Supporting Information

Visual Analysis and Inhibitor Screening of Leucine Aminopeptidase, A Key Virulence for Pathogenic Bacteria Associated Infection

Ming Zhang,[†,‡,#] Zhenhao Tian,[§,#] Jiayue Wang,[†,※,#] Xiangge Tian,[†] Chao Wang,[†,*] Jingnan Cui,[‡] Xiaokui Huo,[†] Lei Feng,[†,ǀ,*] Zhenlong Yu,[†] Xiaochi Ma[†,¤,*]

[†] Dalian Key Laboratory of Metabolic Target Characterization and Traditional Chinese Medicine Intervention, College of Integrative Medicine, College of Pharmacy, Dalian 116044, China

[‡] State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116024, China

[§] School of Life Sciences, Northwestern Polytechnical University, Xi'an 710072, China

[※]Department of Pharmacy, Peking University Shenzhen Hospital, Shenzhen 518036, China

[¤] Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, Xuzhou 221004, China

[ǀ] School of Chemistry and Chemical Engineering, Henan Normal University, Xinxiang 453007, China

* Corresponding authors.

E-mail address: wach_edu@sina.com (C. Wang), leifeng@mail.dlut.edu.cn (L. Feng), maxc1978@163.com (X. Ma)

[#] These authors contributed equally to this work.

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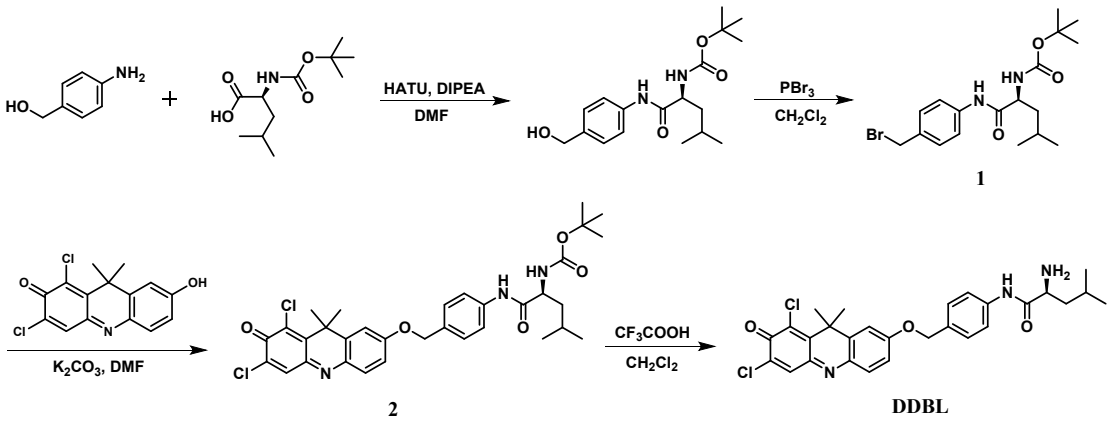
Experimental

Materials and apparatus

All of the constituents for bacterial culture medium such as yeast extracts, tryptone, agar, glucose, and NaCl were purchased from Dalian Meilun Biotechnology Co., Ltd (China). Chemical reagents for the synthesis of fluorescent probe and isolation of natural compounds were produced by Tianjin Kemio Chemical Reagent Co., Ltd (China). Chromatographic reagents, such as acetonitrile and methanol were purchased from sigma-aldrich (MERCK). Leucine aminopeptidase (LAP), Lipase, penicillin G acylase (PGA), bovine serum albumin (BSA), and human albumin (HSA) were purchased from sigma-aldrich (MERCK). Pyroglutamyl aminopeptidase I (PGP-1) was the production of Maibo Pharmaceutical Co., Ltd. CYP450 enzymes (CYP3A5, CYP4F2, CYP3A4) and human carboxylesterases (CE2, CE1c, CE1b) were purchased from Corning Incorporated Life Sciences.

NMR spectra of the synthesized compounds and isolated natural compounds were acquired using a Bruker-500 and 600 with tetramethylsilane (TMS) as the internal standard (Bruker, USA). High resolution mass spectral (HRMS) was measured using a G6224A TOF-MS. HPLC analysis was performed on a Dionex UltiMate 3000 equipped with a DAD detector and a C18 silica gel column (Thermo Scientific, USA). Natural compounds were purified using pre-HPLC manufactured by Agel Technology, which contained an YMC (250 mm × 10 mm) column packed with C18 (5 μm) silica gel, following with a UV detector. A constant temperature incubator shaker (HZQ-C) was obtained from Harbin Donglian Electronic Technology Development Co., LTD (China). A confocal laser scanning microscope was manufactured by Leica (German). The fluorescence spectra and fluorescence intensity was recorded using a BioTek Synergy H1 microplate reader (BioTek, USA). Both fluorescence imaging of native-PAGE gel stained by **DDBL** and 96-well plate was performed using an Amersham Typhoon RGB (GE, USA).

Synthesis of fluorescent probe DDBL



Scheme S1. The synthetic route of the fluorescent probe **DDBL**.

Compound **1** was synthesized according to the previous procedure reported.[1] ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.48 (s, 1H), 7.49 (d, J = 8.4 Hz, 2H), 7.32 (d, J = 8.3 Hz, 2H), 4.93 (s, 1H), 4.47 (s, 2H), 4.23 (s, 1H), 1.76 (ddd, J = 23.4, 13.9, 7.8 Hz, 2H), 1.58 – 1.53 (m, 1H), 1.46 (s, 9H), 0.97 (dd, J = 8.8, 6.4 Hz, 6H).

A solution of compound **1** (398.1 mg, 1.0 mmol), K_2CO_3 (138.2 mg, 1.0 mmol) and 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (**DDAO**, 307.0 mg, 1.0 mmol) in DMF (15 mL) was stirred at room temperature (RT) overnight. Then the reaction solution was diluted with 300 mL CH_2Cl_2 and washed with water and brine. The crude compound **2** was obtained after removing the organic phase by reduced pressure distillation, which was used without further purification. Then, trifluoroacetic acid (5 mL) was added into the solution of the crude product **2** in CH_2Cl_2 (15 mL), and the mixture was stirred overnight at RT for the deprotection. After removing the solvent, the residues were purified over a silica column with CH_2Cl_2/MeOH (16/1 *v/v*) to afford **DDBL** as an orange solid (164.9 mg, yield: 31.4%). ^{1}H NMR (500 MHz, MeOD) δ_H 7.70 (s, 1H), 7.65 (d, J = 8.3 Hz, 2H), 7.62 (d, J = 8.7 Hz, 1H), 7.47 (d, J = 8.3 Hz, 2H), 7.26 (s, 1H), 7.09 (d, J = 8.8 Hz, 1H), 5.22 (s, 2H), 3.56 – 3.52 (m, 1H), 1.90 (s, 6H), 1.78 (dt, J = 13.2, 6.6 Hz, 1H), 1.69 – 1.63 (m, 1H), 1.55 – 1.49 (m, 1H), 1.00 (dd, J = 9.9, 6.6 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$+MeOD) δ_C 175.01, 173.82, 162.83, 147.61, 141.48, 141.22, 139.67, 138.34, 136.73, 136.07, 134.66, 134.39, 131.89, 128.77, 120.36, 114.40, 114.33, 70.57, 54.13, 44.31, 39.54, 26.90, 25.09, 23.20, 21.83. HRMS (ESI negative) calcd. for $[M+CF_3COO]^-$ 638.1442, found

638.1443.

Enzymatic hydrolysis of DDBL mediated by leucine aminopeptidase (LAP)

The enzymatic hydrolysis of **DDBL** mediated by LAP was performed in phosphate buffer (PB, pH 7.4) containing K_2HPO_4 and KH_2PO_4 (K^+ 100 mM). 200 μL PB containing LAP (0.063 U/mL) and **DDBL** (10 μM) were shaken under constant temperature (37 °C) for 1 h. Then, 100 μL acetonitrile was used to terminate the enzymatic reaction before the removing of denatured protein by centrifugation (20000 *g*, 10 min). The supernate was subjected to microplate reader for the measurement of fluorescence spectra (λ_{ex} 600/λ_{em} 660 nm).

In addition, a series enzymatic hydrolysis of **DDBL** (10 μM) were performed in presence of LAP at different concentrations (0 – 0.0945 U/mL) with an incubation at 37 °C for 1 h, as well as the records of fluorescence intensities. The relationship was calculated between fluorescence intensities and LAP concentrations.

The selectivity investigation of **DDBL** toward LAP was performed with the co-incubation of **DDBL** (37 °C, 1 h) and various biological enzymes CYP3A5, CYP4F2, CYP3A4, CE2, CE1c, CE1b, PGP-1, PGA and lipase (3 μg/mL). The interference on the fluorescence intensity of **DDBL** and the enzymatic hydrolysis of **DDBL** was performed for various species, including ions (Mn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Sn^{4+}, Fe^{3+}, Ba^{2+}, Cr^{6+}, 200 μM), amino acids (Ser, Trp, Tyr, Gly, Gys, Arg, Lys, Gln, GSH, 10 μM), even glucose (10 μM).

Identification of intestinal bacteria with active LAP

Fresh human stools were dispersed in sterile water, and coated on nutrient broth (NB) agar plate, which was cultured at 37 °C for about 24 h until the obvious bacterial colonies. Then, **DDBL** (50 μM) was sprayed on these colonies for an incubation at 37 °C for 2 h. Imaged by Amersham Typhoon RGB, the fluorescence image of agar plate was recorded, which displayed fluorescence colonies (λ_{ex} = 635 nm, λ_{em} = 670 ± 15 nm). After further purification, target bacteria strains were obtained as LAP active bacteria species. The isolated bacterial strains were identified by 16S rRNA gene sequencing. The RT-PCR primer was as followed.

1510 R: 5’-ACGGYTACCTTGTTACGACTT-3’

7F: 5'-AGAGTTTGATYMTGGCTCAG-3'

Native-PAGE for bacterial lysates and LAP proteins

The native gel was prepared containing 10% polyacrylamide separation gel and 4% concentrated gel. After solidification at room temperature, bacterial lysates containing equal amounts of protein (18 μg) or the recombinant LAP enzyme with different loading amount (10, 20, 50, 100, 200, 300, 400, 500 ng) were loaded into the gel, respectively. The electrophoresis of native-PAGE was carried out under constant pressure (100 V for 0.5 h and 150 V for 2 h) with ice-water bath until the marker strips are separated and the bromophenol blue was electrophoresed to the bottom of the gel.

When the electrophoresis was finished, the gel was subjected to **DDBL** solution (50 μM) for incubation at 37 °C (4.5 h). After washing by PB solution, the gel was scanned using a fluorescence scanner and fluorescence images were obtained (λ_{ex} = 635 nm, λ_{em} = 670 ± 15 nm).

Investigation about inhibitory effects of natural products on LAP

The high throughput screening for LAP inhibitors were performed in 96-well plate by the co-incubation about LAP (0.063 U/mL), **DDBL** (10 μM) and various herb extractions (100 μg/mL) with the same enzymatic reaction conditions as mentioned above. Then, the plate was subjected to Amersham Typhoon RGB for the fluorescence imaging (λ_{ex} = 635 nm, λ_{em} = 670 ± 15 nm). According to the fluorescence intensity of individual well, the inhibitory effect of each herb was determined. The inhibitory effect of isolated compounds was evaluated as the same protocol.

Spectroscopic data of isolated natural compounds as LAP inhibitors

3-acetyl-11-keto-*β*-boswellic acid. ^{1}H-NMR (600 MHz, DMSO-d_6) δ_H 5.44 (s, 1H), 5.10 (t, J = 2.5 Hz, 1H), 2.38 (m, 1H), 2.36 (m, 1H), 2.09 (m, 2H), 2.04 (s, 3H), 1.82 (m, 2H), 1.65 (m, 2H), 1.56 (d, J = 10.8 Hz, 1H), 1.36-1.46 (m, 4H), 1.30-1.34 (m, 4H), 1.31 (s, 3H), 1.18-1.23 (m, 3H), 1.10 (s, 3H), 1.09 (s, 3H), 1.05 (s, 3H), 0.99 (m, 1H), 0.92 (s, 3H), 0.79 (s, 3H), 0.76 (d, J = 6.4 Hz, 3H). ^{13}C-NMR (150 MHz, DMSO-d_6) δ_C 198.2, 177.4, 169.9, 164.5, 129.6, 72.5, 59.6, 58.2, 49.4, 45.7, 44.5

43.4, 40.4, 38.7, 38.5, 36.8, 34.1, 33.6, 32.2, 30.4, 28.5, 26.9, 26.7, 23.5, 23.1, 21.0, 20.9, 20.1, 18.6, 18.0, 17.0, 13.1. (+)-HR-ESI-MS *m/z* 513.3563 $[M+H]^+$, calcd for $C_{32}H_{49}O_5^+$, *m/z* 513.3575.

Carnosol. ^{1}H-NMR (600 MHz, DMSO-d_6) δ_H 8.37 (s, 1H), 8.13 (s, 1H), 6.70 (s, 1H), 5.46 (dd, J = 4.2, 1.2 Hz, 1H), 3.20 (m, 1H), 2.66 (m, 1H), 2.46 (dt, J = 13.8, 4.2 Hz, 1H), 2.07 (m, 1H), 1.79 (m, 2H), 1.60 (dd, J = 10.8, 6.0 Hz, 1H), 1.52 (m, 1H), 1.45 (m, 1H), 1.23 (m, 1H), 1.19 (d, J = 6.8 Hz, 6H), 0.81 (s, 3H), 0.79 (s, 3H). ^{13}C-NMR (150 MHz, DMSO-d_6) δ_C 175.5, 143.2, 143.0, 134.2, 131.5, 121.9, 111.3, 76.9, 47.8, 44.9, 40.5, 34.1, 31.3, 29.2, 28.8, 26.2, 22.8, 22.6, 19.4, 18.5. (+)-HR-ESI-MS *m/z* 331.1892 $[M+H]^+$, calcd for $C_{20}H_{27}O_4^+$, *m/z* 331.1904.

Carnosic acid. ^{1}H-NMR (600 MHz, DMSO-d_6) δ_H 11.95 (br s, 1H), 7.79 (s, 2H), 6.33 (s, 1H), 3.53 (m, 1H), 3.15 (m, 1H), 2.70 (m, 2H), 2.16 (m, 2H), 1.69 (s, 1H), 1.48 (m, 1H), 1.47 (m, 1H), 1.43 (m, 2H), 1.24 (dt, J = 13.2, 4.8 Hz, 1H), 1.12 (d, J = 7.2 Hz, 3H), 1.09 (d, J = 7.2 Hz, 3H), 0.96 (m, 1H), 0.81 (s, 3H), 0.78 (s, 3H). ^{13}C-NMR (150 MHz, DMSO-d_6) δ_C 176.9, 145.1, 139.0, 134.3, 129.0, 126.8, 116.3, 53.4, 47.0, 40.9, 33.8, 33.6, 32.5, 31.6, 26.0, 22.9, 22.7, 20.1, 19.7, 18.2. (+)-HR-ESI-MS *m/z* 333.2051 $[M+H]^+$, calcd for $C_{20}H_{29}O_4^+$, *m/z* 333.2060.

Fluorescence imaging of *S. aureus* cells by DDBL

S. aureus was cultured in NB medium to get enough cells with OD_{600} values at 0.7-0.8. The medium was removed by centrifugation (5000 rpm, 10 min). The bacterial cells were washed two times by PB (1 mL) and suspended into PB (1 mL) following pre-incubation for 3 min at 37 °C. Then, **DDBL** (50 μM) was added into the bacterial suspension for incubation at 37 °C (6 h). For the inhibitory experiment, inhibitors (50 μM) were added into the bacterial suspension together with the fluorescent probe. After the incubation, the clear bacterial cells were prepared by the washing procedure using PB and centrifugation (5000 rpm). The bacterial cells were resuspended in 30 μL PB for fluorescence imaging experiments. The bacterial suspension was dropped onto a glass slide, which was subjected on confocal laser scanning microscopy to obtain fluorescence images (λ_{ex} = 633 nm, λ_{em} = 645 - 690 nm).

Inhibitory effect of LAP inhibitors against the infection of RAW 264.7 by *S. aureus*

The infection of murine RAW 264.7 macrophage-like cells was performed as previous protocol.[2] RAW 264.7 cells were maintained in tissue culture medium (TCM) consisting of DMEM supplemented with 10% fetal bovine serum. For infection assays cells were seeded into 6-well plates 24 h prior to use and infected at an MOI of 100 by *S. aureus* for 2 h. The *S. aureus* in inhibitor group has been pre-incubated by inhibitors before infection (**AKBA** 25 μM, **CAA** 50 μM). Then, cells were washed with PBS (×2) for the clearance of *S. aureus*, which were subjected into fresh DMEM for a continue culture about 12 h. Meantime, inhibitors and DMSO (control group) were added. Finally, the cells were washed with PBS and lysed using PBS containing 0.5% Triton X-100. Samples were withdrawn to determine bacterial numbers and the remaining bacteria pelleted by centrifugation. The inflammatory cytokines interleukin (IL)-6, tumour necrosis factor-alpha (TNF-α) were also determined using ELISA kits (Elabscience).

Murine sepsis infection model and the attenuation effect of AKBA

Femal six-week old Swiss mice were randomly divided into three groups: vehicle group (n = 7), sepsis model (n = 12), **AKBA** group (n = 12). The mice of three groups were administrated with different samples via intraperitoneal injection: vehicle group (sterile water 0.1 mL), sepsis model (*S. aureus* 3.75×10^8 CFU in 0.1 mL), **AKBA** group (pre-incubated *S. aureus* by 25 μM **AKBA**). Then, the behaviors of experimental mice were recorded and imaged, together with the survival within 24 h. When the death was observed for experimental mice, they were extracted the eyeball blood, as well as the collection of different organs and tissues in aseptic clean bench, including heart, lung, liver, kidney, spleen, kidney, belly. The loaded *S. aureus* in these organs were determined by culture. And, the inflammatory cytokines IL-6, TNF-α, INF-γ were determined using ELISA kits (Elabscience).

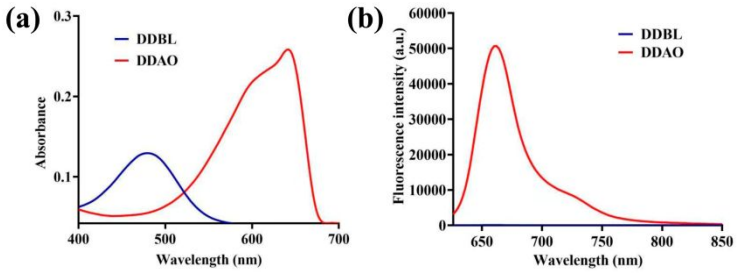


Fig. S1. The absorbance spectra (a) and fluorescence spectra (b) of **DDBL** and **DDAO**.

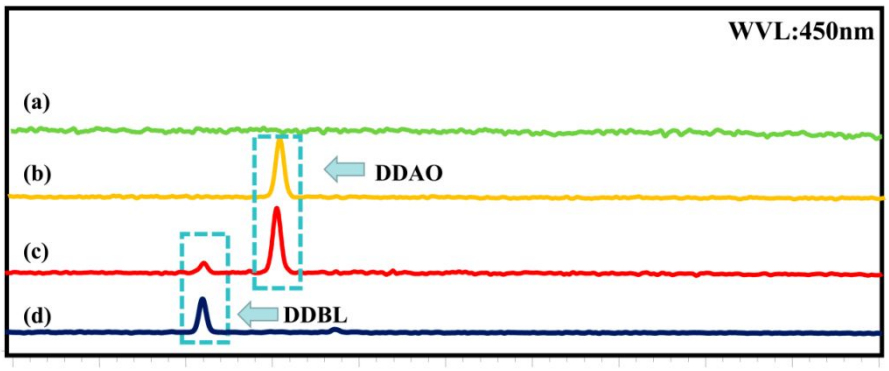


Fig. S2. HPLC analysis for the enzymatic hydrolysis of **DDBL** mediated by LAP. (a) LAP solution. (b) **DDAO** reference standard. (c) The reaction solution about **DDBL** and LAP. (d) **DDBL** reference standard.

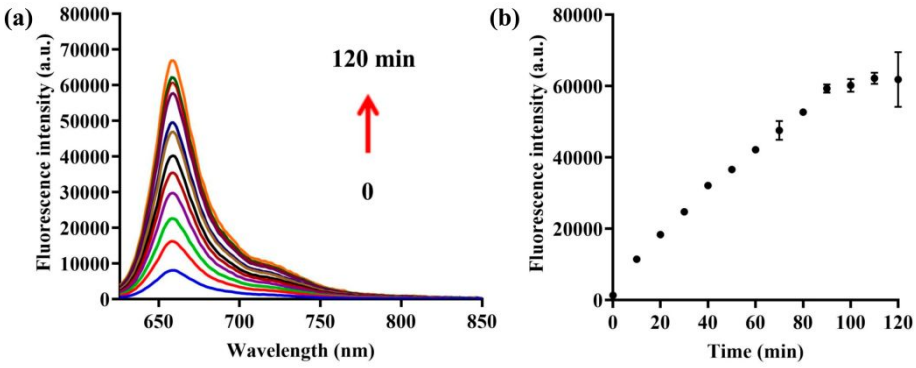


Fig. S3. The fluorescence response of **DDBL** toward LAP at different incubation time (0-120 min). (a) Fluorescence spectra, (b) Relationship between fluorescence intensity and incubation time.

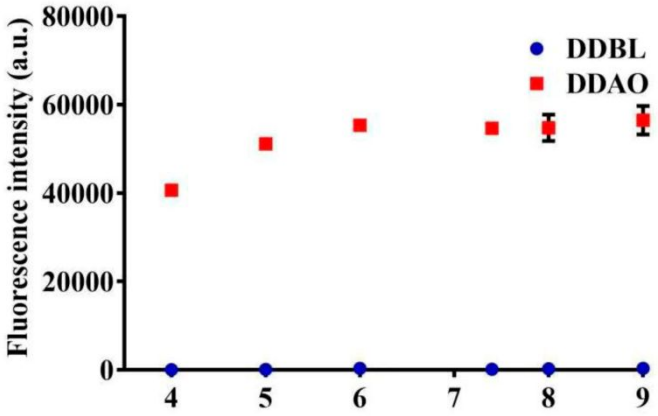


Fig. S4. The fluorescence responses of **DDBL** and **DDAO** in phosphate buffer with different pH values (pH 4-9), λ_{ex} 600 nm, λ_{em} 660 nm.

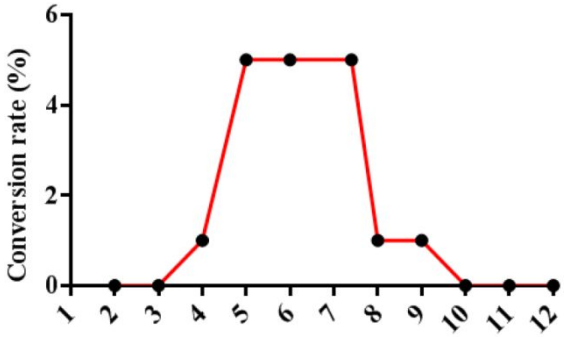


Fig. S5. The inference of phosphate buffer with different pH values on the hydrolysis of **DDBL** mediated by LAP.

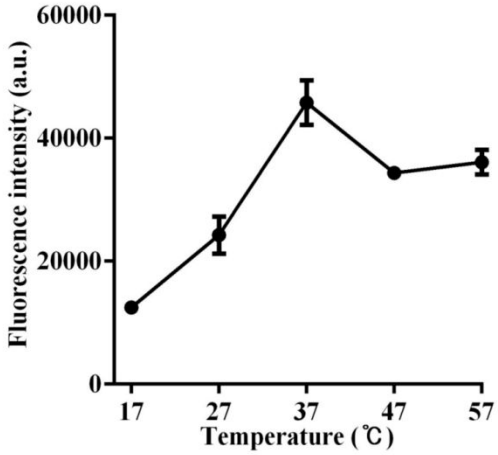


Fig. S6. The fluorescence response of **DDBL** hydrolyzed by LAP under different incubation temperature.

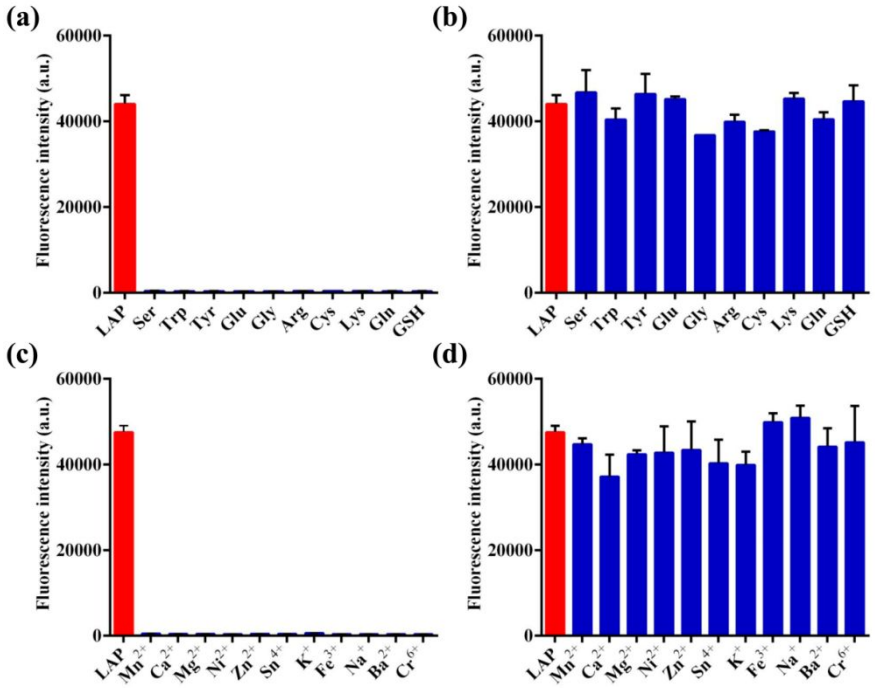


Fig. S7. Inferences of various species on the fluorescence intensity of **DDBL** (a. amino acids, c. ions) and the hydrolysis of **DDBL** mediated by LAP (b. amino acids, d. ions).

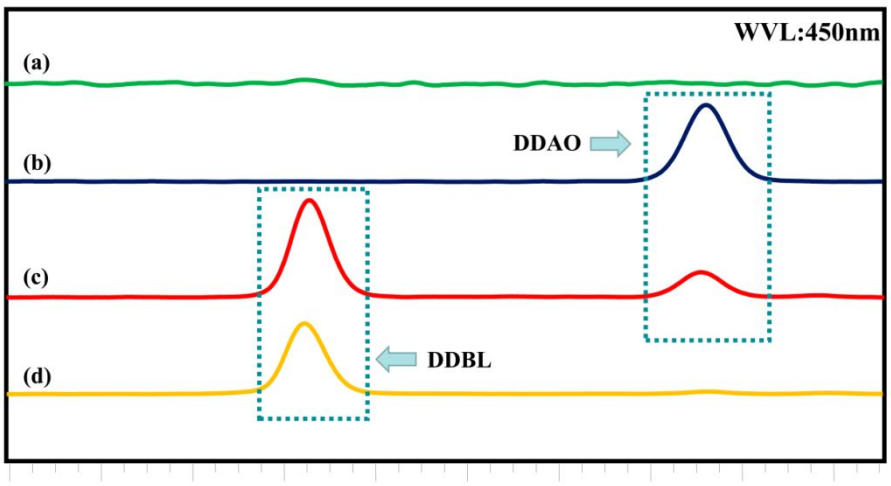


Fig. S8. The analysis of metabolism of **DDBL** by endogenous LAP in *Klebsiella variicola* strain F2R9. (a) The culture of *K. variicola* strain F2R9. (b) **DDAO** reference. (c) The co-incubation of **DDBL** and *K. variicola* strain F2R9. (d) **DDBL** reference.

Table S1. Primers for the RT-PCR of homology LAP in six isolated intestinal bacteria.

Strain	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
P. gergoviae	GCGCATATGATGGAGTTCAGT GTAAAAAG	TATGGATCCTCACTCCTCGCCGTTA TACC
K. variicola	GCGCATATGATGGAGTTCAGT GTAAAAAG	TATGGATCCTCACTCTTCGCCGTTA AA
E. fergusonii	GCGCATATGATGGAGTTTAGT GTAAAAAG	TATGGATCCTTACTCTTCGCCGTTA AA
E.hormaechei	GCGCATATGATGGAGTTCAGT GTAAAAAG	TATGGATCCTCACTCGTCGCCGTTA AA
S. flexneri	GCGCATATGATGGAGTTTAGT GTAAAAAG	TATGGATCCTTACTCTTCGCCGTTA AA
R. planticola	GCGCATATGATGGAGTTCAGT GTAAAAAG	TATGGATCCTCACTCTTCGCCGTTA AA

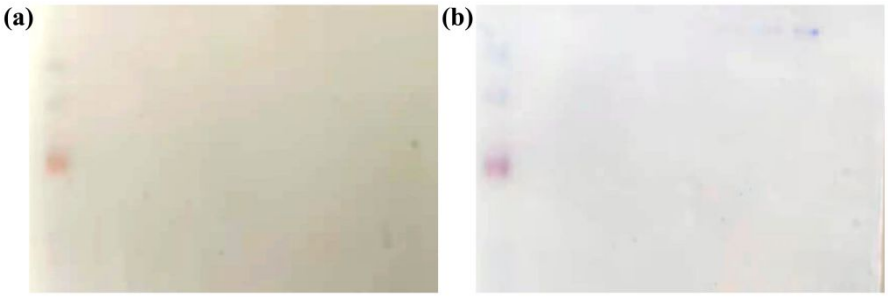


Fig. S9. The native-PAGE analysis of a series LAP at different concentrations stained by silver (a) and Coomassie blue (b).

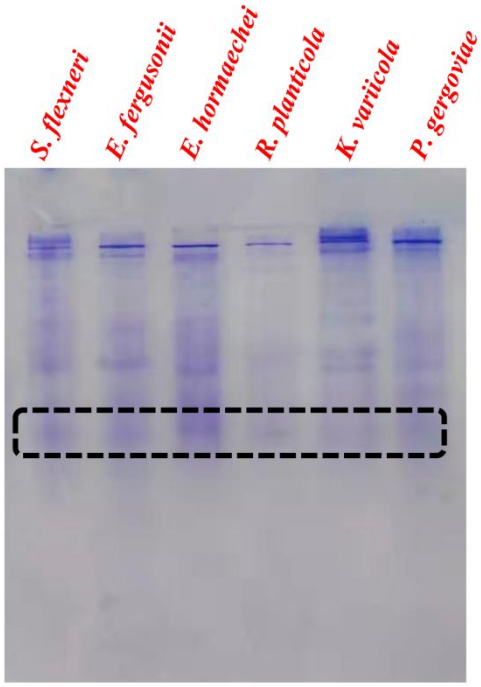


Fig. S10. The native-PAGE analysis for bacterial lysates stained by Commassie blue.

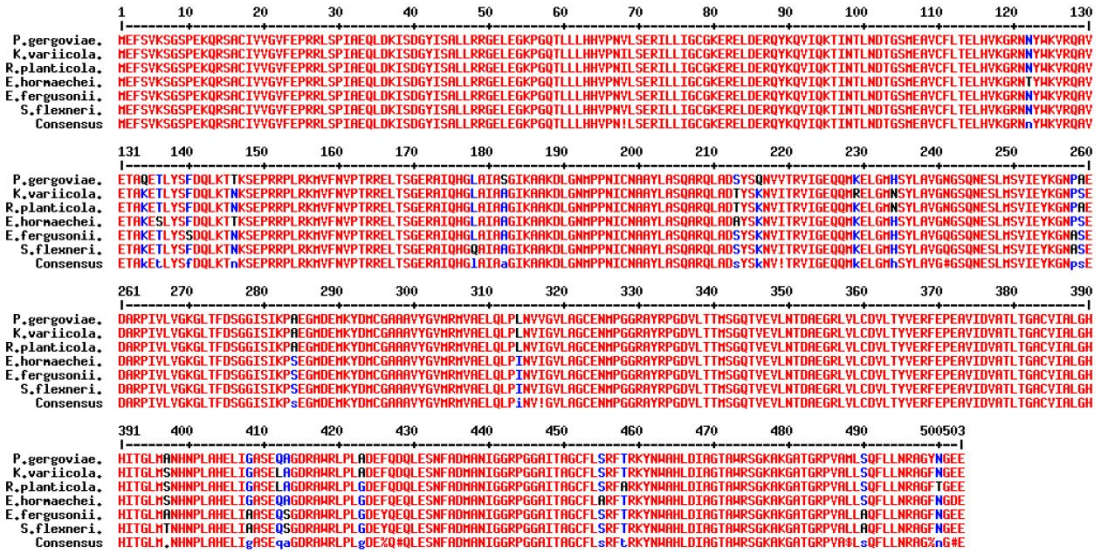


Fig. S11. The amino acids sequence comparison about individual LAP expressed by six bacterial species.

Table S2. Medical herbs for the inhibitory evaluation against LAP.

No.	Name	No.	Name
1	*Rhei radix et rhizoma*	**28**	*Uncariae ramulus cum uncis*
2	*Polygonati rhizoma*	**29**	*Sparganii rhizoma*
3	*Corydalis rhizoma*	**30**	*Leonurifructus*
4	*Stemonae radix*	**31**	*Artemisiae scopariae herba*
5	*Polygoni cuspidati rhizoma et radix*	**32**	*Rubiae radix et rhizoma*
6	*Euodiae fructus*	**33**	*Angelicae dahuricae radix*
7	*Lycopi herba*	**34**	*Vaccariae semen*
8	*Olibanum*	**35**	*Erigerontis herba*
9	*Imperatae rhizoma*	**36**	*Cistanches herba*
10	*Asari radix et rhizoma*	**37**	*Houttuynlae herba*
11	*Aucklandlae radix*	**38**	*Radix bupleuri*
12	*Polygoni multiflori radix*	**39**	*Eucommiae cortex*
13	*Solanum nigrum L.*	**40**	*Zingiberis rhizoma*
14	*Notopterygii rhizoma et radix*	**41**	*Ephedrae herba*
15	*Dryopteridis crassirhizomatis rhizoma*	**42**	*Cannabis fructus*
16	*Aconiti lateralis radix praeparaia*	**43**	*Stachyuri medulla*
17	*Agrimonlae herba*	**44**	*Galla chinensis*
18	*Forsythiae fructus*	**45**	*Hedyotis diffusa*
19	*Ganoderma*	**46**	*Aconiti radix*
20	*Angelicae sinensis radix*	**47**	*Curcumae rhizoma*
21	*Angelicae pubescentis radix*	**48**	*Dipsaci radix*
22	*Prunellae spica*	**49**	*Curcumae longae rhizoma*
23	*Sophorae flavescentis radix*	**50**	*Astragali radix*
24	*Arecae pericarpium*	**51**	*Anemonealtaica fisch.ex C.A.Mey*
25	*Euphorbia fischeriana steud.*	**52**	*Scrophulariae radix*
26	*Houttuynia cordata thunb.*	**53**	*Gastrodlae rhizoma*
27	*Sinapis semen*	**54**	*Rosmarinus officinalis*

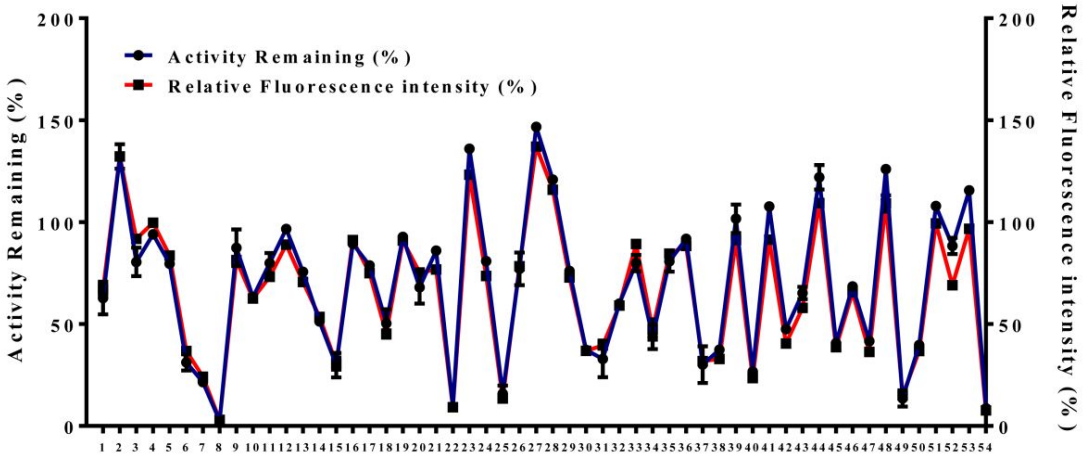


Fig. S12. The inhibitory effects of herb medicines 1-54 against LAP.

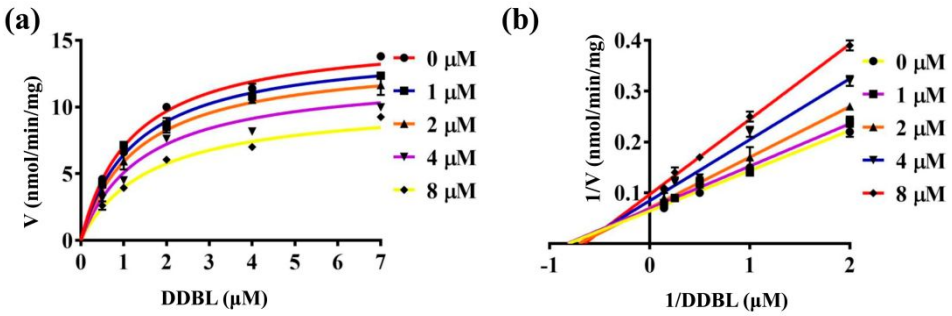


Fig. S13. (a) Inhibitory kinetics about **AKBA** (0-8 μM) against LAP. (b) Lineweaver−Burk plot.

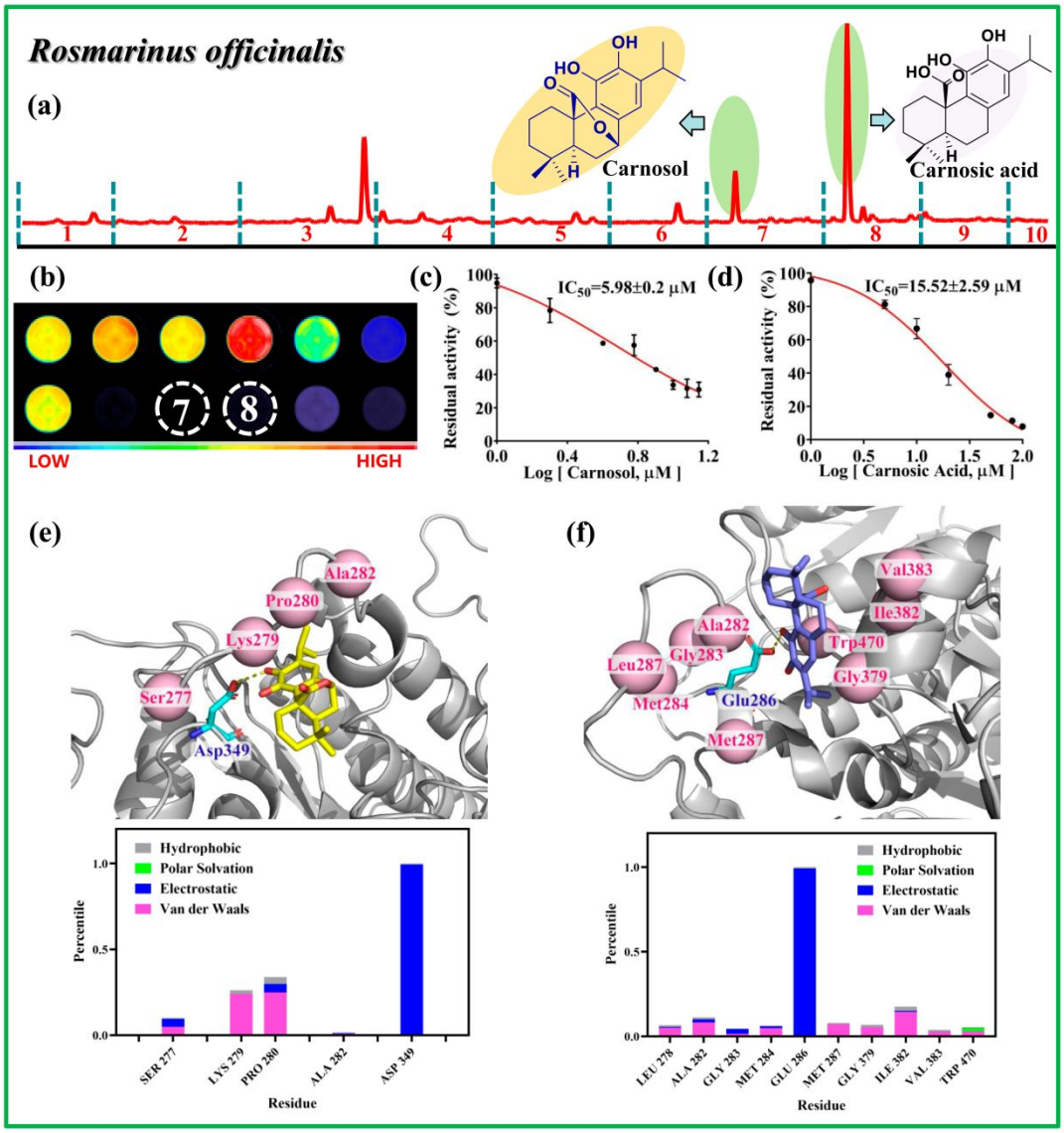


Fig. S14. Preparation of LAP inhibitors from *R. officinalis*. (a) HPLC chromatogram of *R. officinalis* and preparative fractions. (b) Inhibitory effects of fractions on LAP. (c) Inhibitory activity of carnosol. (d) Inhibitory activity of carnosic acid. (e) Simulated interactions between carnosol and LAP. (f) Simulated interactions between carnosic acid and LAP.

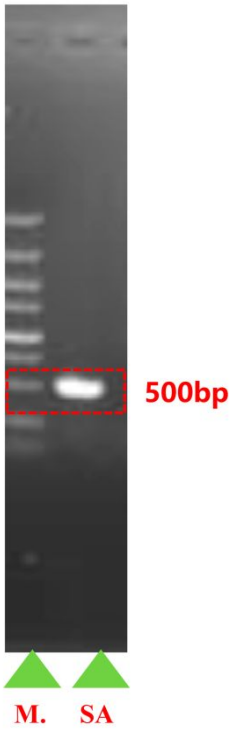


Fig. S15. RT-PCR for LAP in *S. aureus*.

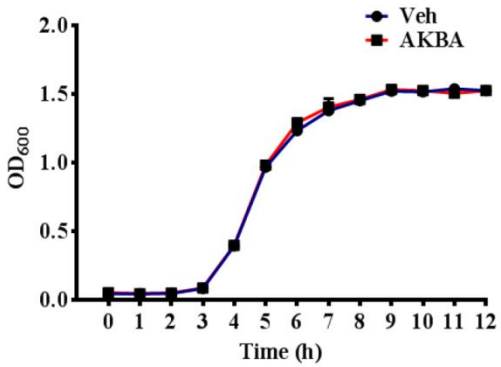


Fig. S16. Interference evaluation about **AKBA** on the proliferation of *S. aureus*.

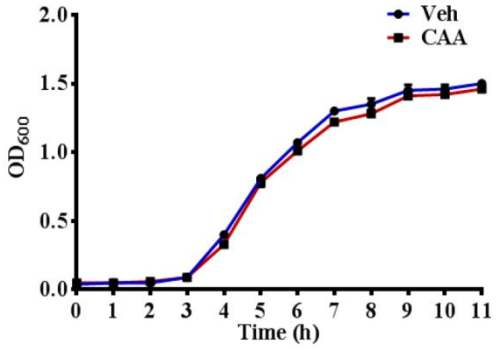


Fig. S17. Interference evaluation about carnosic acid (**CAA**) on the proliferation of *S. aureus*.

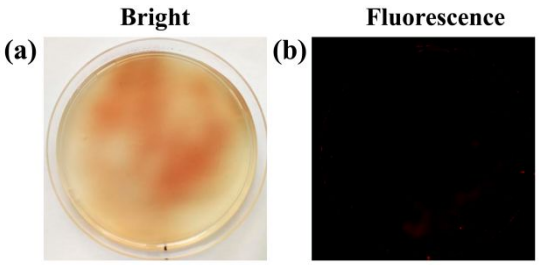


Fig. S18. Images of blood cultured agar plate stained by **DDBL** from mice as the vehicle group. (a) Bright image, (b) Fluorescence image.

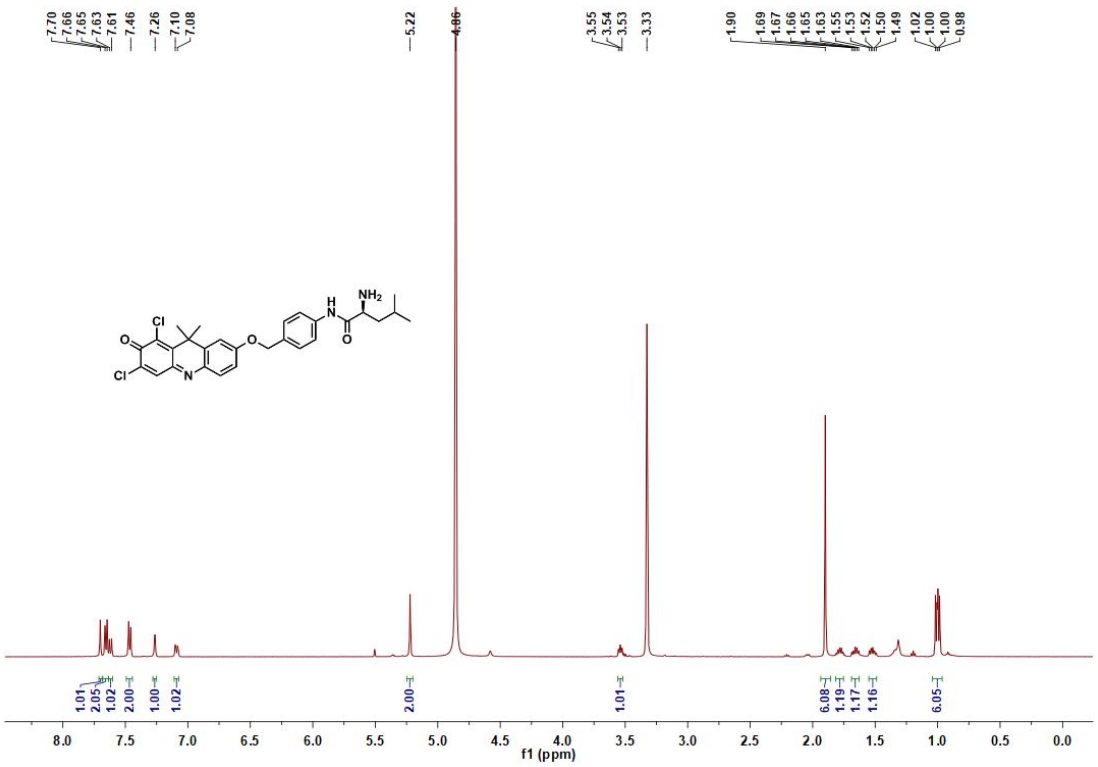


Fig. S19. ^{1}H NMR spectrum of **DDBL** in MeOD.

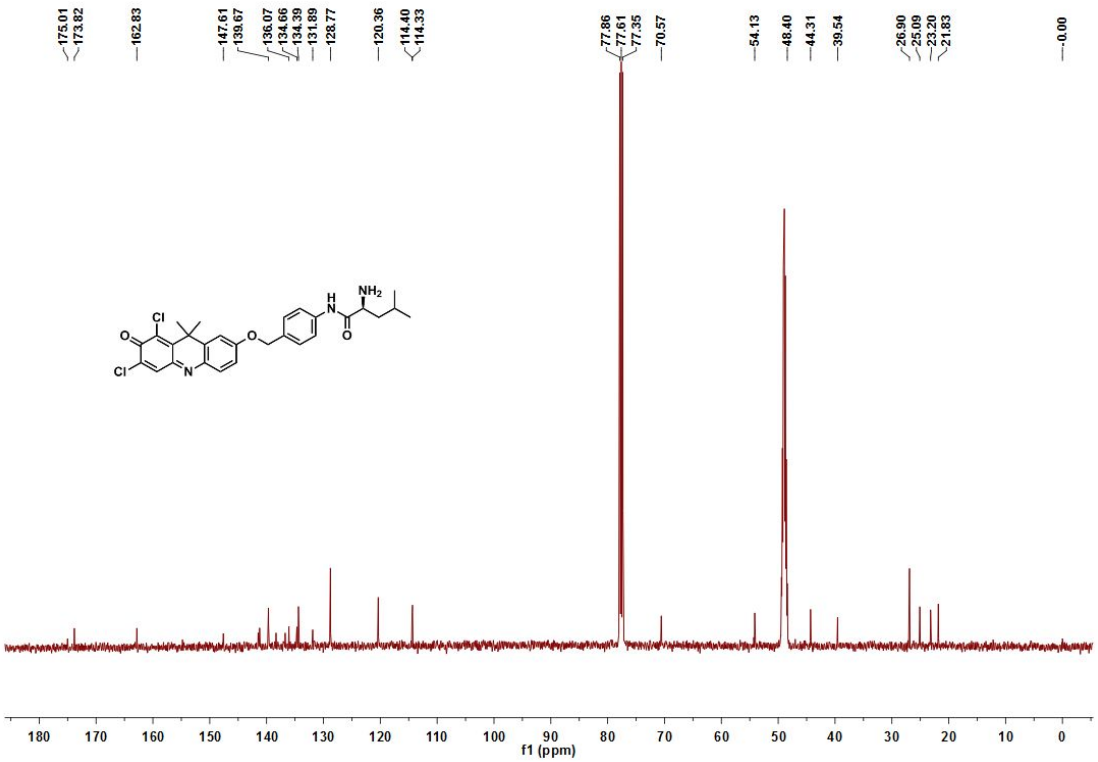


Fig. S20. ^{13}C NMR spectrum of **DDBL** in $CDCl_3$+MeOD.

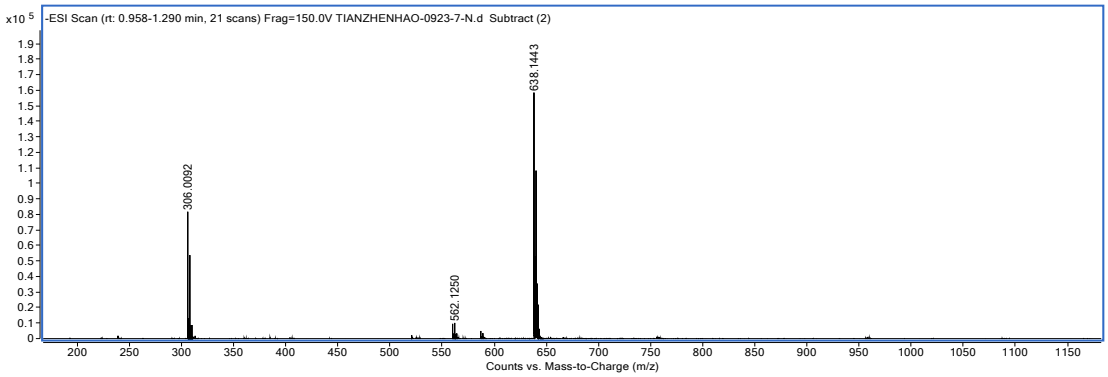


Fig. S21. HRMS spectrum of **DDBL**.

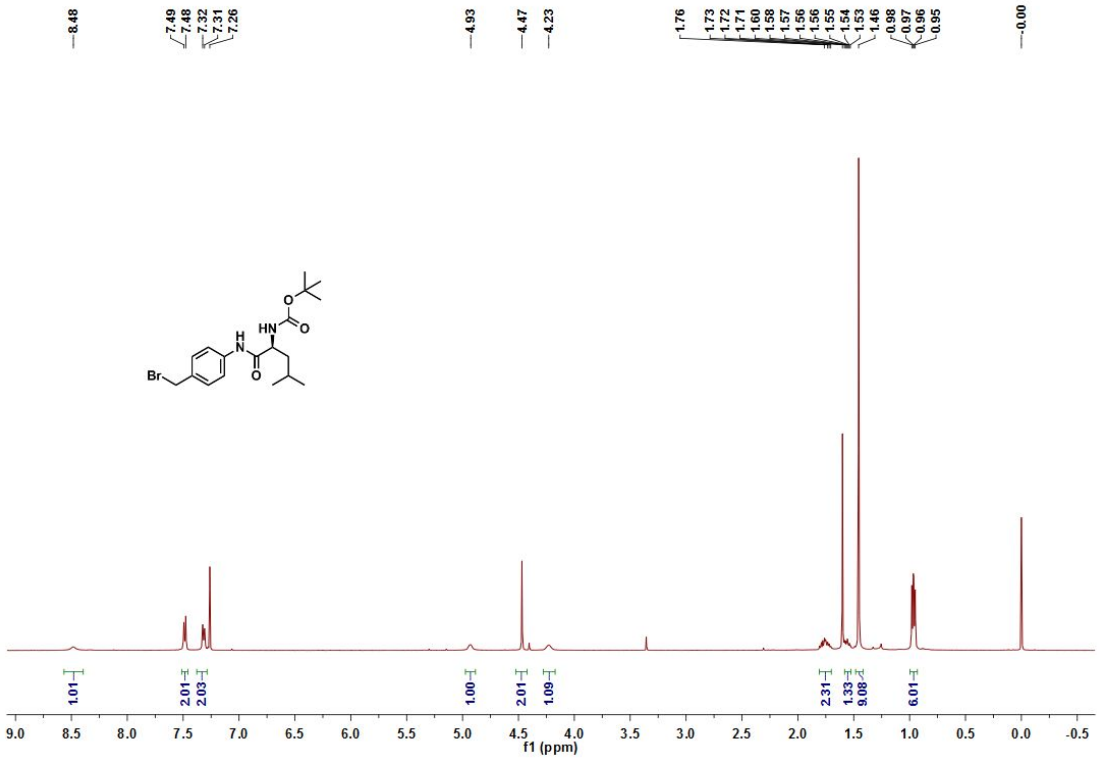


Fig. S22. ^{1}H NMR spectrum of compound **1** in $CDCl_3$.

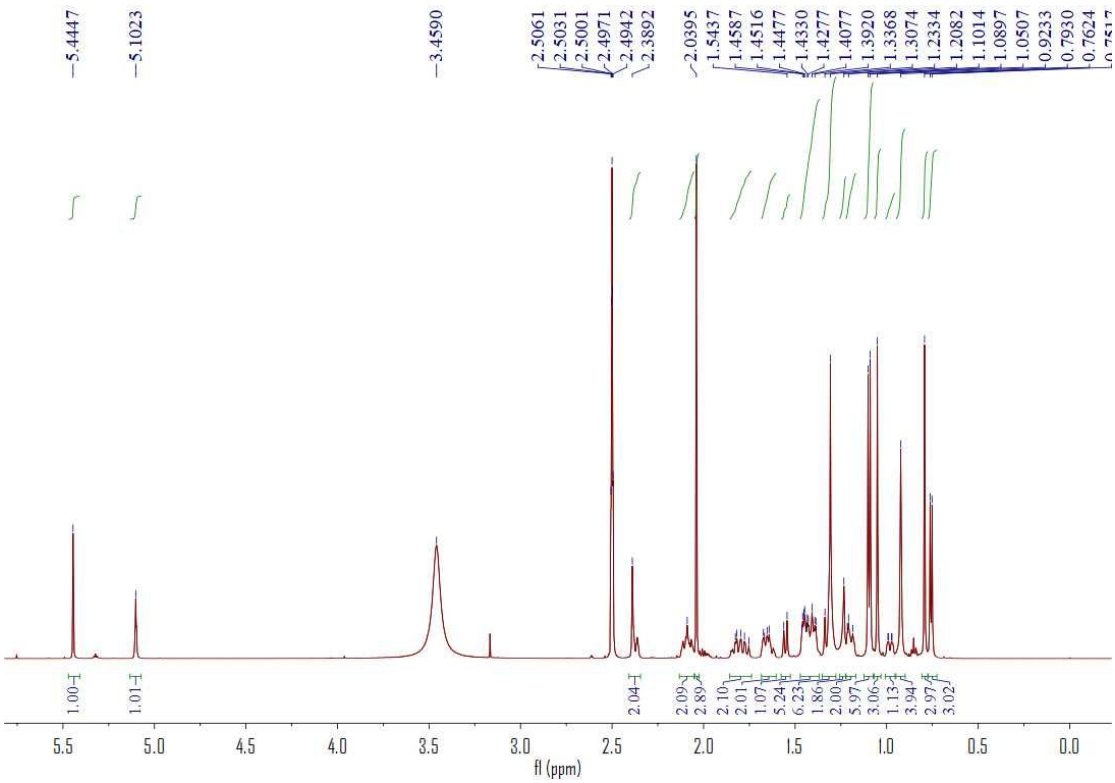


Fig. S23.^{1}H NMR spectrum of **AKBA** in DMSO-d_6.

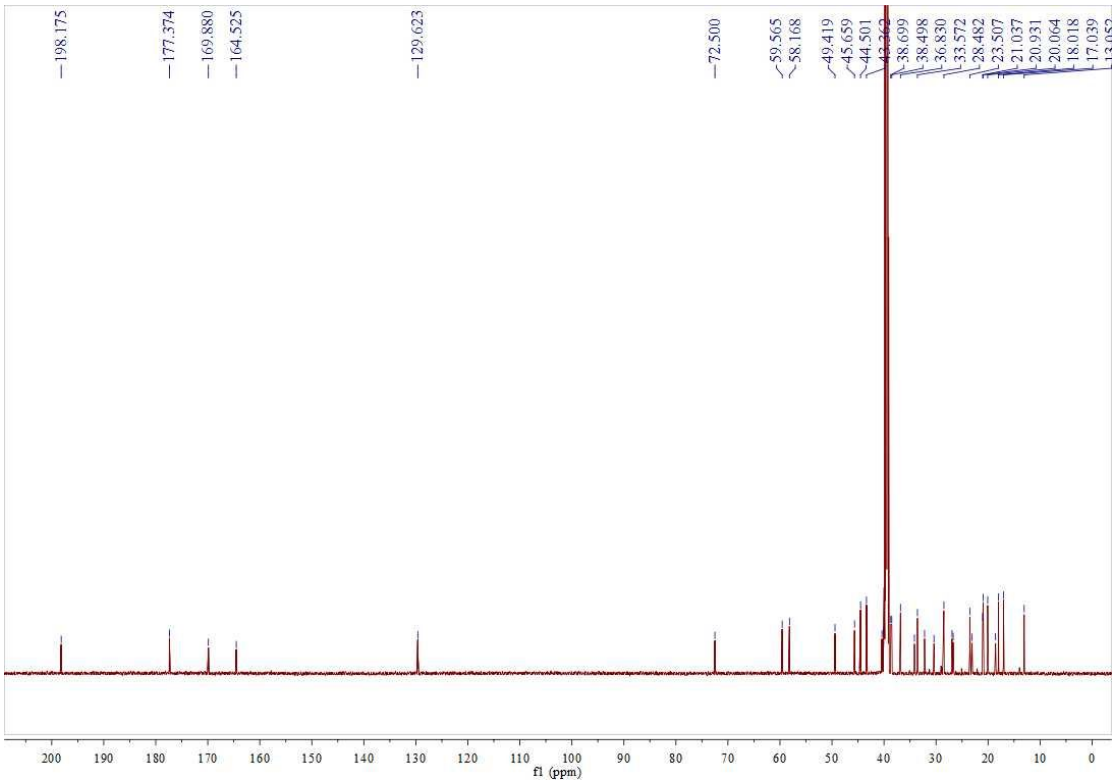


Fig. S24.[13]C NMR spectrum of **AKBA** in DMSO-d_6.

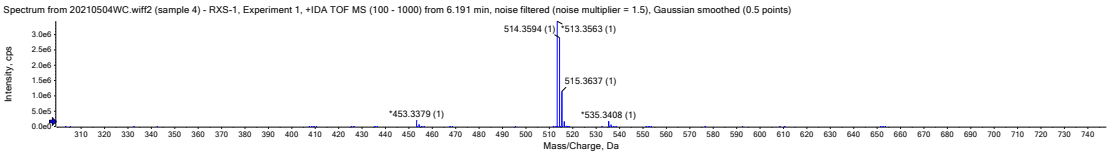


Fig. S25. HRMS of **AKBA**.

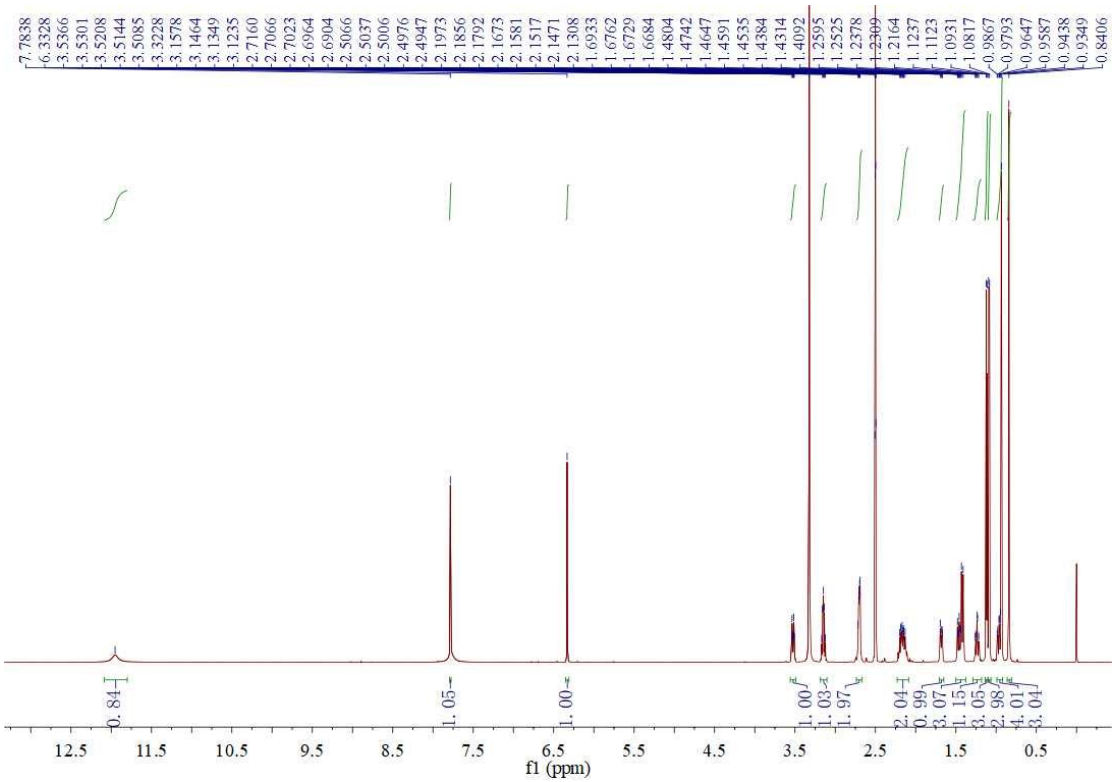


Fig. S26. ^{1}H NMR spectrum of carnosic acid in DMSO-d_6.

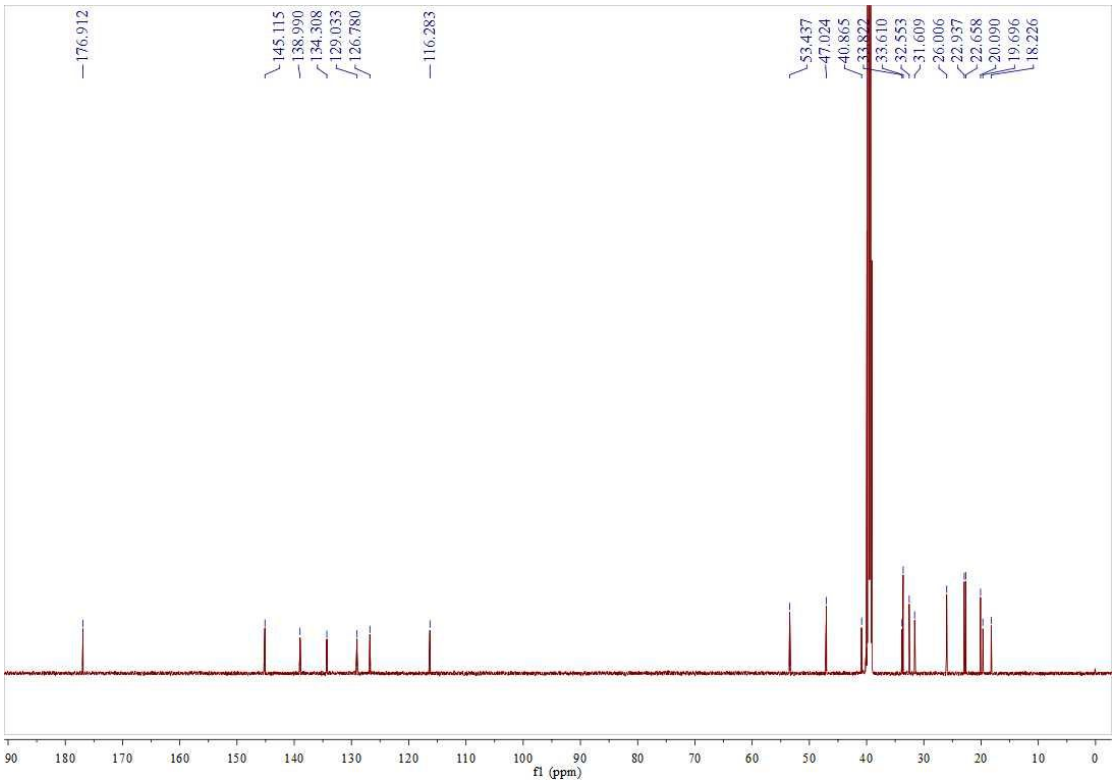


Fig. S27. ^{13}C NMR spectrum of carnosic acid in DMSO-d_6.

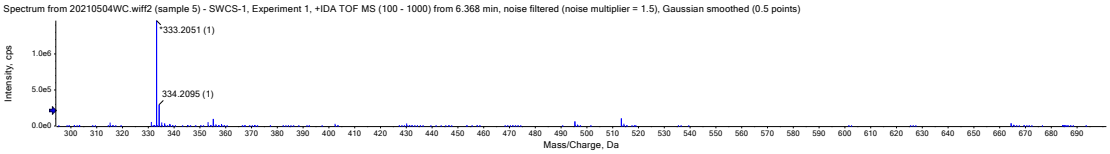


Fig. S28. HRMS of carnosic acid.

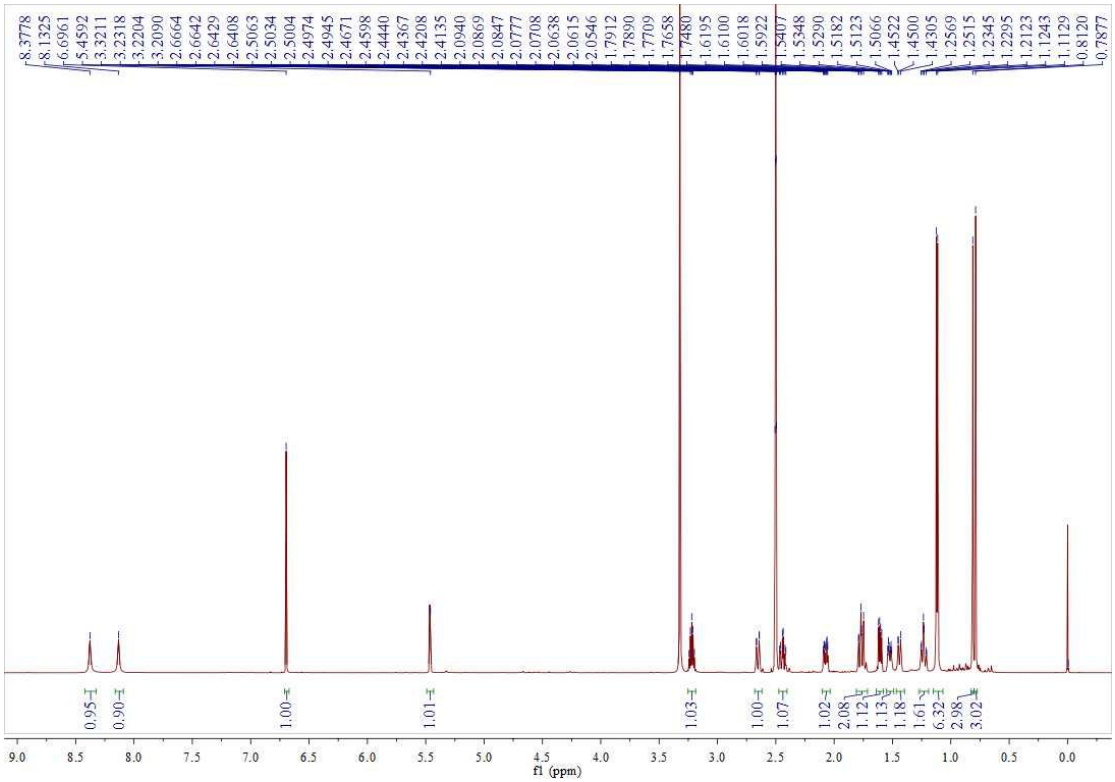


Fig. S29. ^{1}H NMR spectrum of carnosol in DMSO-d_6.

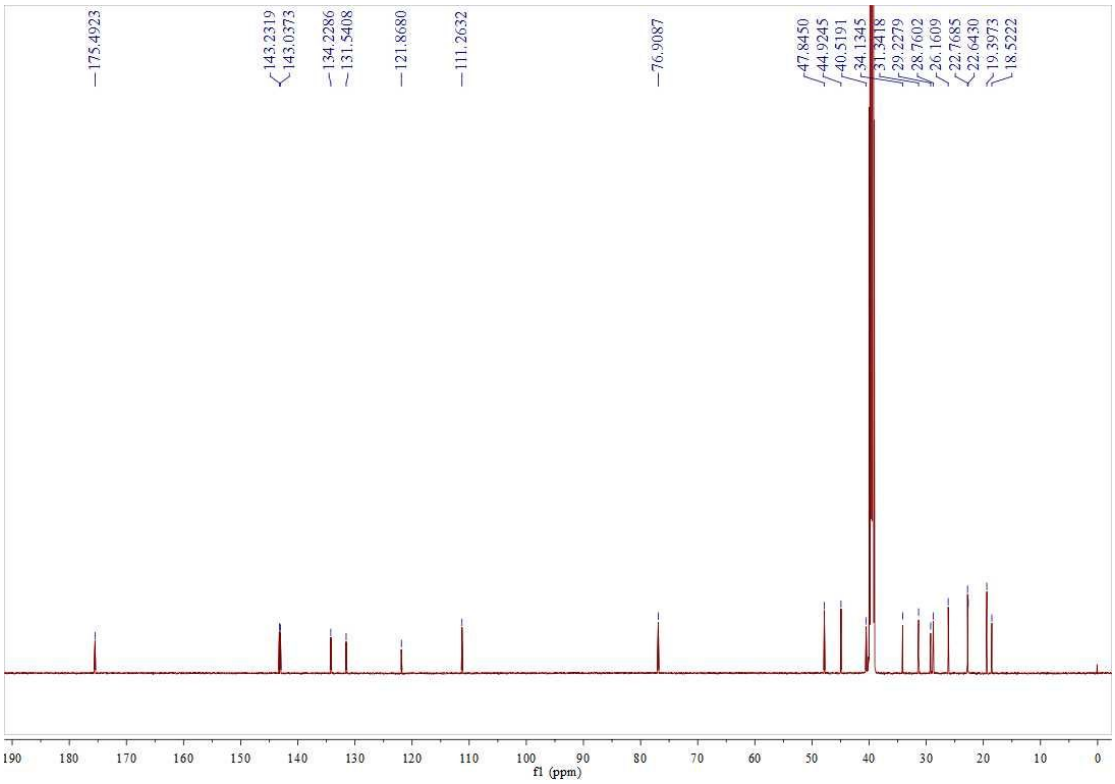


Fig. S30. ^{13}C NMR spectrum of carnosol in DMSO-d_6.

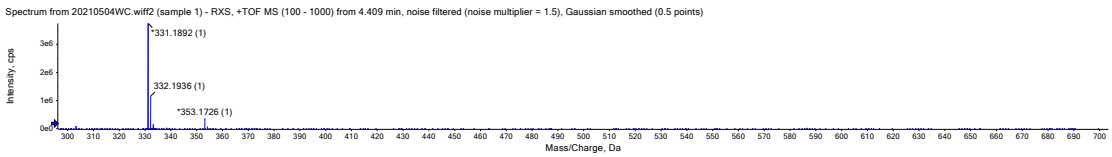


Fig. S31. HRMS of carnosol.

Reference

[1] Huang, Y.; Qi, Y.; Zhan, C. Y.; Zeng, F.; Wu, S. Z. Diagnosing drug-induced liver injury by multispectral optoacoustic tomography and fluorescence imaging using a leucine-aminopeptidase-activated probe. *Anal. Chem*. **2019**, *91*, 8085–8092.

[2] Carroll, R. K.; Robison, T. M.; Rivera, F. E.; Davenport, J. E.; Jonsson, I. M.; Florczyk, D.; Tarkowski, A.; Potempa, J.; Koziel, J.; Shaw, L. N. Identification of an intracellular M17 family leucine aminopeptidase that is required for virulence in Staphylococcus aureus. *Microbes Infect.* **2012**, *14*, 989–999.