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Molecular engineering of thiostrepton via single "base"-based mutagenesis to generate side ring-derived variants

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1. Supplementary Methods

1.1. General materials and methods

Bacterial strains, plasmids, and reagents. The bacterial strains and plasmids used in this study are summarized in Table S6. The primer sequences are listed in Table S7. The biochemicals, chemicals, media, restriction enzymes, and other molecular biological reagents were purchased from standard commercial sources unless otherwise stated.

DNA isolation, manipulation, and sequencing. DNA isolation and manipulation in *Escherichia coli* and *streptomyces* were performed according to standard protocols.[1,2] PCR amplifications were carried out on an Authorized Thermal TM Cycler (AG 22331; Eppendorf, Hamburg, Germany) using PrimeStar HS DNA polymerase (TaKaRa). Primer synthesis and DNA sequencing were performed at Shanghai GeneCore Biotechnology Inc. .

Sequence Analysis. Protein comparison was carried out by available BLAST methods (http://www.ncbi.nlm.nih.gov/blast/). Amino acid sequence alignment was performed by the CLUSTALW method, and the DRAWTREE and DRAWGRAM methods, respectively, from BIOLOGYWORKBENCH 3.2 software (http://workbench.sdsc.edu). DNA hairpin was determined by using the programs from Vector NTI Advance 11. 0 software (Invitrogen).

Compound Analysis. High performance liquid chromatography (HPLC) analysis was carried out on an Agilent™ 1200 HPLC system (Agilent Technologies Inc., USA). Measurement of UV-visible absorbance was carried out on a JASCO V-530 spectrophotometer (Tokyo, Japan). Electrospray ionization MS (ESI-MS) was performed on a Thermo Fisher LTQ Fleet ESI-MS S4 spectrometer (Thermo Fisher Scientific Inc., USA). NMR data were recorded on Bruker AV500 (Bruker Co. Ltd., Germany) with tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in parts per million (δ); coupling constants (J) in Hz; assignments were supported by COSY, HSQC, HMBC, and NOESY experiments. Electrospray ionization MS (ESIMS) was performed on a Thermo Fisher LTQ Fleet ESIMS spectrometer (Thermo Fisher Scientific Inc. , USA).

1.2. Mutant construction

Construction of SL-I1X (producing the precursor peptide TsrH-Ile1X). Using the recombinant plasmid pSL2050[3] as the template, PCR amplification with the primers I1X-F/I1X-R was carried out for site-specific mutation of ATC (coding for Ile1 of the precursor peptide TsrH) into NNS(coding for X1), yielding the recombinant plasmid pSL-I1X. Site-specific integration of pSL-I1X onto the chromosome of the thiostrepton non-producing strain SL2051[3] took place after the introduction via *E.coli-Streptomyces* conjugation, yielding the recombinant strain SL-I1X for producing the precursor peptide TsrH-I1X.

1.3. Chemical purification and characterization

Cultivation, production and analysis of thiostrepton variants were performed according to the methods described previously.[4,5] Isolation and purification of the thiostrepton variants were performed according to the methods described previously.[3]

1.4. Physical Property and Bioactivity of Tested Thiopeptides

Water solubility. The solubility was measured according to a saturation shake-flask method described previously.[4]To determine the solubility of **TSR, SIO, SIO-Dha2Ser, TSR-Ile1Phe, TSR-Ile1Leu, TSR-Ile11Met,** or **TSR-Ile11Ala**, an excess amount of solid was added into 1 mL of water in a vial. The vial was gently stirred over a 24-hour period at the room temperature to ensure the equilibrium of the solution. A 0.22 μm centrifugal filter unit (Millipore Utrafree-MC, USA) was used for filtration to remove the excess solid. The saturated solution was then subjected to HPLC analysis to determine the concentration of the sample based on the established calibration curve.

To establish the calibration curve, a set of the solutions for **TSR, SIO, SIO-Dha2Ser, TSR-Ile1Phe, TSR-Ile1Leu, TSR-Ile11Met,** or **TSR-Ile11Ala** varying in concentration were analyzed independently by HPLC. The peak areas correlated with the concentrations were fitted to a regression equation, resulting in a linear standard curve for estimating the concentration.

Minimum Inhibitory Concentrations (MICs). The MICs were measured by broth dilution using a modified method described previously.[6] Each tested compound was dissolved in DMSO to produce a stock solution (100 μg/ml), which was serially diluted into 100 μL of Mueller-Hinton broth (Qingdao Hope Bio-Technology Co. Ltd., China) in a 96-well microtiter plate to a final concentration ranging from 1 to 0 μg/ml. Given the poor solubility of **TSR, SIO, SIO-Dha2Ser, TSR-Ile1Phe, TSR-Ile1Leu, TSR-Ile11Met,** or **TSR-Ile11Ala** in the aqueous medium, 2% DMSO (v/v) was added into the broth. 100 μL of the testing strain (10^7-10^8 cfu/ml, calculated according to the 0.5 McFarland standard [7]) was then added into each well of the microtiter plate. After incubation at 37℃ for 18-24 hr(for *Mycobacterium. marinum*, the incubation was 7 days), The MIC was determined to be the lowest concentration of compound that inhibited visible bacterial growth. All testings were carried out in duplicate.

1.5. Preparation of *M. marinum* Single Cells for Infection

The *M. marinum* M strain (ATCC BAA-535), in which the GFPmut3 protein is constitutively expressed, was incubated in 7H9C medium at 32 ℃ for 5 to 7 d until the OD_{600} reached 0.5 to 1.0. The pellet was re-suspended in 7H9OADC medium, sheared by passage through a syringe several times, and then filtered to remove bacterial aggregates. The prepared single cells were diluted in 7H9OADC medium to the final CFU of 2×10^8.

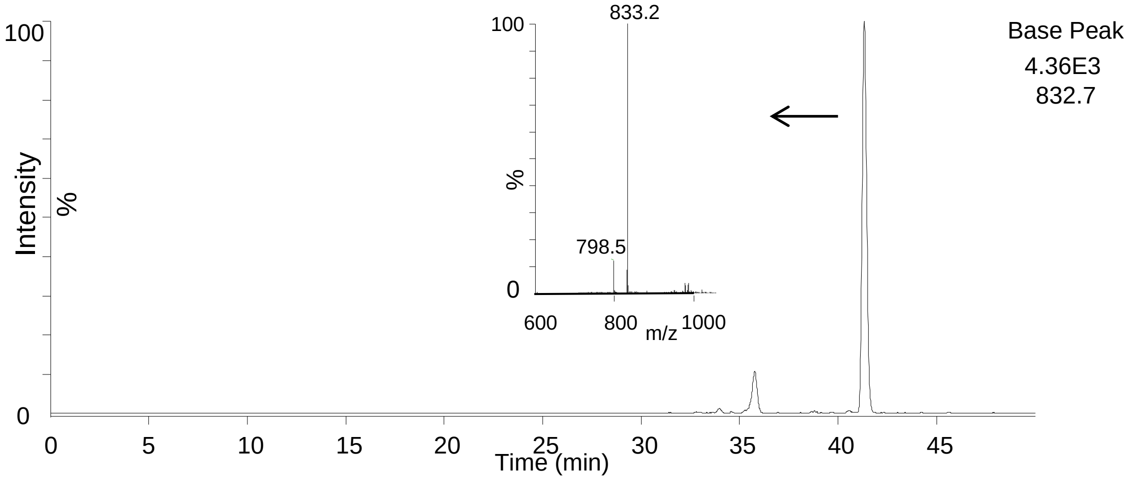
1.6. Detection of Biomarkers Involved in ER Stress-Mediated Autophagy Pathways

The murine macrophage cell line RAW 264.7 (ATCC TIB-71) was maintained at 37 ℃ with 5% CO2 in DMEM supplemented with 10% fetal bovine serum and 10 mM HEPES. The cells, plated in 12-well plates (2×10^5 cells per well), were infected with 2 3 106 cells of *M. marinum* for 2 hr at 32 ℃. After treatment with 1 mM of each drug, the cells were maintained at 32 ℃ for 2 hr and then lysed in mammalian cell total protein lysis buffer containing 1% PMSF. After 5× protein loading buffer was added, the lysate was separated by 15% SDS-PAGE and transferred onto a PVDF membrane (Millipore) under a voltage of 100 V for 90 min. The primary antibodies used in this study included anti-phosphoPERK (Cell Signaling Technology, 1:500), anti-phospho-eIF2a (Cell Signaling Technology, 1:200), anti-LC3 (Sigma, 1:1,000), anti-IRE1 (Cell Signaling Techn-ology, 1:500), and anti-ATF6 (Gene Tex, 1:500). The immunoreactive bands were visualized by using a chemiluminescent detection reagent after incubation with the HRP-conjugated secondary antibody (Cell Signaling Technology, 1:5,000).

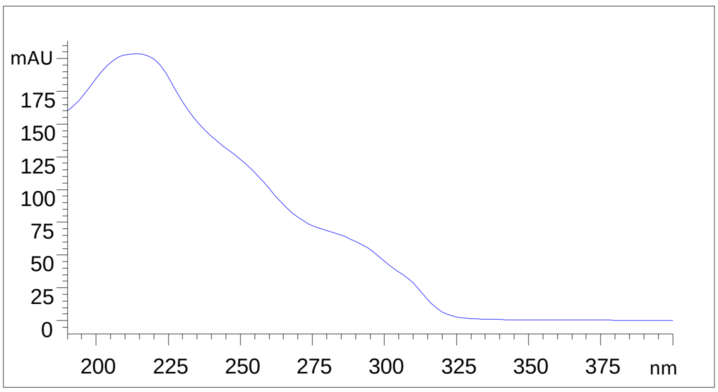
2. Supplemental Results

Fig. S1. HPLC-MS analysis, Ultraviolet absorption pattern and structure of TSR-Ile1Leu

(A) Chromatogram extracted for *m/z* 832.7, the calculated $[M+2H]^{2+}$ ion of TSR-Ile1Leu.



(B) Ultraviolet absorption pattern of TSR-Ile1Leu



(C) Structure of TSR-Ile1Leu

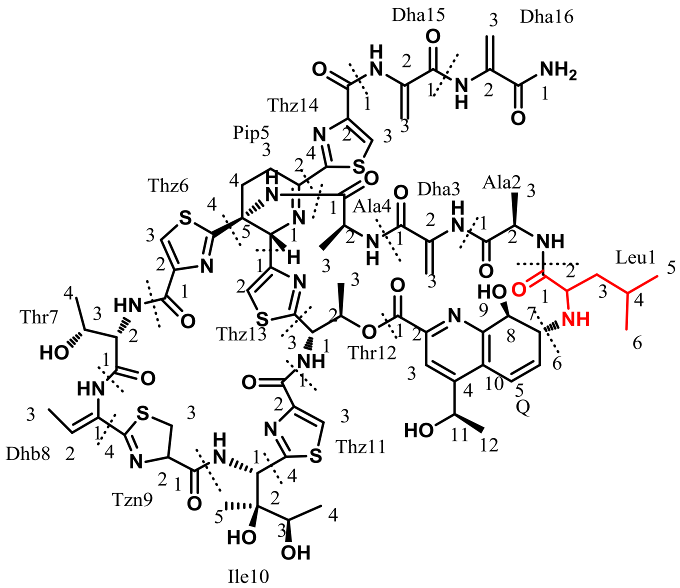
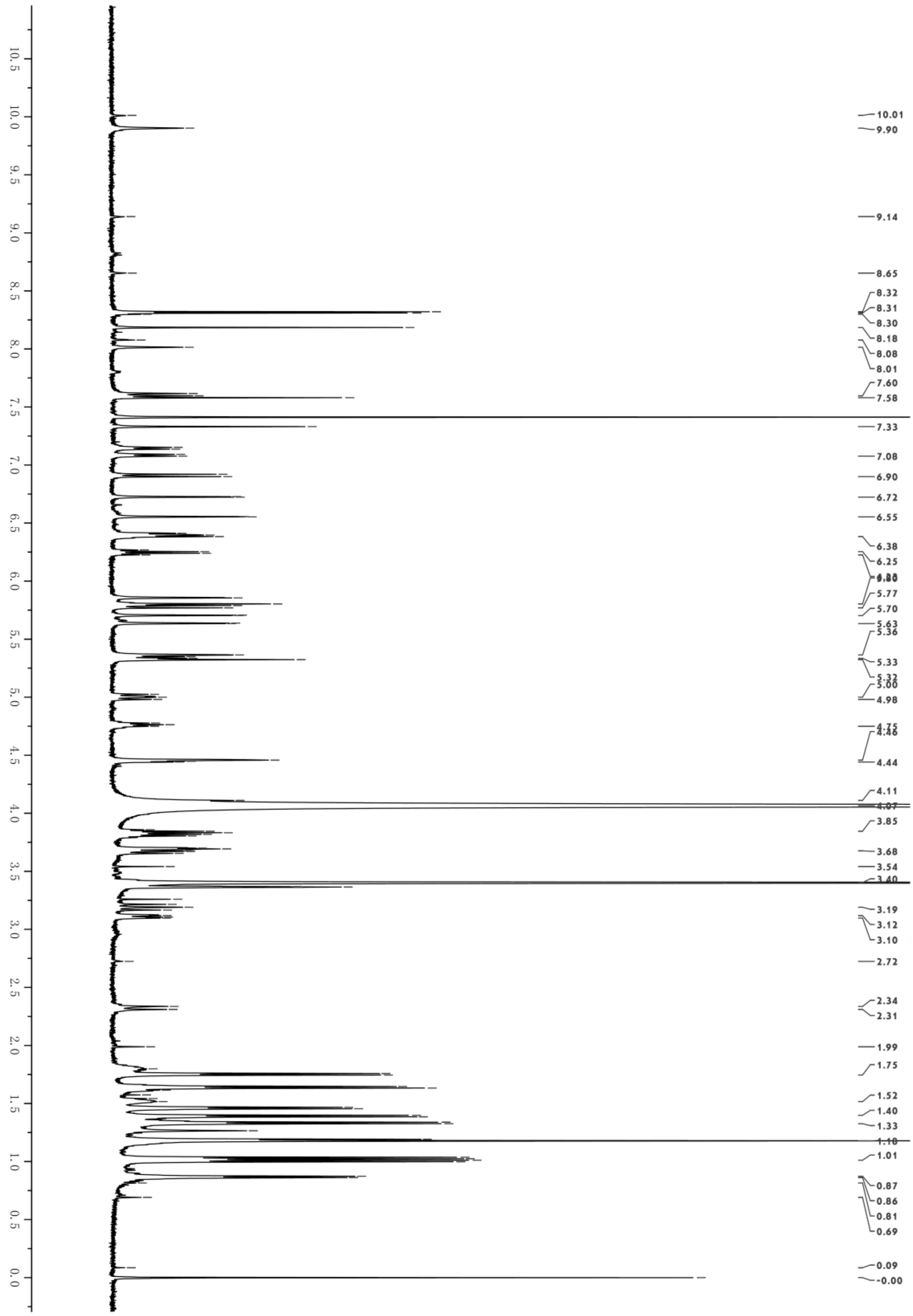
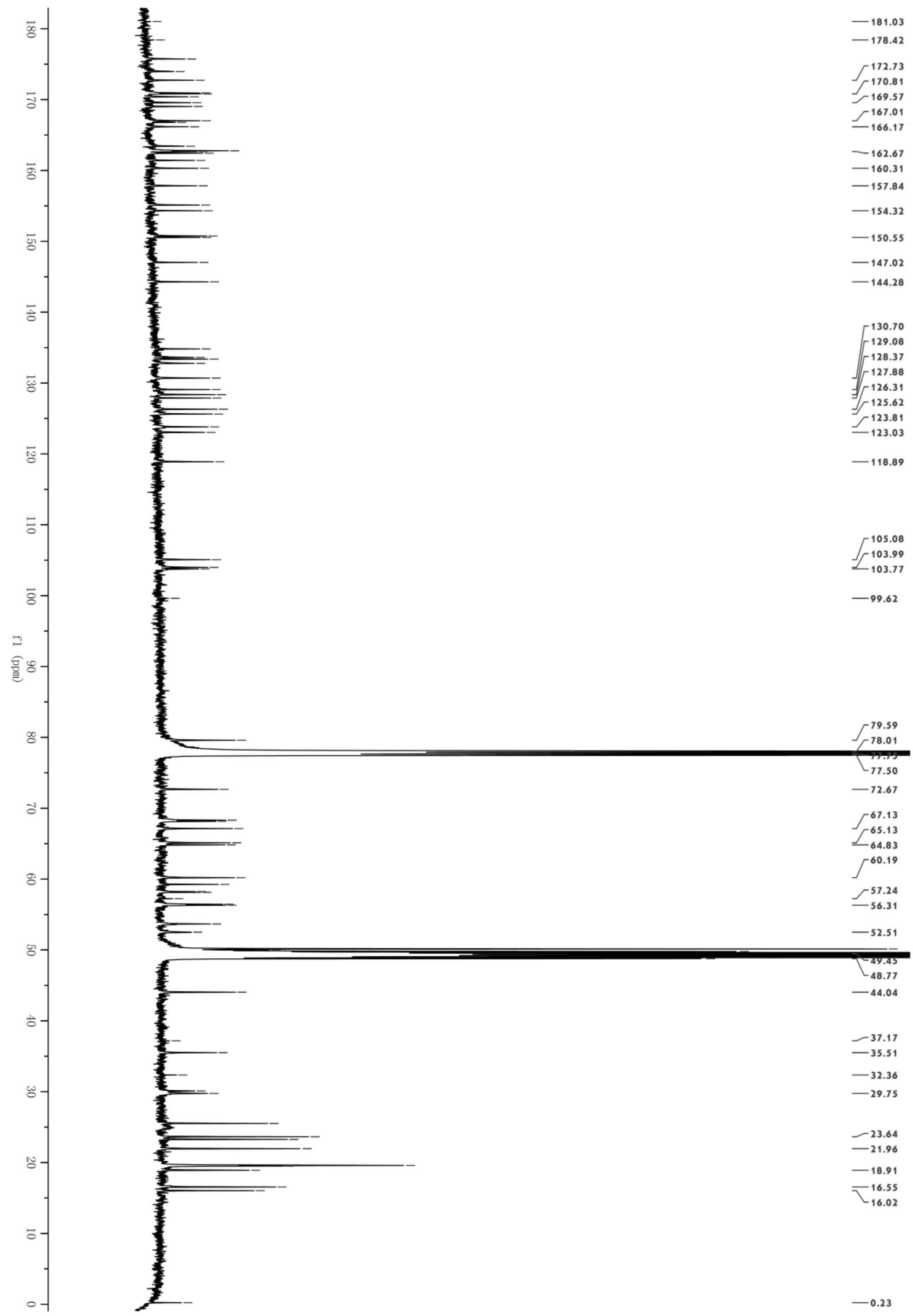


Fig. S2. 1D and 2D NMR spectra of TSR-Ile1Leu

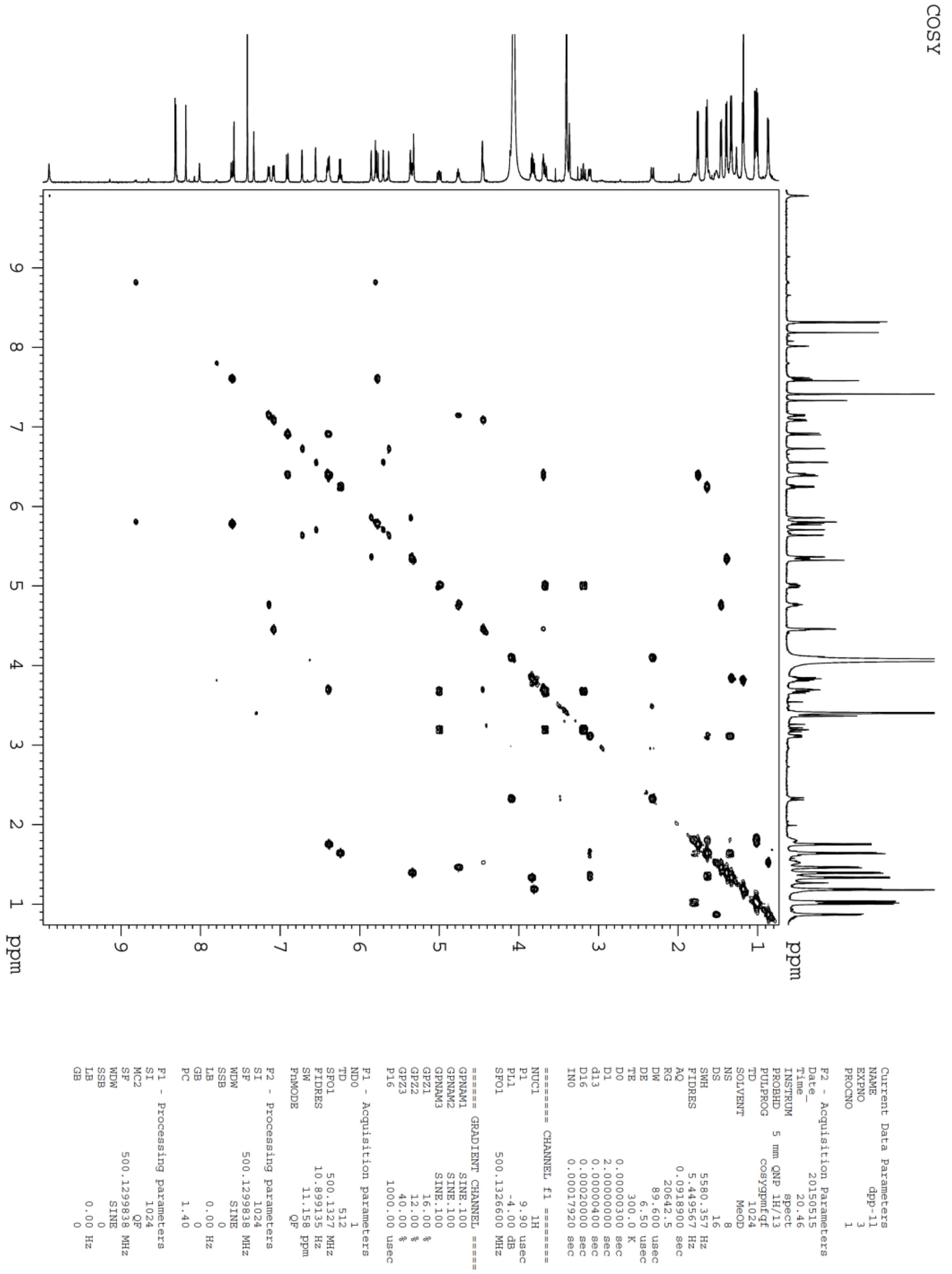
(A) ^{1}H spectrum of TSR-Ile1Leu



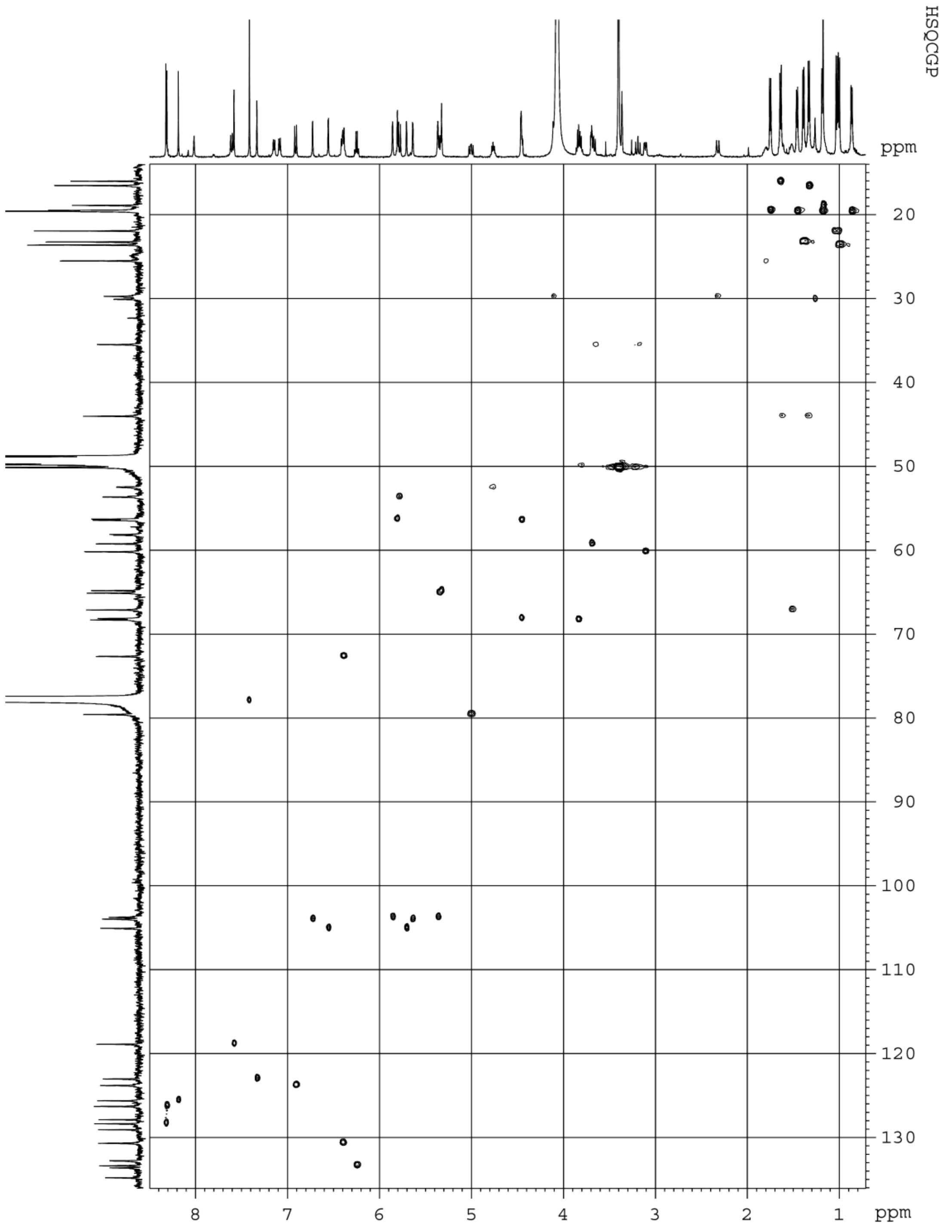
(B) ^{13}C NMR spectrum of TSR-Ile1Leu



(C) COSY spectrum of TSR-Ile1Leu



(D) HSQC spectrum of TSR-Ile1Leu



(E) HMBC spectrum of TSR-Ile1Leu

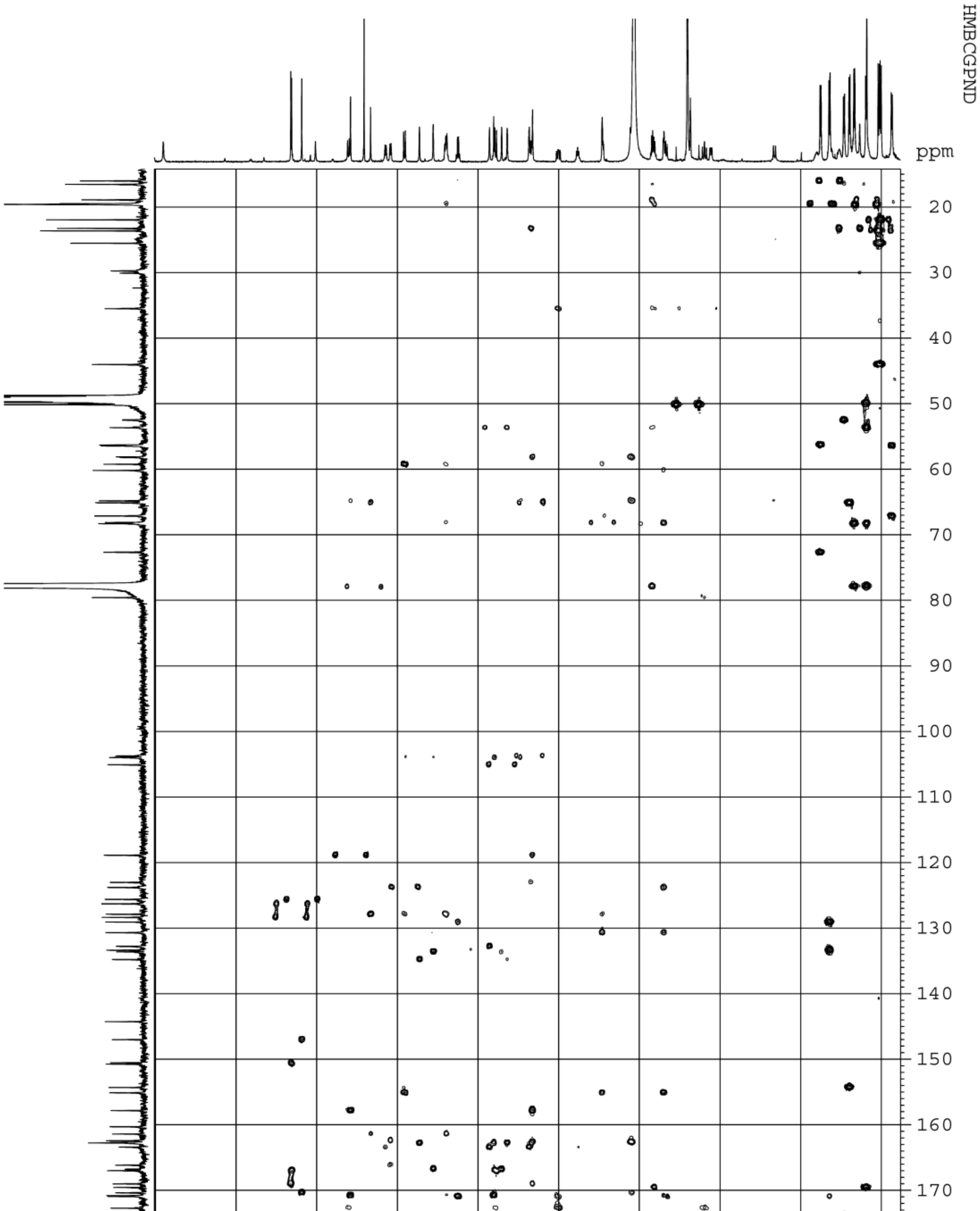

HMBCGPND
ppm
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
10
9
8
7
6
5
4
3
2
1
ppm

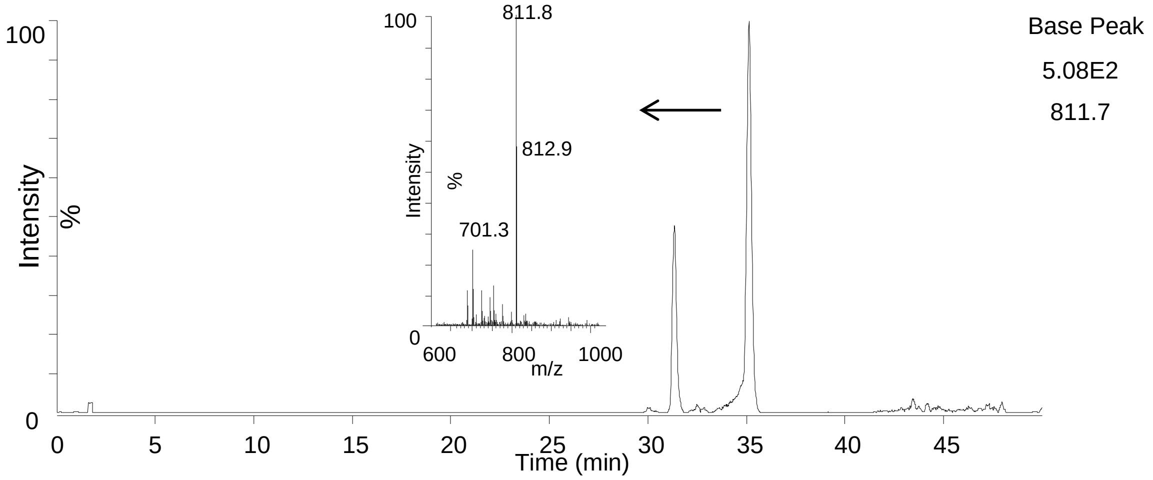
Table S1. ^{1}H and ^{13}C NMR assignments of TSR-Ile1Leu（*δ* in ppm, *J* in Hz）

Assigment	δ_H	δ_C	HMBC	COSY
Leu1-1		175.7		
Leu1-2	3.11 m	60.3	Leu1-3	Leu1-3-H_a; Leu1-3-H_b
Leu1-3	H_a 1.61 m	44.2		Leu1-2; Leu1-3-H_b; Leu1-4
	H_b 1.32 m			Leu1-2; Leu1-3-H_a; Leu1-4
Leu1-4	1.80 m	25.6		Leu1-3-H_a; Leu1-3-H_b; Leu1-5; Leu1-6
Leu1-5	1.03 d (6.52)	22.0	Leu1-3; Leu1-4	Leu1-4
Leu1-6	1.00 d (6.60)	23.7	Leu1-3; Leu1-4	Leu1-4
Ala2-1		169.5		
Ala2-2	3.81 m	50.0	Leu1-1; Ala2-1; Ala2-3	Ala2-3
Ala2-3	1.18 d (6.73)	19.4	Ala2-1; Ala2-2	Ala2-2
Ala2-NH	7.61 bs			
Dha3-1		163.5		
Dha3-2		132.8		
Dha3-3	H_a 5.85 bs;	103.8	Dha3-1; Dha3-2	Dha3-3- H_b
	H_b 5.36 bs		Dha3-1; Dha3-2	Dha3-3- H_a
Dha3-NH				
Ala4-1		174.0		
Ala4-2	4.76 m	52.8	Ala4-1; Dha3-1	Ala4-3; Ala4-NH
Ala4-3	1.46 d (6.52)	19.8	Ala4-1; Ala4-2	Ala4-2
Ala4-NH	7.14 d (7.94)		Dha3-1	Ala4-2
Pip5-1	5.32 s	64.9	Pip5-2; Pip5-5; Thz13-1; Thz13-2; Thz14-4	
Pip5-2		162.6		
Pip5-3	H_a 2.94 m	24.5		Pip5-4- H_a; Pip5-4- H_b;
	H_b 3.47 m			Pip5-4- H_a
Pip5-4	H_a 2.32 d (12.64)	29.8	Pip5-3; Pip5-1	Pip5-3- H_a; Pip5-3- H_b; Pip5-4- H_b;
	H_b 4.10 m		Pip5-3; Pip5-5; Pip5-1; Pip5-2; Thz6-4	Pip5-3- H_a; Pip5-4- H_a
Pip5-NH	9.90 bs			
Pip5-5		58.3		
Thz6-1		162.4		
Thz6-2		147.0		
Thz6-3	8.18 s	125.6	Thz6-1; Thz6-2; Thz6-4	
Thz6-4		170.5		
Thr7-1		166.3		
Thr7-2	4.44 m	56.6	Thr7-1; Thr7-3	Thr7-3; Thr7-NH;
Thr7-3	1.51 m	67.3		Thr7-2; Thr7-4
Thr7-4	0.87 d (6.02)	19.8	Thr7-2; Thr7-3	Thr7-3
Thr7-NH	7.08 d (7.57)		Thz6-1; Thr7-1	Thr7-3
Dhb8-1		129.1		
Dhb8-2	6.24 q (6.80)	133.4	Dhb8-1; Dhb8-3; Thr7-1; Tzn9-4	Dhb8-3
Dhb8-3	1.64 d (6.80)	16.0	Dhb8-1; Dhb8-2; Tzn9-4	Dhb8-2
Dhb8-NH	8.69 bs			
Tzn9-1		172.8		
Tzn9-2	5.00 m	79.7	Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3- H_a; Tzn9-3- H_b

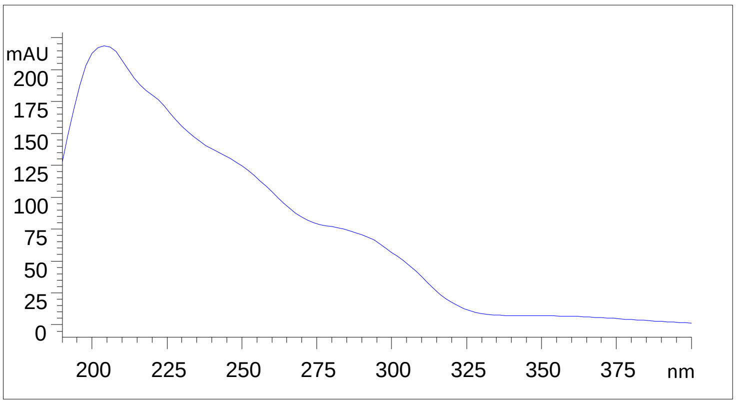
Assigment	δ_H	δ_C	HMBC	COSY
Tzn9-3	H_a 3.19 m H_b 3.66 m	35.6	Tzn9-1; Tzn9-2; Tzn9-4	Tzn9-2; Tzn9-3- H_b Tzn9-2; Tzn9-3- H_a
Tzn9-4		170.9		
Ile10-1	5.77 d (10.00)	53.6	Ile10-2; Tzn9-1; Thz11-4	Ile10-NH
Ile10-2		77.8		
Ile10-3	3.83 m	68.3	Ile10-1; Ile10-2; Ile10-4	Ile10-4
Ile10-4	1.33 d (6.38)	16.6	Ile10-2; Ile10-3	Ile10-3
Ile10-5	1.18 s	18.9	Ile10-1; Ile10-2; Ile10-3	
Ile10-NH	7.60 bs			Ile10-1
Thz11-1		162.7		
Thz11-2		150.8		
Thz11-3	8.31 s	126.3	Thz11-1; Thz11-2; Thz11-4	
Thz11-4		167.1		
Thr12-1	5.80 s	56.5	Thz11-1; Thz13-3	
Thr12-2	6.39 m	72.6	Thr12-1; Thz13-3; Q-1	Thr12-3
Thr12-3	1.75 d (6.55)	19.5	Thr12-1; Thr12-2	Thr12-2
Thr12-NH	8.82 m			
Thz13-1		157.8		
Thz13-2	7.58 s	118.9	Thz13-1; Thz13-3; Pip5-1	
Thz13-3		170.8		
Thz14-1		160.3		
Thz14-2		150.6		
Thz14-3	8.32 s	128.4	Thz14-2; Thz14-4	
Thz14-4		169.0		
Dha15-1		162.7		
Dha15-2		134.8		
Dha15-3	H_a 6.72 d (2.23) H_b 5.64 d (2.20)	104.0	Dha15-1; Dha15-2 Dha15-1; Dha15-2	Dha15-3- H_b Dha15-3- H_a
Dha15-NH	10.00 bs			
Dha16-1		166.8		
Dha16-2		133.4		
Dha16-3	H_a 6.55 d (1.60) H_b 5.70 d (1.72)	105.1	Dha16-1; Dha16-2 Dha16-1; Dha16-2	Dha16-3- H_b Dha16-3- H_a
Q-1		161.4		
Q-2		144.4		
Q-3	7.33 s	123.1	Q-1; Q-10; Q-11	
Q-4		154.4		
Q-5	6.90 d (10.09)	123.9	Q-7; Q-9; Q-10	Q-6
Q-6	6.39 m	130.8	Q-7; Q-8; Q-10	Q-5; Q-7
Q-7	3.69 m	59.3	Q-5; Q-6; Q-8; Q-9; Leu1-2	Q-6; Q-8
Q-8	4.45 m	68.2	Q-6; Q-7; Q-9; Q-10	Q-7
Q-9		155.1		
Q-10		127.9		
Q-11	5.35 m	65.1	Q-3; Q-12	Q-12
Q-12	1.39 d (6.54)	23.3	Q-4; Q-11	Q-11

Fig. S3. HPLC-MS analysis, Ultraviolet absorption pattern and structure of TSR-Ile1Ala

(A) Chromatogram extracted for *m/z* 811.7, the calculated $[M+2H]^{2+}$ ion of TSR-Ile1Ala.



(B) Ultraviolet absorption pattern of TSR-Ile1Ala



(C)Structure of TSR-Ile1Ala

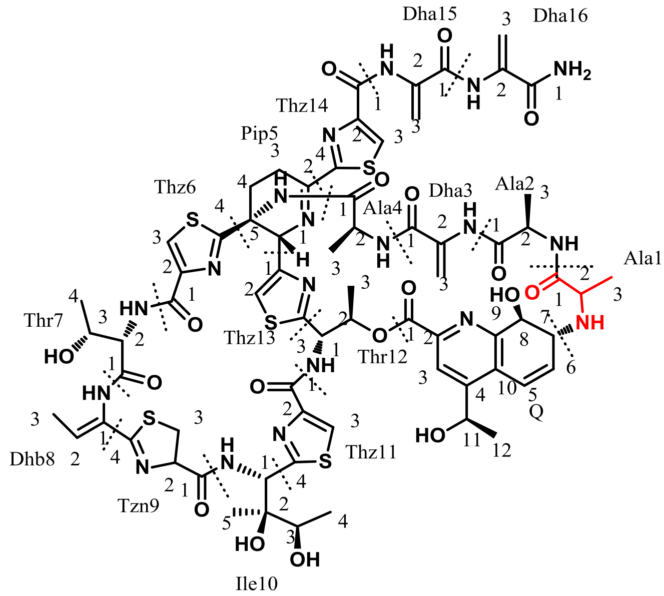
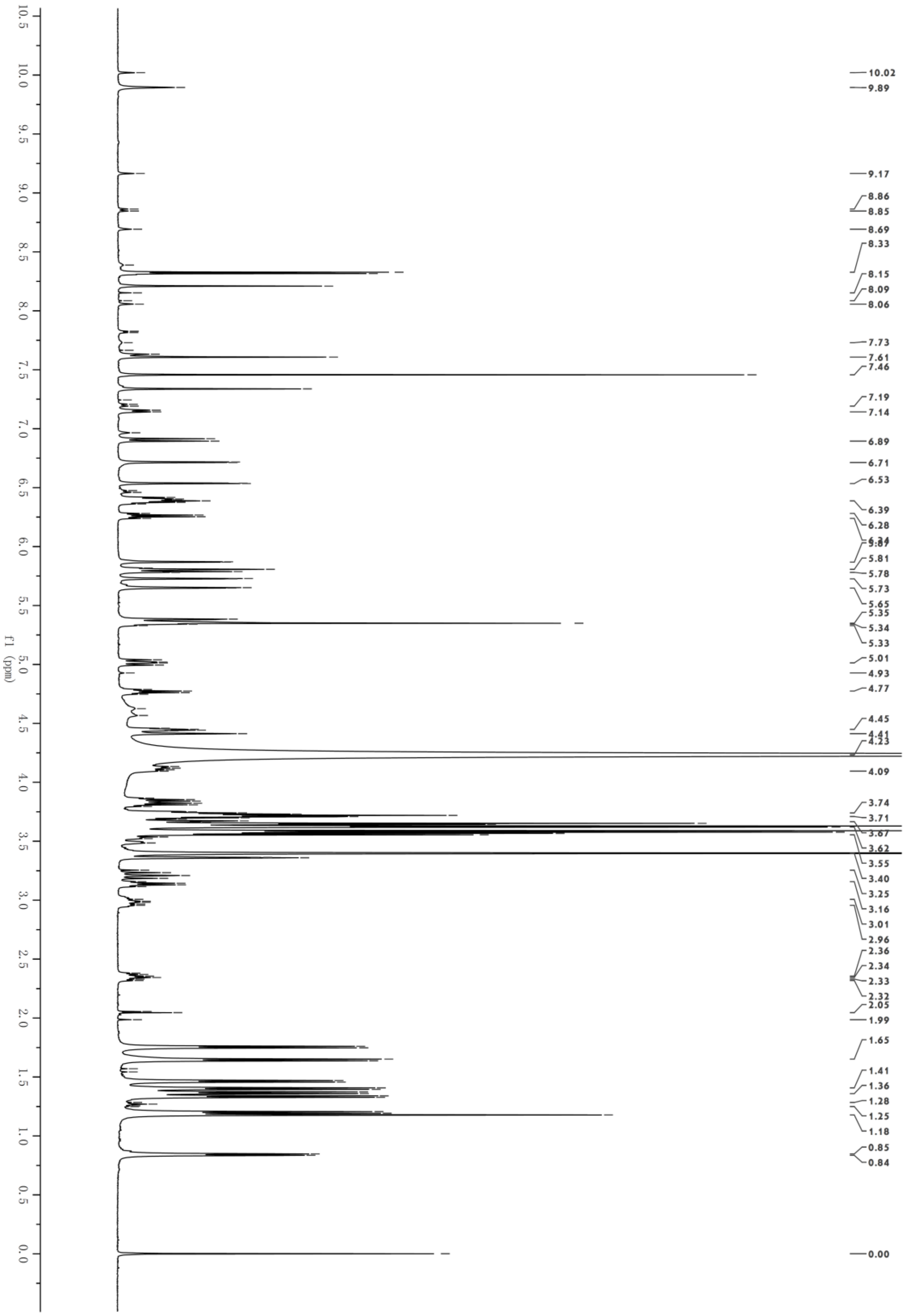
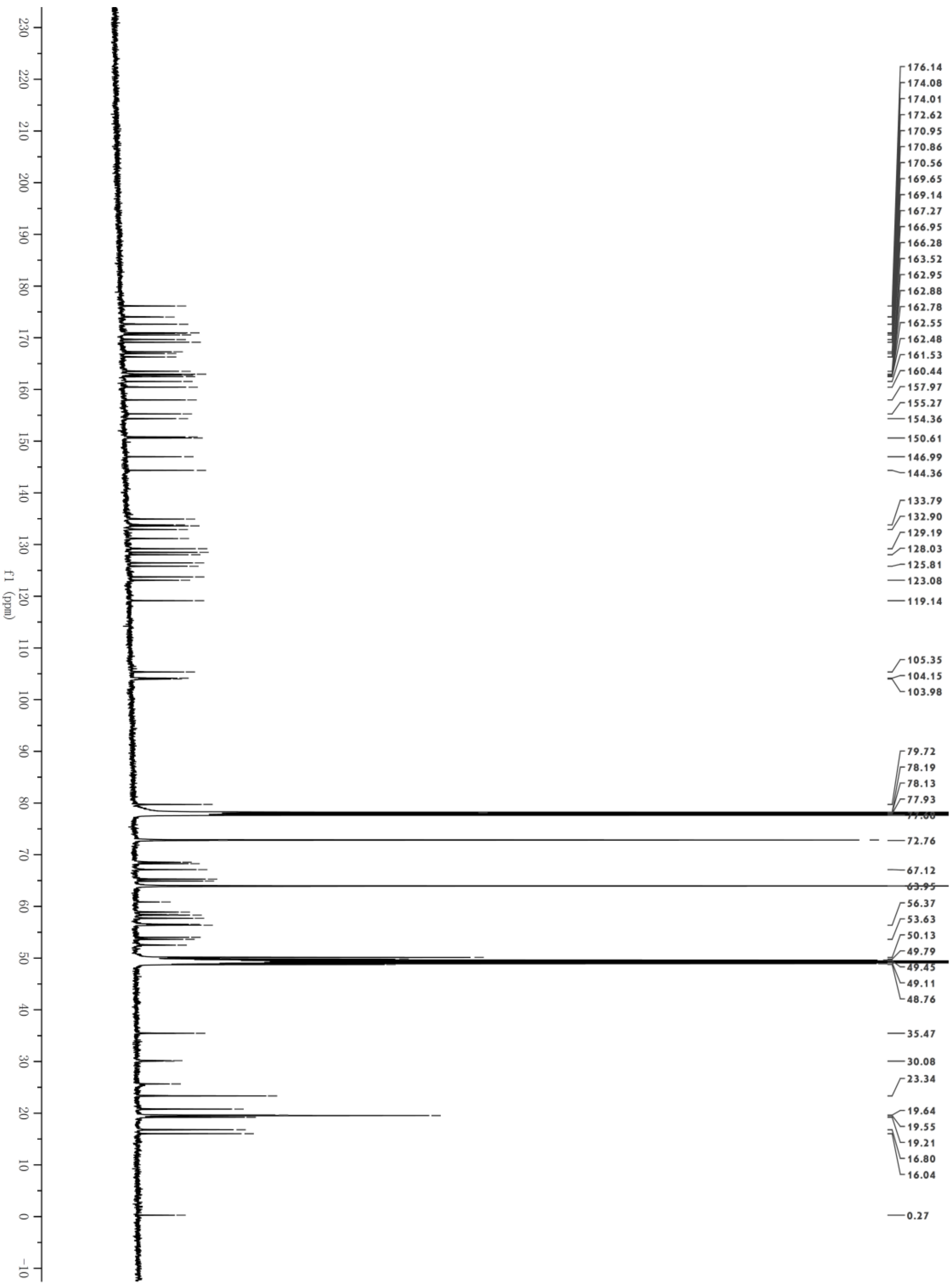


Fig. S4. 1D and 2D NMR spectra of TSR-Ile1Ala

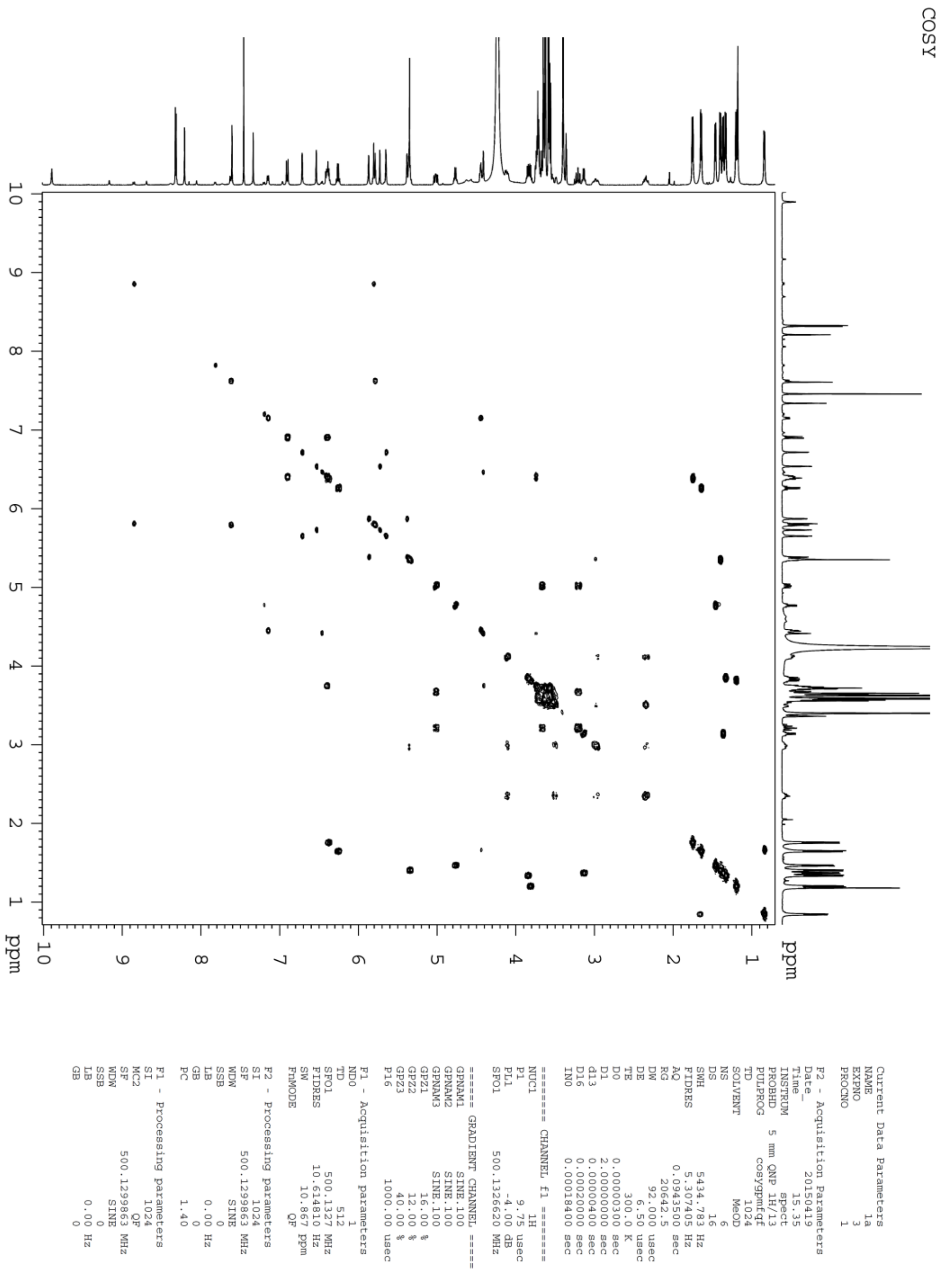
(A)[1]H spectrum of TSR-Ile1Ala



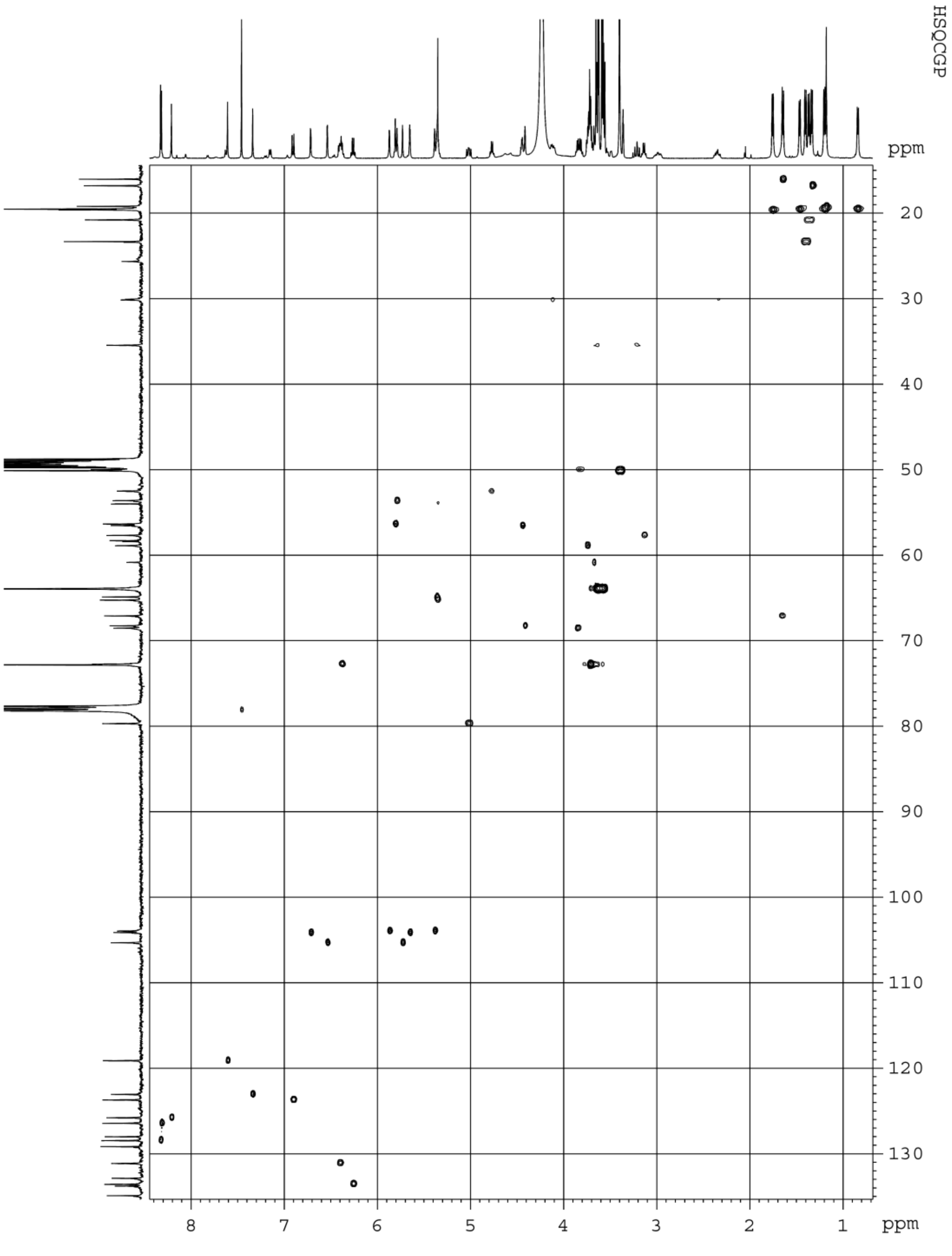
(B) ^{13}C spectrum of TSR-Ile1Ala



(C) COSY spectrum of TSR-Ile1Ala



(D) HSQC spectrum of TSR-Ile1Ala



(E) HMBC spectrum of TSR-Ile1Ala

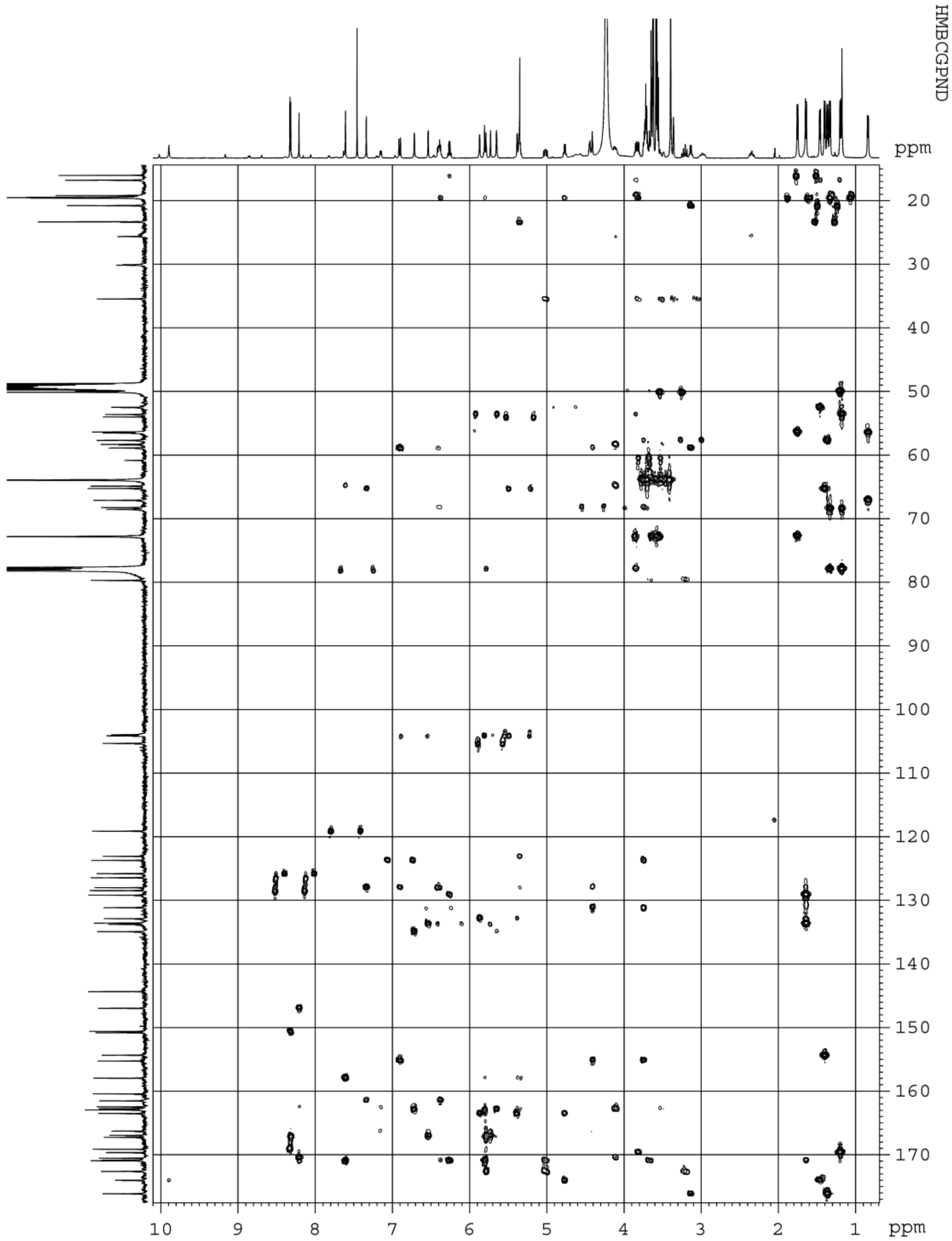


Table S2. [1]H and [13]C NMR assignments of TSR-Ile1Ala（*δ* in ppm, *J* in Hz）

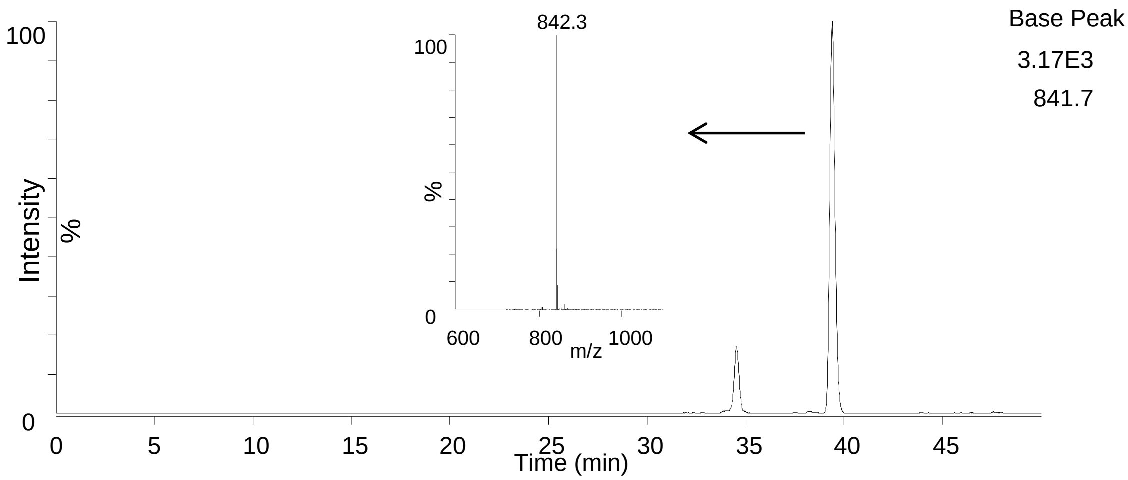
Assigment	δ_H	δ_C	HMBC	COSY
Ala1-1		176.2		
Ala1-2	3.15 m	57.8	Ala1-3; Q-7	Ala1-3
Ala1-3	1.36 d (7.10)	20.9	Ala1-1; Ala1-2	Ala1-2
Ala2-1		169.7		
Ala2-2	3.83 m	50.0	Ala2-1; Ala2-3	Ala2-3
Ala2-3	1.19 d (6.70)	19.4	Ala2-1; Ala2-2	Ala2-2
Ala2-NH	7.63 s			
Dha3-1		163.5		
Dha3-2		132.7		
Dha3-3	H_a 5.87 d (1.80),	103.7	Dha3-1; Dha3-2	
	H_b 5.38 d (1.70)		Dha3-1; Dha3-2	
Dha3-NH				
Ala4-1		174.1		
Ala4-2	4.77 q (6.60)	52.5	Dha3-1; Ala4-1; Ala4-3	Ala4-3
Ala4-3	1.45 d (6.60)	19.4	Ala4-1; Ala4-2	Ala4-2
Ala4-NH				
Pip5-1	5.25 s	64.9	Thz13-1; Thz13-2; Pip5-2	
Pip5-2		162.9		
Pip5-3	H_a 2.98 m	25.7		Pip5-3-H_b; Pip5-4-H_a; Pip5-4-H_b
	H_b 3.48 m		Pip5-2;	Pip5-3-H_a; Pip5-4-H_a
Pip5-4	H_a 2.34 m	30.3	Pip5-3	Pip5-3-H_a; Pip5-3-H_b; Pip5-4-H_b
	H_b 4.10 m		Pip5-1; Pip5-2; Pip5-3; Pip5-5; Thz6-4; Thz13-1; Thz14-4	Pip5-3-H_a; Pip5-4-H_a
Pip5-5		58.3		
Pip5-NH	9.89 s		Ala4-1	
Thz6-1		162.6		
Thz6-2		147.1		
Thz6-3	8.21 s	125.8	Thz6-1; Thz6-2; Thz6-4	
Thz6-4		170.7		
Thr7-1		166.3		
Thr7-2	4.44 m	56.6	Thr7-1	Thr7-3; Thr7-NH
Thr7-3	1.63 m	67.2		Thr7-2; Thr7-4
Thr7-4	0.84 d (6.10)	19.6	Thr7-2; Thr7-3	Thr7-3
Thr7-NH	7.15 d (7.30)		Thz6-1; Thr7-1	Thr7-2
Dhb8-1		129.3		
Dhb8-2	6.26 q (7.00)	133.6	Dhb8-1; Dhb8-3; Tzn9-4	Dhb8-3
Dhb8-3	1.64 d (7.00)	16.0	Dhb8-1; Dhb8-2; Tzn9-4	Dhb8-2
Dhb8-NH	8.69 s		Dhb8-2	
Tzn9-1		172.6		
Tzn9-2	5.01 dd (9.25,12.65)	79.7	Dhb8-1; Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3-H_a; Tzn9-3-H_b
Tzn9-3	H_a 3.21 t (12.05)	35.6	Tzn9-1; Tzn9-2	Tzn9-2; Tzn9-3-H_b
	H_b 3.67 m		Tzn9-2	Tzn9-2; Tzn9-3-H_a

Assigment	δ_H	δ_C	HMBC	COSY
Tzn9-4		170.7		
Ile10-1	5.79 s	53.7	Tzn9-1; Ile10-2; Thz11-4	Ile10-NH
Ile10-2		77.8		
Ile10-3	3.85 m	68.4	Ile10-1; Ile10-2; Ile10-4; Ile10-5	Ile10-4
Ile10-4	1.33 d (6.40)	16.8	Ile10-2; Ile10-3	Ile10-3
Ile10-5	1.18 s	19.4	Ile10-1; Ile10-2; Ile10-3	
Ile10-NH	7.63 s			Ile10-1
Thz11-1		162.8		
Thz11-2		150.6		
Thz11-3	8.32 s	126.5	Thz11-1; Thz11-2; Thz11-4	
Thz11-4		167.3		
Thr12-1	5.81 s	56.4	Thr12-3; Thz11-1; Thz13-1; Thz13-3	Thr12-NH
Thr12-2	6.38 m	72.8	Thr12-3; Thz13-3; Q-1	Thr12-3
Thr12-3	1.74 d (6.55)	19.8	Thr12-1; Thr12-2; Thz13-3	Thr12-2
Thr12-NH	8.85 d (8.60)			Thr12-1
Thz13-1		158.1		
Thz13-2	7.60 s	119.3	Thz13-1; Thz13-3; Pip5-1	
Thz13-3		171.0		
Thz14-1		160.5		
Thz14-2		150.8		
Thz14-3	8.33 s	128.5	Thz14-1; Thz14-2; Thz14-4	
Thz14-4		169.1		
Dha15-1		162.8		
Dha15-2		135.0		
Dha15-3	H_a 6.71 d (2.15),	104.2	Dha15-1; Dha15-2	Dha15-3- H_b
	H_b 5.64 d (2.10)		Dha15-1; Dha15-2	Dha15-3- H_b
Dha15-NH	10.02s			
Dha16-1		167.0		
Dha16-2		133.8		
Dha16-3	H_a 6.54 d (1.55),	106.4	Dha16-1; Dha16-2	Dha16-3- H_b
	H_b 5.73 d (1.50)		Dha16-1; Dha16-2	Dha16-3- H_a
Q-1		161.5		
Q-2		144.4		
Q-3	7.34 s	123.1	Q-1; Q-10; Q-11	
Q-4		154.5		
Q-5	6.90 d (9.95)	123.7	Q-7; Q-9; Q-10	Q-6
Q-6	6.40 m	131.2	Q-7; Q-8; Q-10	Q-5; Q-7
Q-7	3.74 m	58.9	Q-5; Q-6; Q-8; Q-9; Ala1-2	Q-6; Q-8
Q-8	4.41 s	68.3	Q-6; Q-7; Q-9; Q-10	Q-7

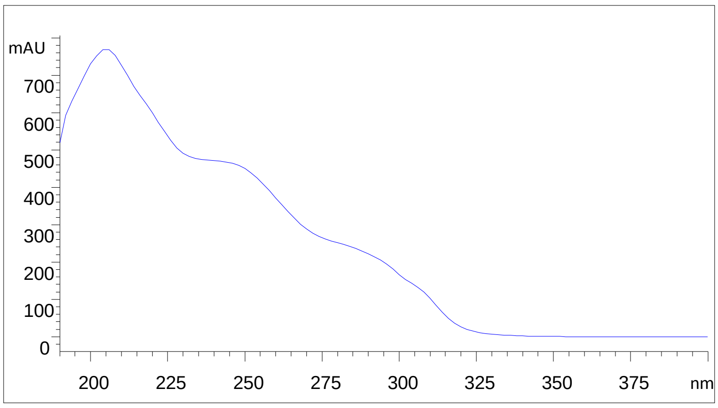
Assigment	δ_H	δ_C	HMBC	COSY
Q-9		155.4		
Q-10		128.1		
Q-11	5.34 m	65.3	Q-3; Q-10; Q-12	Q-12
Q-12	1.40 d (6.57)	23.4	Q-4; Q-11	Q-11

Fig. S5. HPLC-MS analysis, Ultraviolet absorption pattern and structure of TSR-Ile1Met

(A)Chromatogram extracted for *m/z* 842.3, the calculated $[M+2H]^{2+}$ ion of TSR-Ile1Met.



(B) Ultraviolet absorption pattern of TSR-Ile1Met



(C) structure of TSR-Ile1Met

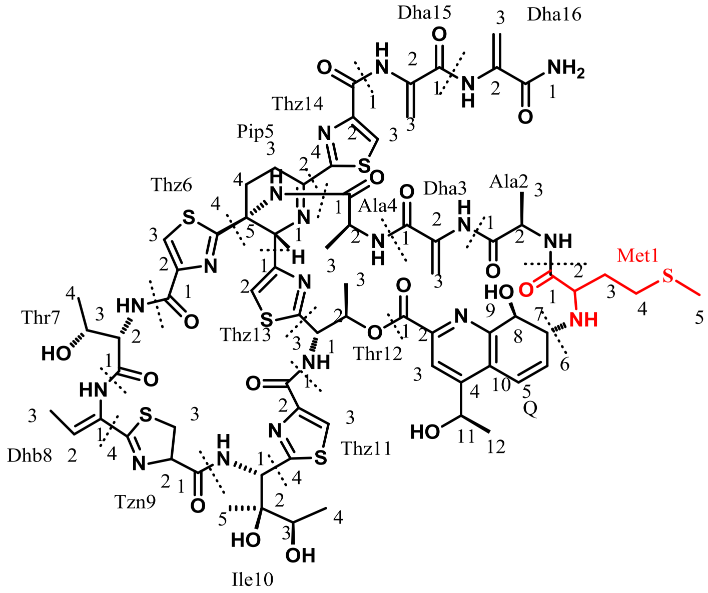
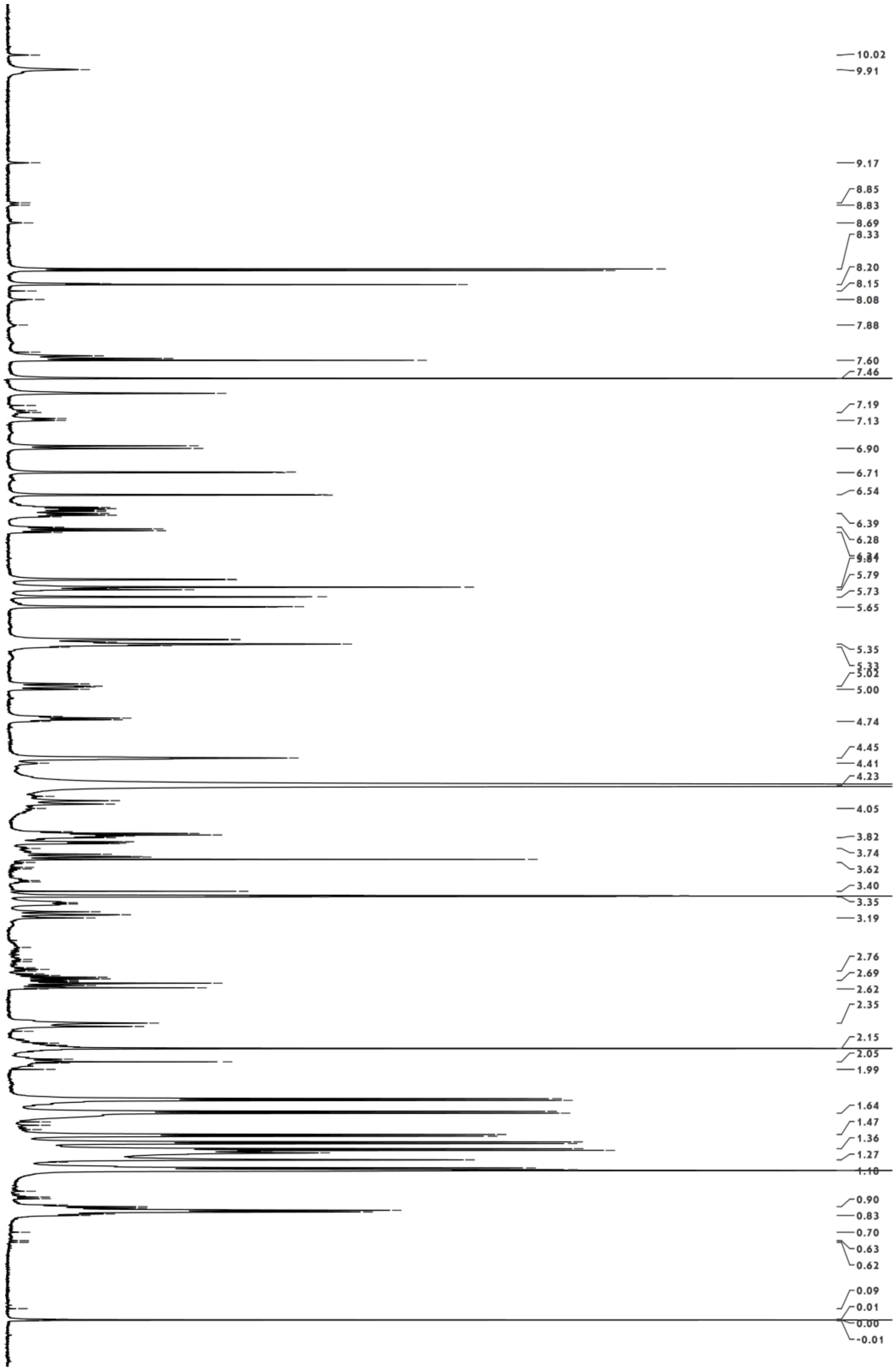
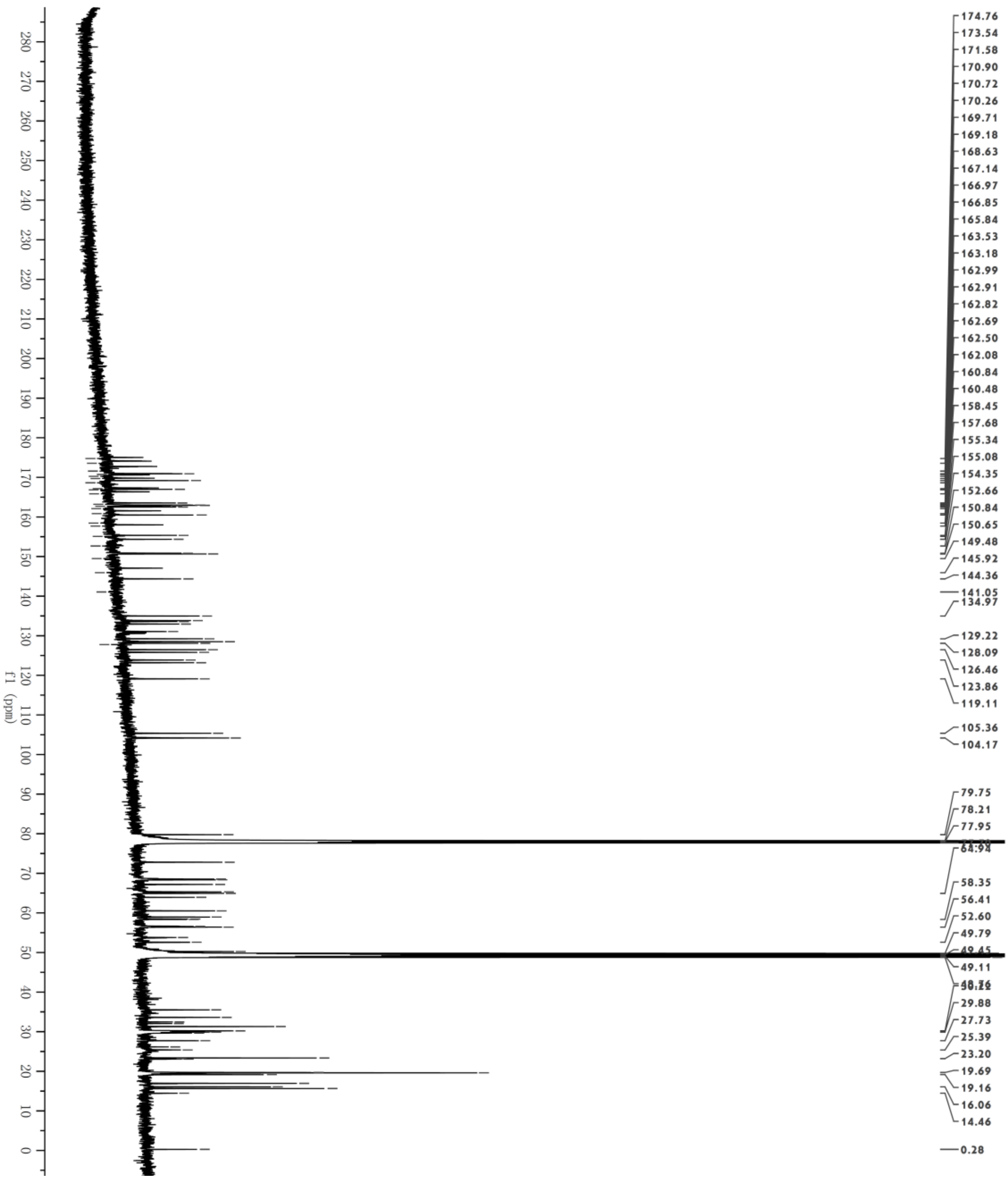


Fig. S6. 1D and 2D NMR spectra of TSR-Ile1Met

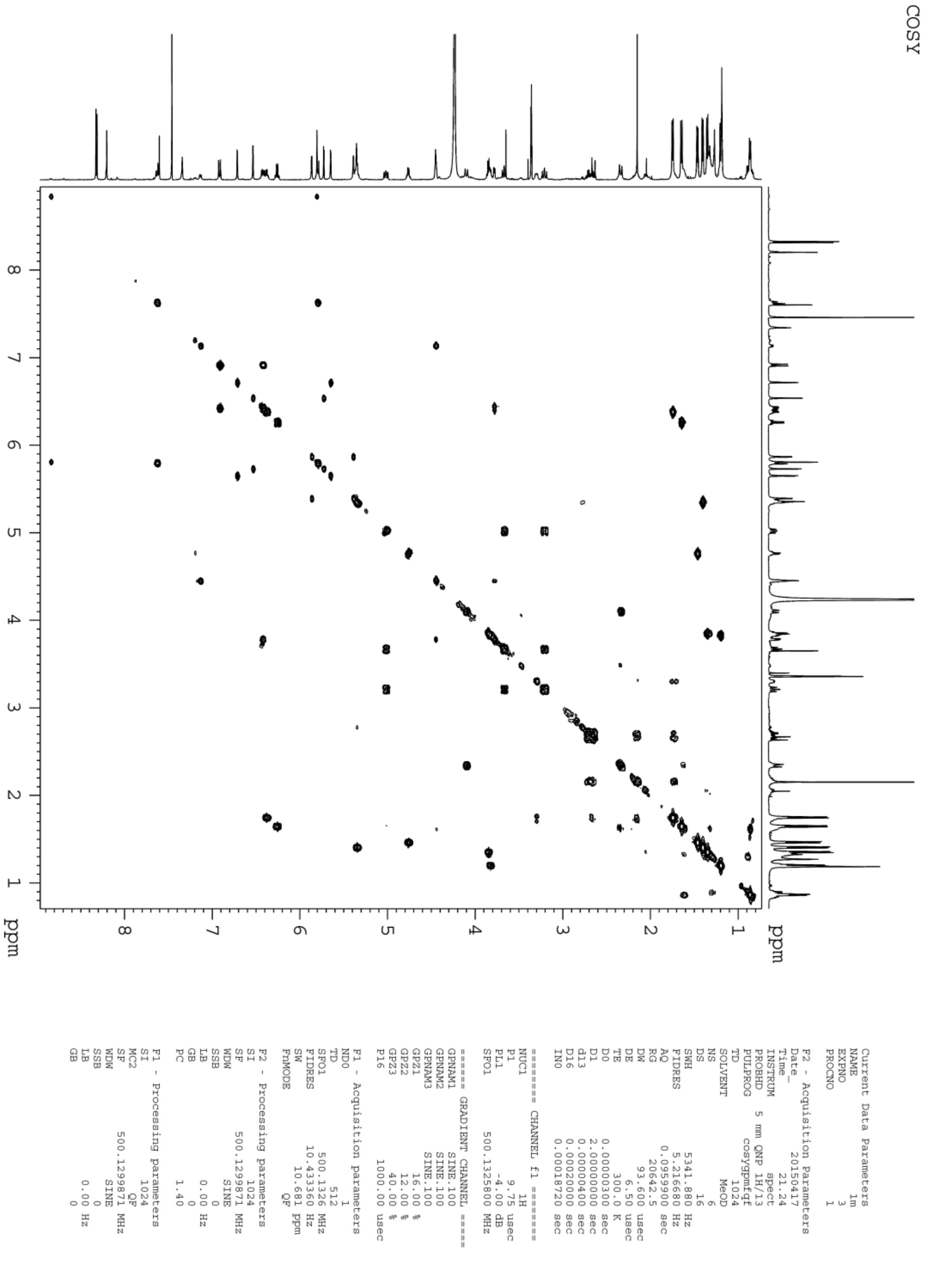
(A)^{1}H spectrum of TSR-Ile1Met



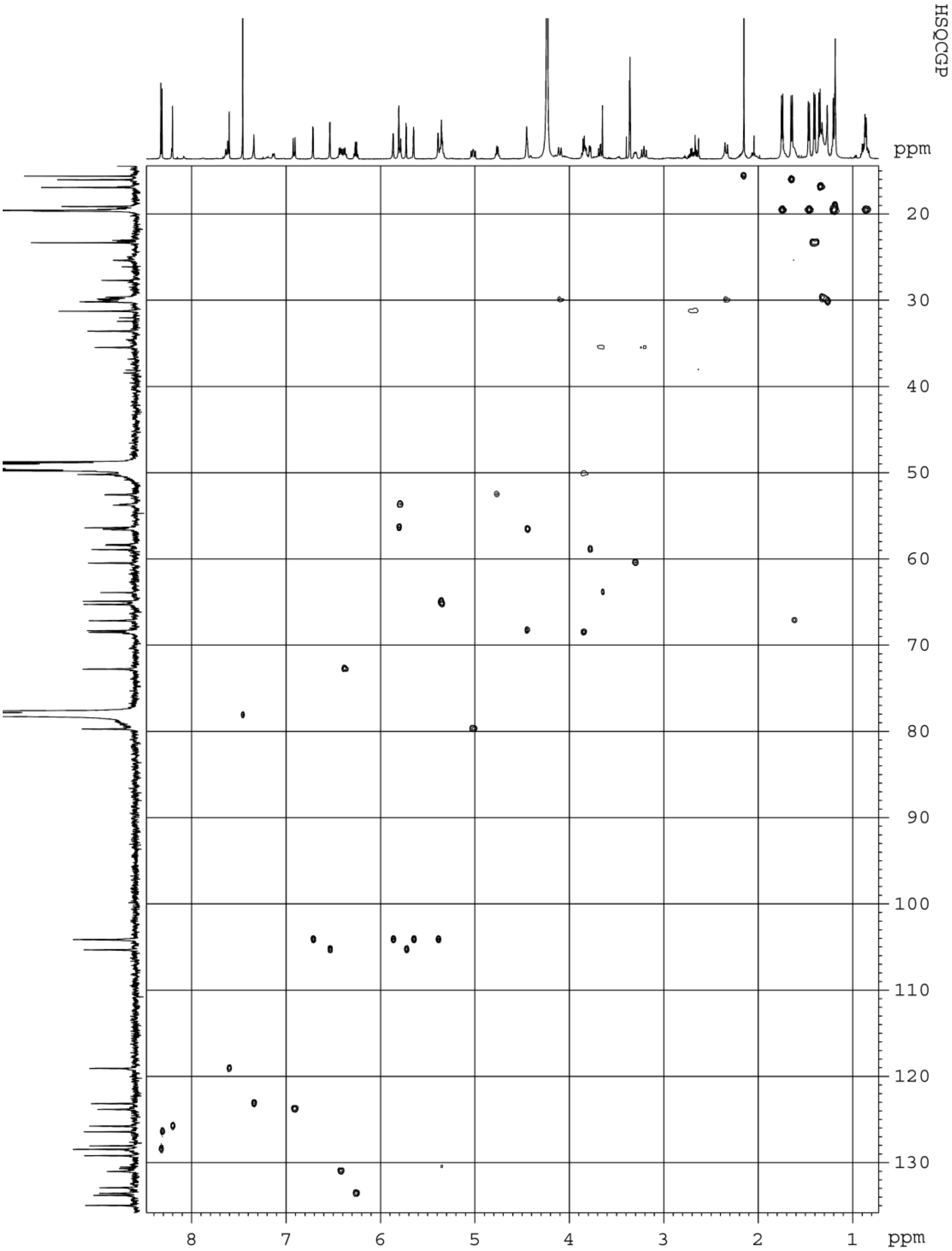
(B)^{13}C spectrum of TSR-Ile1Met



(C) COSY spectrum of TSR-Ile1Met



(D)HSQC spectrum of TSR-Ile1Met



(E)HMBC spectrum of TSR-Ile1Met

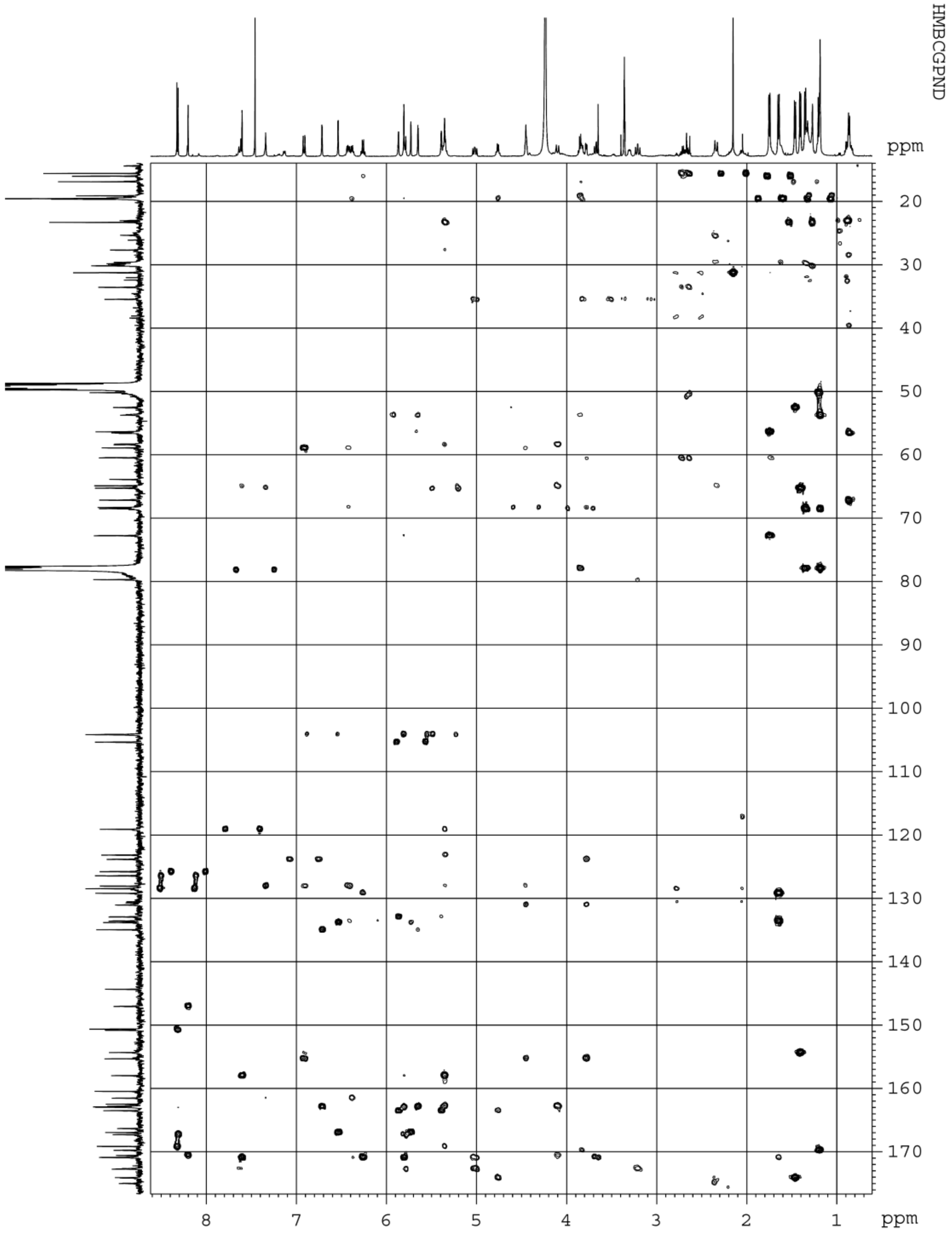


Table S3. ^{1}H and ^{13}C NMR assignments of TSR-Ile1Met（δ in ppm, J in Hz）

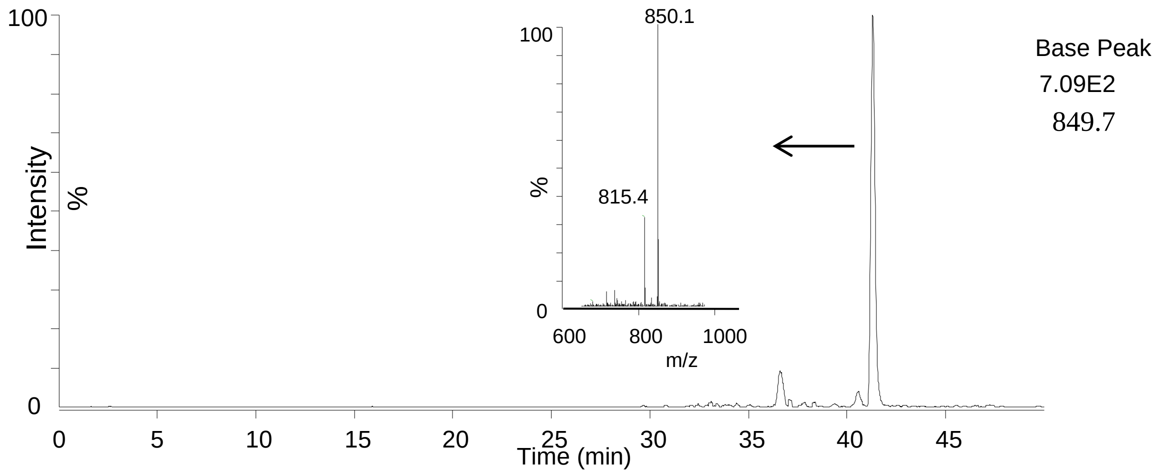
Assigment	δ_H	δ_C	HMBC	COSY
Met1-1		175.0		
Met1-2	3.29 m	60.6		Met1-3- H$_a$
Met1-3	H$_a$ 1.72 m H$_b$ 2.13 m	33.5	Met1-2	Met1-2; Met1-3-H$_b$; Met1-4- H$_a$ Met1-2; Met1-3-H$_a$; Met1-4- H$_a$
Met1-4	H$_a$ 2.63 m H$_b$ 2.71 m	31.3	Met1-2; Met1-3; Met1-5 Met1-2; Met1-5	Met1-3-H$_a$; Met1-3-H$_b$; Met1-4- H$_b$ Met1-4- H$_a$
Met1-5	2.15 s	15. 6	Met1-4	
Ala2-1		169.9		
Ala2-2	3.84 m	50.1	Ala2-1; Ala2-3	Ala2-3
Ala2-3	1.20 d (6.84)	19.6	Ala2-1; Ala2-2	Ala2-2
Ala2-NH	7.63 bs			
Dha3-1		163.5		
Dha3-2		133.0		
Dha3-3	H$_a$ 5.86 s H$_b$ 5.39 s	104.1	Dha3-1; Dha3-2 Dha3-1; Dha3-2	Dha3-3- H$_b$ Dha3-3- H$_b$
Dha3-NH				
Ala4-1		174.2		
Ala4-2	4.76 q (6.42)	52.5	Ala4-1; Ala4-3; Dha3-1	
Ala4-3	1.45 d (6.56)	19.5	Ala4-1; Ala4-2	
Ala4-NH				
Pip5-1	5.35 m	65.1	Pip5-2; Pip5-5; Thz13-1; Thz13-2; Thz14-4	
Pip5-2		162.8		
Pip5-3	H$_a$ 2.95 m H$_b$ 3.48 m	25.4		Pip5-4-H$_a$; Pip5-4-H$_b$ Pip5-4-H$_a$
Pip5-4	H$_a$ 2.32 m H$_b$ 4.10 m	29.7	Pip5-1; Pip5-3; Ala4-1 Pip5-1; Pip5-2; Pip5-3; Pip5-5; Thz6-4	Pip5-3-H$_a$; Pip5-3-H$_b$; Pip5-4-H$_b$ Pip5-3-H$_a$; Pip5-4-H$_a$
Pip5-5		58.5		
Pip5-NH				
Thz6-1		162.6		
Thz6-2		147.1		
Thz6-3	8.20 s	125.8	Thz6-2; Thz6-4	
Thz6-4		170.7		
Thr7-1		166.4		
Thr7-2	4.44 m	56.7	Thr7-1; Thr7-3	Thr7-3; Thr7-NH
Thr7-3	1.61 m	67.3		Thr7-2; Thr7-4
Thr7-4	0.85 d (6.25)	19.7	Thr7-2; Thr7-3	Thr7-3
Thr7-NH	7.13 d (7.84)			Thr7-2
Dhb8-1		129.2		
Dhb8-2	6.26 q (6.88)	133.8	Dhb8-1; Dhb8-3; Tzn9-4	Dhb8-3
Dhb8-3	1.64 d (7.04)	16.1	Dhb8-1; Dhb8-2; Tzn9-4	Dhb8-2
Dhb8-NH	8.69 s			
Tzn9-1		172.8		

Assigment	δ_H	δ_C	HMBC	COSY
Tzn9-2	5.01 dd (9.08,12.69)	79.8	Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3-H_a; Tzn9-3-H_b
Tzn9-3	H_a 3.20 m H_b 3.67 m	35.6	Tzn9-1; Tzn9-4 Tzn9-2; Tzn9-4	Tzn9-2; Tzn9-3-H_b Tzn9-2; Tzn9-3-H_a
Tzn9-4		171.0		
Ile10-1	5.78 s	53.8	Tzn9-1; Thz11-4	Ile10-NH
Ile10-2		78.1		
Ile10-3	3.84 m	68.6	Ile10-1; Ile10-2; Ile10-4; Ile10-5	
Ile10-4	1.33 d (6.25)	17.0	Ile10-2; Ile10-3	
Ile10-5	1.19 s	19.4	Ile10-1; Ile10-2; Ile10-3	
Ile10-NH	7.61 m			Ile10-1
Thz11-1		163.0		
Thz11-2		150.8		
Thz11-3	8.31 s	126.5	Thz11-1; Thz11-2; Thz11-4	
Thz11-4		167.3		
Thr12-1	5.80 m	56.5	Thz11-1; Thz13-1; Thz13-3	Thr12-NH
Thr12-2	6.36 m	72.8	Thr12-3; Q-1	
Thr12-3	1.74 d (6.58)	19.5	Thr12-1; Thr12-2; Thz13-3	
Thr12-NH	8.84 d (8.86)			Thr12-1
Thz13-1		158.1		
Thz13-2	7.60 s	119.2	Thz13-1; Thz13-3; Pip5-1	
Thz13-3		170.9		
Thz14-1		160.6		
Thz14-2		150.7		
Thz14-3	8.32 s	128.6	Thz14-2; Thz14-4	
Thz14-4		169.0		
Dha15-1		163.0		
Dha15-2		135.0		
Dha15-3	H_a 6.71 bs H_b 5.64 bs	104.1	Dha15-1; Dha15-2 Dha15-1	Dha15-3- H_b Dha15-3- H_a
Dha15-NH	10.02 s			
Dha16-1		167.0		
Dha16-2		133.9		
Dha16-3	H_a 6.53 d (1.74) H_b 5.72 d (1.72)	106.4	Dha16-1; Dha16-2 Dha16-1	Dha16-3- H_b Dha16-3- H_a
Dha16-NH				
Q-1		161.6		
Q-2		144.3		
Q-3	7.34 s	123.3	Q-1; Q-10; Q-11	
Q-4		158.1		
Q-5	6.91 d (10.08)	124.0	Q-7; Q-9; Q-10	Q-6

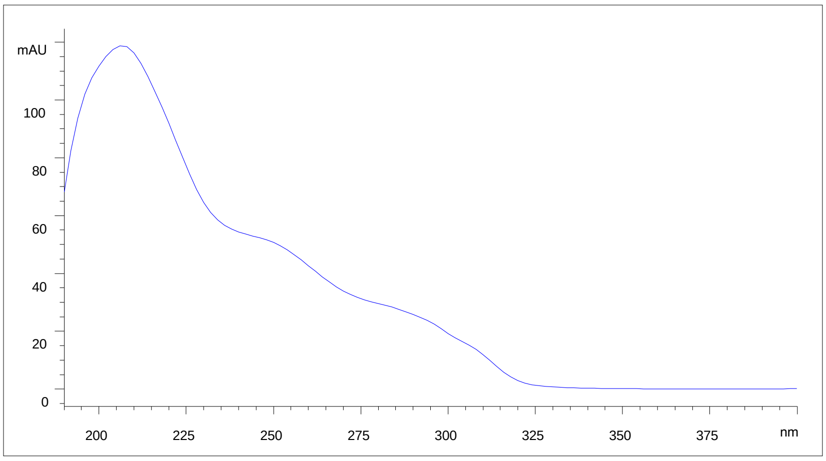
Assigment	δ_H	δ_C	HMBC	COSY
Q-6	6.41 m	131.1	Q-7; Q-10	Q-5; Q-7
Q-7	3.77 dd (1.92,5.68)	59.2	Met1-2; Q-5; Q-6; Q-8; Q-9	Q-6; Q-8
Q-8	4.44 m	68.5	Q-6; Q-7; Q-9; Q-10	Q-7
Q-9		155.5		
Q-10		128.6		
Q-11	5.35 m	65.0	Q-12	Q-12
Q-12	1.40 d (6.60)	23.5	Q-4; Q-11	Q-11

Fig. S7. HPLC-MS analysis, Ultraviolet absorption pattern and structure of TSR-Ile1Phe

(A) Chromatogram extracted for *m/z* 850.1, the calculated $[M+2H]^{2+}$ ion of TSR-Ile1Phe.



(B) Ultraviolet absorption pattern of TSR-Ile1Phe



（C）structure of TSR-Ile1Phe

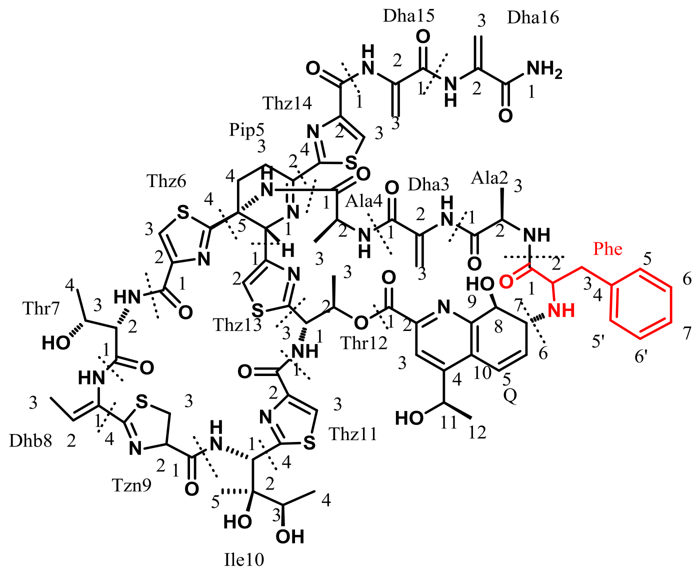
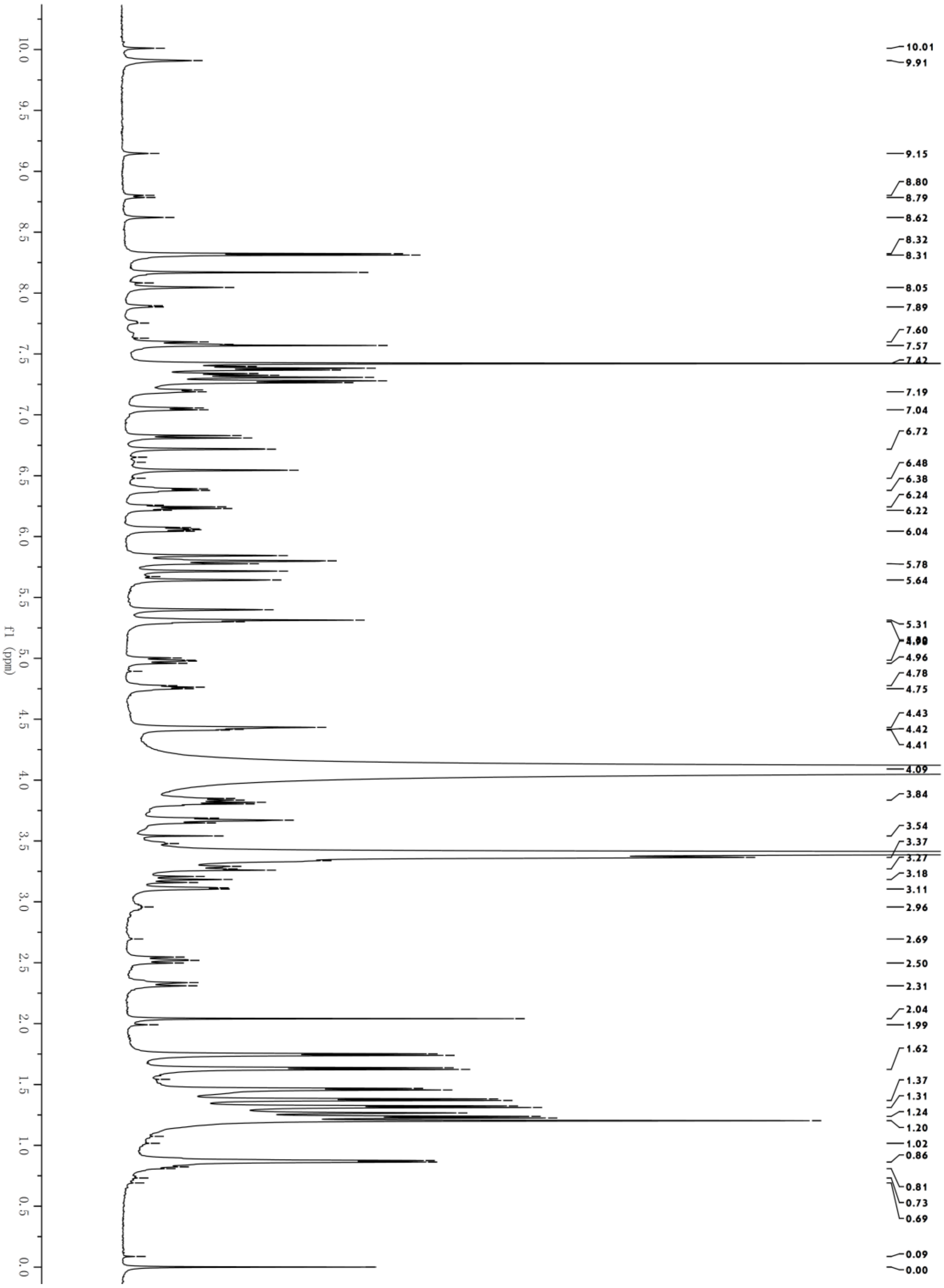
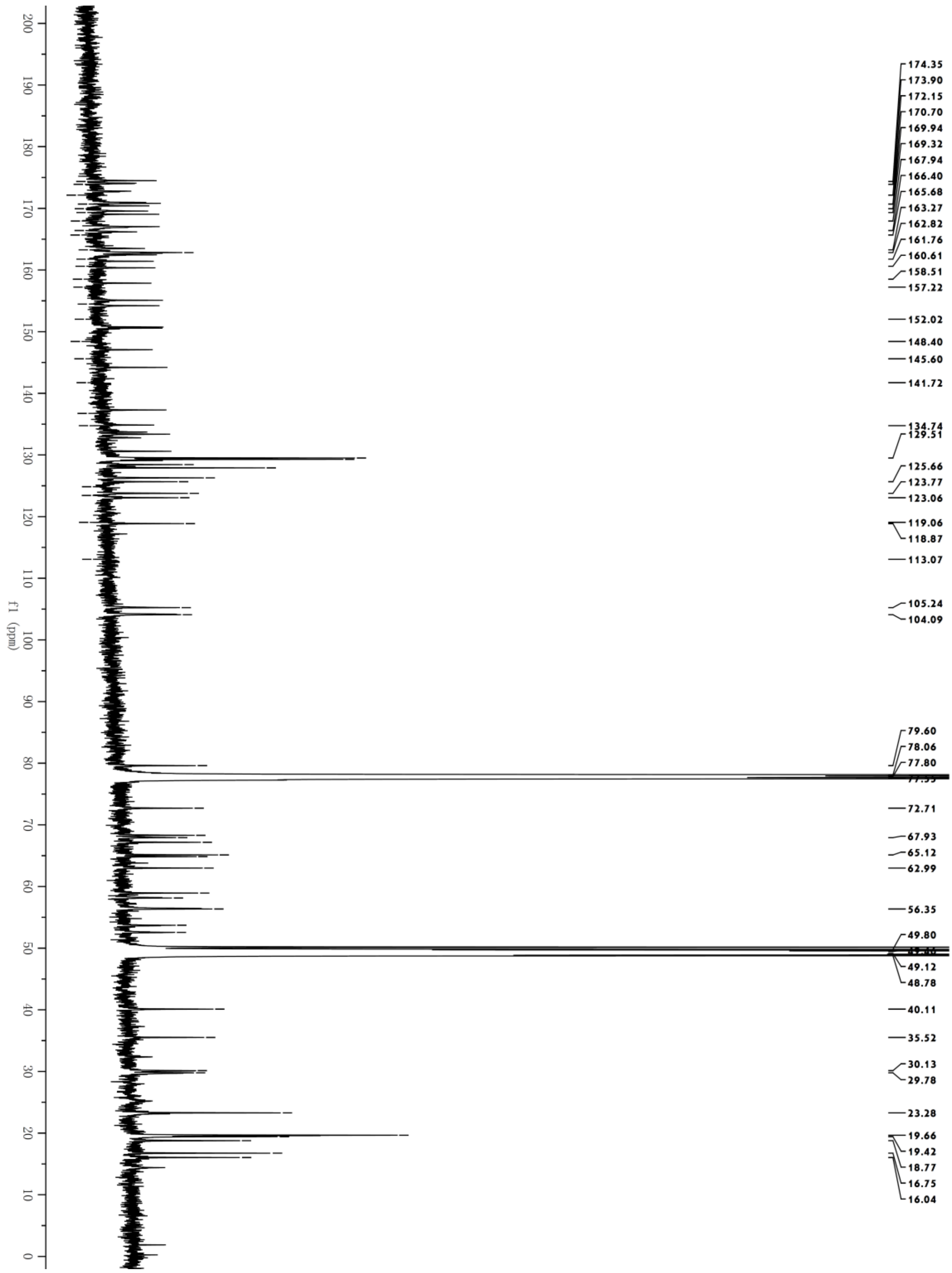


Fig. S8. 1D and 2D NMR spectra of TSR-Ile1Phe

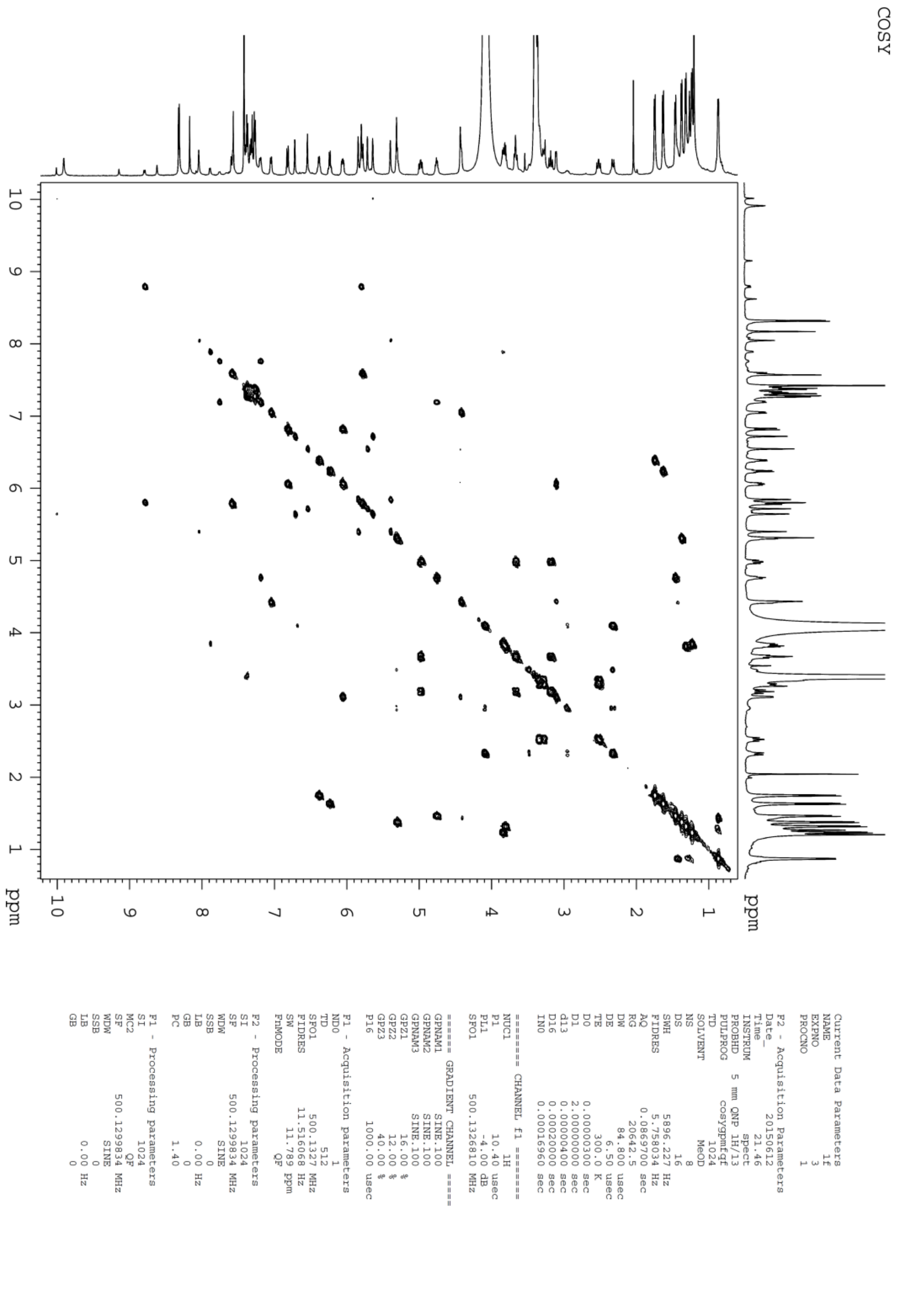
(A) ^{1}H spectrum of TSR-Ile1Phe



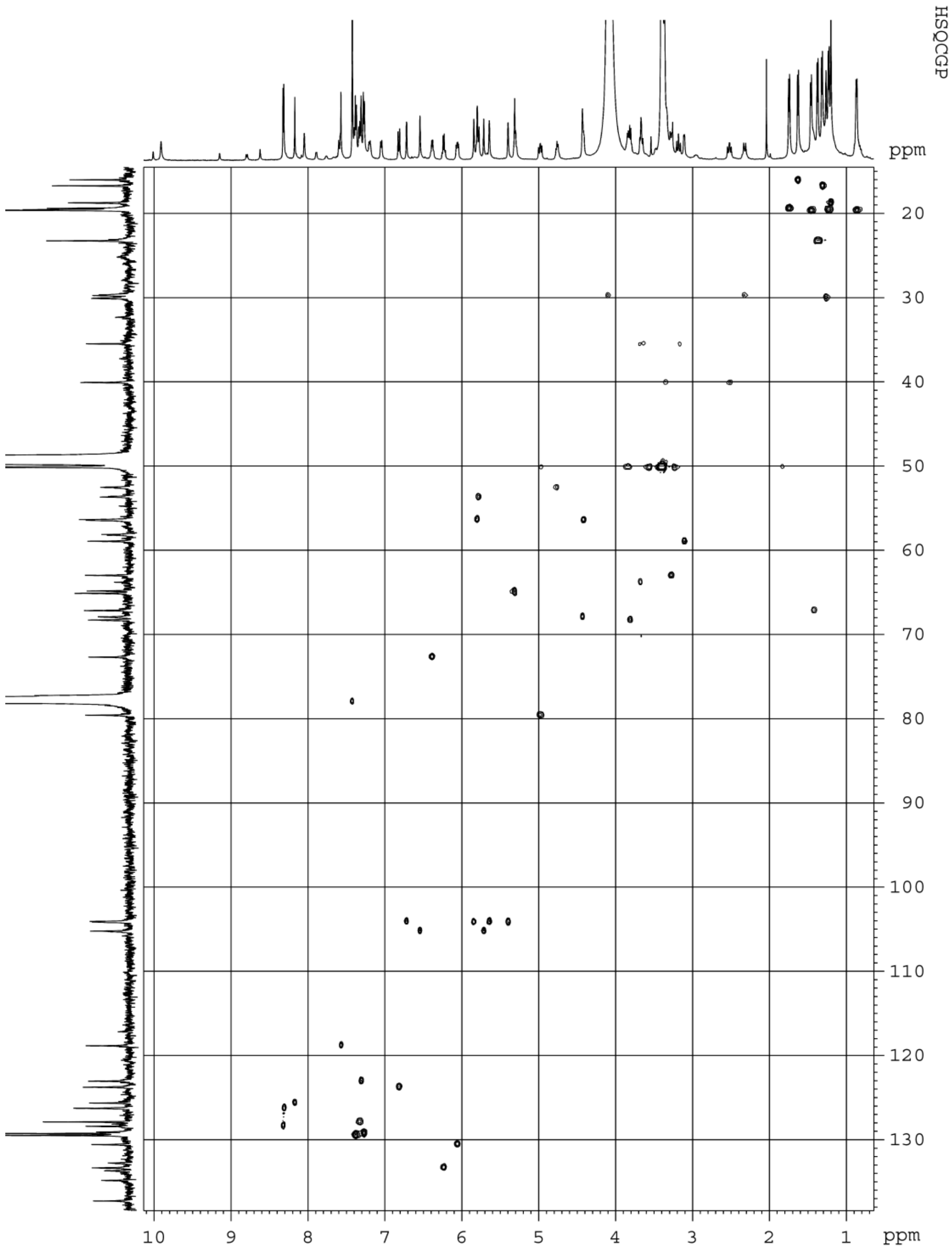
(B)^{13}C spectrum of TSR-Ile1Phe



(C) COSY spectrum of TSR-Ile1Phe



(C) HSQC spectrum of TSR-Ile1Phe



(D) HMBC spectrum of TSR-Ile1Phe

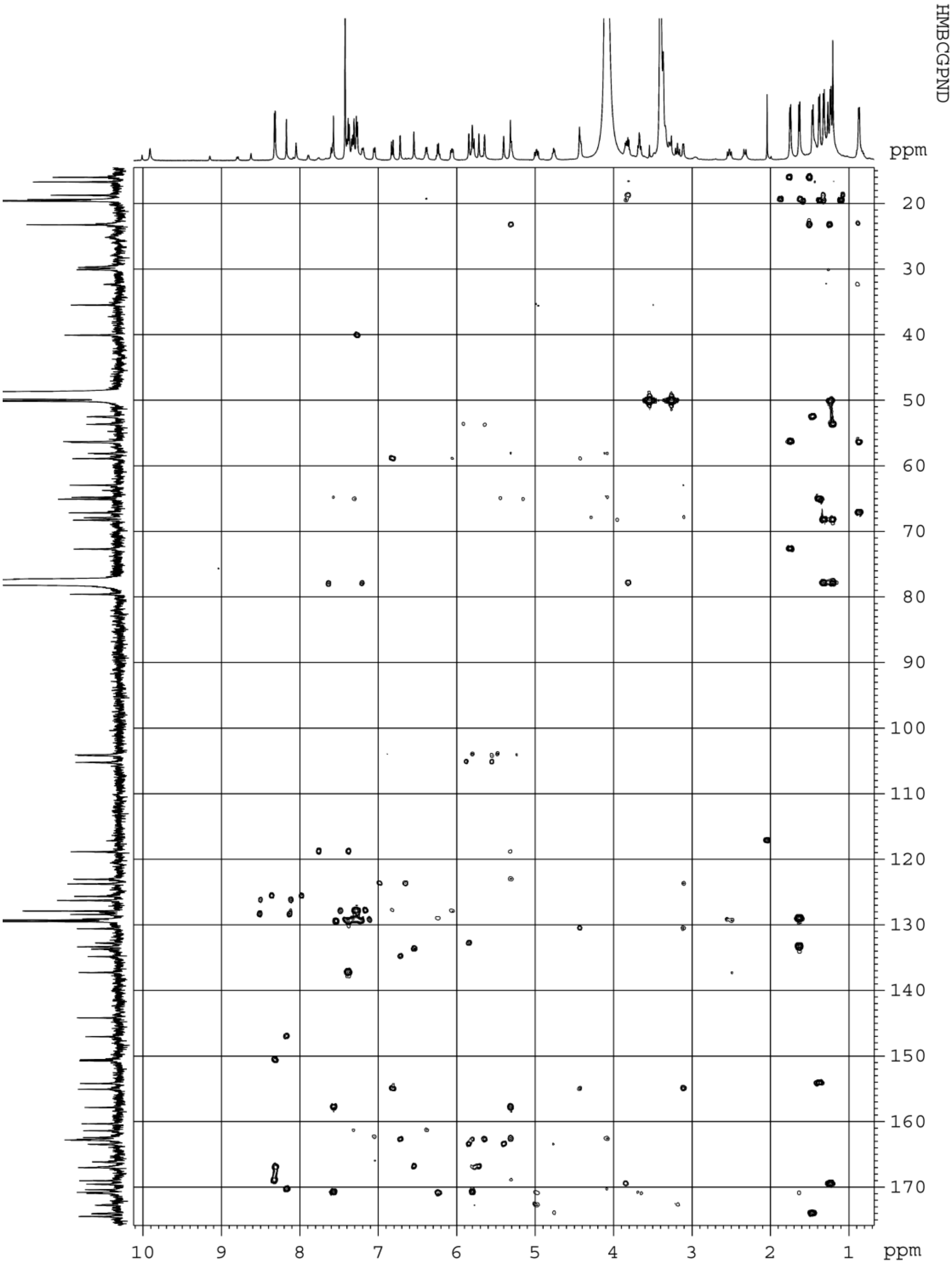


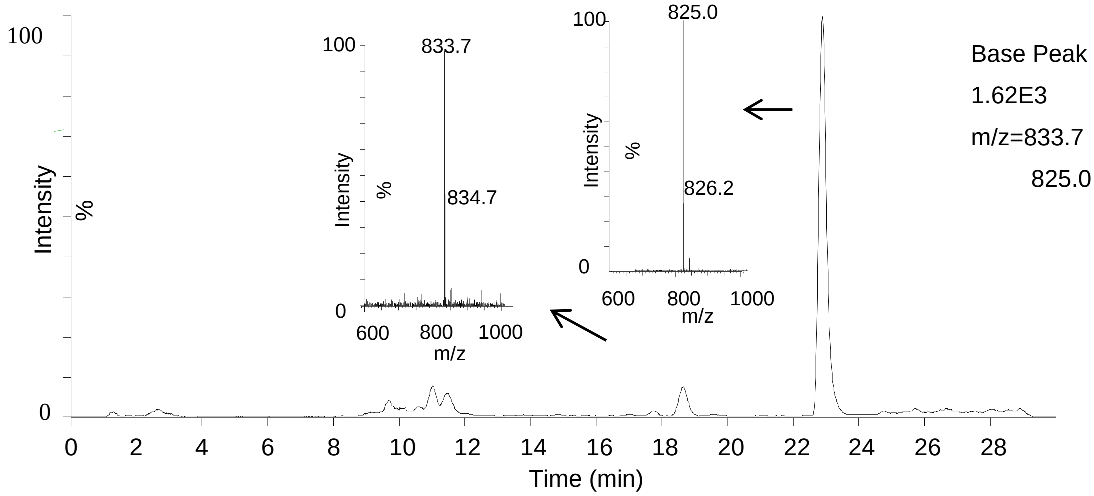
Table S4. ^{1}H and ^{13}C NMR assignments of TSR-Ile1Phe（δ in ppm, J in Hz）

Assigment	δ_H	δ_C	HMBC	COSY
Phe1-1		174.6		
Phe1-2	3.26 m	63.1		Phe1-3-H_a; Phe1-3-H_b
Phe1-3	H_a 2.52 m H_b 3.34 m	40.2	Phe1-2; Phe1-(5,5') Phe1-2	Phe1-2; Phe1-3-H_b Phe1-2; Phe1-3-H_a
Phe1-4		137.3		
Phe1-(5,5')	7.27 m	129.3	Phe1-3; Phe1-4;	Phe1-(6,6')
Phe1-(6,6')	7.37 m	129.5	Phe1-7;	Phe1-(5,5'); Phe1-7
Phe1-7	7.31 m	127.9		Phe1-(6,6')
Ala2-1		169.6		
Ala2-2	3.83 m	50.0	Ala2-1; Ala2-3;	Ala2-3
Ala2-3	1.20 d (6.65)	19.8	Ala2-1; Ala2-2;	Ala2-2
Dha3-1		163.6		
Dha3-2		132.8		
Dha3-3	H_a 5.84 s; H_b 5.40 s	104.2	Dha3-1; Dha3-2 Dha3-2;	Dha3-3-H_b; Dha3-3-H_a
Dha3-NH				
Ala4-1		174.1		
Ala4-2	4.75 q	52.6	Dha3-1; Ala4-1;	Ala4-3
Ala4-3	1.46 d (6.40)	19.7	Ala4-1; Ala4-2	Ala4-2
Ala4-NH				
Pip5-1	5.31 s	64.9	Pip5-2; Pip5-5; Thz13-1; Thz14-4	
Pip5-2		162.5		
Pip5-3	H_a 2.95 m; H_b 3.47 m	25.5		Pip5-3-H_b; Pip5-4-H_a; Pip5-4-H_b Pip5-3-H_a; Pip5-4-H_a;
Pip5-4	H_a 2.32 m; H_b 4.10 m	29.8	 Pip5-2; Pip5-5; Thz6-4	Pip5-3-H_a; Pip5-3-H_b; Pip5-4-H_b Pip5-3-H_a; Pip5-4-H_a
Pip5-5		58.2		
Pip5-NH				
Thz6-1		162.7		
Thz6-2		147.3		
Thz6-3	8.16 s	125.7	Thz6-2; Thz6-4	
Thz6-4		170.5		
Thr7-1		166.2		
Thr7-2	4.41 m	56.4	Thr7-3	Thr7-NH
Thr7-3	1.43 m	67.2		Thr7-4
Thr7-4	0.87 d (5.88)	19.8	Thr7-2; Thr7-3	Thr7-3
Thr7-NH	7.04 d (7.44)		Thz6-1; Thr7-1	Thr7-2
Dhb8-1		129.3		
Dhb8-2	6.24 q	133.4	Dhb8-1; Tzn9-4	Dhb8-3
Dhb8-3	1.63 d (6.96)	16.1	Dhb8-1; Dhb8-2; Tzn9-4	Dhb8-2
Dhb8-NH	8.62 s			
Tzn9-1		172.8		
Tzn9-2	4.98 dd (9.20,12.55)	79.6	Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3- H_a; Tzn9-3- H_b
Tzn9-3	H_a 3.17 m, H_b 3.66 t (9.92)	35.6	Tzn9-1 Tzn9-4	Tzn9-2; Tzn9-3- H_b Tzn9-2; Tzn9-3- H_a

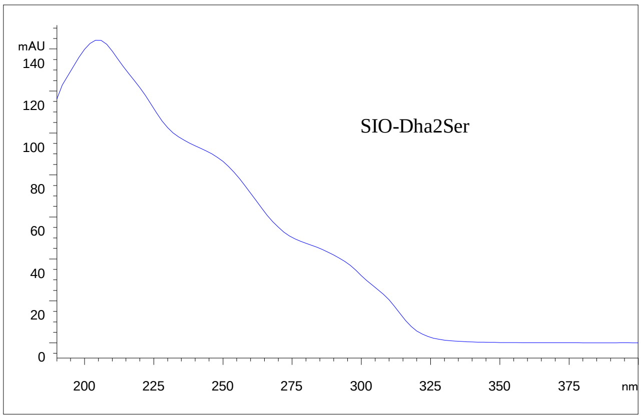
Assigment	δ_H	δ_C	HMBC	COSY
Tzn9-4		171.0		
Ile10-1	5.78 s	53.8	Ile10-2; Thz11-4	Ile10-NH
Ile10-2		78.0		
Ile10-3	3.81 m	68.3	Ile10-2; Ile10-4; Ile10-5	Ile10-4
Ile10-4	1.31 d (6.31)	16.8	Ile10-1; Ile10-2; Ile10-3	Ile10-3
Ile10-5	1.20 s	18.8	Ile10-2; Ile10-3	
Ile10-NH	7.60 s			Ile10-1
Thz11-1		162.8		
Thz11-2		150.7		
Thz11-3	8.31 s	126.3	Thz11-2; Thz11-4	
Thz11-4		167.1		
Thr12-1	5.80 s	56.7	Thz11-1; Thz13-3	Thr12-NH
Thr12-2	6.38 m	72.9	Q-1	Thr12-3
Thr12-3	1.74 d (6.45)	19.6	Thr12-1; Thr12-2	Thr12-2
Thr12-NH	8.79 d (8.61)			Thr12-1
Thz13-1		158.0		
Thz13-2	7.57 s	118.8	Thz13-1; Thz13-3	
Thz13-3		170.8		
Thz14-1		160.4		
Thz14-2		150.8		
Thz14-3	8.32 s	128.4	Thz14-2; Thz14-4	
Thz14-4		169.0		
Dha15-1		162.8		
Dha15-2		134.9		
Dha15-3	H_a 6.72 bs;	104.2	Dha15-1; Dha15-2	Dha15-3- H_b
	H_b 5.64 bs		Dha15-1	Dha15-3- H_a
Dha15-NH	10.01 s			
Dha16-1		167.1		
Dha16-2		133.8		
Dha16-3	H_a 6.54 bs;	105.3	Dha16-1; Dha16-2	Dha16-3- H_b
	H_b 5.71 bs		Dha16-1	Dha16-3- H_a
Q-1		161.5		
Q-2		144.3		
Q-3	7.31 s	123.1	Q-1; Q-10; Q-11	
Q-4		154.2		
Q-5	6.82 d (9.93)	123.9	Q-7; Q-9; Q-10	Q-6
Q-6	6.05 m	130.7	Q-7; Q-10	Q-5; Q-7
Q-7	3.11 d (4.91)	59.0	Phe1-2; Q-5; Q-6; Q-8; Q-9	Q-6; Q-8
Q-8	4.43 m	68.0	Q-6; Q-7; Q-9; Q-10	Q-7
Q-9		155.0		
Q-10		127.9		
Q-11	5.30 m	65.1	Q-12	Q-12
Q-12	1.37 d (6.49)	23.5	Q-4; Q-11	Q-11

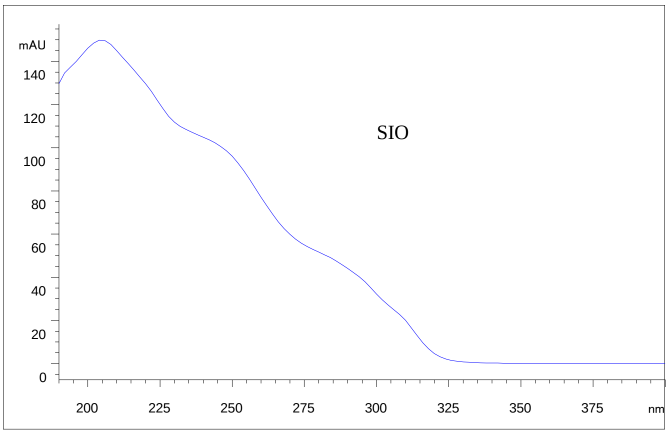
Fig. S9. HPLC-MS analysis, Ultraviolet absorption patterns and structures of SIO-Dha2Ser and SIO

(A) Chromatogram extracted for *m/z* 833.7 and 825.0, the calculated $[M+2H]^{2+}$ ion of SIO-Dha2Ser and SIO.



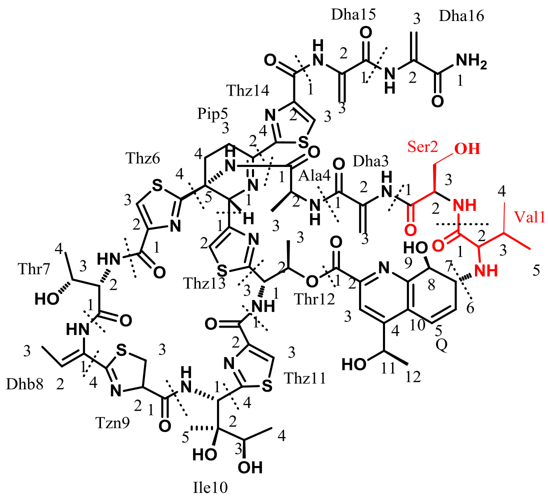
(B) Ultraviolet absorption patterns of SIO-Dha2Ser and SIO





(C) structures of SIO-Dha2Ser and SIO

SIO-Dha2Ser



SIO

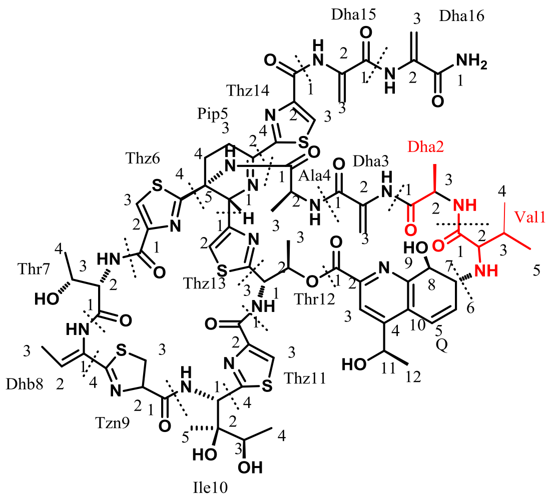
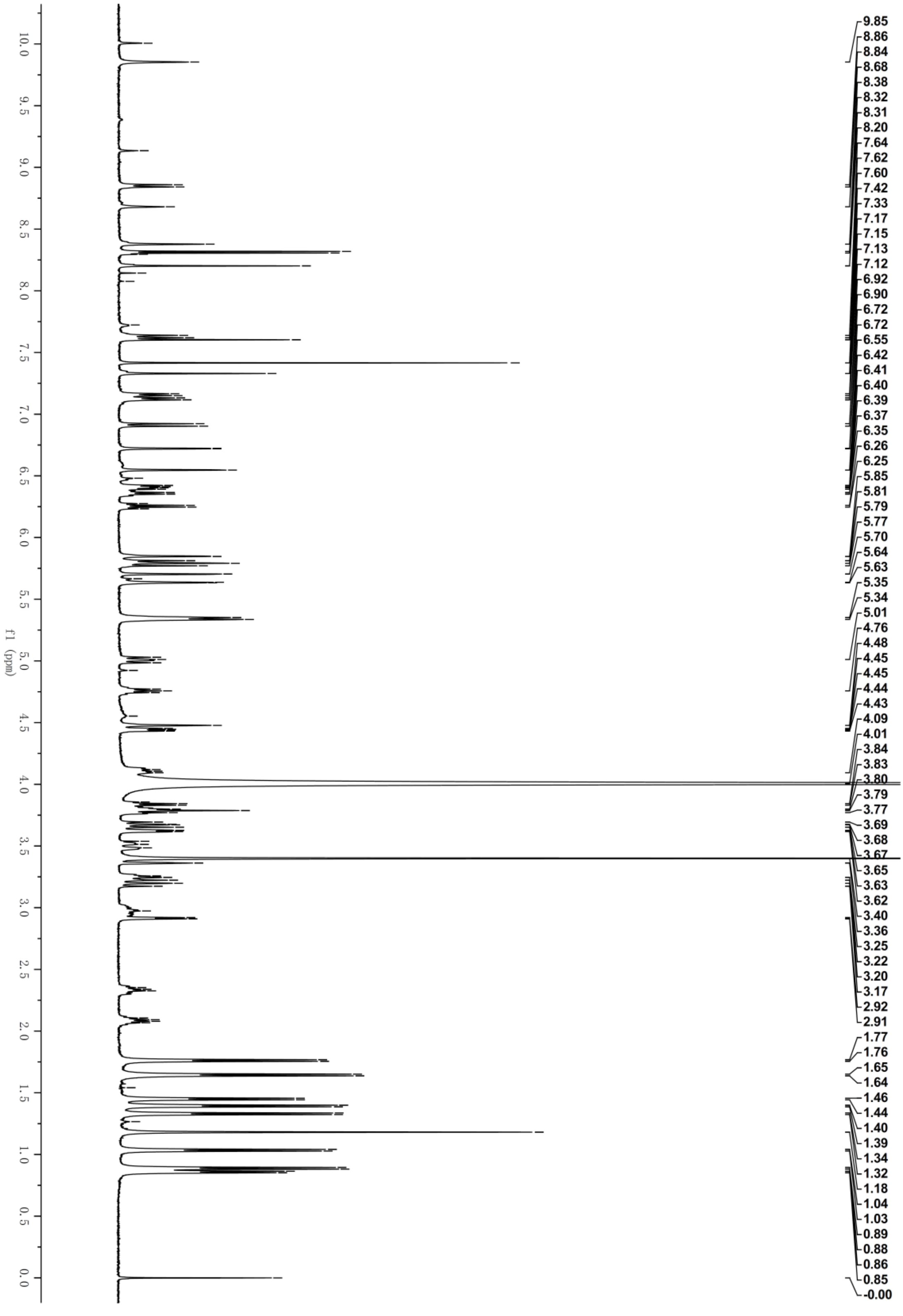
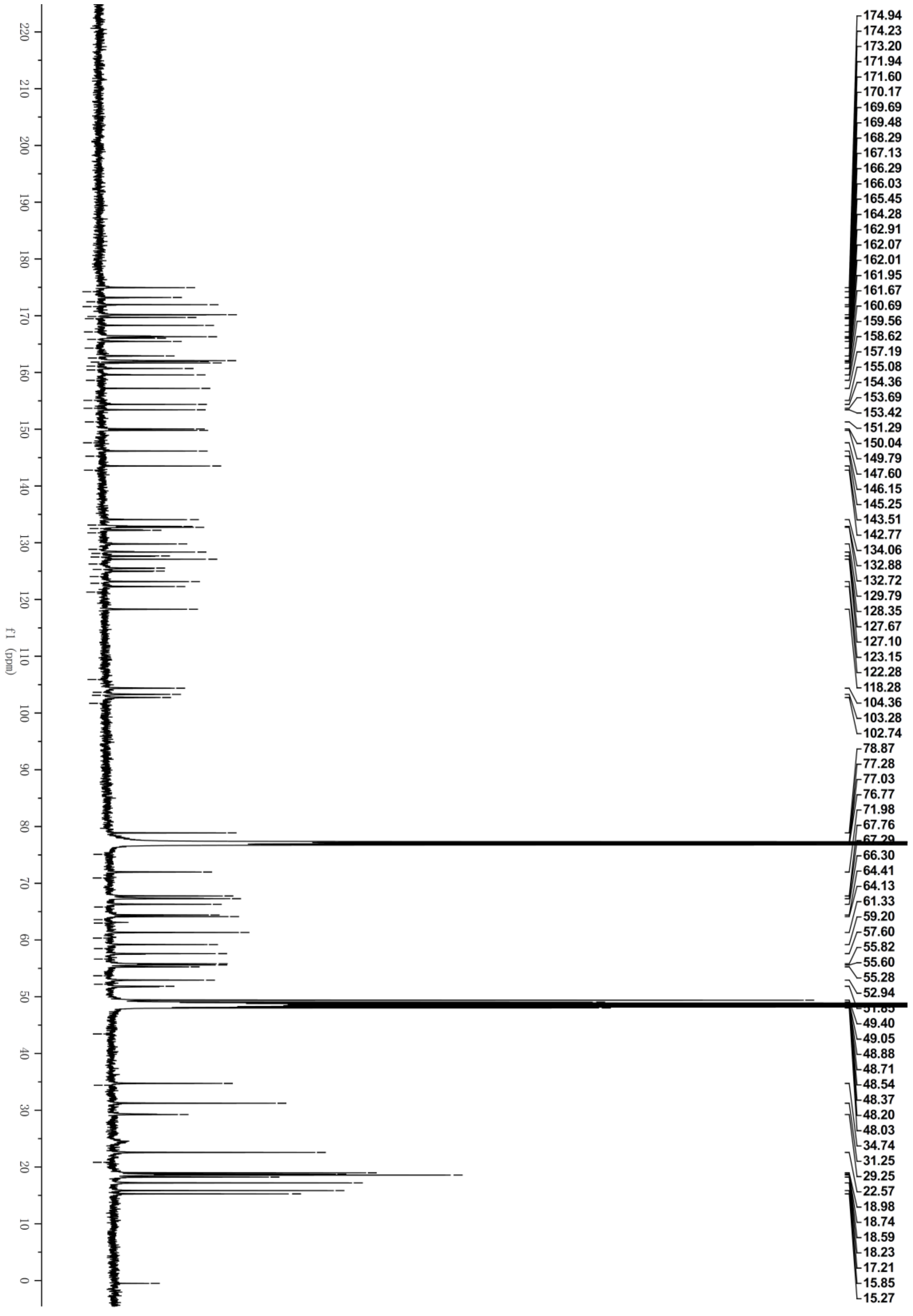


Fig. S10. 1D and 2D NMR spectra of SIO-Dha2Ser

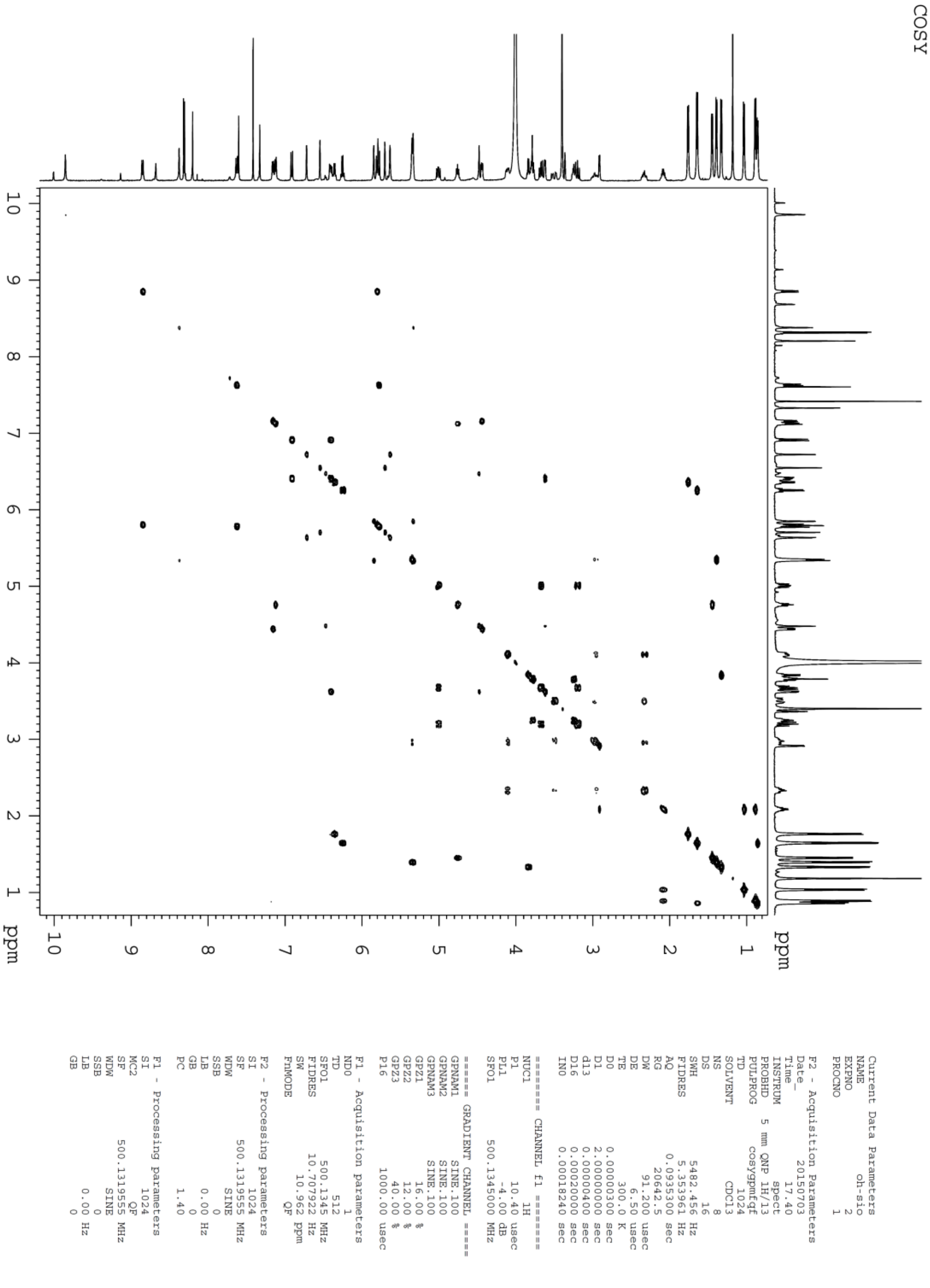
(A) ^{1}H spectrum of SIO-Dha2Ser



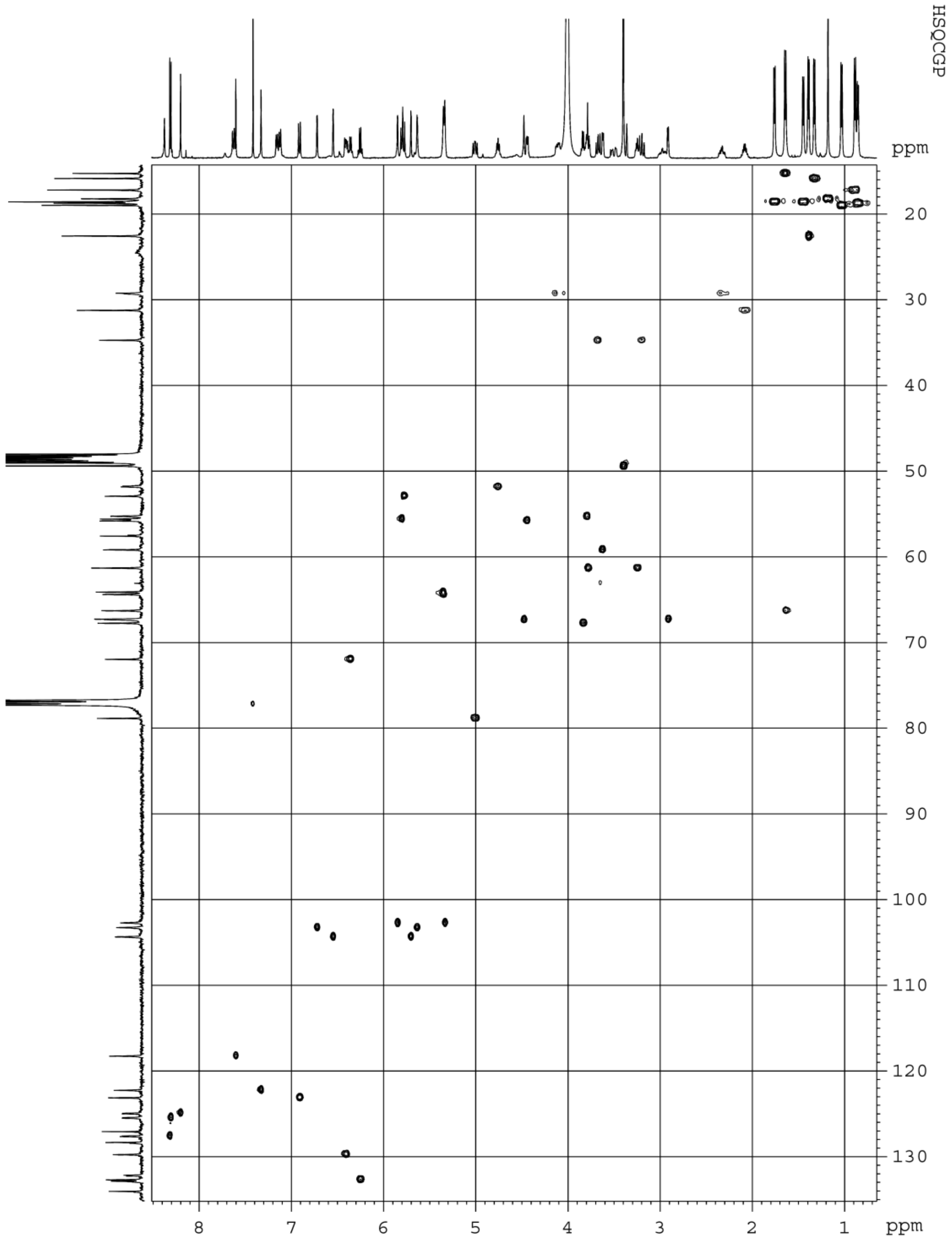
(B) ^{13}C spectrum of SIO-Dha2Ser



(C) COSY spectrum of SIO-Dha2Ser



(D) HSQC spectrum of SIO-Dha2Ser



(E) HMBC spectrum of SIO-Dha2Ser

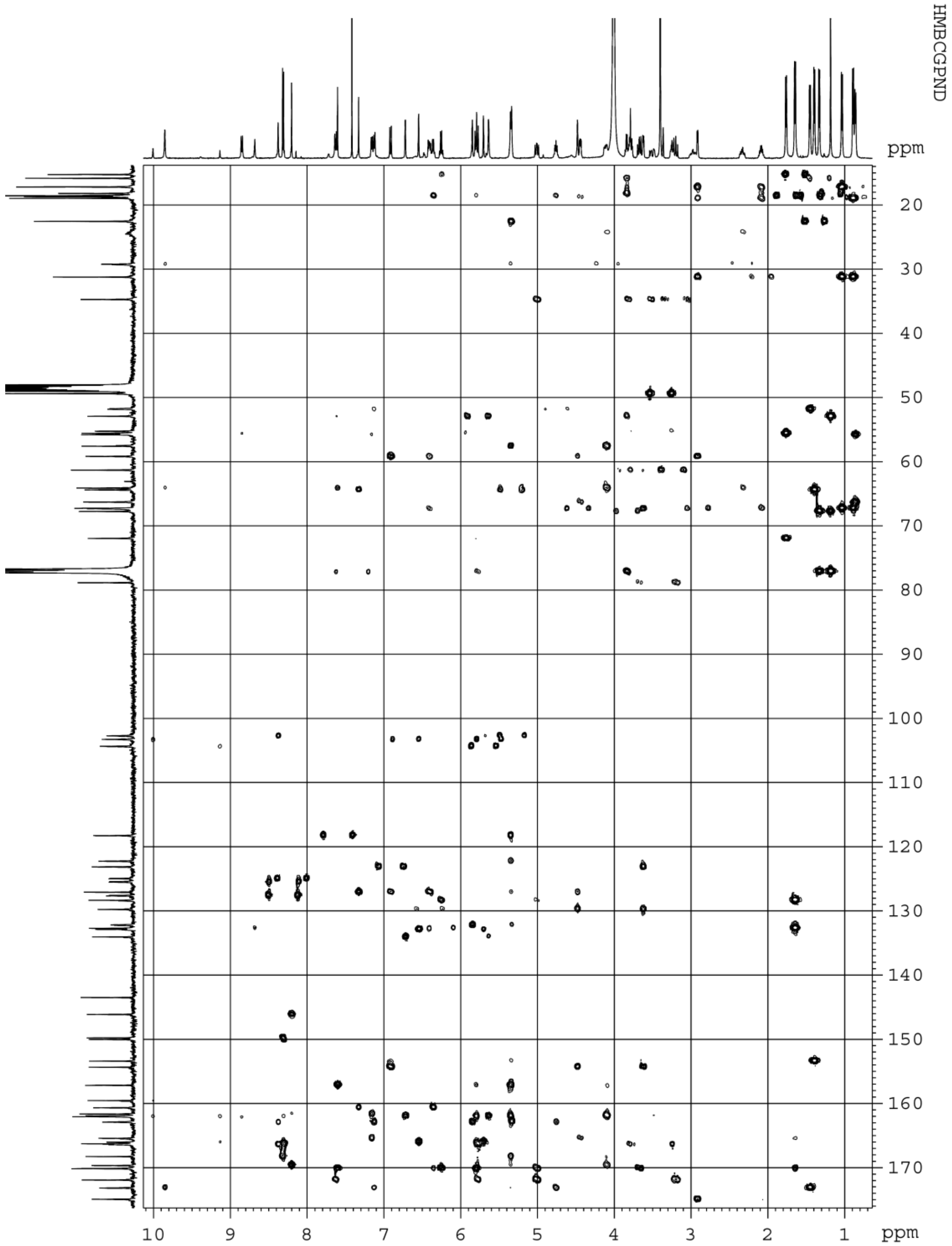


Table S5. [1]H and [13]C NMR assignments of SIO-Dha2Ser（δ in ppm, J in Hz）

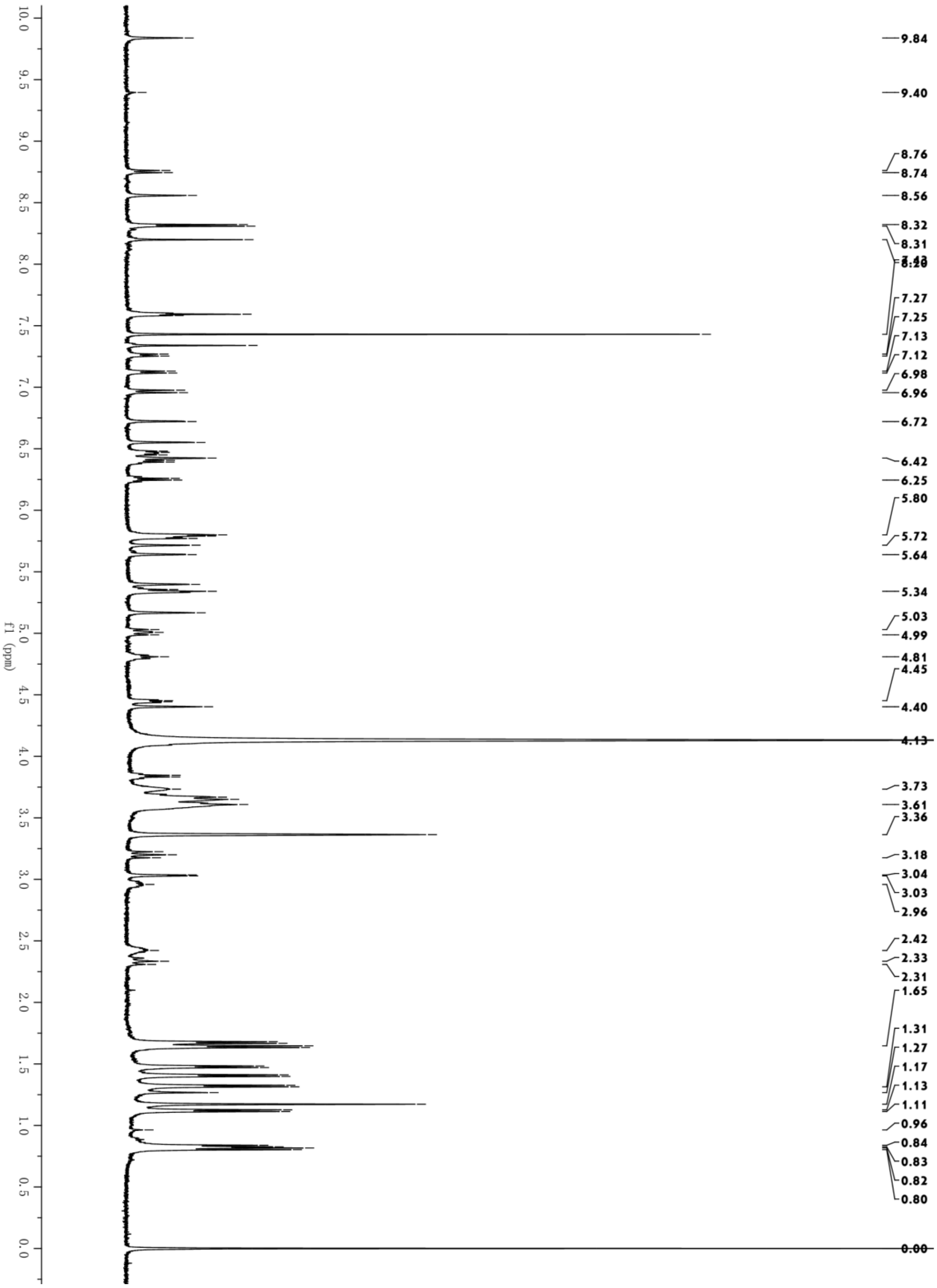
Assigment	δ_H	δ_C	HMBC	COSY
Val1-1		174.9		
Val 1-2	2.91 d (5.00)	67.4	Val1-1; Val1-3; Val1-4; Val 1-5; Q-7	Val 1-3
Val 1-3	2.08 m	31.3	Val 1-2; Val 1-4; Val 1-5	Val 1-2; Val 1-4; Val 1-5
Val 1-4	0.88 d (6.85)	17.2	Val 1-2; Val 1-3; Val 1-5	Val 1-3
Val 1-5	1.03 d (6.80)	18.6	Val 1-2; Val 1-3; Val 1-4	Val 1-3
Ser2-1		166.5		
Ser2-2	3.80 m	55.4	Ser2-1; Ser2-3	
Ser2-3	H_a 3.77 m H_b 3.25 m	61.3	Ser2-1; Ser2-2 Ser2-1; Ser2-2	Ser2-2-H_b Ser2-2-H_b
Dha3-1		162.9		
Dha3-2		132.1		
Dha3-3	H_a 5.86 s H_b 5.34 s	102.7	Dha3-1; Dha3-2 Dha3-1; Dha3-2	Dha3-3-H_b Dha3-3-H_a
Dha3-NH	8.37 bs		Ser2-1; Dha3-1; Dha3-3	
Ala4-1		173.1		
Ala4-2	4.76 m	51.8	Dha3-1; Ala4-1; Ala4-3	Ala4-3
Ala4-3	1.44 d	18.5	Ala4-1; Ala4-2	Ala4-2
Ala4-NH	7.12 d		Dha3-1; Ala4-1	
Pip5-1	5.35 s	64.2	Pip5-2; Pip5-4; Pip5-5; Ala4-1; Thz6-4; Thz13-1; Thz13-2	
Pip5-2		161.8		
Pip5-3	H_a 2.97 m H_b 3.50 m	24.1		Pip5-3-H_b; Pip5-4-H_a; Pip5-4-H_b Pip5-3-H_a; Pip5-4- H_a
Pip5-4	H_a 2.32 m H_b 4.11 m	29.2	Pip5-1; Pip5-3; Pip5-5 Pip5-1; Pip5-2; Pip5-3; Pip5-5; Thz6-4; Thz13-1	Pip5-3-H_a; Pip5-3-H_b; Pip5-4-H_b Pip5-3-H_a; Pip5-4- H_a
Pip5-5		57.6		
Pip5-NH	9.84 bs		Ala4-1	
Thz6-1		161.6		
Thz6-2		146.1		
Thz6-3	8.20 s	125.1	Thz6-1; Thz6-2; Thz6-4	
Thz6-4		169.8		
Thr7-1		165.3		
Thr7-2	4.44 m	55.8	Thr7-1; Thr7-3; Thr7-4; Thz6-1	Thr7-3; Thr7-NH
Thr7-3	1.60 m	66.2		Thr7-2; Thr7-4
Thr7-4	0.86 d (6.05)	18.8	Thr7-2; Thr7-3	Thr7-3
Thr7-NH	7.16 d (7.65)		Thz6-1; Thr7-1	Thr7-2
Dhb8-1		128.2		
Dhb8-2	6.25 q (6.85)	132.9	Dhb8-1; Dhb8-3; Tzn9-4	Dhb8-3
Dhb8-3	1.64 d (7.05)	15.2	Dhb8-1; Dhb8-2; Tzn9-4	Dhb8-2

Assigment	δ_H	δ_C	HMBC	COSY
Dhb8-NH	8.68 bs		Dhb8-2	
Tzn9-1		171.9		
Tzn9-2	5.01 dd (8.75, 13.25)	78.9	Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3-H_a; Tzn9-3-H_b
Tzn9-3	H_a 3.20 m H_b 3.68 m	35.0	Tzn9-1; Tzn9-2 Tzn9-1; Tzn9-4	Tzn9-2; Tzn9-3-H_b Tzn9-2; Tzn9-3-H_a
Tzn9-4		170.2		
Ile10-1	5.78 s	52.8	Tzn9-1; Ile10-2; Thz11-4	Ile10-NH
Ile10-2		77.1		
Ile10-3	3.83 m	67.9	Ile10-1; Ile10-2; Ile10-4; Ile10-5	Ile10-4
Ile10-4	1.32 d (6.30)	15.8	Ile10-2; Ile10-3	Ile10-3
Ile10-5	1.19 s	18.2	Ile10-1; Ile10-2; Ile10-3; Ile10-4	
Ile10-NH	7.63 d (9.70)		Tzn9-1	Ile10-1
Thz11-1		162.0		
Thz11-2		149.9		
Thz11-3	8.30 s	125.5	Thz11-1; Thz11-2;	
Thz11-4		166.4		
Thr12-1	5.79 m	55.5	Thz11-1; Thr12-2; Thr12-3; Thz13-3	Thr12-NH
Thr12-2	6.35 m	72.0	Thr12-3; Thz13-3; Q-1	Thr12-3
Thr12-3	1.76 d (6.50)	18.6	Thr12-1; Thr12-2	Thr12-2
Thr12-NH	8.85 d (8.80)			Thr12-1
Thz13-1		157.2		
Thz13-2	7.60 s	118.5	Thz13-1; Thz13-3; Pip5-1	
Thz13-3		170.2		
Thz14-1		159.6		
Thz14-2		150.1		
Thz14-3	8.31 s	127.8	Thz14-1; Thz14-2; Thz14-4	
Thz14-4		168.4		
Dha15-1		162.1		
Dha15-2		134.3		
Dha15-3	H_a 5.63 s H_b 6.72 s	103.3	Dha15-1; Dha15-2 Dha15-1; Dha15-2	Dha15-3- H_b Dha15-3- H_a
Dha15-NH	10.01 bs		Thz14-1; Dha15-1; Dha15-3	
Dha16-1		166.1		
Dha16-2		133.0		
Dha16-3	H_a 6.54 bs H_b 5.70 bs	104.4	Dha16-1; Dha16-2 Dha16-1; Dha16-2	Dha16-3- H_b Dha16-3- H_a
Dha16-NH	9.13 bs		Dha15-1; Dha16-1; Dha16-3	
Q-1		160.7		
Q-2		143.6		
Q-3	7.32 s	122.3	Q-1; Q-10; Q-11	
Q-4		153.5		
Q-5	6.90 d (10.00)	123.2	Q-6; Q-7; Q-9; Q-10	Q-6
Q-6	6.40 dd (5.30, 9.65)	129.8	Q-6; Q-7; Q-8	Q-5; Q-7
Q-7	3.62 d (4.70)	59.3	Q-5; Q-6; Q-8; Q-9; Val 1-2	Q-6; Q-8
Q-8	4.47 s	67.4	Q-6; Q-7; Q-9; Q-10	Q-7

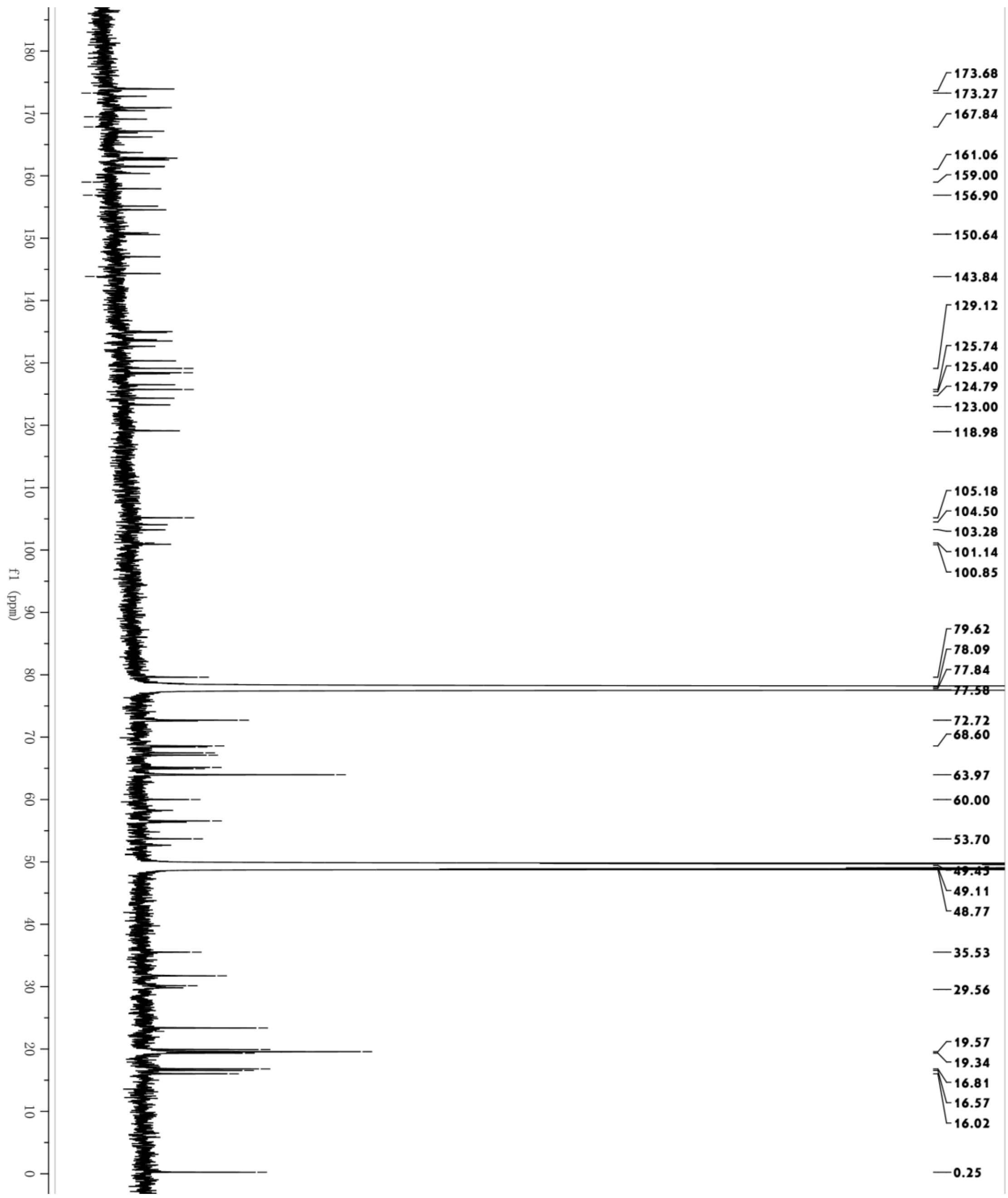
Assigment	δ_H	δ_C	HMBC	COSY
Q-9		154.4		
Q-10		127.1		
Q-11	5.33 m	64.5	Q-3; Q-10; Q-12	Q-12
Q-12	1.39 d (6.55)	22.7	Q-4; Q-11	Q-11

Fig. S11. 1D and 2D NMR spectra of SIO

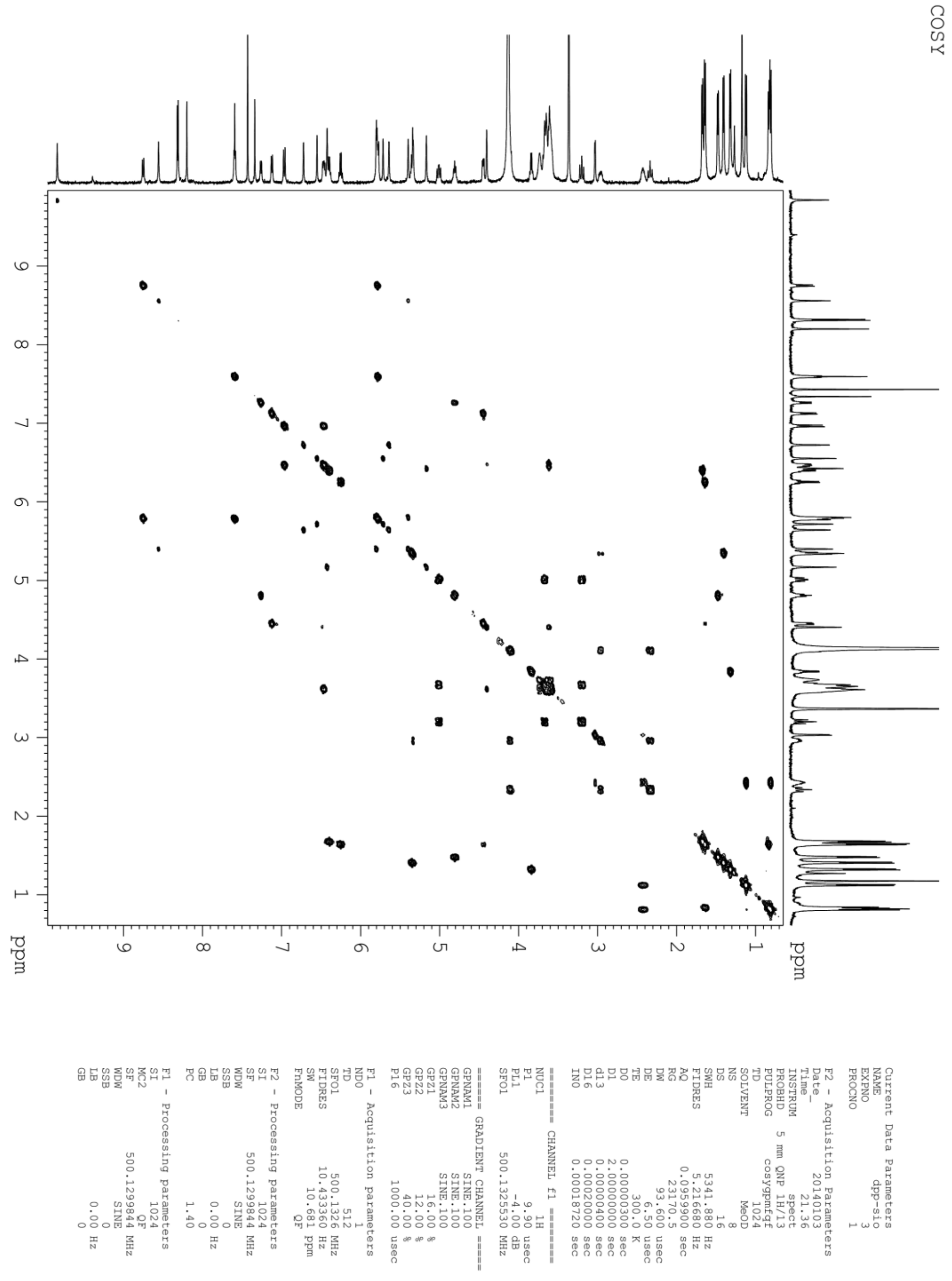
(A) ^{1}H spectrum of SIO



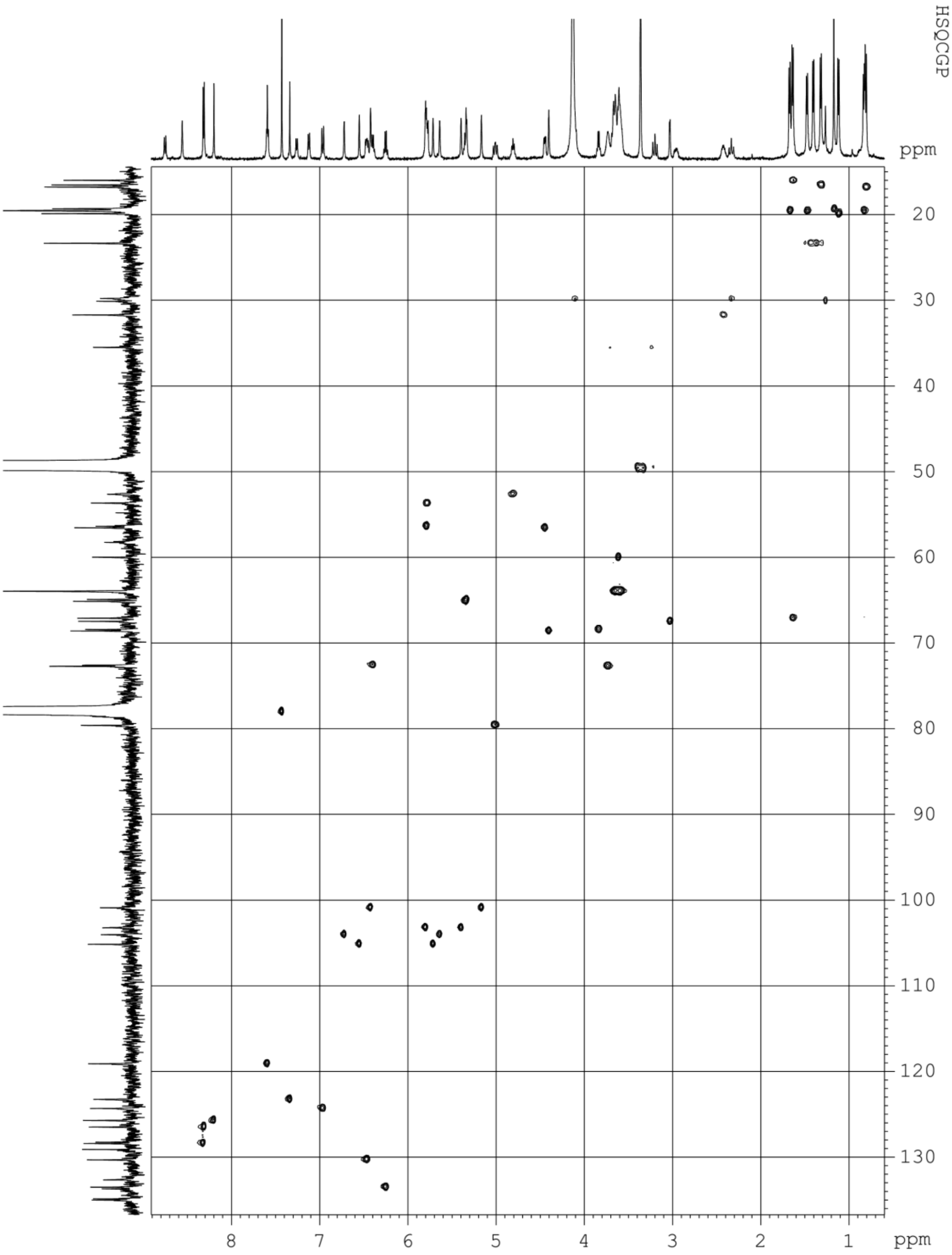
(B) ^{13}C spectrum of SIO



(B) COSY spectrum of SIO



(C) HSQC spectrum of SIO



(D) HMBC spectrum of SIO

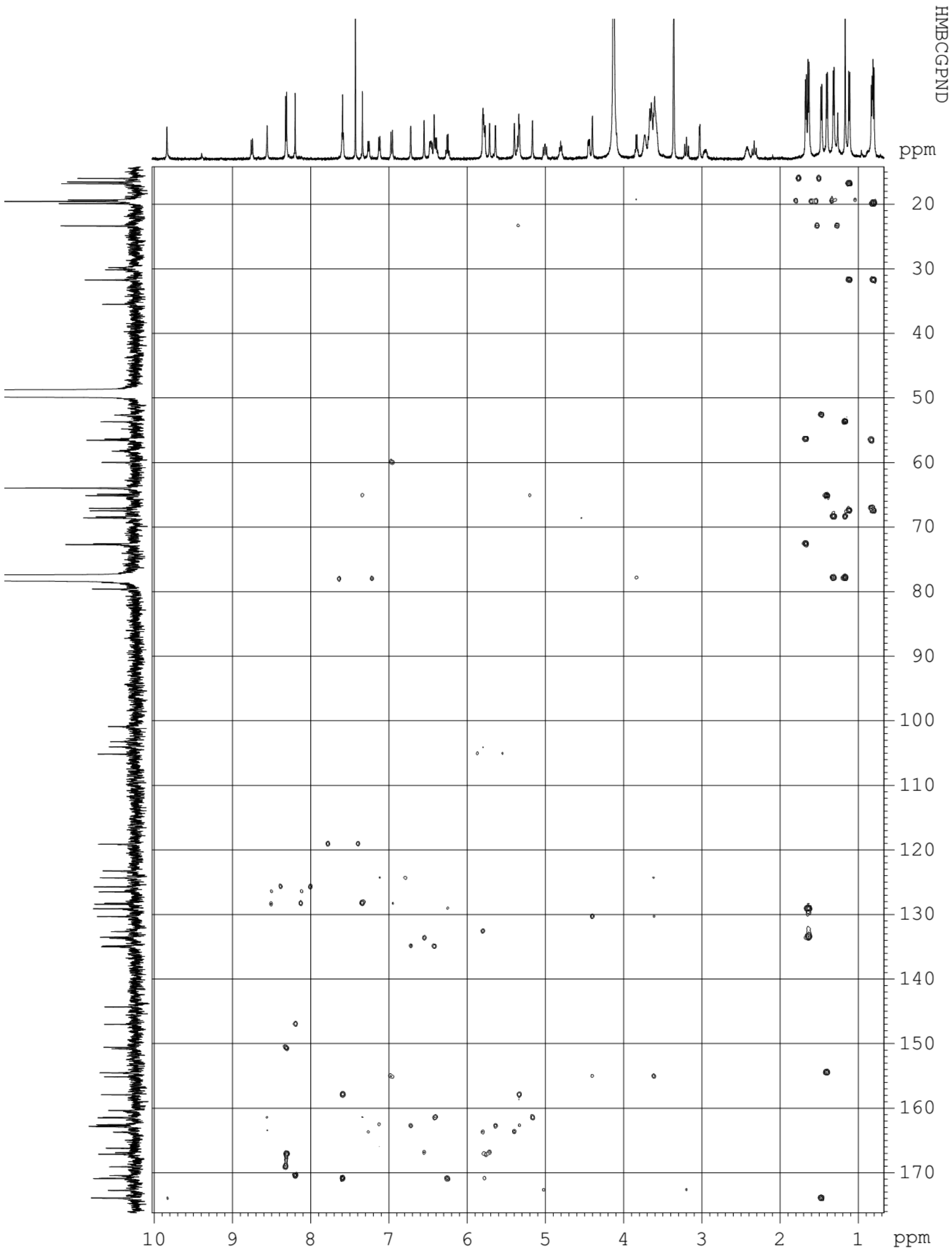


Table S6. ^{1}H and ^{13}C NMR assignments of SIO（δ in ppm, J in Hz）

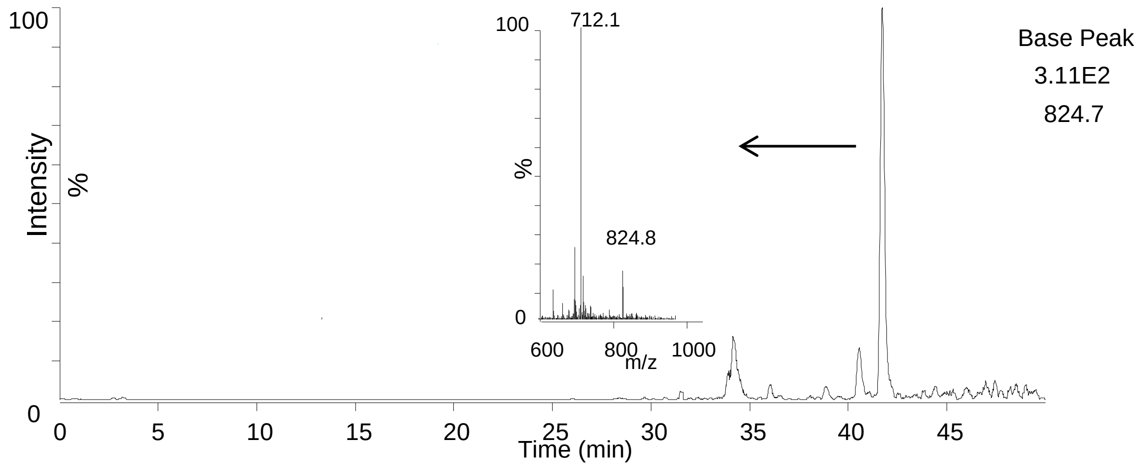
Assigment	δ_H	δ_C	HMBC	COSY
Val1-1		173.8		
Val 1-2	2.98 d (5.00)	67.4	Val1-1; Val1-3; Val1-4; Val 1-5; Q-7	Val 1-3
Val 1-3	2.38 m	31.5	Val 1-2; Val 1-4; Val 1-5	Val 1-2; Val 1-4; Val 1-5
Val 1-4	0.76 d (6.85)	16.8	Val 1-2; Val 1-3; Val 1-5	Val 1-3
Val 1-5	1.08 d (6.80)	19.6	Val 1-2; Val 1-3; Val 1-4	Val 1-3
Dha2-1		161.3		
Dha 2-2		135.0		
Dha 2-3	H_a 6.40 m H_b 5.16 m	100.7	Dha2-1; Dha2-2 Dha2-1; Dha2-2	Dha2-3-H_b Dha2-3-H_a
Dha3-1		162.9		
Dha3-2		132.1		
Dha3-3	H_a 5.79 s H_b 5.40 s	102.7	Dha3-1; Dha3-2 Dha3-1; Dha3-2	Dha3-3-H_b Dha3-3-H_a
Dha3-NH	8.37 bs		Ser2-1; Dha3-1; Dha3-3	
Ala4-1		173.1		
Ala4-2	4.76 m	51.8	Dha3-1; Ala4-1; Ala4-3	Ala4-3
Ala4-3	1.44 d	18.5	Ala4-1; Ala4-2	Ala4-2
Ala4-NH	7.12 d		Dha3-1; Ala4-1	
Pip5-1	5.35 s	64.2	Pip5-2; Pip5-4; Pip5-5; Ala4-1; Thz6-4; Thz13-1; Thz13-2	
Pip5-2		161.8		
Pip5-3	H_a 2.97 m H_b 3.50 m	24.1		Pip5-3-H_b; Pip5-4-H_a; Pip5-4-H_b Pip5-3-H_a; Pip5-4- H_a
Pip5-4	H_a 2.32 m H_b 4.11 m	29.2	Pip5-1; Pip5-3; Pip5-5 Pip5-1; Pip5-2; Pip5-3; Pip5-5; Thz6-4; Thz13-1	Pip5-3-H_a; Pip5-3-H_b; Pip5-4-H_b Pip5-3-H_a; Pip5-4- H_a
Pip5-5		57.6		
Pip5-NH	9.84 bs		Ala4-1	
Thz6-1		161.6		
Thz6-2		146.1		
Thz6-3	8.20 s	125.1	Thz6-1; Thz6-2; Thz6-4	
Thz6-4		169.8		
Thr7-1		165.3		
Thr7-2	4.44 m	55.8	Thr7-1; Thr7-3; Thr7-4; Thz6-1	Thr7-3; Thr7-NH
Thr7-3	1.60 m	66.2		Thr7-2; Thr7-4
Thr7-4	0.86 d (6.05)	18.8	Thr7-2; Thr7-3	Thr7-3
Thr7-NH	7.16 d (7.65)		Thz6-1; Thr7-1	Thr7-2
Dhb8-1		128.2		
Dhb8-2	6.25 q (6.85)	132.9	Dhb8-1; Dhb8-3; Tzn9-4	Dhb8-3
Dhb8-3	1.64 d (7.05)	15.2	Dhb8-1; Dhb8-2; Tzn9-4	Dhb8-2

Assigment	δ_H	δ_C	HMBC	COSY
Dhb8-NH	8.68 bs		Dhb8-2	
Tzn9-1		171.9		
Tzn9-2	5.01 dd (8.75, 13.25)	78.9	Tzn9-1; Tzn9-3; Tzn9-4	Tzn9-3-H_a; Tzn9-3-H_b
Tzn9-3	H_a 3.20 m	35.0	Tzn9-1; Tzn9-2	Tzn9-2; Tzn9-3-H_b
	H_b 3.68 m		Tzn9-1; Tzn9-4	Tzn9-2; Tzn9-3-H_a
Tzn9-4		170.2		
Ile10-1	5.78 s	52.8	Tzn9-1; Ile10-2; Thz11-4	Ile10-NH
Ile10-2		77.1		
Ile10-3	3.83 m	67.9	Ile10-1; Ile10-2; Ile10-4; Ile10-5	Ile10-4
Ile10-4	1.32 d (6.30)	15.8	Ile10-2; Ile10-3	Ile10-3
Ile10-5	1.19 s	18.2	Ile10-1; Ile10-2; Ile10-3; Ile10-4	
Ile10-NH	7.63 d (9.70)		Tzn9-1	Ile10-1
Thz11-1		162.0		
Thz11-2		149.9		
Thz11-3	8.30 s	125.5	Thz11-1; Thz11-2;	
Thz11-4		166.4		
Thr12-1	5.79 m	55.5	Thz11-1; Thr12-2; Thr12-3; Thz13-3	Thr12-NH
Thr12-2	6.35 m	72.0	Thr12-3; Thz13-3; Q-1	Thr12-3
Thr12-3	1.76 d (6.50)	18.6	Thr12-1; Thr12-2	Thr12-2
Thr12-NH	8.85 d (8.80)			Thr12-1
Thz13-1		157.2		
Thz13-2	7.60 s	118.5	Thz13-1; Thz13-3; Pip5-1	
Thz13-3		170.2		
Thz14-1		159.6		
Thz14-2		150.1		
Thz14-3	8.31 s	127.7	Thz14-1; Thz14-2; Thz14-4	
Thz14-4		168.4		
Dha15-1		162.1		
Dha15-2		134.3		
Dha15-3	H_a 5.63 s	103.3	Dha15-1; Dha15-2	Dha15-3- H_b
	H_b 6.72 s		Dha15-1; Dha15-2	Dha15-3- H_a
Dha15-NH				
Dha16-1		166.1		
Dha16-2		133.0		
Dha16-3	H_a 6.54 bs	104.4	Dha16-1; Dha16-2	Dha16-3- H_b
	H_b 5.70 bs		Dha16-1; Dha16-2	Dha16-3- H_a
Dha16-NH				
Q-1		160.7		
Q-2		143.6		
Q-3	7.32 s	122.3	Q-1; Q-10; Q-11	
Q-4		153.5		
Q-5	6.90 d (10.00)	123.2	Q-6; Q-7; Q-9; Q-10	Q-6
Q-6	6.40 dd (5.30, 9.65)	130.3	Q-6; Q-7; Q-8	Q-5; Q-7
Q-7	3.62 d (4.70)	59.3	Q-5; Q-6; Q-8; Q-9; Val 1-2	Q-6; Q-8
Q-8	4.47 s	67.4	Q-6; Q-7; Q-9; Q-10	Q-7

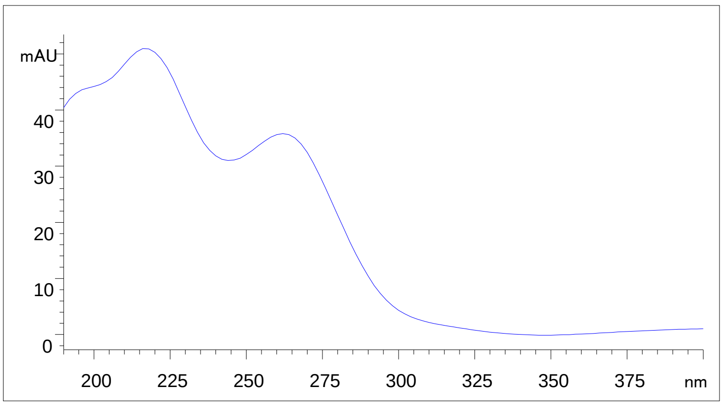
Assigment	δ_H	δ_C	HMBC	COSY
Q-9		154.4		
Q-10		127.1		
Q-11	5.33 m	64.5	Q-3; Q-10; Q-12	Q-12
Q-12	1.39 d (6.55)	22.8	Q-4; Q-11	Q-11

Fig. S12. HPLC-MS analysis, Ultraviolet absorption pattern and structure of TSR-Ile1Pro

(A) Chromatogram extracted for *m/z* 824.7, the calculated $[M+2H]^{2+}$ ion of TSR-Ile1Pro.



(B) Ultraviolet absorption pattern of TSR-Ile1Pro



(C) Structure of TSR-Ile1Pro

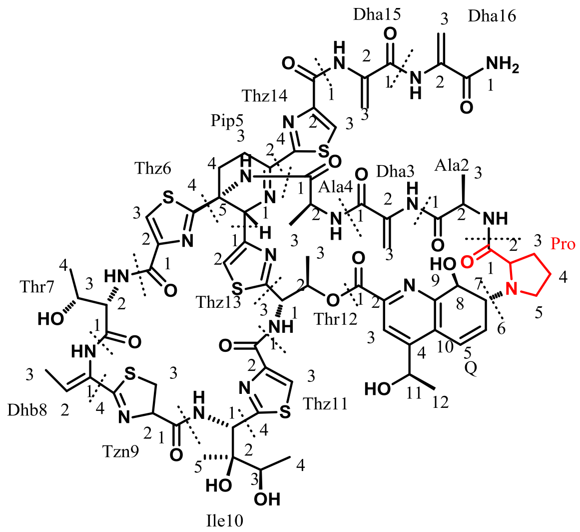
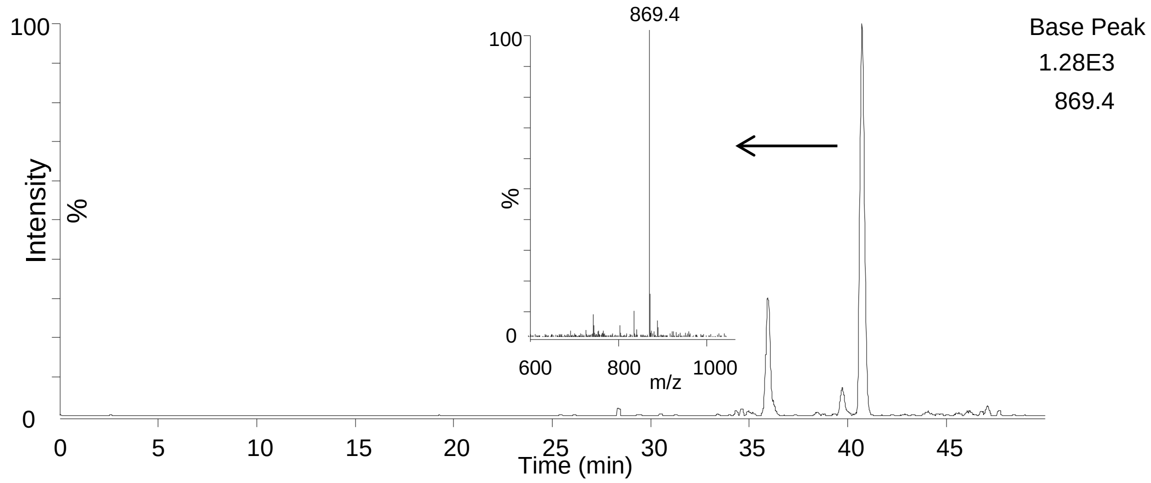
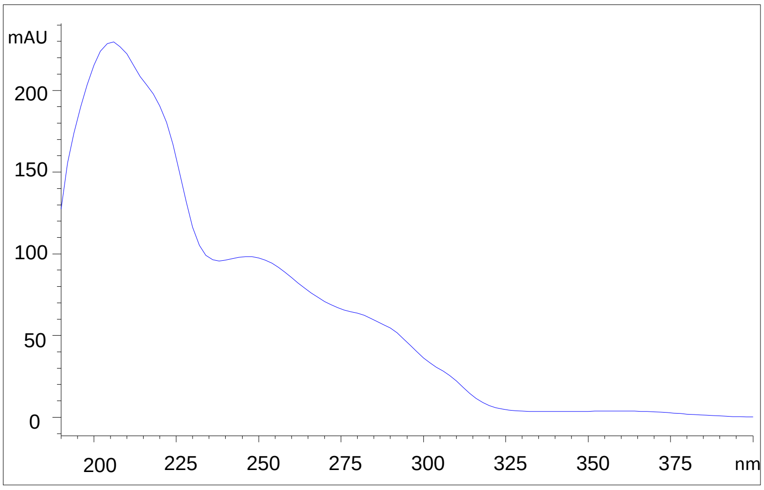


Fig. S13. HPLC-MS analysis, Ultraviolet absorption pattern and structure of TSR-Ile1Trp

(A) Chromatogram extracted for *m/z* 869.4, the calculated $[M+2H]^{2+}$ ion of TSR-Ile1Trp.



(B) Ultraviolet absorption pattern of TSR-Ile1Trp



(C)Structure of TSR-Ile1Trp

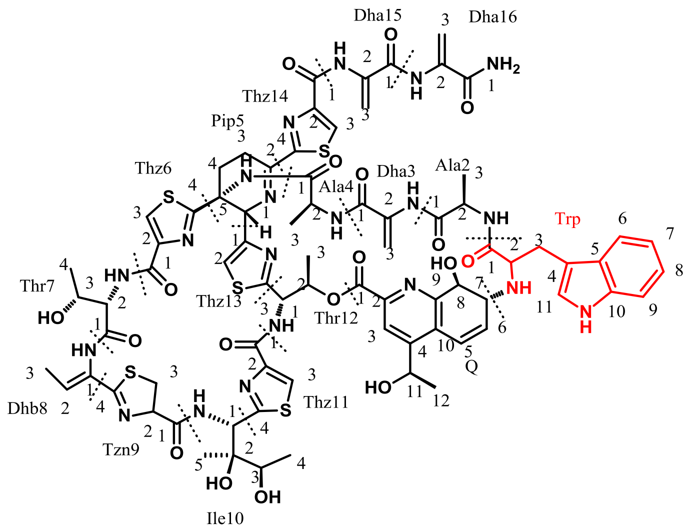


Table S7. MALDI High Resolution MS DATA of TSR analogs

Compound	TSR-Ile1Leu	TSR-Ile1Phe	TSR-Ile1Ala	TSR-Ile1Met	TSR-Ile1Pro	TSR-Ile1Trp	SIO	SIO-Dha2Ser
Found	1664.4983	1698.4841	1622.4550	1682.4600	1648.4624	1759.4681	1648.4626	1666.4771
Calculated	1664.4996	1698.4840	1622.4527	1682.4560	1648.4683	1759.4768	1648.4683	1666.4789
Formula	$C_{72}H_{86}O_{18}N_{19}S_5$	$C_{75}H_{84}O_{18}N_{19}S_5$	$C_{69}H_{80}O_{18}N_{19}S_5$	$C_{71}H_{84}O_{18}N_{19}S_6$	$C_{71}H_{82}O_{18}N_{19}S_5$	$C_{77}H_{84}N_{20}O_{18}NaS_5$	$C_{71}H_{82}O_{18}N_{19}S_5$	$C_{71}H_{84}O_{19}N_{19}S_5$

Table S8. Strains and plasmids used in this study

Strain/Cell line/Plasmid	Description	Source / Reference
Strains		
Escherichia coli		
DH5α	Host for general cloning	Invitrogen
ET12567 (pUZ8002)	Donor strain for conjugation between *E. coli* and *Streptomyces*	[1]
Streptomyces		
laurentii	Thiostrepton-producing	ATCC
SL2051	*S. laurentii* derivative, in which the codon GAG for Glu-7	[3]

	of TsrH was mutated into the stop codon TAG	
SL-I1G	SL2051 derivative, containing pSL-I1G	This study
SL-I1A	SL2051 derivative, containing pSL-I1A	This study
SL-I1L	SL2051 derivative, containing pSL-I1L	This study
SL-I1F	SL2051 derivative, containing pSL-I1F	This study
SL-I1P	SL2051 derivative, containing pSL-I1P	This study
SL-I1S	SL2051 derivative, containing pSL-I1S	This study
SL-I1T	SL2051 derivative, containing pSL-I1T	This study
SL-I1W	SL2051 derivative, containing pSL-I1W	This study
SL-I1C	SL2051 derivative, containing pSL-I1C	This study
SL-I1D	SL2051 derivative, containing pSL-I1D	This study
SL-I1E	SL2051 derivative, containing pSL-I1E	This study
SL-I1K	SL2051 derivative, containing pSL-I1K	This study
SL-I1Y	SL2051 derivative, containing pSL-I1Y	This study
SL-I1M	SL2051 derivative, containing pSL-I1M	This study
SL-I1Q	SL2051 derivative, containing pSL-I1Q	This study
SL-I1R	SL2051 derivative, containing pSL-I1R	This study
SL-1N	SL2051 derivative, containing pSL-1N	This study
SL-1H	SL2051 derivative, containing pSL-1H	This study
SL-I1V-A2S	SL2051 derivative, containing pSL-I1V-A2S	This study
Strains used for antimicrobial assays		
Streptococcus pneumoniae **PRSP1063**	Penicillin-resistant	ATCC
Streptococcus pneumoniae **PRSP2831**	Penicillin-resistant	ATCC
Streptococcus pneumoniae **PRSP224588**	Penicillin-resistant	ATCC
Staphylococcus aureus **MRSA-S1**	Methicillin-resistant	ATCC
Staphylococcus aureus **MRSA-SAU3**	Methicillin-resistant	ATCC
Staphylococcus aureus **MRSA-SAU5**	Methicillin-resistant	ATCC
Enterococcus faecium **VRE3**	Vancomycin-resistant	ATCC
Enterococcus faecium **VRE73**	Vancomycin-resistant	ATCC
Enterococcus faecium **VRE83**	Vancomycin-resistant	ATCC
Mycobacterium. marinum	GFPmut3-expressing bacteria for MIC test and infection to cells	ATCC
Cell line		
RAW 264.7 ATCC TIB71	Macrophage cells for infection by *M. marinum*	ATCC
Plasmids		
pSET152	*E. coli-Streptomyces* shuttle vector containing the *aac(3)IV* gene and the C31 *attP* site and integrase gene	[8]
pSL2050	pSET152 derivative for *in trans* expressing *tsrH* with the 580 bp upstream sequence and 288 bp downstream region	[3]
pSL-I1G	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH-Ile1Gly	This study
pSL-I1L	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Leu	This study
pSL-I1F	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Phe	This study
pSL-I1P	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Pro	This study
pSL-I1S	pSL2050 derivative for *in trans* expressing the gene that	This study

	encodes TsrH- Ile1Ser	
pSL-I1T	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Thr	This study
pSL-I1W	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Trp	This study
pSL-I1C	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Cys	This study
pSL-I1D	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Asp	This study
pSL-I1E	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Glu	This study
pSL-I1K	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Lys	This study
pSL-I1Y	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Tyr	This study
pSL-I1M	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Met	This study
pSL-I1Q	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Gln	This study
pSL-I1R	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Arg	This study
pSL-1N	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Asn	This study
pSL-1H	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1His	This study
pSL-I1V-A2S	pSL2050 derivative for *in trans* expressing the gene that encodes TsrH- Ile1Val-Ala2Ser	This study

Table S9. Primers used in this study

Primer	Sequence	Description
I1G-F I1G-R	GACCTGACCGTCACGATG<u>GGC</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>GCC</u>CATCGTGACGGTCAGGTC	Underlined codon encodes Gly at the 1st position of the TsrH core peptide
I1A-F I1A-R	GACCTGACCGTCACGATG<u>GCC</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>GGC</u>CATCGTGACGGTCAGGTC	Underlined codon encodes Ala at the 1st position of the TsrH core peptide
I1L-F I1L-R	GACCTGACCGTCACGATG<u>CTC</u>GCGTCCGCCTCCTGCACC GGTGCAGGAGGCGGACGC<u>GAG</u>CATCGTGACGGTCAGGTC	Underlined codon encodes Leu at the 1st position of the TsrH core peptide
I1F-F I1F-R	ACCTGACCGTCACGATG<u>TTC</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>GAA</u>CATCGTGACGGTCAGGT	Underlined codon encodes Phe at the 1st position of the TsrH core peptide
I1P-F I1P-R	ACCTGACCGTCACGATG<u>CCT</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>AGG</u>CATCGTGACGGTCAGGT	Underlined codon encodes Pro at the 1st position of the TsrH core peptide
I1S-F I1S-R	GACCTGACCGTCACGATG<u>TCC</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>GGA</u>CATCGTGACGGTCAGGTC	Underlined codon encodes Ser at the 1st position of the TsrH core peptide
I1T-F I1T-R	GACCTGACCGTCACGATG<u>ACT</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>AGT</u>CATCGTGACGGTCAGGTC	Underlined codon encodes Thr at the 1st position of the TsrH core peptide
I1W-F I1W-R	GACCTGACCGTCACGATG<u>TGG</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>CCA</u>CATCGTGACGGTCAGGTC	Underlined codon encodes Trp at the 1st position of the TsrH core peptide
I1C-F I1C-R	GACCTGACCGTCACGATG<u>TGC</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>GCA</u>CATCGTGACGGTCAGGTC	Underlined codon encodes Cys at the 1st position of the TsrH core peptide
I1D-F I1D-R	GACCTGACCGTCACGATG<u>GAC</u>GCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGC<u>GTC</u>CATCGTGACGGTCAGGTC	Underlined codon encodes Asp at the 1st position of the TsrH core peptide

I1E-F I1E-R	GACCTGACCGTCACGATGGAAGCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGCTTCCATCGTGACGGTCAGGTC	Underlined codon encodes Glu at the 1^{st} position of the TsrH core peptide
I1K-F I1K-R	GACCTGACCGTCACGATGAAGGCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGCCTTCATCGTGACGGTCAGGTC	Underlined codon encodes Lys at the 1^{st} position of the TsrH core peptide
I1Y-F I1Y-R	GACCTGACCGTCACGATGTACGCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGCGTACATCGTGACGGTCAGGTC	Underlined codon encodes Tyr at the 1^{st} position of the TsrH core peptide
I1M-F I1M-R	GACCTGACCGTCACGATGATGGCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGCCATCATCGTGACGGTCAGGTC	Underlined codon encodes Met at the 1^{st} position of the TsrH core peptide
I1Q-F I1Q-R	GACCTGACCGTCACGATGCAAGCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGCTTGCATCGTGACGGTCAGGTC	Underlined codon encodes Gln at the 1^{st} position of the TsrH core peptide
I1R-F I1R-R	GACCTGACCGTCACGATGCGAGCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGCTCGCATCGTGACGGTCAGGTC	Underlined codon encodes Arg at the 1^{st} position of the TsrH core peptide
I1N-F I1N-R	GACCTGACCGTCACGATGAATGCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGCATTCATCGTGACGGTCAGGTC	Underlined codon encodes Asn at the 1^{st} position of the TsrH core peptide
I1H-F I1H-R	GACCTGACCGTCACGATGCATGCGTCCGCCTCCTGCAC GTGCAGGAGGCGGACGCATGCATCGTGACGGTCAGGTC	Underlined codon encodes His at the 1^{st} position of the TsrH core peptide
I1V-A2S-F I1V-A2S-R	CTGACCGTCACGATGGTCTCGTCCGCCTCCTGCACCAC GTGGTGCAGGAGGCGGACGAGACCATCGTGACGGTCAG	Underlined codon encodes Val at the 1^{st} position and Ser at the 2^{nd} of the TsrH core peptide

Table S10. Aqueous solubilities of thiostrepton analogues

Compound	TSR	1F	1M	1A	1L	SIO	OH-SIO
Water solubility(μg/mL)	9.38±0.48	3.52±0.12	11.17±0.65	15.08±0.73	5.47±0.24	0.95±0.09	17.09±0.85

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