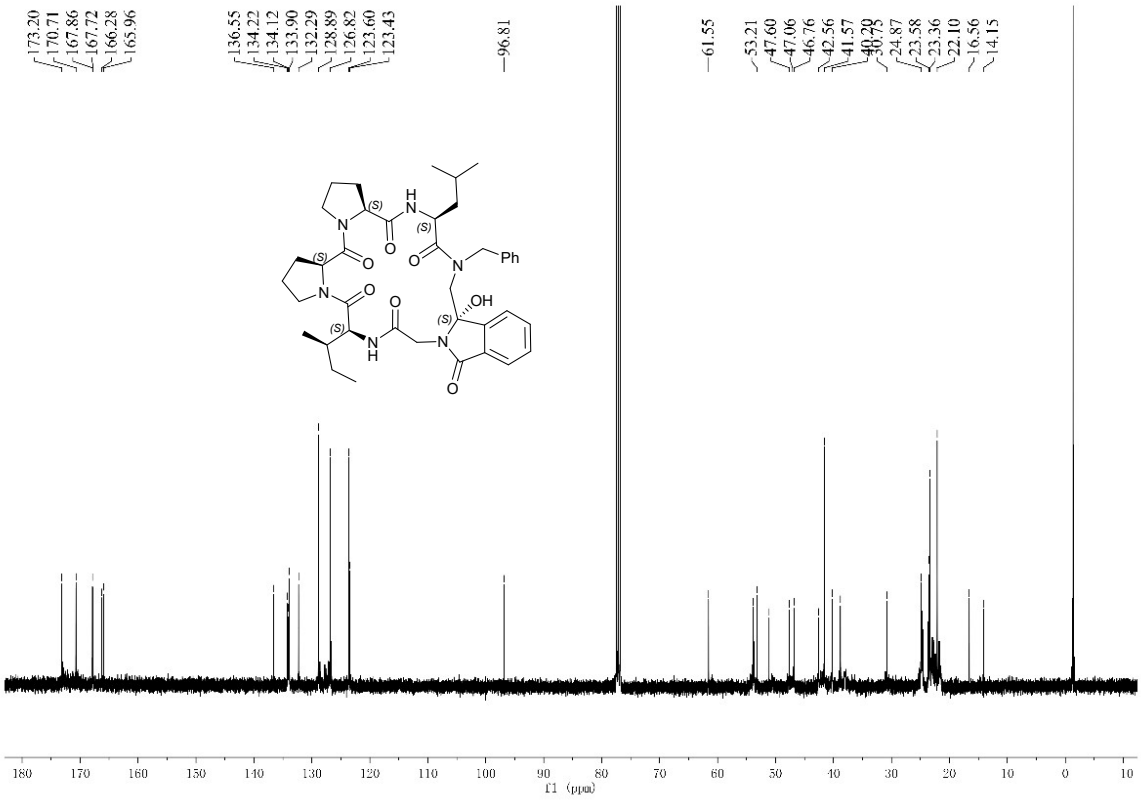






6.2 ^{1}H-NMR, ^{13}C-NMR and MS of compound **2**

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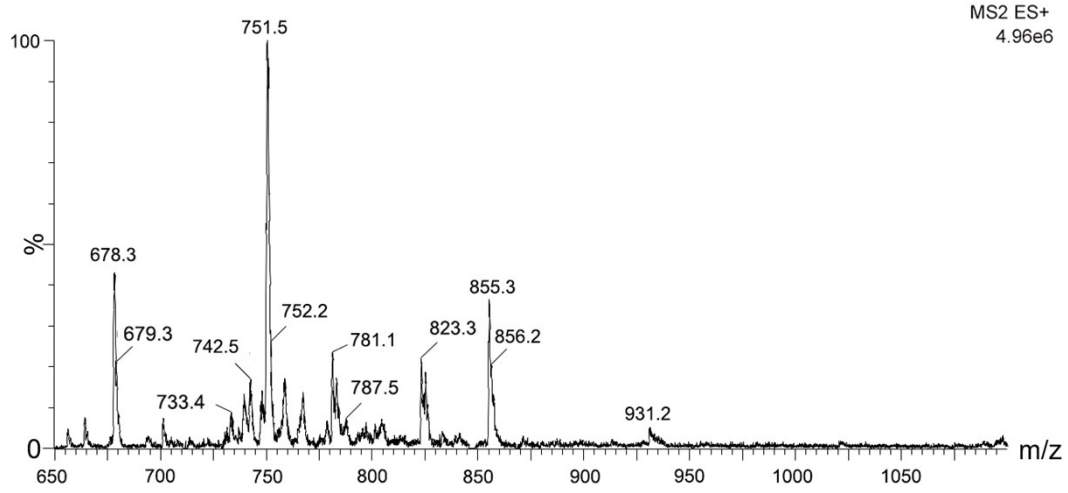
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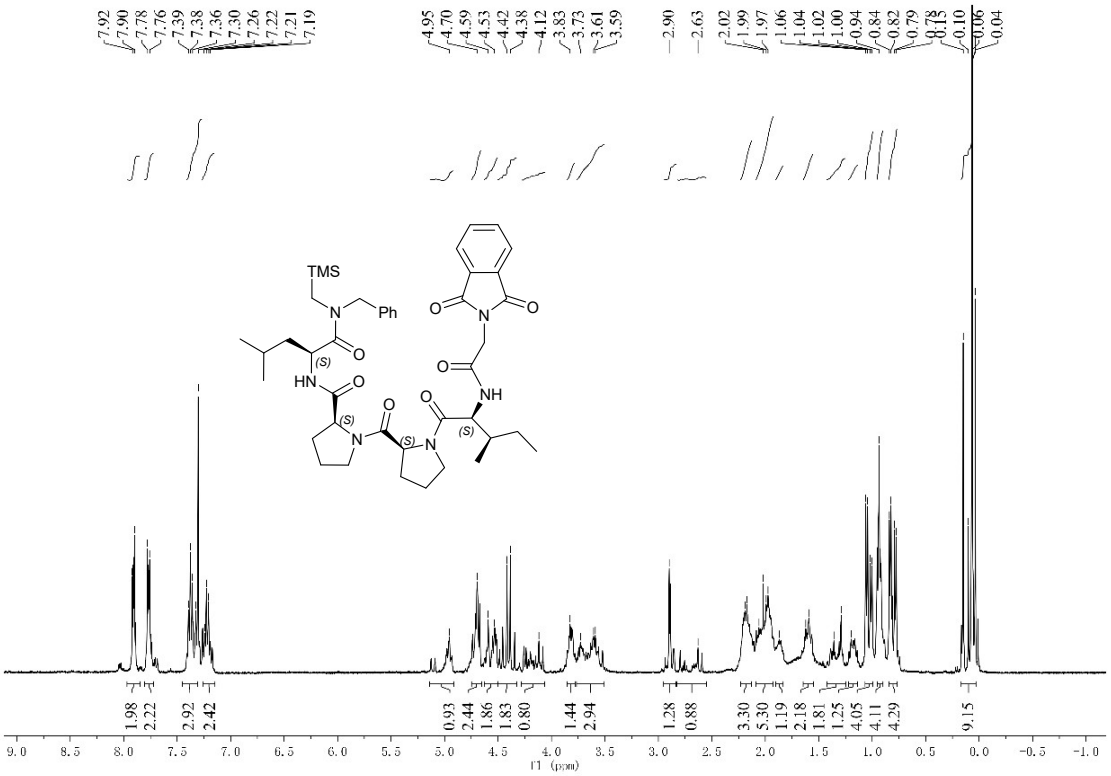
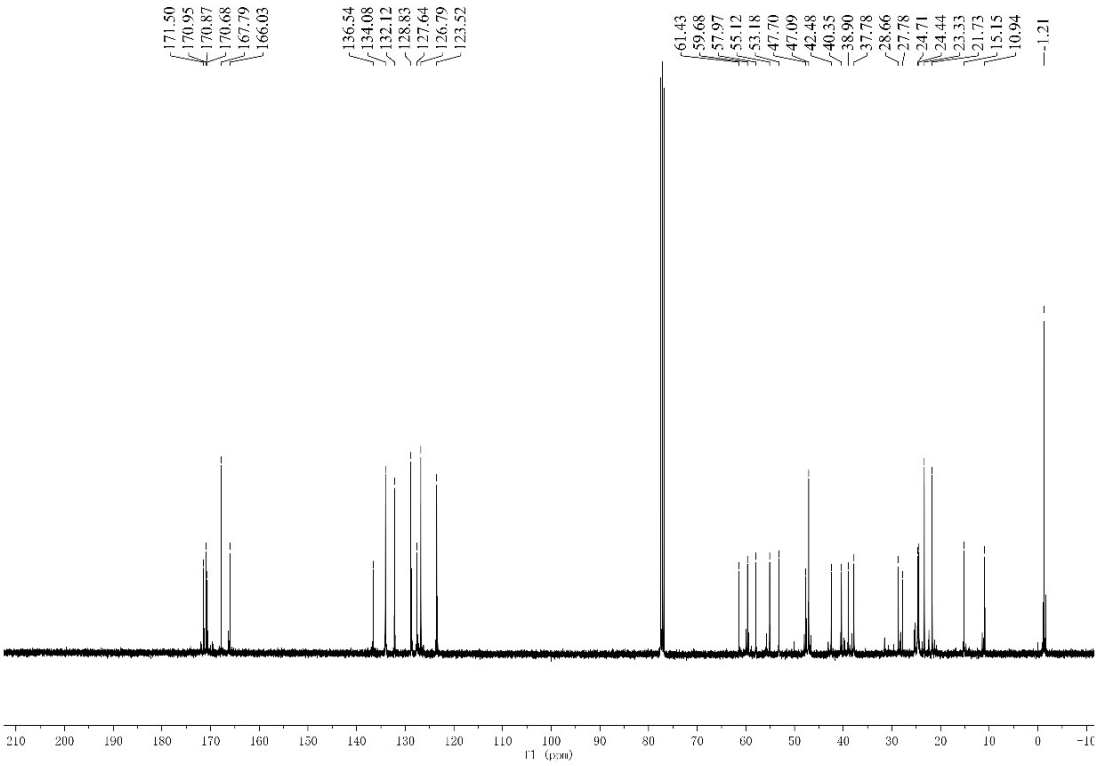
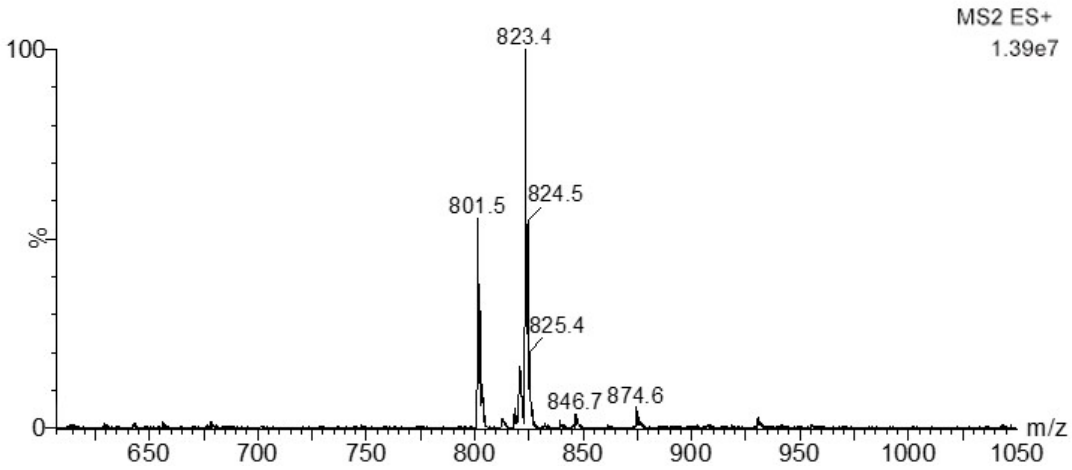
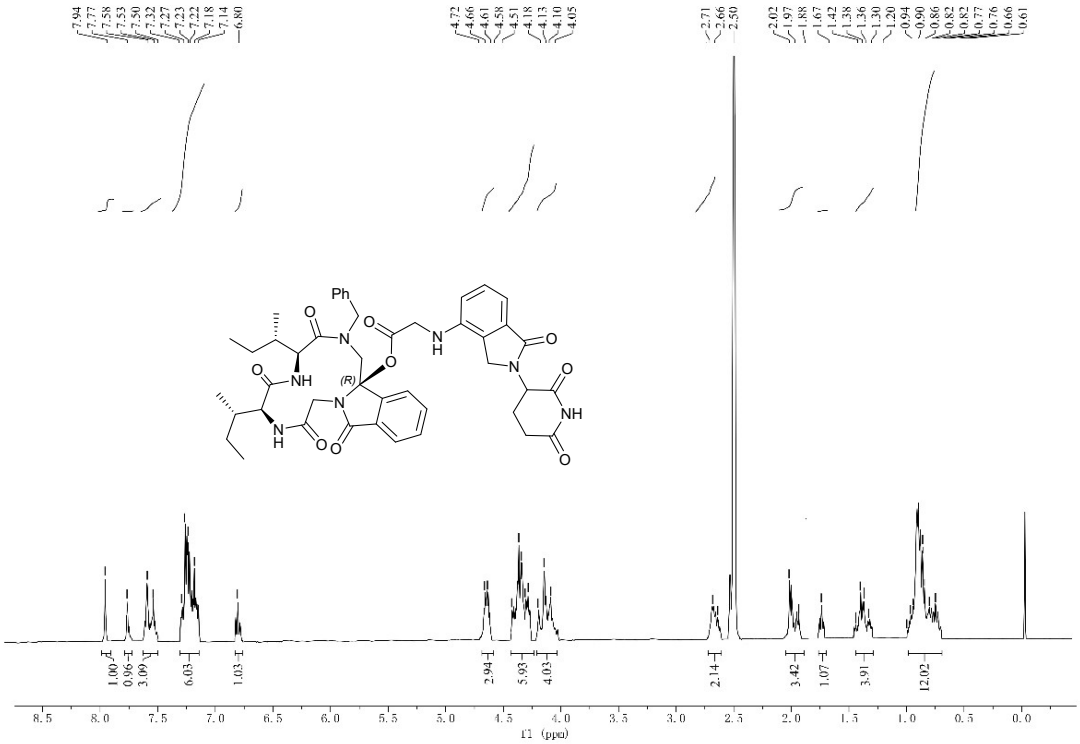
6.3 ^{1}H-NMR, ^{13}C-NMR and MS of compound **2a**

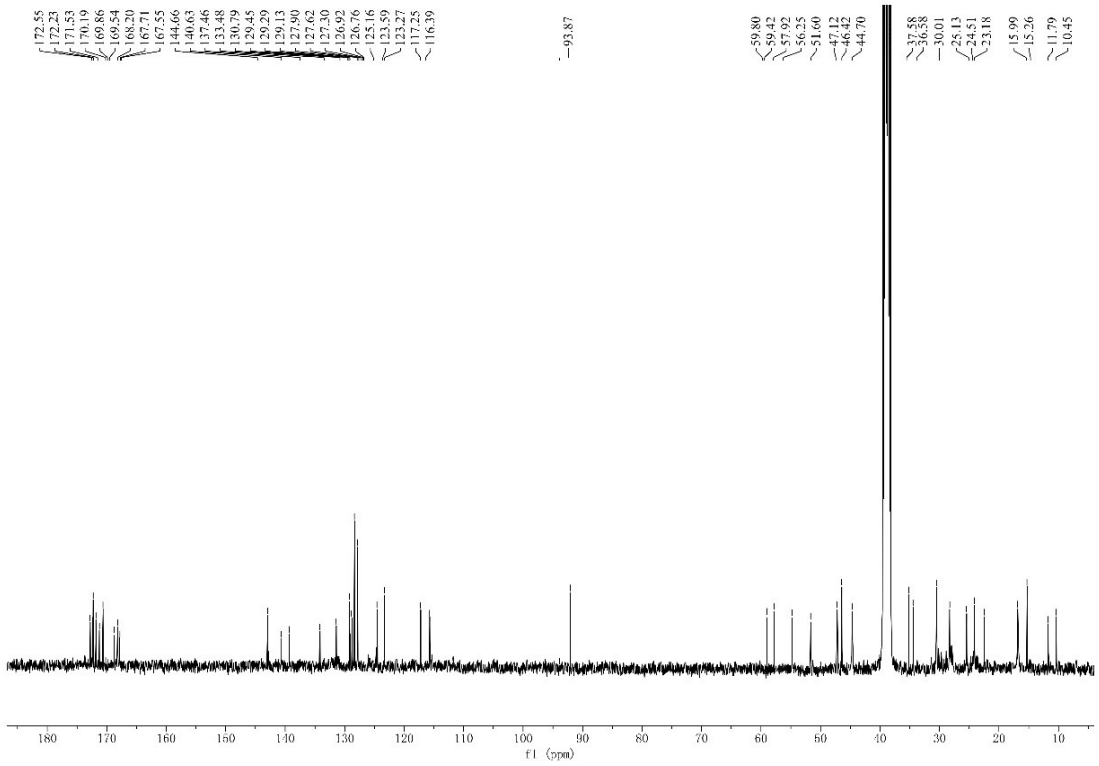


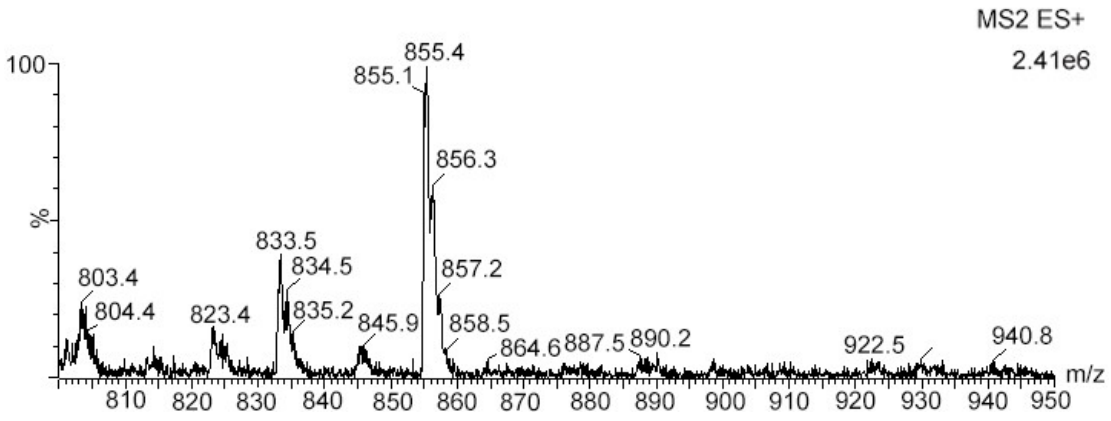




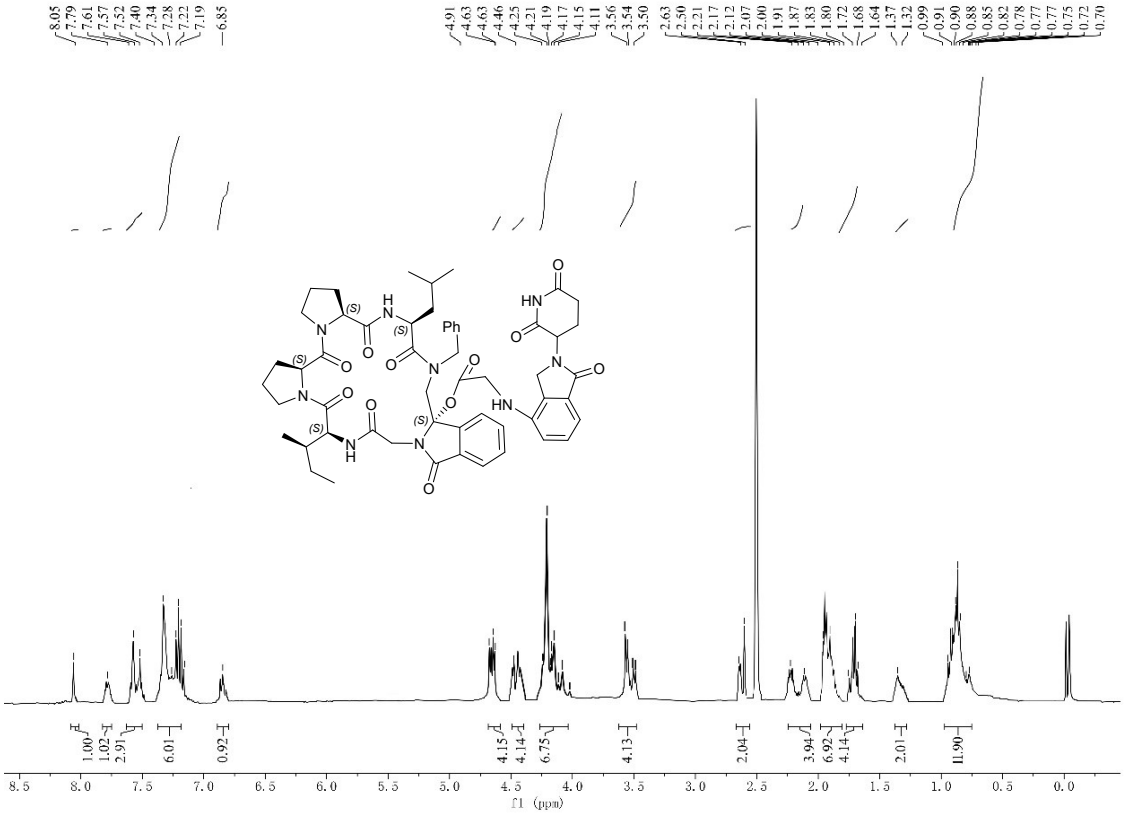
6.4 ^{1}H-NMR, ^{13}C-NMR and MS of compound **5**

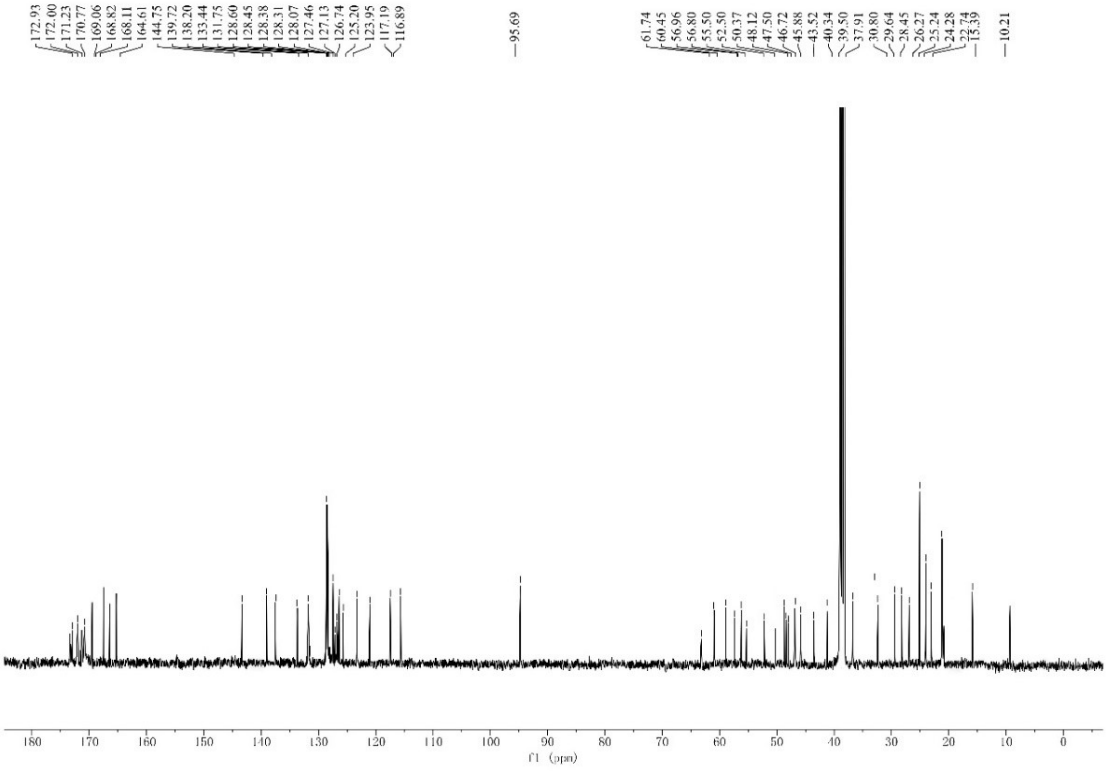


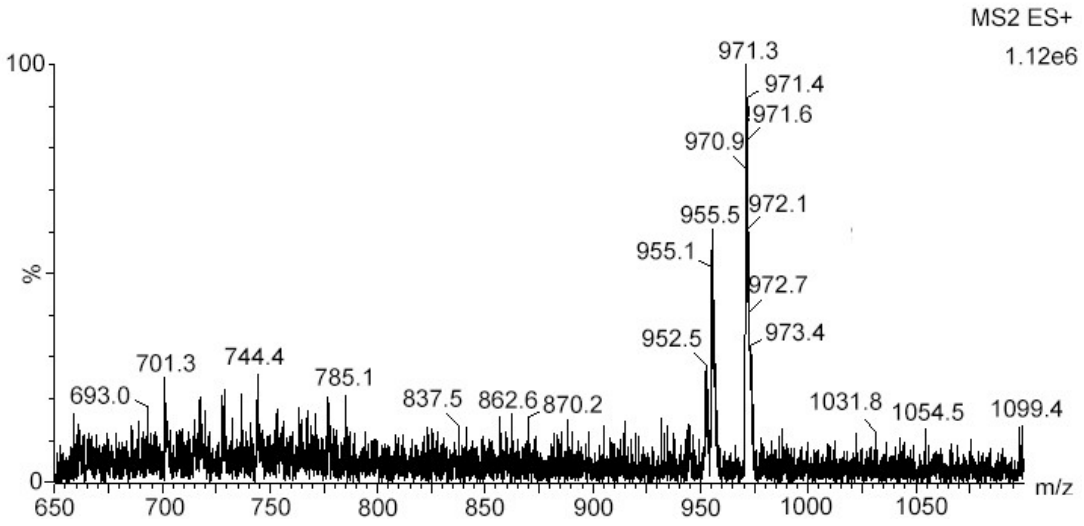

172.55
172.23
171.53
170.19
169.86
169.54
168.20
167.71
167.55
144.66
140.63
137.46
133.48
130.79
129.45
129.29
129.13
127.90
127.62
127.30
126.92
126.76
125.16
123.59
123.27
117.25
116.39
93.87
59.80
59.42
57.92
56.25
51.60
47.12
46.42
44.70
37.58
36.58
30.01
25.13
24.51
23.18
15.99
15.26
11.79
10.45
f1 (ppm)


MS2 ES+
2.41e6
100
%
855.4
855.1
856.3
833.5
834.5
857.2
803.4
804.4
823.4
835.2
845.9
858.5
864.6
887.5
890.2
922.5
940.8
m/z
810
820
830
840
850
860
870
880
890
900
910
920
930
940
950

6.5 ^{1}H-NMR, ^{13}C-NMR and MS of compound **6**






MS2 ES+
1.12e6
100
%
0
971.3
971.4
971.6
970.9
972.1
955.5
955.1
972.7
973.4
952.5
701.3
744.4
693.0
785.1
837.5
862.6
870.2
1031.8
1054.5
1099.4
650
700
750
800
850
900
950
1000
1050
m/z