Supporting Information

Materials and Methods

Agents and chemicals were purchased from Sigma-Aldrich Corporation (USA) or TCI (shanghai), Sinopharm Chemical Reagent Co., Ltd (China). Reagents were purchased commercially and used without further purification. Enzymes used for genetic manipulation were purchased from Genescript Co., Ltd (China), Epicentre Co., Ltd (USA), Thermo Fisher Scientific Co., Ltd (USA), Takara Biotechnology (Dalian) Co., Ltd (China) and New England Biolabs (USA). Enzymes used for PCR amplifications and mutagenesis were Taq DNA polymerase (Vazyme Biotech Co., Ltd) and Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd). sgRNA was *in vitro* transcribed using a MEGAscript Kit (Ambion) and then purified with a MEGAclear Kit (Ambion). NMR spectra were recorded on an Agilent 500/54/ASP instrument and calibrated using residual protonated solvent (δH = 7.26 and δC = 77.16 for $CDCl_3$, δH = 3.31 and δC = 49.15 for CD_3OD) as an internal reference. Data for ^1H NMR were recorded as follows: chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet or unresolved. Coupling constants (J) are reported in Hertz. Mass spectra were recorded by ESI-MS. All the strains and plasmids used are in Supplementary Table S1. The primers used are in Supplementary Table S2. Construction of gene in-frame deletion and complementation mutant, fermentation, analysis of production were followed the published methods in references 1 and 2.

Fermentation and isolation of compounds 2 and 3

S. coelicolor TG1108 and *S. coelicolor* TG1109 were inoculated into 2 and 5 liters of GYM medium (glucose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L, peptone 1 g/L, NaCl 2 g/L) and fermented at 30℃, 220 rpm for 5 days. The cultures were centrifuged at 4000 rpm, the supernatant was then extracted with ethyl acetate, while the cells were extracted with acetone for 3 times. The extracted residue was put together and submitted to the Sephadex LH-20 chromatography for twice with the washing solution as chloroform mixed with methanol (1:1) and methanol (100%) sequentially, the targeted compounds were eluted within the first group. Pure compound **2** (12 mg) and **3** (20 mg) were obtained after the second Sephadex LH-20 chromatography and subjected to NMR experiments.

Acid degradation and FDAA derivatization of compound 2 and 3

Compound **2** and **3** were prepared as 50 mM of DMSO solutions. 50 μL of compound solution, 450 μL of ddH_2O and 500 μL of HCl were added sequentially to the sealing tude and reacted at 110℃ for 10 hours. The reaction mixtures were evaporated until the water and HCl were totally removed, then 50 μL H_2O were added to the residue for re-dissolvation. The derivations were performed as the following ingredient: 50 μL of the degradation solution or standard compounds (50 mM of *D-allo*-Ile or *L*-Ile); 50 μL of the 400 mM $NaHCO_3$ in water; 100 μL of 50 mM FDAA in acetone. The reactions were performed at 50℃ for 1 hour, then evaporated and dissolved in methanol for the LC-MS analysis.

HPLC and LC-MS experiments

Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific) equipped with a LTQ XL mass spectrometer (Thermo Fisher Scientific) and ionization was carried out with electrospray in positive ion mode. GraceSmart RP C18 column (5 μm, 250 × 4.6 mm) was used for the analysis with flow rate of 1 mL/min. Compound **2** and **3** or the fermentation extract were analyzed with the following method: mobile phase as 0 - 5 min, 10% acetonitrile/water; 5 – 25 min, 10% - 95% acetonitrile/water; 25 – 28 min, 95% - 10% acetonitrile/water; 28 – 30 min, 10% acetonitrile/ water; selected UV length: 287 nm. The FDAA derivatization products were analyzed with the following method: mobile phase as 0 - 1 min, 10% acetonitrile/water; 1 – 28 min, 10% - 50% acetonitrile/water; 28 – 30 min, 50% - 10% acetonitrile/water; selected UV length: 338 nm.

Cytotoxicity assay for compound 2, compound 3 and YM-216391

All cell-culture work was conducted in a class II biological safety cabinet. Hela cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS). Jurkat cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were plated at a density of 1 × 103 cells per well in a 96-well plate, followed by incubating with compounds for 48 h. After incubation, cell counting kit-8 reagent (CCK-8, VazymeTMCCK-8 Cell Counting Kit) was added to each well with 10 μl. Incubated for 4 h, the optical density at 450 nm was read out with a microplate reader and the background was subtracted at 650 nm. Cells incubated with 0.1% DMSO served as control. Three independent replicates were conducted.

Table S1. Strains and plasmids used in this study

Strain/Plasmid	Characteristics	Reference
E. coli DH5α	Host for general cloning	Invitrogen
E. coli S17-1	Donor strain for conjugation between *E. coli* and *S. lividans*	3
E. coli ET12567/pUZ8002	Donor strain for conjugation between *E. coli* and *S. coelicolor*	3
E. coli BW 25114	Host for PCR-targeting gene replacement on fosmids	3
S. lividans TG1104	*S. lividans* 1326 containing pTG1104, YM-216391 producing	1
S. albus TG1105	*S. albus* containing pTG1105 (Δ*ymA* gene replacement mutant), YM-216391 non-producing	This work
S. albus TG1106	*S. albus* containing pTG1105 and *ymA* gene complementation plasmid pTG1106, YM-216391 producing	This work
S. albus TG1107	*S. albus* containing pTG1105 and *ymA** gene complementation plasmid pTG1107, new compound **3** producing	This work
S. coelicolor M1452	Host for expression of gene cluster	2
S. coelicolor TG1108	*S. coelicolor* M1452 containing 4 x pTG1108 (*ymA**), new compound **3** producing	This work
S. coelicolor TG1109	*S. coelicolor* M1452 containing 4 x pTG1109 (*ymA***), new compound **2** producing	This work
pCB003	A universal plasmid containing the synthetic guide RNA (sgRNA)	4
pTG1104	pJTU2554-based fosmid containing YM-216391 gene cluster with Δ*ymR3*	1
pTG1105	pTG1104 derivative with Δ*ymA*	This work
pTG1106	pSET152-derivative for *ymA* gene complementation in-trans	This work
pTG1107	pTG1106-derivative for mutated-*ymA* gene complementation in-trans (S20C)	This work
pTG1108	pTG1104 derivative with mutated-*ymA* gene in-situ (S20C)	This work
pTG1109	pTG1108 derivative with mutated-*ymA* gene in-situ (V16I and S20C)	This work

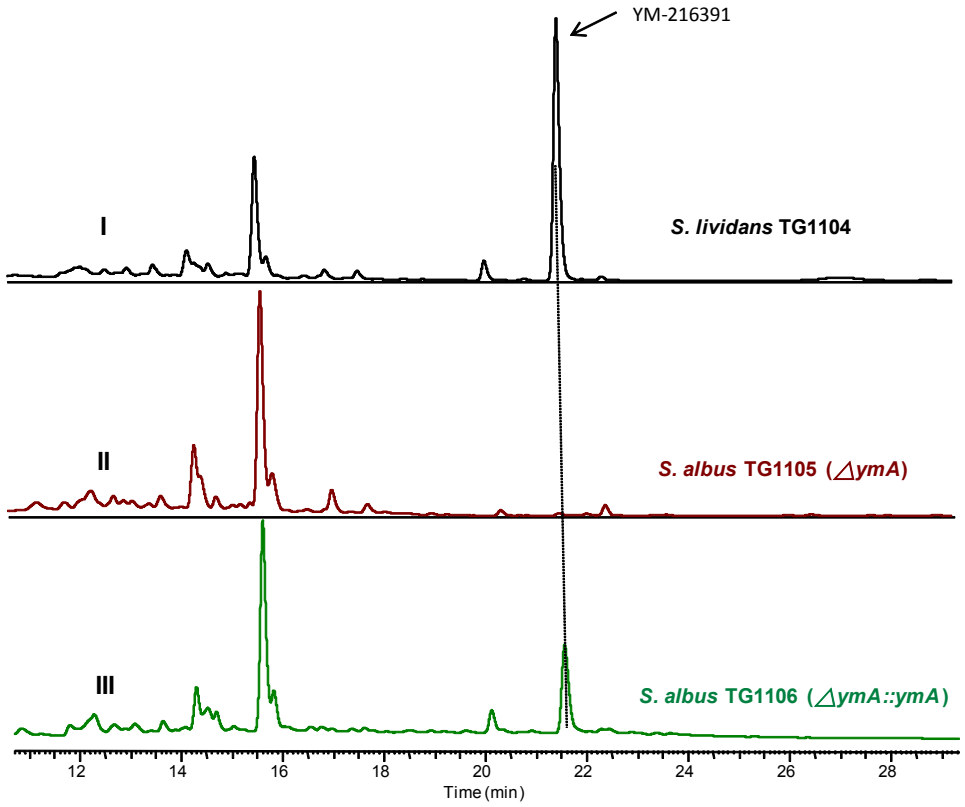
Table S2. Oligonucleotide sequences used in this study

Primers	**Sequences (5'-3')**
ymA-up-fw	agaagctgtcagaacggcggagaa
ymA-up-S20C-rev	agctcgagggagcagcaggaggagccgacgat
ymA-down-S20C-fw	atcgtcggctcctcctgctgctccctcgagct
ymA-down-rev	acaagtaccggacgtacggcaca
ymA-up-V16I/S20C-rev	agctcgagggagcagcaggaggagccgatgatgaatccgacct
ymA-down-V16I/S20C-fw	aggtcggattcatcatcggctcctcctgctgctccctcgagct
sgRNA-up-fw	gactgacactgataatacgactcactataggcggagaaccgtggagacgagttttagagctaga aatagc
sgRNA-down-fw	gactgacactgataatacgactcactataggcacaccttggagggcctcggttttagagctagaa atagc
sgRNA-rev	ggaattcagatctcaaaaaaagcaccgac

Table S3. NMR assignments for compound 3 in dimethyl sulfoxide-d6

NO.	δ_H (multiplicity; *J* in Hz)	δ_C	HMBC	COSY
1		160.6		
2	8.14 (d, 7.6, 1H)		C-1, C-8	3-H
3	4.82 (dd, 7.6, 4.1, 1H)	56.9	C-8	2-H, 4-H
4	1.96 (m, 1H)	39.4		5-H
5	0.90	15.2	C-4, C-6, C-8	4-H
6	1.60 (m), 1.06 (m)	26.0	C-3, C-4, C-5, C-7	5-H
7	0.87	12.5	C-4	6-H
8		170.6		
9	8.54 (d, 8.7, 1H)		C-8	10-H
10	4.37 (dd, 8.5, 5.8, 1H)	58.4	C-12, C-14	9-H
11	2.10 (m, 1H)	31.3	C-13	10-H, 13-H
12	0.93	18.4	C-10, C-11, C-13	11-H, 13-H
13	0.91	20.1	C-10, C-11, C-12	
14		171.3		
15	8.72 (dd, 8.9,1.7, 1H)			16-H
16	4.16 (d, 15.0, 1H)	35.6	C-14, C-17	15-H
	5.02 (dd, 16.7, 9.0, 1H)			
17		163.4		
19	8.90 (s, 1H)	140.4	C-17, C-20	
20		129.8		
22		155.8		
24	9.06 (s, 1H)	139.9	C-22	
25		136.2		
27		157.6		
29	8.58 (s, 1H)	121.4	C-27, C-30, C-32	
30		148.4		
32		161.7		
34	8.67 (s, 1H)	123.4	C-30, C-32, C-35, C-37	
35		142.3		
37		154.6		
39		151.1		
40		127.1		
41/45	8.35 (d, 5.3, 2H)	128.1	C-39, C-45/41, C-43	42/44-H
42/44	7.56 (m, 2H)	129.1	C-40, C-44/42	41/45-H
43	7.50 (m, 1H)	130.5	C-41/45	
46		131.1		

Figure S1. Construction of heterologous expression system for YmA-expressed *in-trans*.



I), YM-216391 gene cluster with Δ*ymR3* (pTG1104, see reference 1 for detail) expressed in *S. lividans* 1326, with YM-216391 production of 3-5 mg/L; II), pTG1105 (YM-216391 gene cluster with Δ*ymR3::*Δ*ymA*) in *S. albus*; III), pTG1106 (*ymA* expressed in-trans) in *S. albus*, with YM-216391 production of ~1 mg/L.

Figure S2. Production of new compound 3 based on heterologous expression system in *S. albus*.

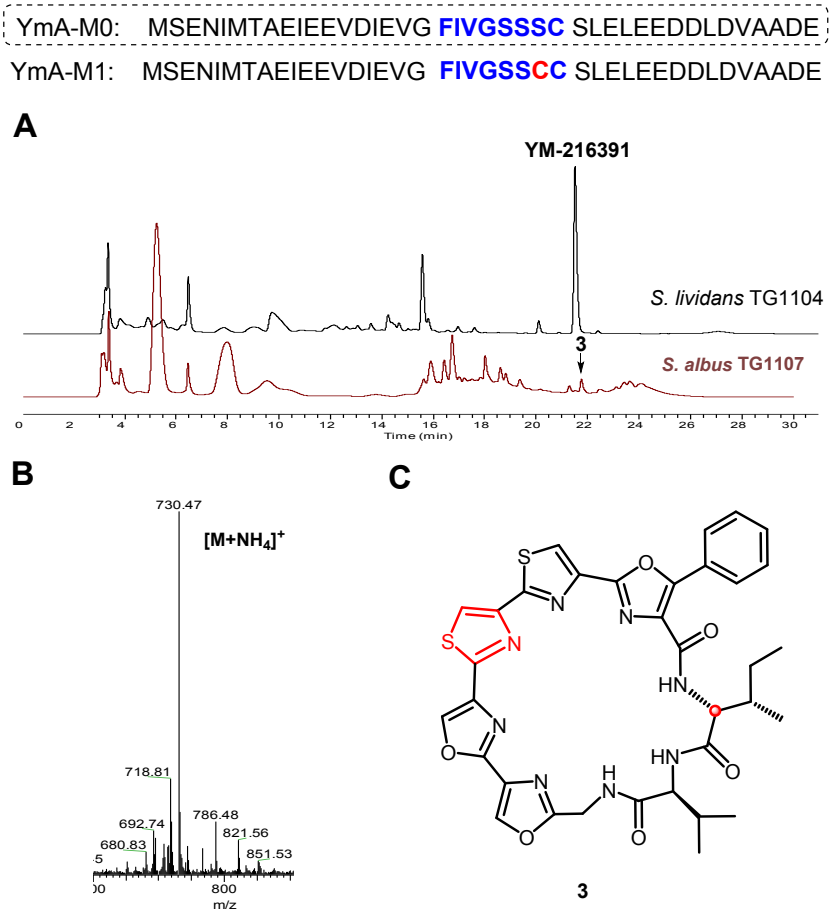
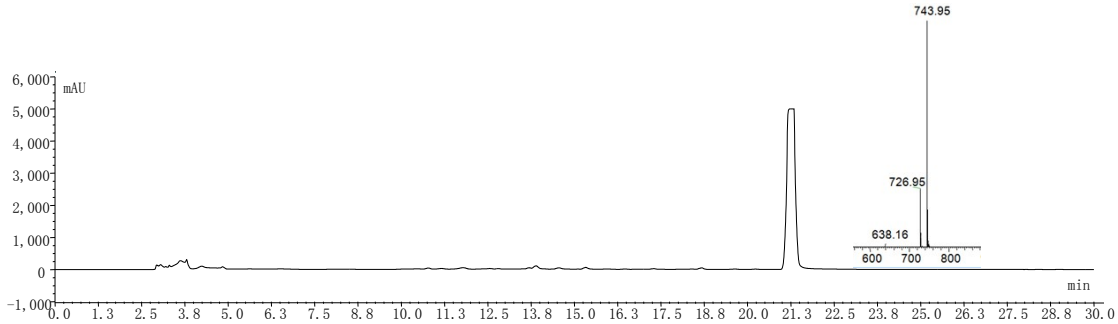
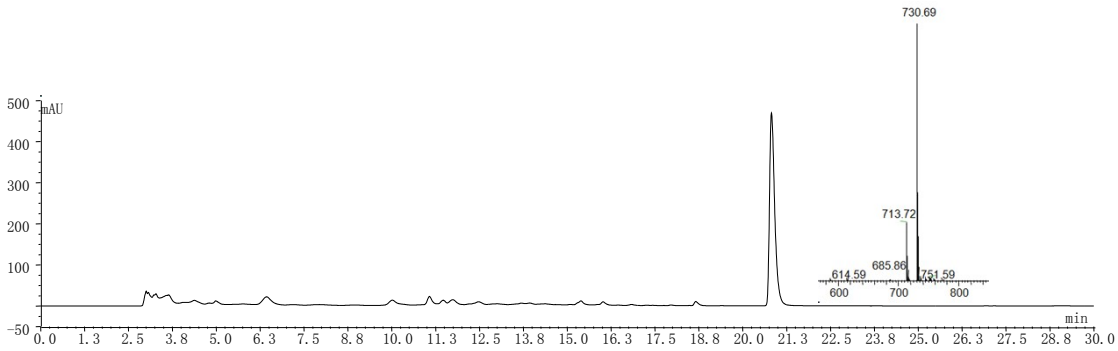


Figure S3. Production of new compounds 2 and 3 via heterologous expression in super *S. coelicolor* host by multiplexed site-specific genome engineering.

A



B



HPLC and MS analysis of *S. coelicolor* TG1108 and *S. coelicolor* TG1109 fermentations. Peaks are corresponding to compound **2** (**A**) and **3** (**B**); spectrums were performed at UV 287 nm.

Fig. S4 ^{1}H-NMR spectrum of compound 2 (600 MHz) in DMSO-D_6.

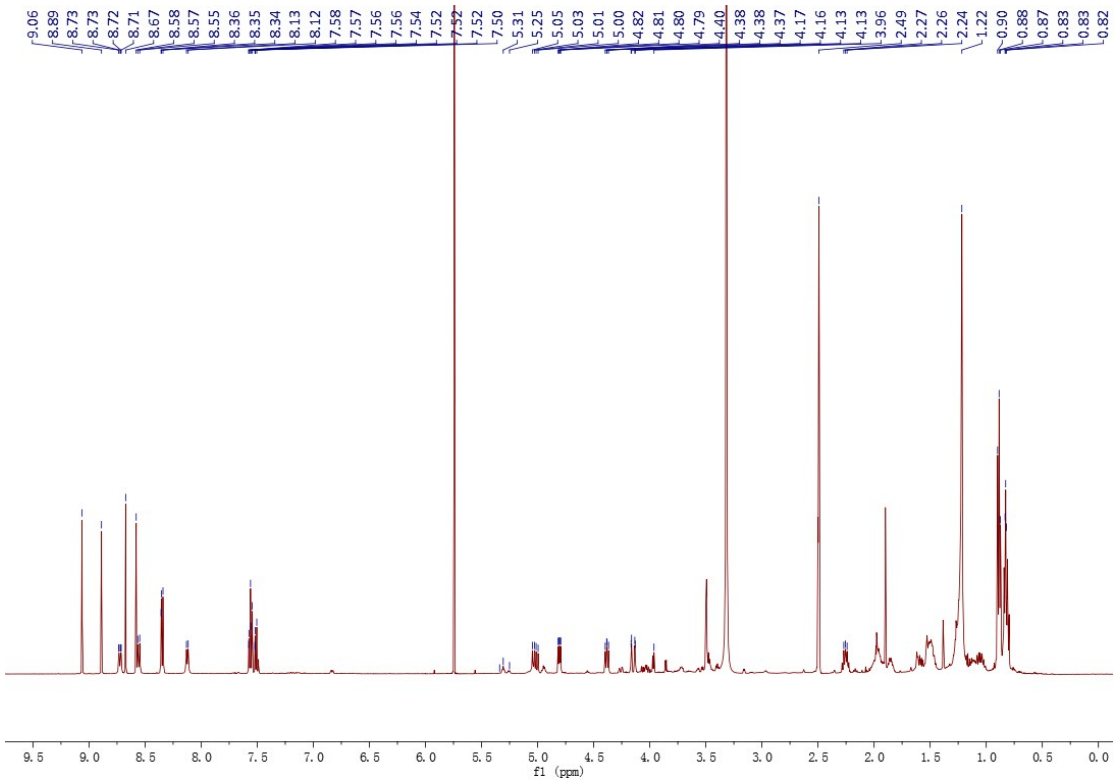


Fig. S5 ^{13}C-NMR spectrum of compound 2 (150 MHz) in DMSO-D_6.

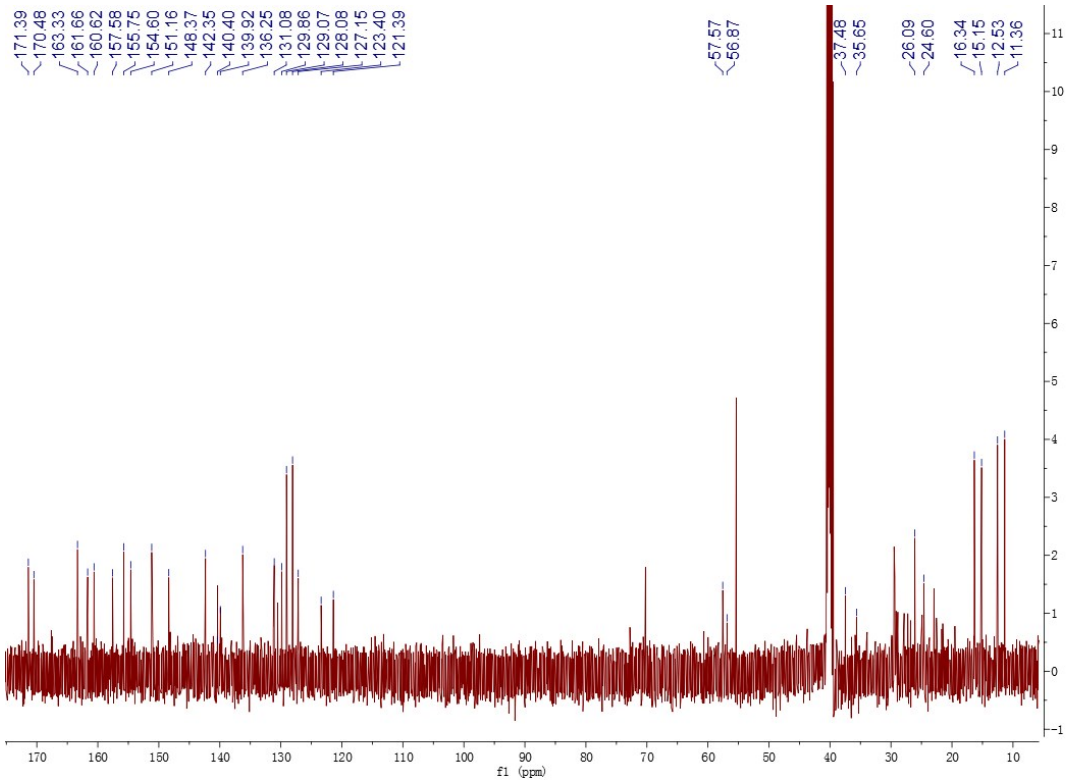


Fig. S6 ^{1}H-^{1}H COSY spectrum of compound 2 (600 MHz) in DMSO-D_6.

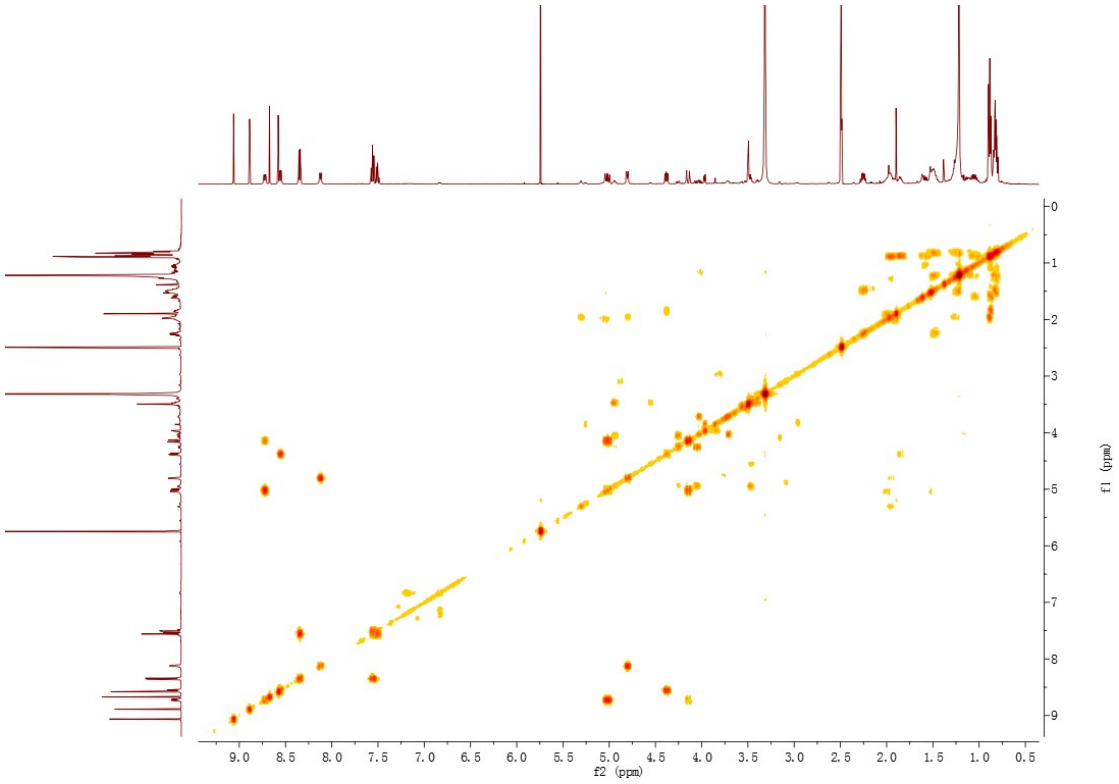


Fig. S7 ^{1}H-^{13}C HSQC spectrum of compound 2 (600 MHz) in DMSO-D_6.

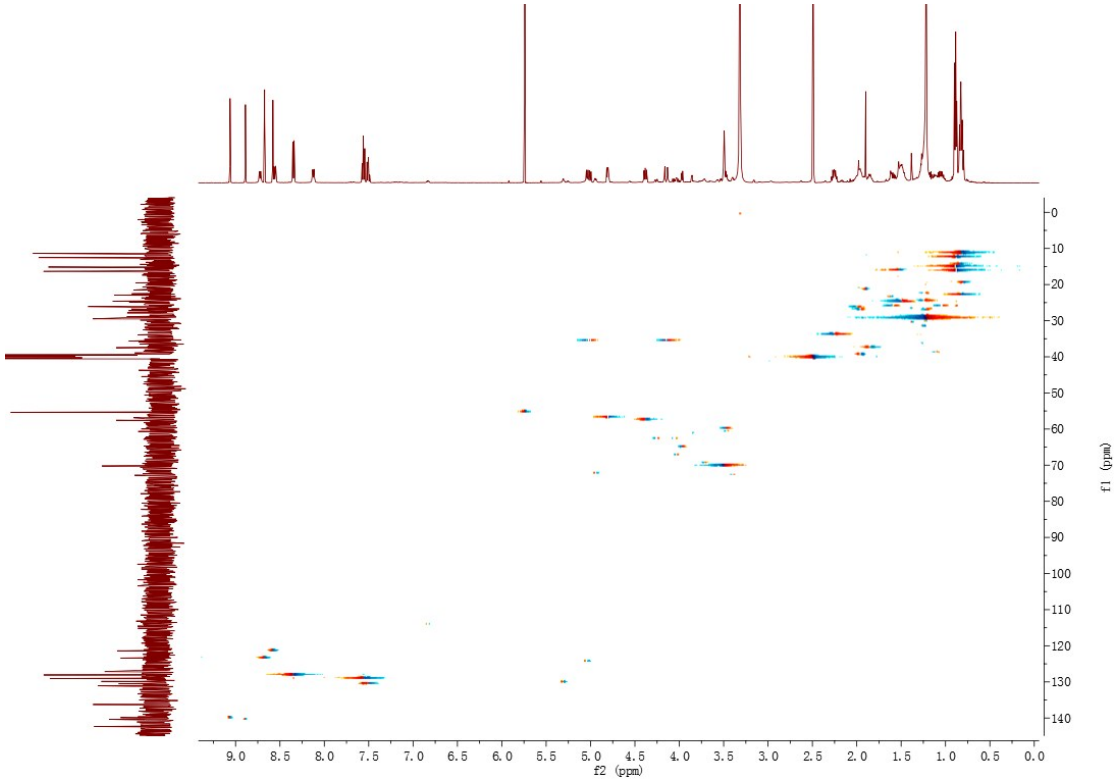


Fig. S8 ^{1}H-^{13}C HMBC spectrum of compound 2 (600 MHz) in DMSO-D_6.

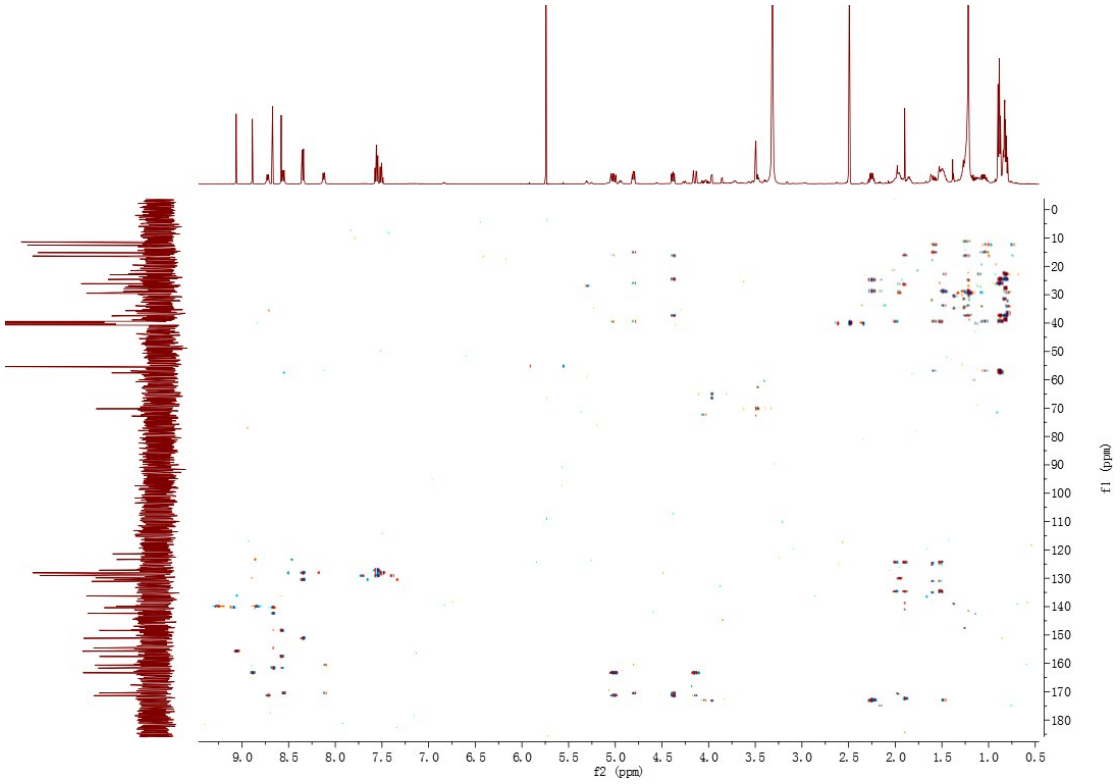


Fig. S9 ^{1}H-NMR spectrum of compound 3 (600 MHz) in DMSO-D_6.

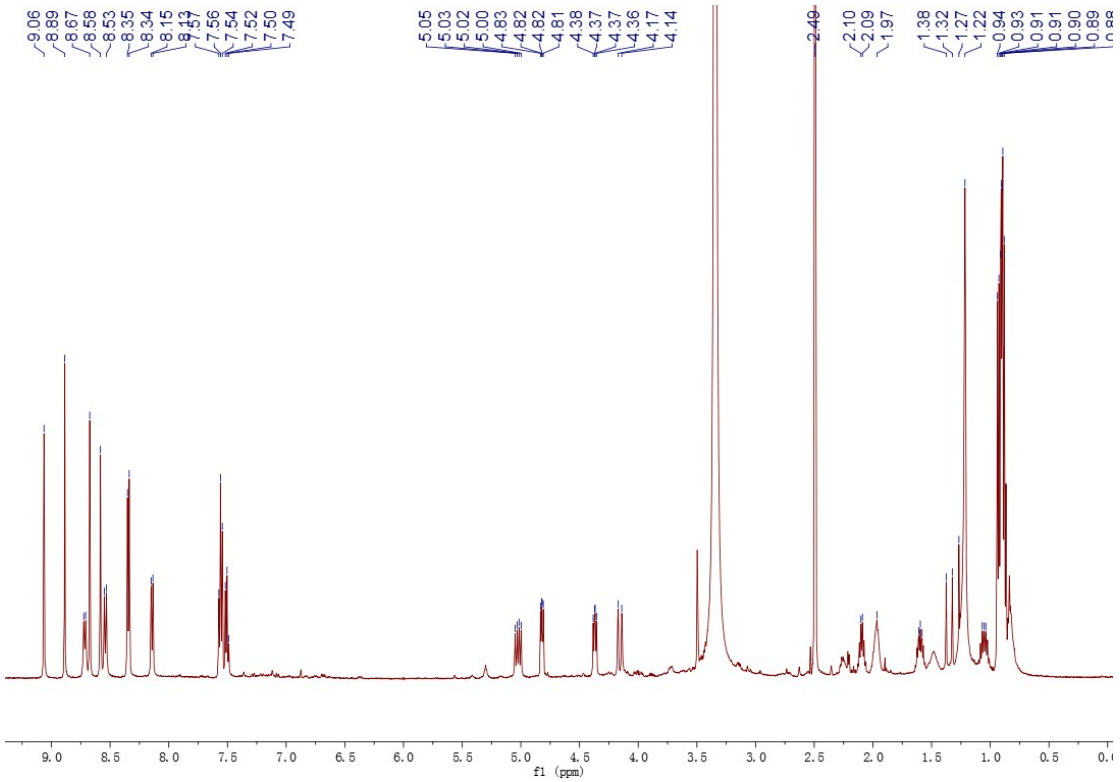


Fig. S10 ^{13}C-NMR spectrum of compound 3 (150 MHz) in DMSO-D$_6$.

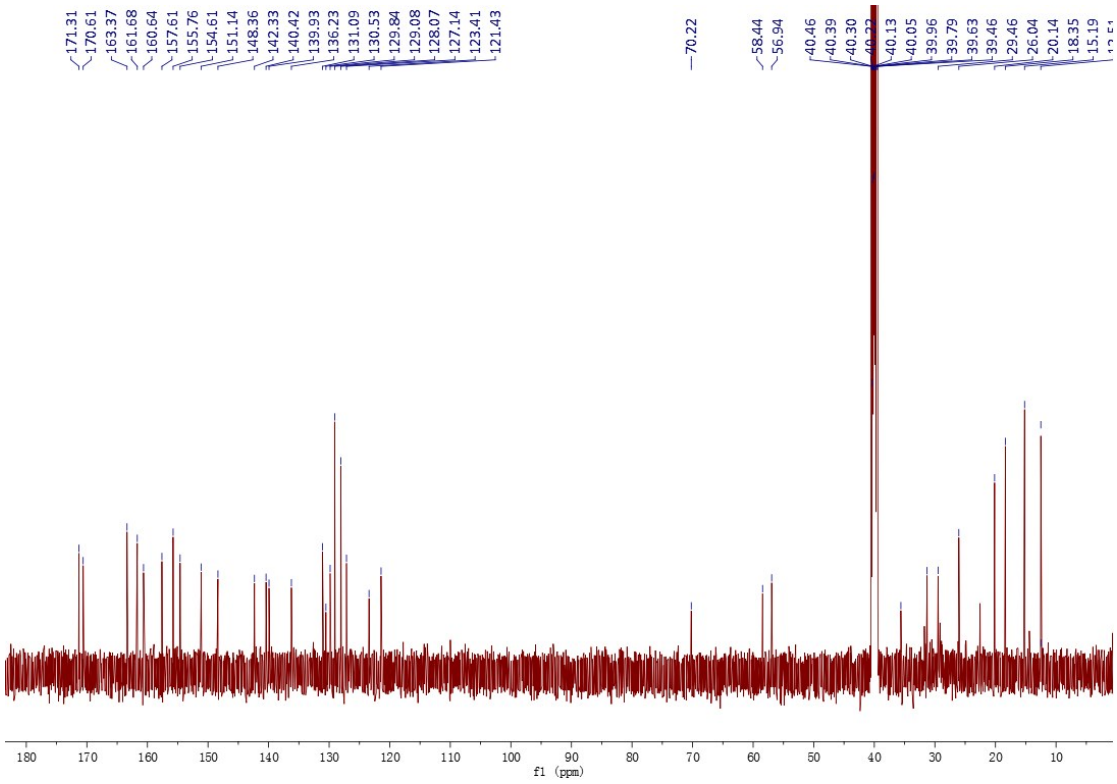


Fig. S11 ^{1}H-^{1}H COSY spectrum of compound 3 (600 MHz) in DMSO-D$_6$.

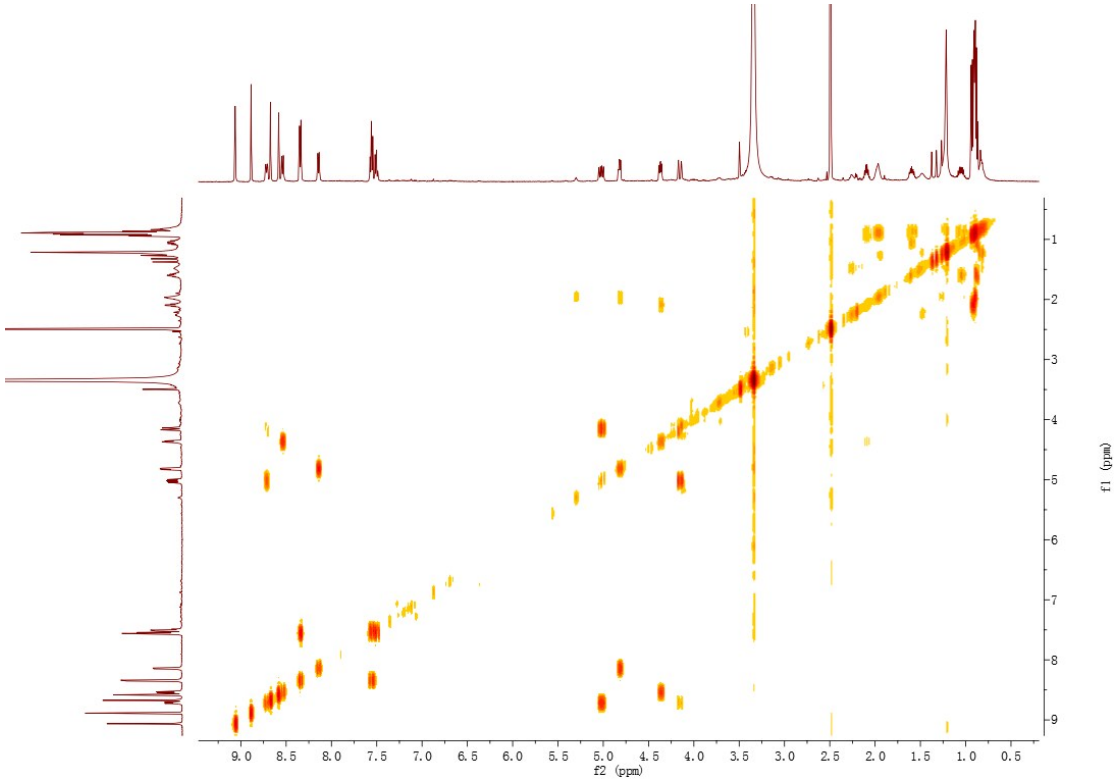


Fig. S12 ^{1}H-^{13}C HSQC spectrum of compound 3 (600 MHz) in DMSO-D_6.

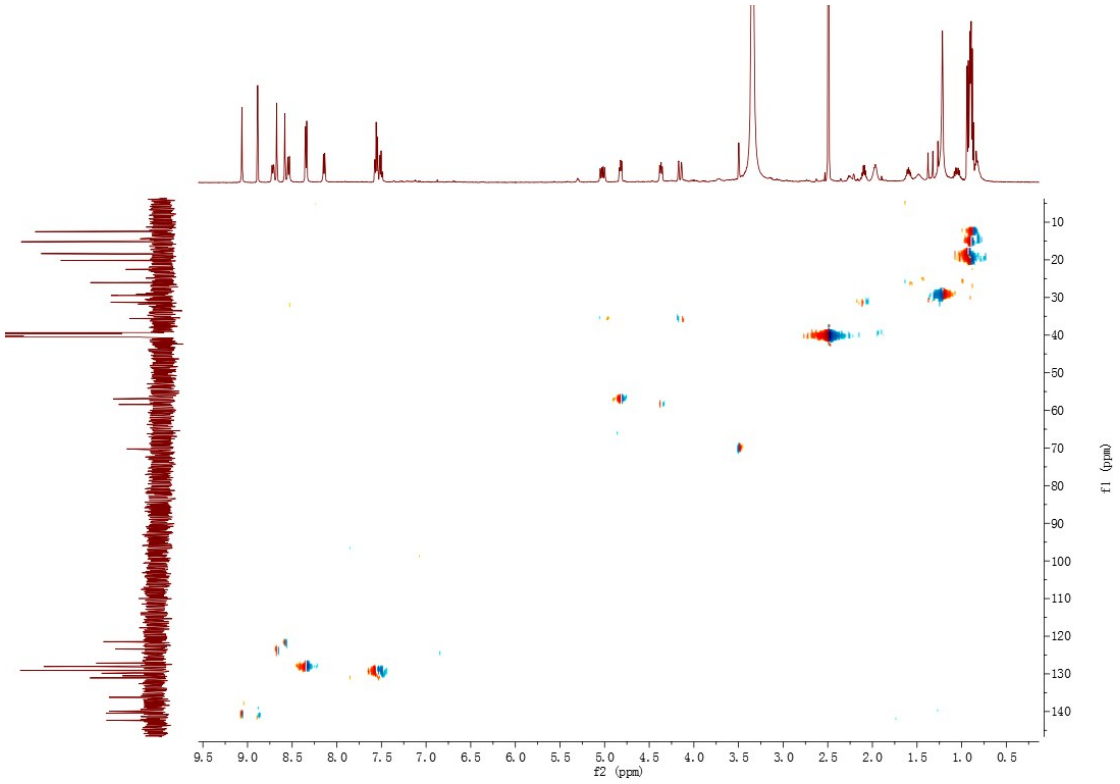
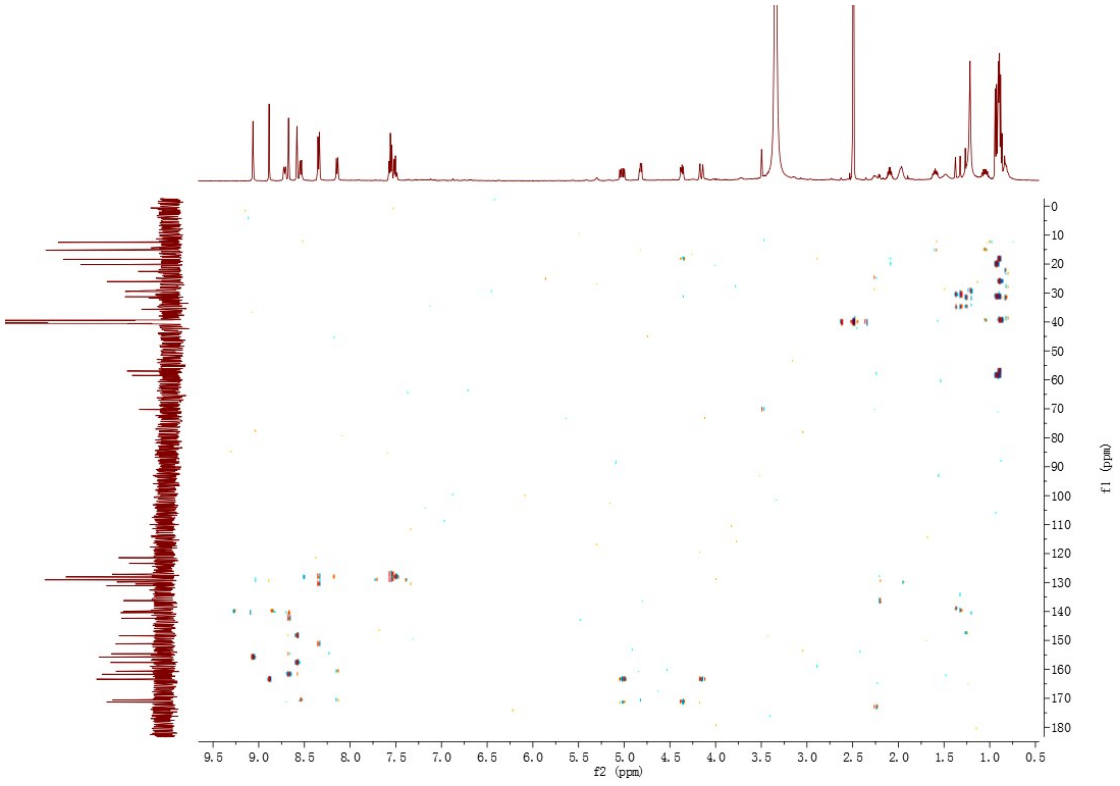


Fig. S13 ^{1}H-^{13}C HMBC spectrum of compound 3 (600 MHz) in DMSO-D_6.



References

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