Supporting Information

Marine-Derived Piericidin Diglycoside S18 Alleviates Inflammatory Responses in the Aortic Valve *via* Interaction with Interleukin 37

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Contents

Supplementary Figures and Tables

Methods and Materials.

1.Cell Viability Analysis. HAVICs (3×10^3) were cultured in each well of a 96-well plate in M199 growth medium supplemented with 10% FBS for 24 hours. Then, the cells were starved in serum-free medium for 12 hours. Subsequently, the cells were treated with different final concentrations of S18 (0-4 μM) for 48 hours. Cell viability was analyzed using CKK-8 assay kits (Glpbio, GK10005, USA). CCK8 solution was added to each well of the plate according to the manufacturer's instructions, and the plate was incubated in an incubator with 5% CO_2 at 37°C for 3 hours. The absorbance of the samples was determined spectrophotometrically at 450 nm using a microplate reader.

2.**Immunoblotting.** Immunoblotting was applied to analyze ICAM-1 (Santa Cruz, Sc-8439), IL-8 (Proteintech, 27095-1-AP), MCP-1 (Abclonal, A7277), IL-37 (Abcam, ab278499), phosphorylated NF-κB p65(Cell Signaling Technology, 3033), total p65 (Cell Signaling Technology, 8242) levels. Briefly, cells were lysed in lysis buffer (Solarbio, R0010) containing phosphatase and protease inhibitors (Solarbio, P6730, P1260) and quantified with a bicinchoninic acid protein assay. The protein samples were transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% skim milk in TBST solution (Boster, AR0031) at room temperature for 1 hour, the blocked membranes were incubated overnight at 4°C with primary antibodies. After washing with TBST, the membranes were incubated with HRP-conjugated secondary antibodies specific to the primary antibodies for 1 hour at room temperature. GAPDH (Protechtein, 60004-1-Ig) and β-actin (Protechtein, 66009-1-Ig) was analyzed for normalizing protein loading. In the phosphorylation assay, total NF-κB were used for normalization. Finally, immunoreactive bands were detected with FDbio-Dura ECL Kit (Fdbio Science, FD8020) and GeneGnome imaging system (Syngene, Frederick, MD, USA). The images were analyzed with ImageJ software.

3.ELISA. Cell culture supernatants were obtained. The levels of IL-8 and MCP-1 were detected using ELISA kits (R&D, DY208, DY279) as described in the manufacturer's protocol.

4.RNA Purification and Quantitative Real-time Polymerase Chain Reaction(qRT-PCR). Total RNA was extracted from HAVICs using EZ-press RNA Purification Kit (EZBioscience, B004D) following the manufacturer's instructions. The cDNA for each

sample was reverse transcribed using the EVO M-MLV First Strand cDNA Synthesis Kit (Accurate Biotechnology(Hunan)Co.,Ltd, AG11706). PCR was performed using SYBR Green premix Pro Taq HS qPCR Kit (Accurate Biotechnology(Hunan)Co.,Ltd, AG11701) and LightCycler 480 (Roche Diagnostics, Basel, Switzerland). The following primers were used to amplify specific cDNA fragments: human ICAM-1, human IL-8, human MCP-1, GAPDH, and the sequences were listed in Table S2. The mean relative gene expression was calculated with the 2-ΔΔCt method after normalization of the cycle threshold (Ct) values to GAPDH gene expression.

5. ROS and MMP Detection. ROS levels were detected using DCFH-DA (Beyotime, S0033S) as described in the manufacturer's protocol. The MMP was determined using the JC-1 Kit and TMRE Kit (Beyotime, C2001S) following the manufacturer's instructions. For mitochondrial staining, cells were incubated in M199 medium containing 100 nmol/L MitoTracker Green (Beyotime, C1048) in dark at 37°C for 30 minutes. Cell nuclei were stained with Hoechst 33342 (Beyotime, C1022) for 10 min. The images were acquired using a Leica TCS SP8 and were analyzed using ImageJ software.

6. Histopathological and Immunofluorescence Staining. After cell treatment, HAVICs were rinsed briefly with PBS, fixed in 4% paraformaldehyde for 15 minutes, and permeabilized with PBS containing 0.3% Triton X-100 for 10 minutes at room temperature. Nonspecific immunoreactions were blocked using 5% BSA in PBS for 1 hour at room temperature. HAVICs were incubated overnight with primary antibodies against NF-κB p65 (Cell Signaling Technology, 8242). After secondary antibody (Proteintech, SA00013-2) incubation, the cell nuclei were stained with DAPI (Solarbio, C0060) for 15 minutes, and then confocal images were visualized using Leica TCS SP8.

A similar protocol was performed to stain the tissue sections. The mouse valves and human valves were deparaffinized with xylene and grades of ethanol, followed by antigen retrieval. After permeabilization and blocking with 5% BSA for 1 hour, the sections were incubated with primary antibodies against ALP (ABclonal, A1080), BMP2 (Abcam, ab214821). After washing with PBS, the sections were incubated with secondary antibodies conjugated to Alexa Fluor 552 (Proteintech, SA00009-1, SA00009-2). Cell

nuclei were stained with DAPI for 15 minutes, and images were acquired using a Leica TCS SP8.

7.Von Kossa Staining. Calcium deposition staining in mouse aortic valve was stained using Von Kossa Kit (Servicebio, G1042) according to the manufacturer's protocol. Calcium depositions in the mouse aortic valves were evaluated in vivo using ImageJ software.

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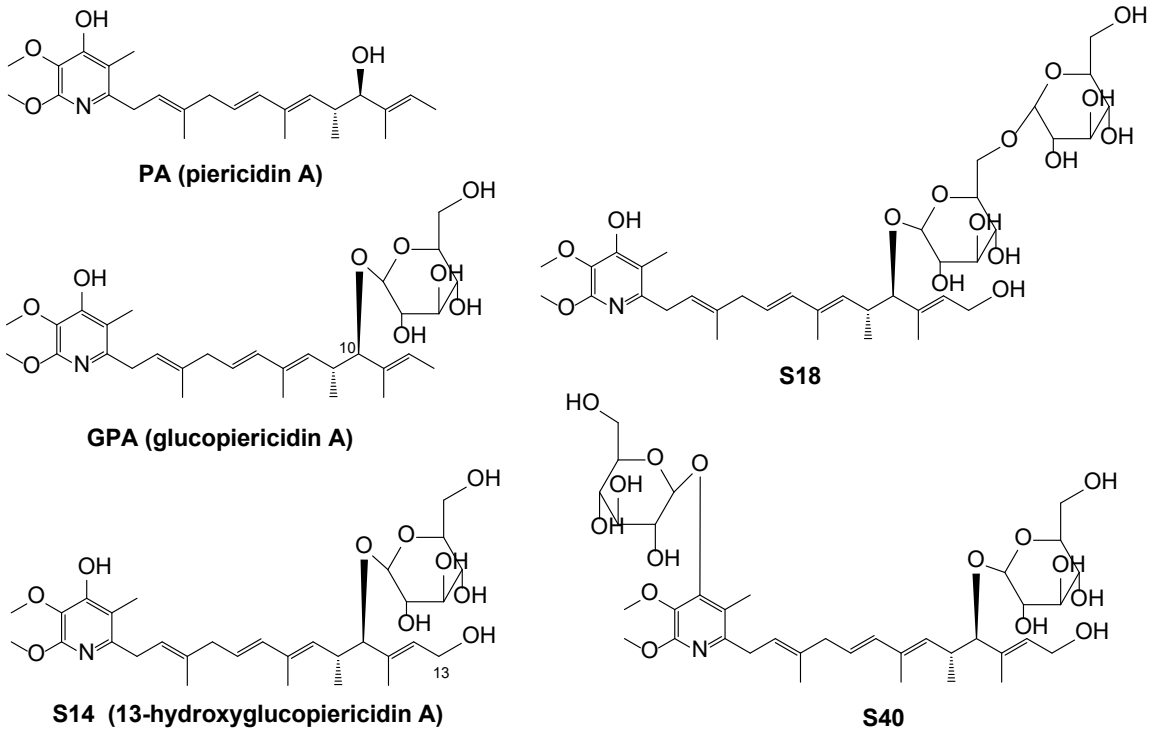

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139 **Figure S1** Structures of piericidin analogues PA, GPA, S14, S18 and S40

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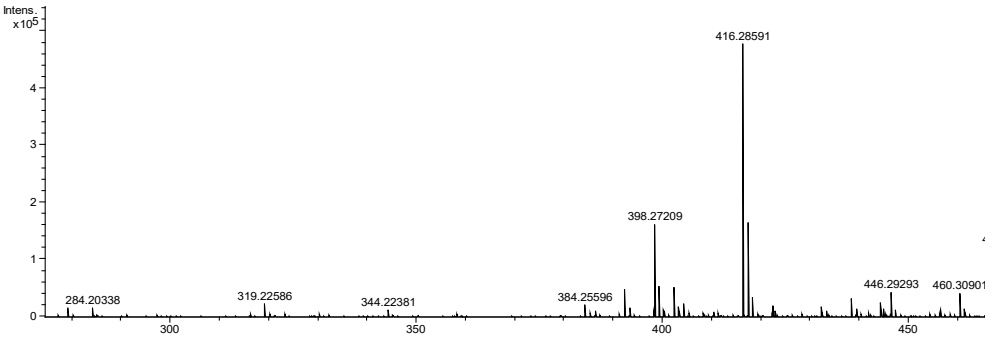
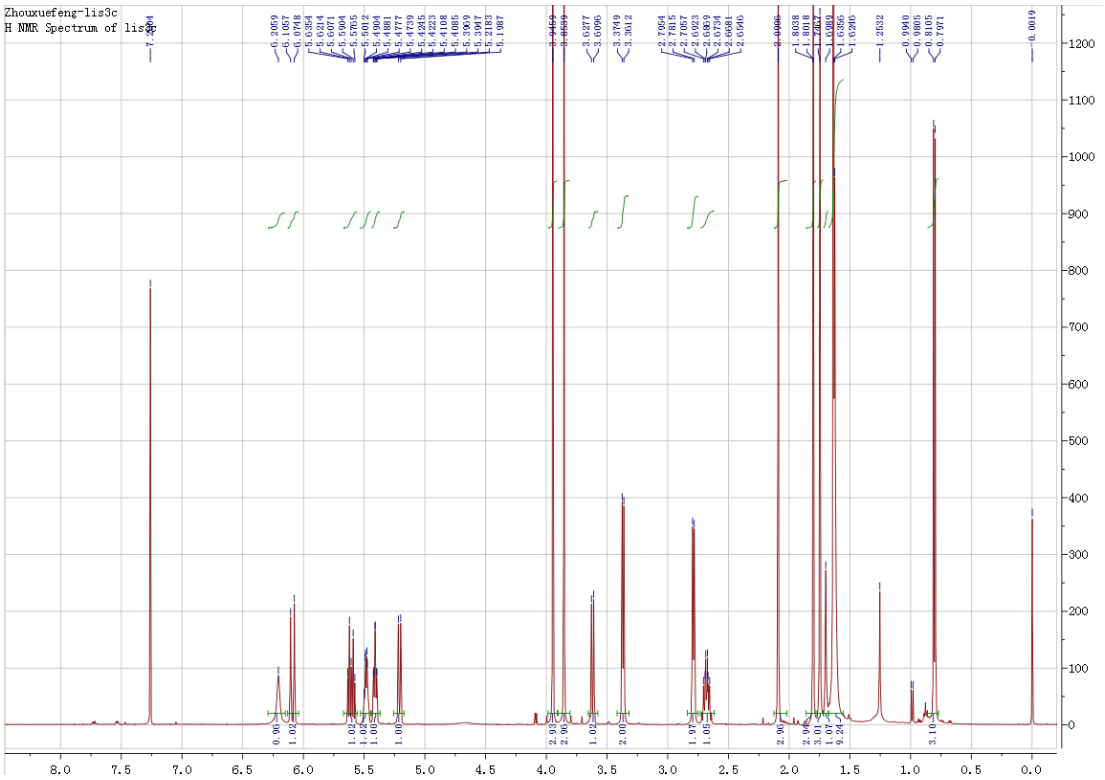
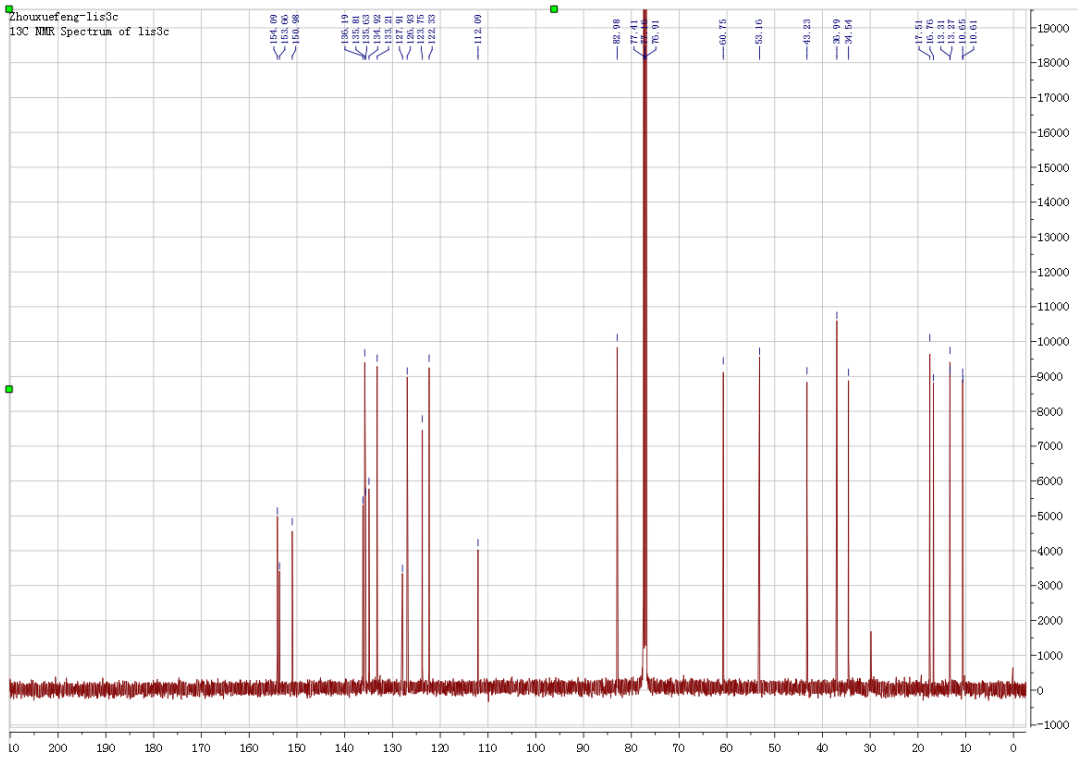


A) HR-MS (+) spectrum of **PA**.

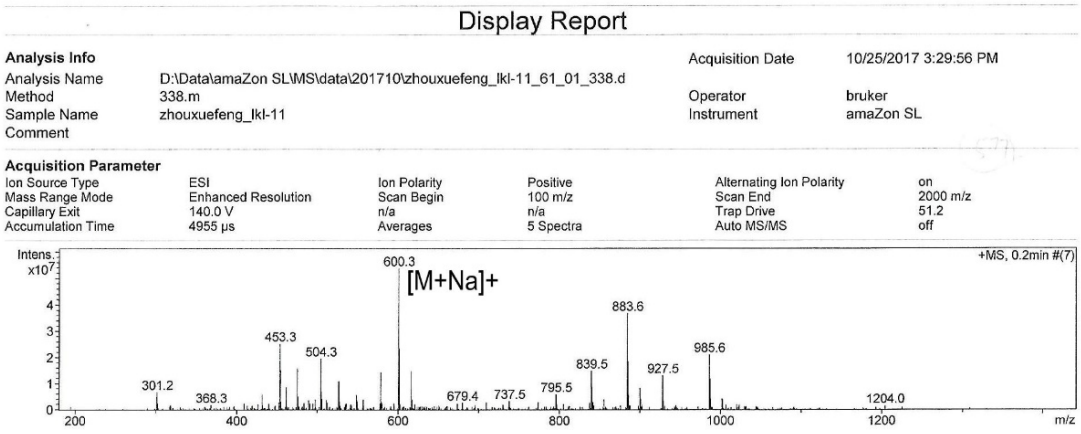


B) ^{1}H NMR spectrum of **PA** (in $CDCl_3$, 500 MHz).

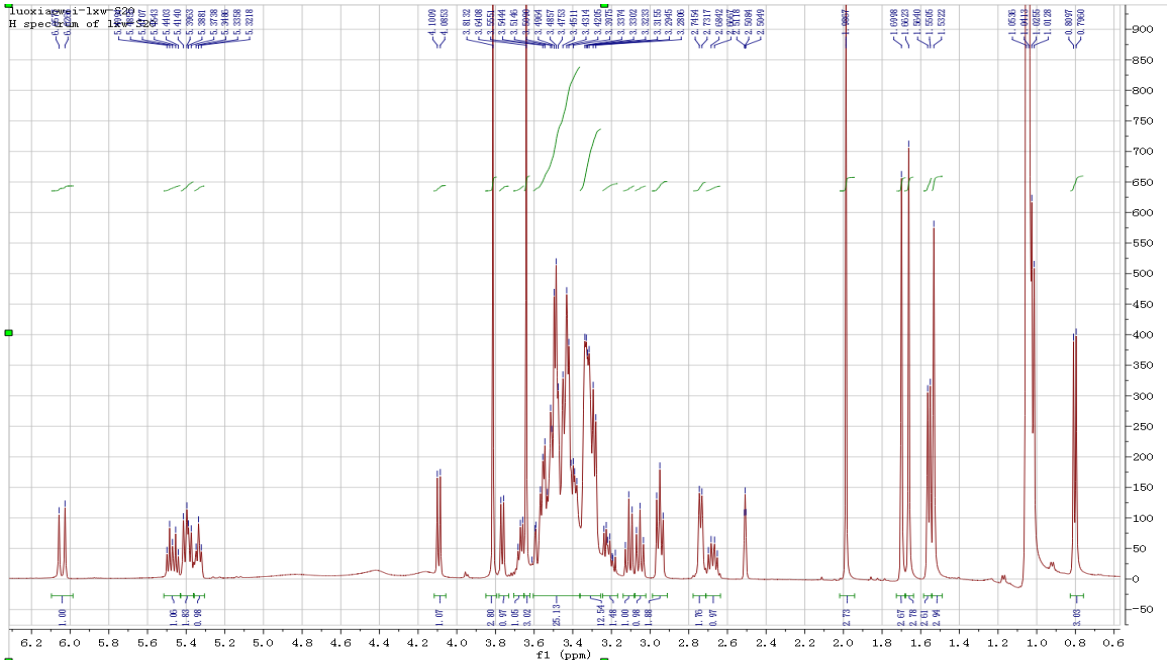


C) ^{13}C NMR spectrum of **PA** (in $CDCl_3$, 125 MHz).

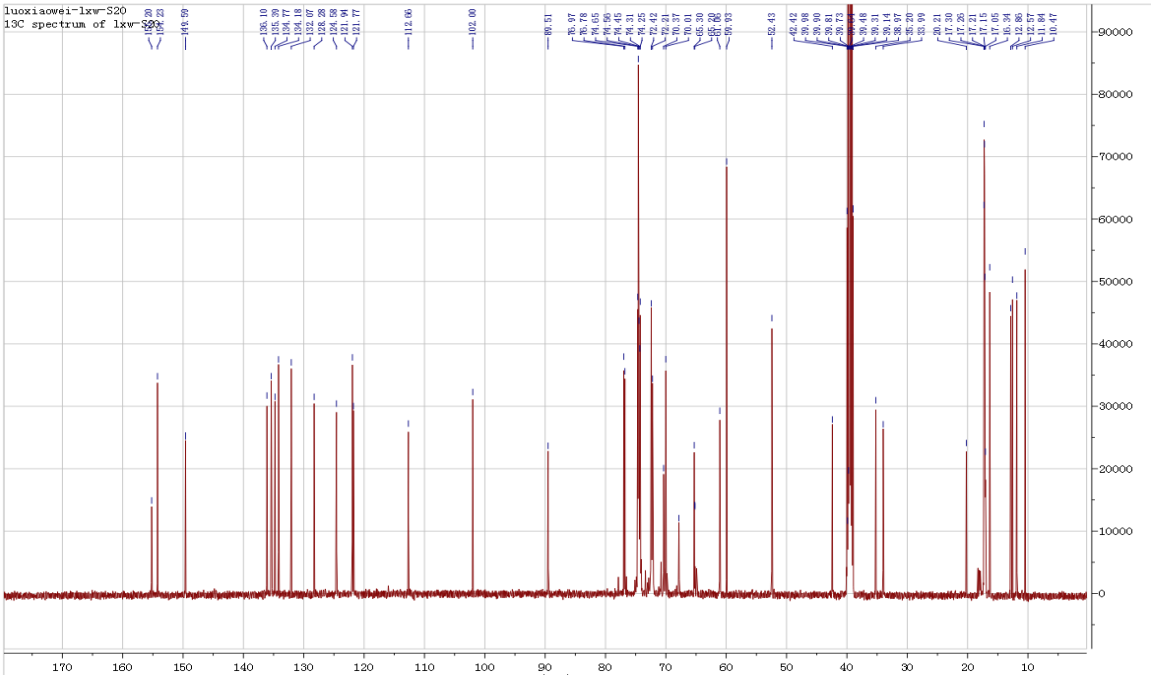
Figure S2. MS, ^{1}H-, ^{13}C-NMR spectra of **PA**.



A) HR-MS (+) spectrum of **GPA**.

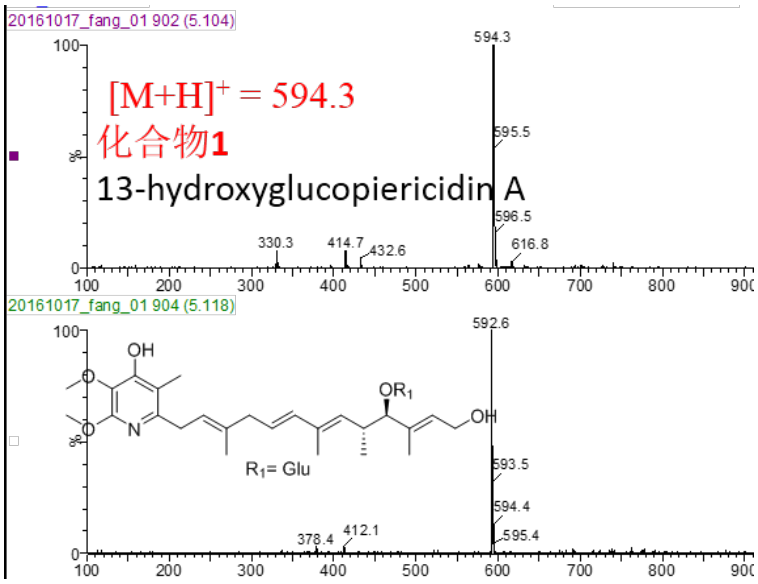


B) ^{1}H NMR spectrum of **GPA** (in DMSO-d_6, 700 MHz).

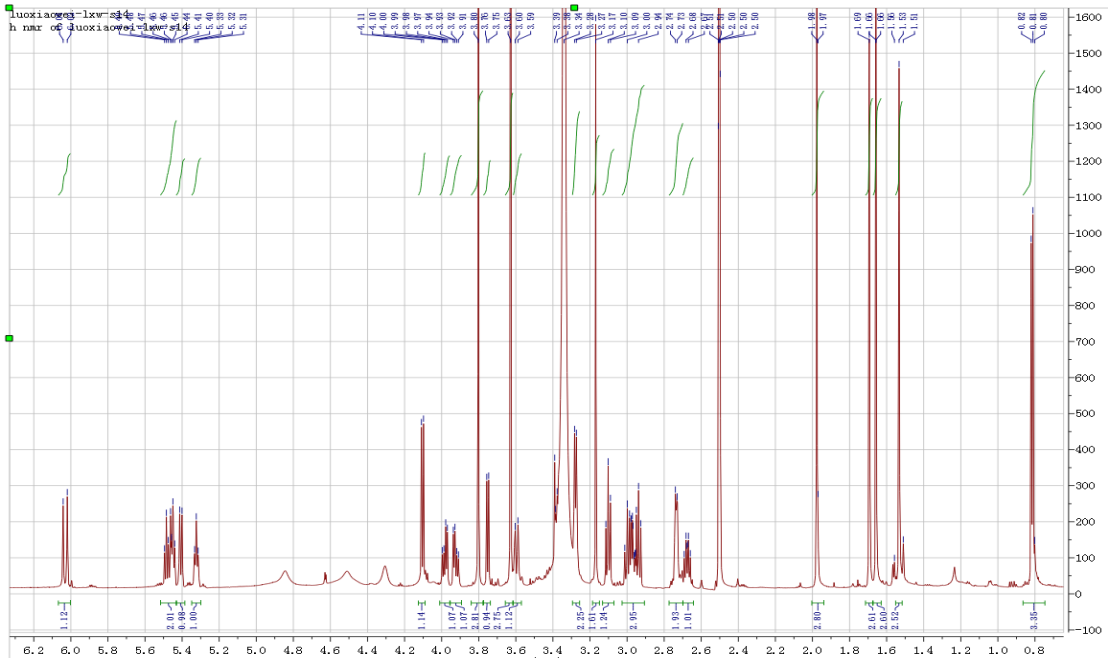


C) ^{13}C NMR spectrum of **GPA** (in DMSO-d_6, 175 MHz).

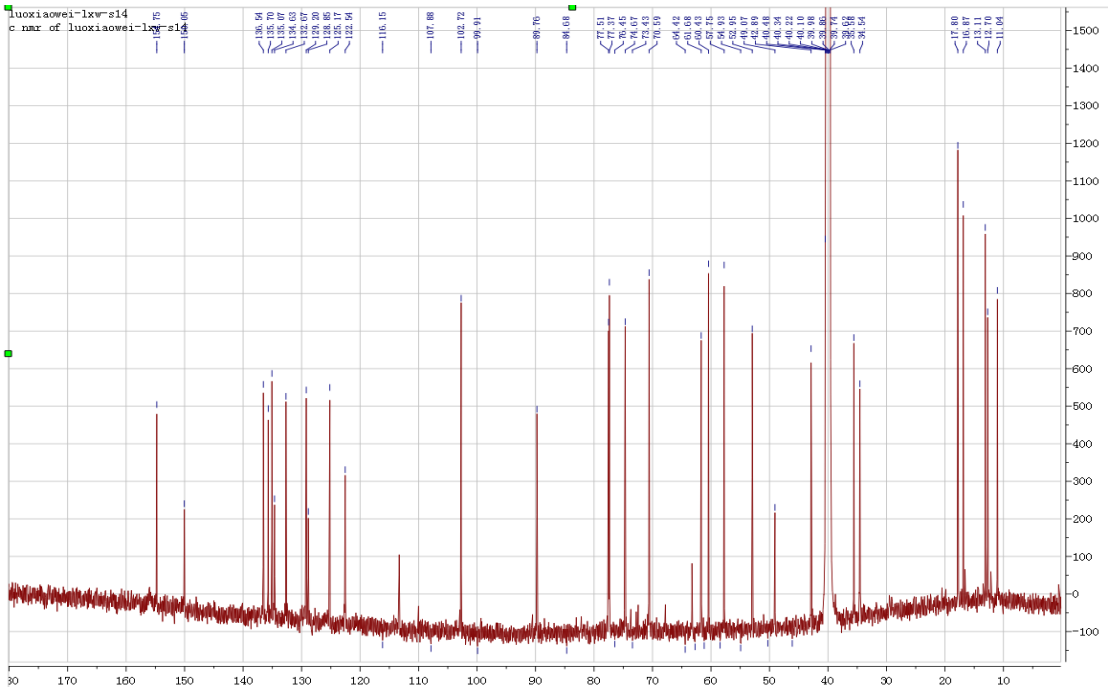
Figure S3. MS, ^{1}H-, ^{13}C-NMR spectra of **GPA.**



A) ESI-MS (+) and ESI-MS (-) spectra of **S14**.

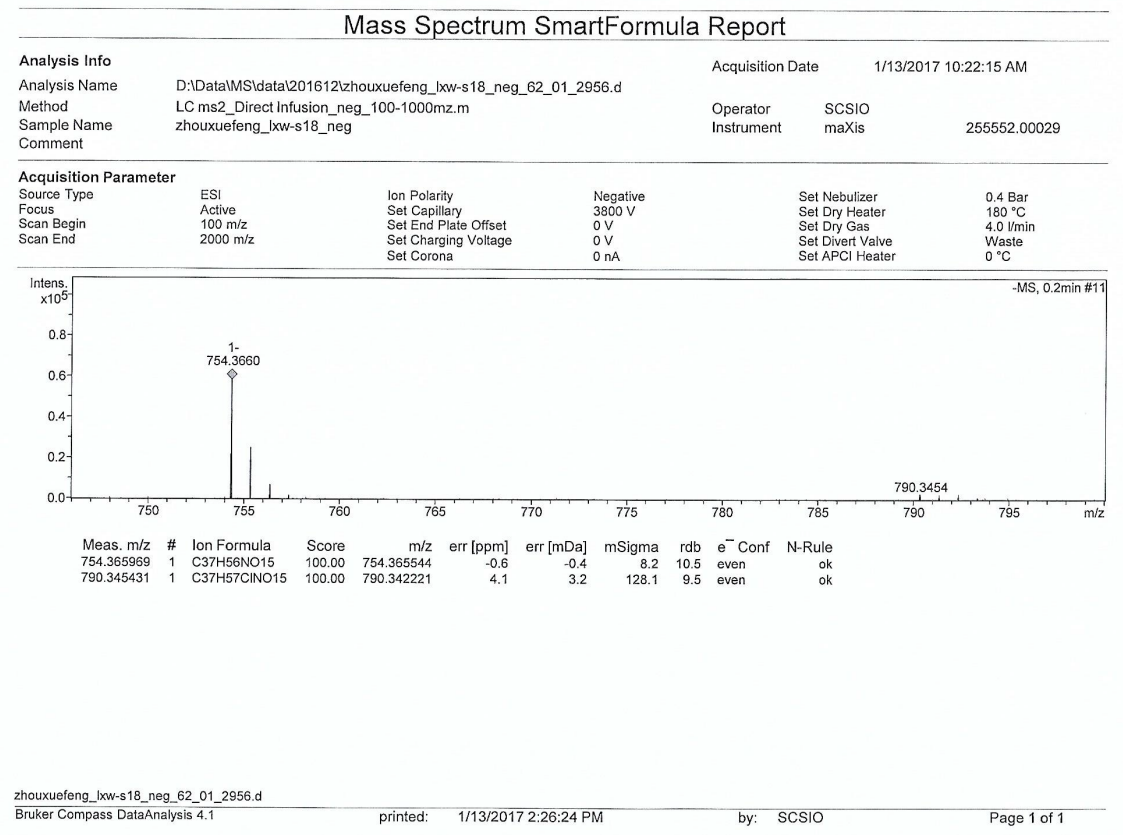


B) ^{1}H NMR spectrum of **S14** (in DMSO-d_6, 700 MHz).

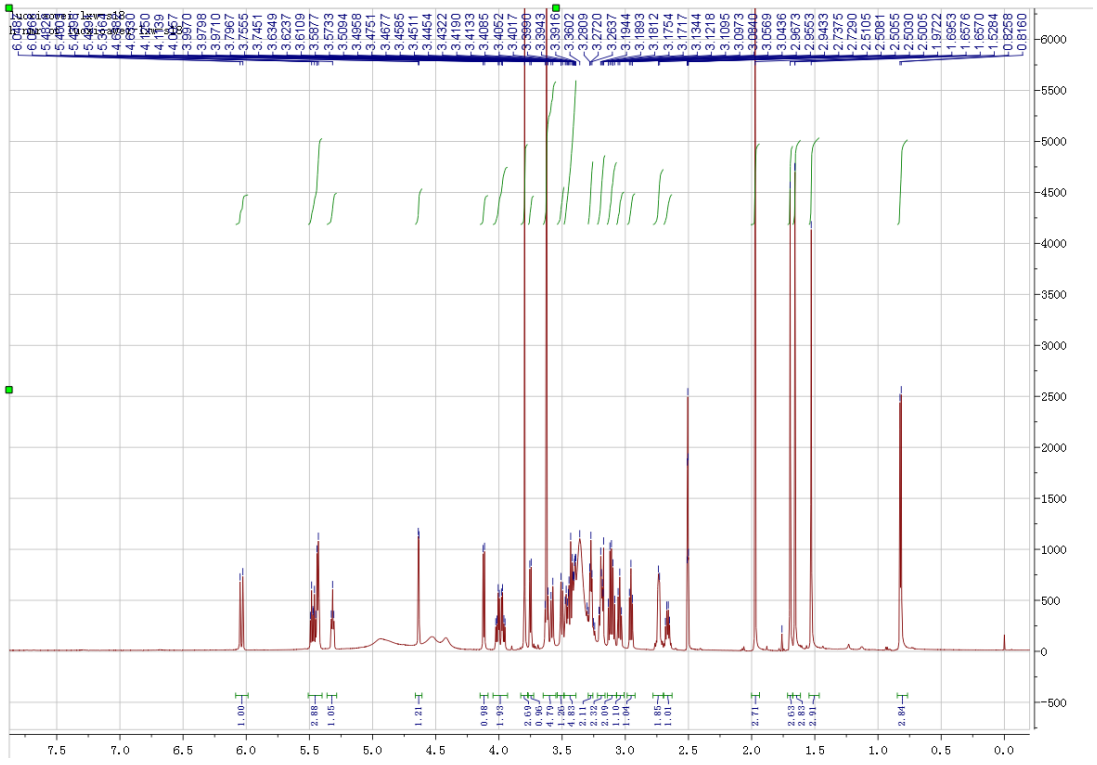


C) ^{13}C NMR spectrum of **S14** (in DMSO-d_6, 175 MHz).

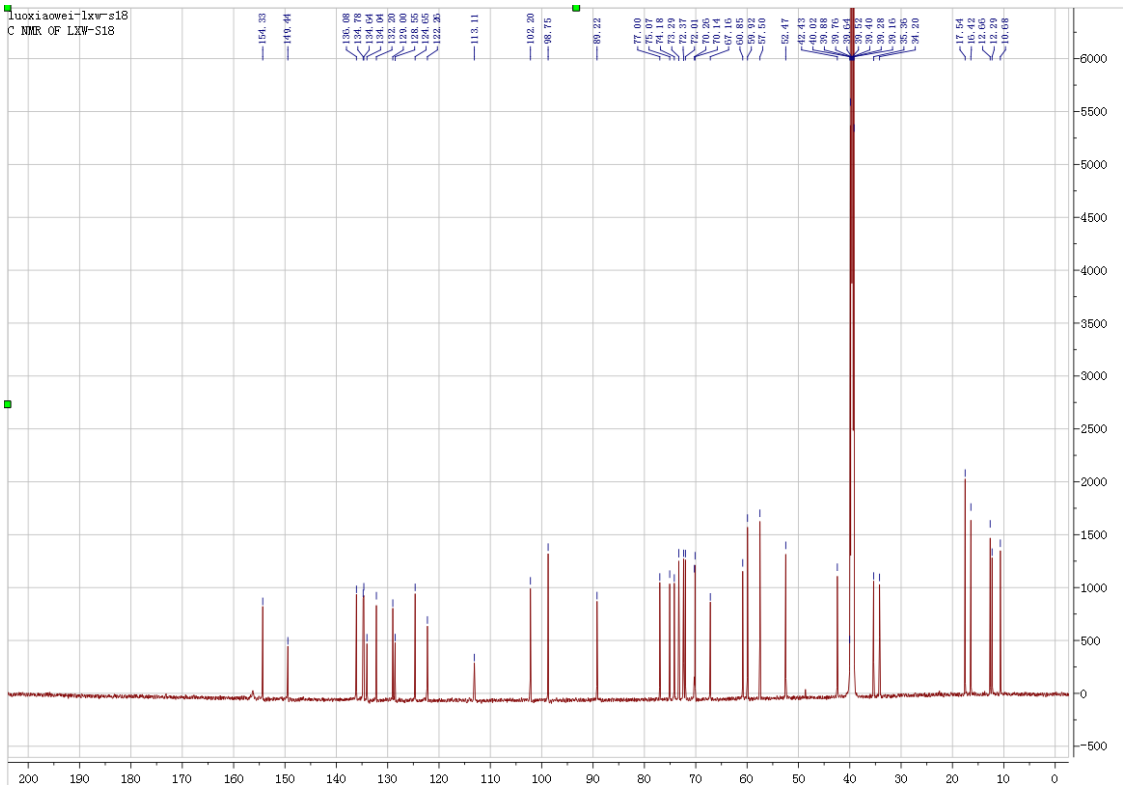
Figure S4. MS, ^{1}H-, ^{13}C-NMR spectra of **S14.**



A) HRESIMS (-) spectrum of **S18**.

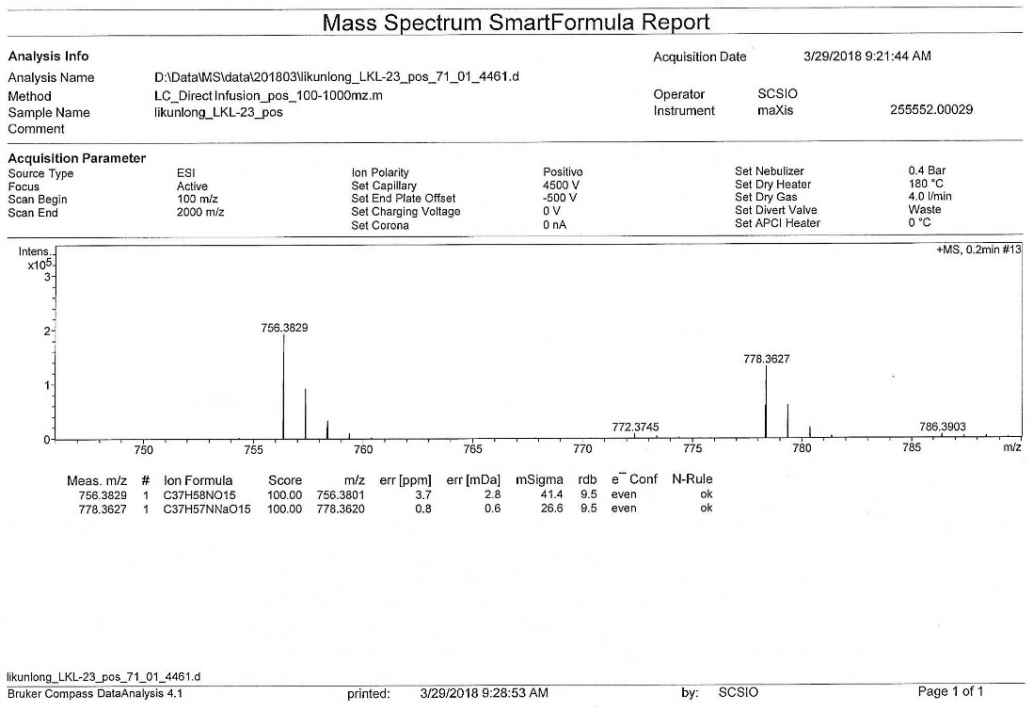


B) ^{1}H NMR spectrum of **S18** (in DMSO-d_6, 700 MHz).



C) ^{13}C NMR spectrum of **S18** (in DMSO-d_6, 175 MHz).

Figure S5. MS, ^{1}H-, ^{13}C-NMR spectra of **S18.**



Mass Spectrum SmartFormula Report

Analysis Info

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Method	LC_Direct Infusion_pos_100-1000mz.m	Operator	SCSIO
Sample Name	likunlong_LKL-23_pos	Instrument	maXis 255552.00029
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
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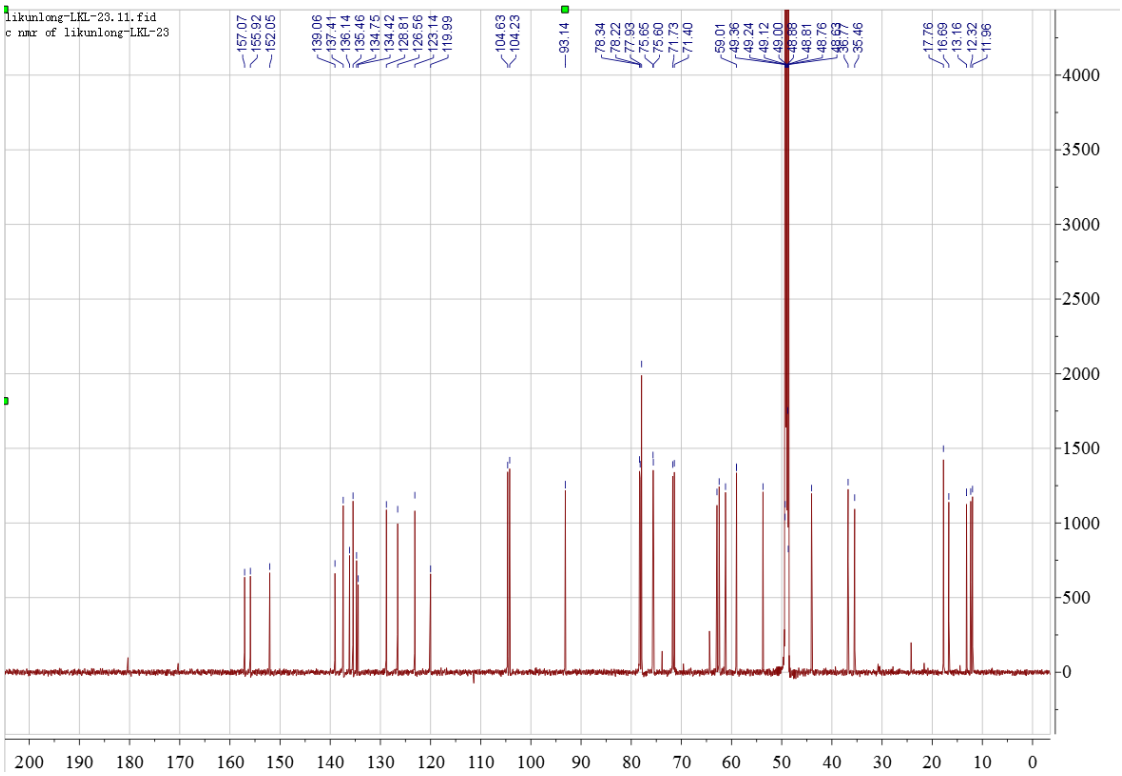
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	2000 m/z	Set Charging Voltage	0 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	0 °C

Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	err [mDa]	mSigma	rdb	e⁻ Conf	N-Rule
756.3829	1	C37H58NO15	100.00	756.3801	3.7	2.8	41.4	9.5	even	ok
778.3627	1	C37H57NNaO15	100.00	778.3620	0.8	0.6	26.6	9.5	even	ok

likunlong_LKL-23_pos_71_01_4461.d
Bruker Compass DataAnalysis 4.1 printed: 3/29/2018 9:28:53 AM by: SCSIO Page 1 of 1

A) HRESIMS (+) spectrum of **S40.**

B) ^{1}H NMR spectrum of **S40** (in CD_3OD, 700 MHz).



C) ^{13}C NMR spectrum of **S40** (in CD_3OD, 175 MHz).

Figure S6. MS, ^{1}H-, ^{13}C-NMR spectra of **S40.**

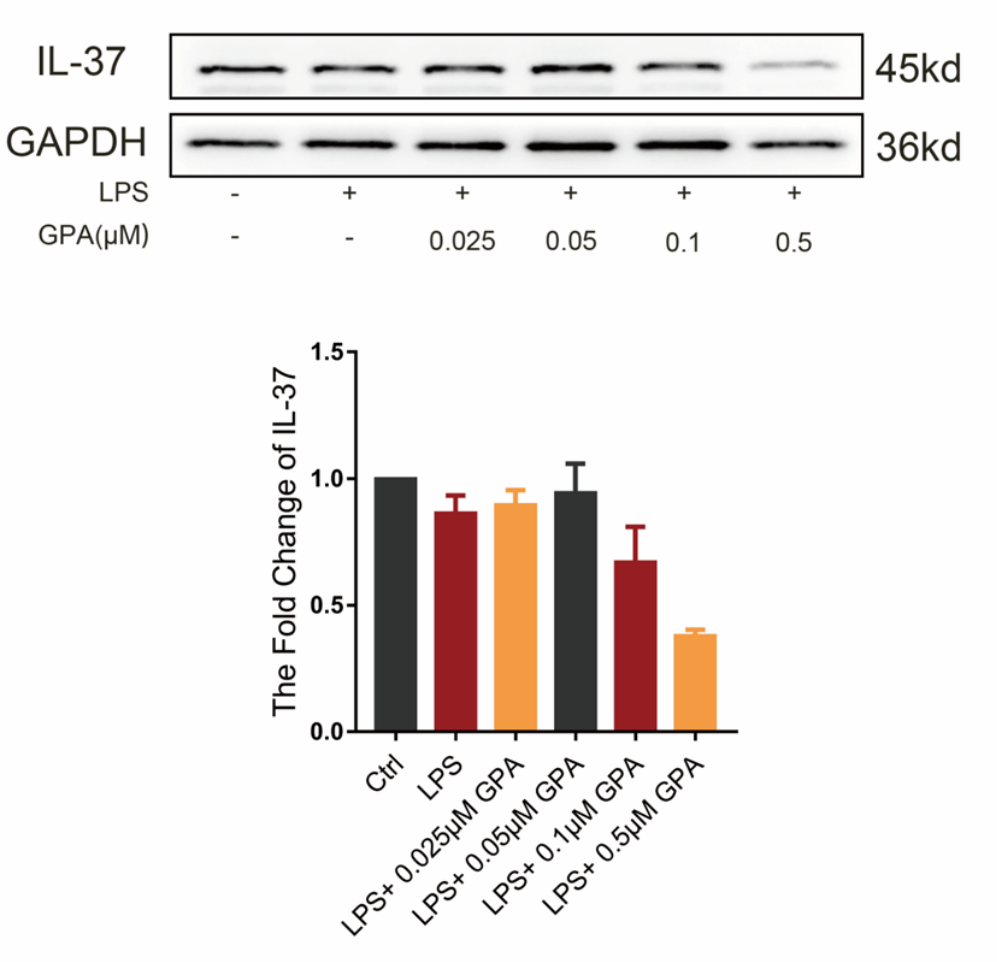
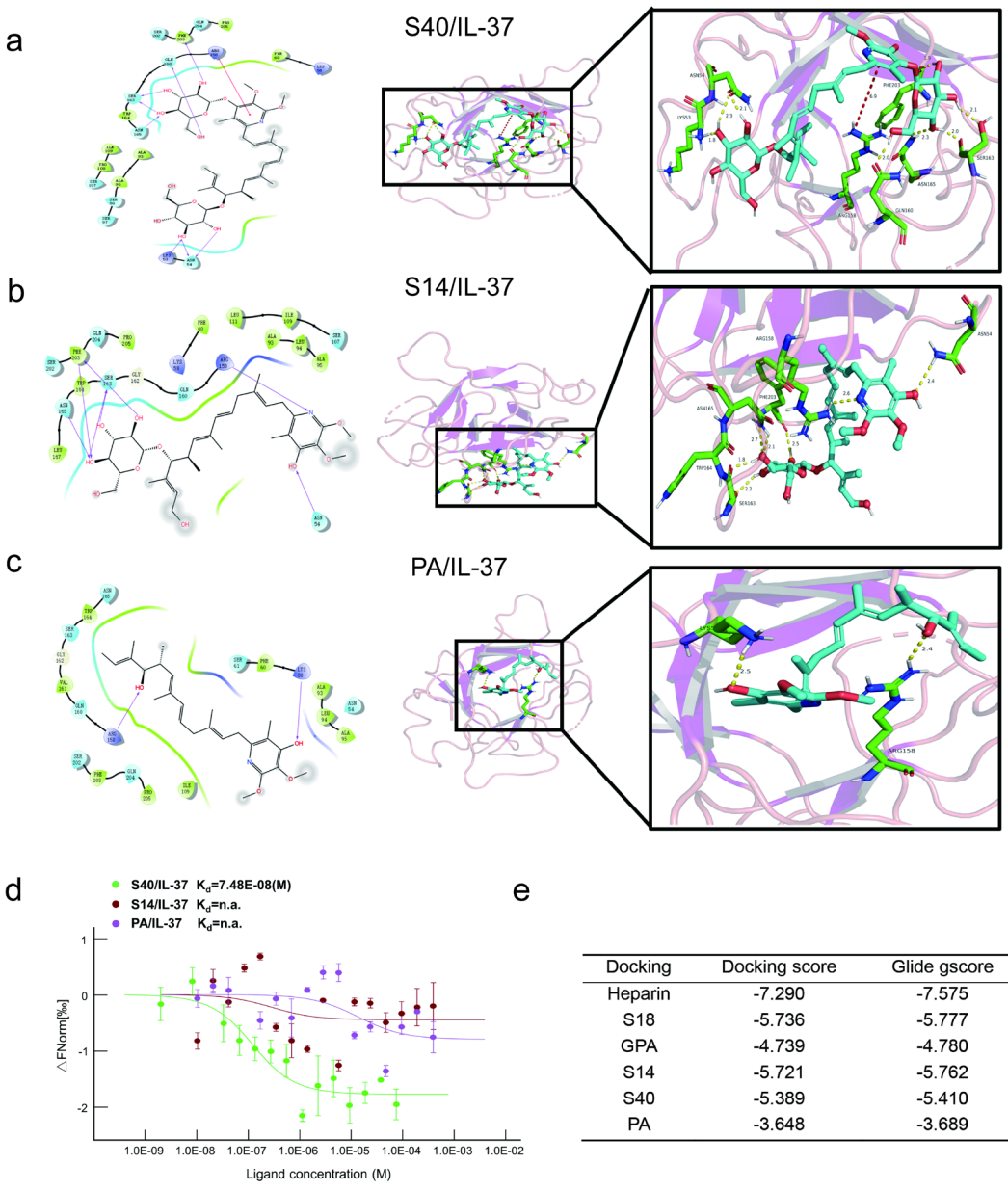


Figure S7. Effect of GPA on expression of IL-37 protein in HAVICs. (A) Human AVICs were stimulated with 200 ng/ml LPS and then treated or not treated with GPA for 24 hours. Immunoblotting revealed the expression of Expression in human AVICs. n=4.

Figure S8. Docking results of some piericidin aglycones binding with IL-37 (6NCU).



Docking	Docking score	Glide gscore
Heparin	-7.290	-7.575
S18	-5.736	-5.777
GPA	-4.739	-4.780
S14	-5.721	-5.762
S40	-5.389	-5.410
PA	-3.648	-3.689

Figure S8 The binding affinity was identified by MST assay and the binding mode as well as sites of ligands were predicted by molecular docking analysis, indicating that S40 could perfectly combine with Il-37 protein but S14 and PA displayed weak binding ability. The detailed docking mode of (A) S40/IL-37, (B) S14/IL-37 and (C) PA/IL-37 with docking pock. (D) Affinity activity of S40/IL-37, S14/IL-37 and PA/IL-37 binding analyzed by MST assay, n = 3. (E) Docking scores of heparin, S18, GPA, S14, S40 and PA.

Figure S9

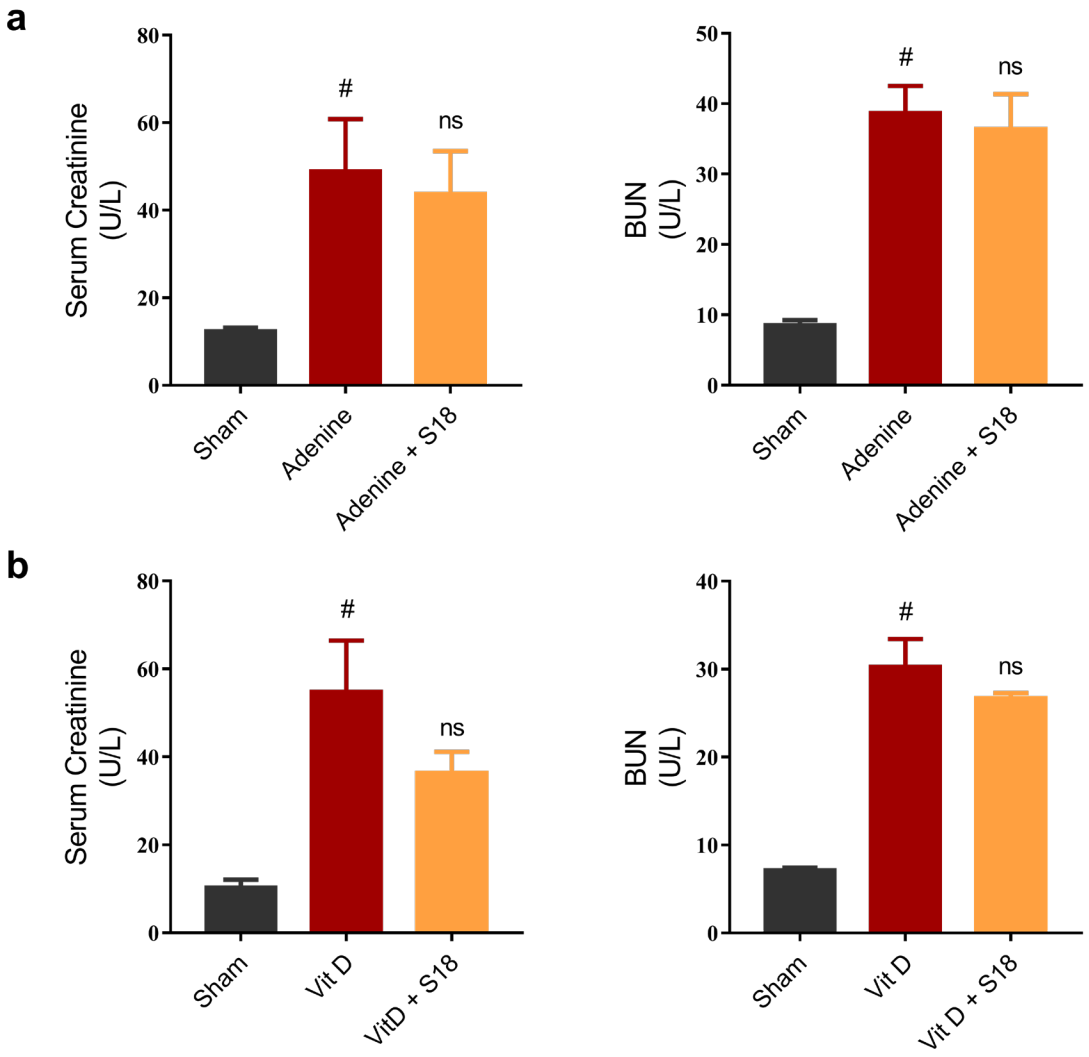


Figure S9. Renal function in mice fed an adenine diet or after vitamin D injection. The IL-37 overexpression mice were fed adenine diet for 21 days. (A) The levels of serum creatinine and BUN were increased. But no difference was found between adenine group and S18 group. (B) The content of serum creatinine and BUN also have high levels with no difference between Vit D group and S18 group. n=5, ns $p>0.05$, #$p<0.05$.

Table S1. Demographic Characteristic of Enrolled Patients

	Non-CAVD patients (n=4)	CAVD patients (n=5)
Age (years)	56.0 ± 4.79	63.6 ± 6.04
Male, n (%)	2 (50%)	3 (60%)
BMI (Kg/m^2)	27.7 ± 1.34	25.7 ± 1.79
Hypertension, n (%)	3 (75%)	3 (60%)
Dyslipidaemia, n (%)	1 (25%)	2 (40%)
Diabetes mellitus, n (%)	1 (25%)	1 (20%)
Smoking history, n (%)	1 (25%)	2 (40%)
β-blockers, n (%)	3 (75%)	1 (20%)
ACE inhibitors/ARB, n (%)	3 (75%)	4 (80%)
Statins, n (%)	2 (50%)	3 (60%)
LVEF (%)	57.4 ± 7.72	62.8 ± 2.73
AO (cm/s)	-	367.4 ± 30.31

Data are presented as mean ± SEM, n (%), or median (interquartile range). ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; AO, aorta valve peak flow velocity; BMI, body mass index; CAVD, calcific aortic valve disease; LVEF, left ventricle Ejection fraction;

Table S2. The primer sequences used for RT-qPCR.

Species	Gene name		Primer (5'- 3')
human	ICAM-1	F	TGACCGTGAATGTGCTCTCC
		R	TCCCTTTTTGGGCCTGTTGT
	IL-8	F	GGAGAAGTTTTTGAAGAGGGCTG
		R	ACAGACCCACACAATACATGAAG
	MCP-1	F	CAGCCAGATGCAATCAATGCC
		R	TGGAATCCTGAACCCACTTCT
	IL-37	F	TTCTTTGCATTAGCCTCATCCTT
		R	CGTGCTGATTCCTTTTGGGC
	GAPDH	F	GGCAAGGTCATCCCAGAGCT
		R	CCCAGGATGCCCTTTAGTGG