Supporting Information

Amphiphilic Lysine-based Glycopeptides Exert Antibacterial Effect on Pseudomonas aeruginosa

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S1. Experimental section

1.1 Methods

1.1.1 Studies on the self-assembly behavior of SA-L-Gal, OA-L-Gal, and LOA-L-Gal

To study the self-assembly behaviour of the compounds, we first prepared a 0.5 mM solution of Nile Red acetone. This was done by transferring 50 μL of Nile Red solution into a 5 mL volumetric flask and then using a hair dryer to

accelerate the volatilisation of acetone. Next, we prepared a 1 mM masterbatch solution and took different volumes of the masterbatch solution and added water to volume to 5 mL according to different experimental needs to form a solution containing Nile Red and the compound to be tested. These solutions were sonicated for 4 h to ensure that the compounds were homogeneously mixed, and subsequently left to stand for 12 h to form the self-assembly solution. To assess the effect of self-assembly, we measured the fluorescence intensity of the solutions at an excitation wavelength of 560 nm using a fluorescence photometer and plotted the fluorescence intensity versus the concentration of the compounds, thus determining the self-assembly concentration of the compounds. In addition, we prepared 100 μM aqueous solutions of SA-L-Gal, OA-L-Gal, and LOA-L-Gal, and 2 mL of each was taken into a washed transparent syringe bottle. These solutions were subjected to 4 h of sonication and 12 h of standing to promote self-assembly formation. Finally, we used laser irradiation of these solutions to further verify the effect of self-assembly.

1.1.2 Strain freezing and resuscitation

For strain preservation, select bacteria in the logarithmic growth phase, mix with 20% glycerol and 80% MHB broth, and store at -80°C. Thaw the frozen strains at room temperature, mix evenly, and inoculate onto TSA solid medium, then culture at 37°C for 12 hours. Pick single colonies from the TSA medium and inoculate into broth, culture at 37°C with a shaking speed of 150 rpm for 4-6 hours to reach the logarithmic growth phase.

1.1.3 Antimicrobial activity studies

We prepared a 100 μM masterbatch of the compound in an autoclaved container with PBS buffer to a total volume of 3 mL. The masterbatch was then diluted with PBS to twice the target concentration, with approximately 300 μL taken at each dilution step. next, we processed the bacterial broth to the logarithmic growth phase. After centrifugation to remove the supernatant, it was washed with an equal volume of PBS and centrifuged again. Afterwards, the resulting bacterial solution was diluted until the McFarland standard read 0.5, and 30 μL was taken and added to 4.5 mL of PBS and mixed well with an oscillator to obtain a bacterial concentration of about 10^6 CFU/mL and set aside. The diluted compound was mixed well with an equal volume of bacterial solution, and then incubated for 5 hours in a 37°C incubator. At the end of the incubation, 40 μL of the mixture was taken and spread evenly onto the TSA solid medium using a sterile applicator stick or applicator glass bead. The remaining mixture (100 μL per well) was added to a 96-well plate with MHB liquid medium and continued to incubate for 12 h in a 37°C incubator. After the incubation was completed, we photographed and recorded the TSA solid medium and took optical density readings of the samples in 96-well plates in an spectrophotometer at 600 nm to assess the antimicrobial effect of the compounds.

Bacterial survival rate of 96-well plate readings was calculated by the formula:

$$Survival\,rate\,(\%) = (ODe - ODo)/(ODp - ODo) \times 100\%$$

ODe: the OD value of the compound sample set;

ODo: the OD value of the blank group;

ODp: OD value of the culture solution.

1.1.4 Bactericidal Mechanism Research

To study the bactericidal mechanism, we observe the morphology of bacteria before and after treatment with a compound using SEM. Mix 1 mL of bacterial suspension (McFarland standard 3.0) with an equal volume of 1 mM compound solution and incubate at 37°C for 5 hours. After incubation, centrifuge and discard the supernatant, wash the bacteria with pure water, then fix with 2.5% glutaraldehyde and refrigerate at 4°C overnight. After fixation, centrifuge and discard the supernatant, wash with pure water, and dehydrate through a gradient of ethanol concentrations from 10% to 100%, finally dehydrate with anhydrous ethanol for 10 minutes. After dehydration, drop the bacterial suspension onto a silicon wafer and allow to dry at room temperature overnight. The next day, after sputter-coating the samples with gold, observe the bacterial morphology using SEM.

1.1.5 Crystal violet staining of biological periplasm

Biofilm disruption

The bacteria in the logarithmic growth phase were diluted to a turbidity meter reading of 0.5, 200 μL was added to 2800 μL of MHB medium, and shaken well to obtain about 10^7 CFU/mL of bacterial solution. After adding the bacterial solution to a 96-well plate at 100 μL per well and incubating at 37°C overnight

to form a homogeneous biofilm, the medium was removed and washed three times with purified water. Compounds were diluted to predetermined concentrations with PBS and added to wells with 6 parallel wells per concentration, including PBS control, for a total of 12 h incubation. After incubation, the compounds were washed off with purified water, the biofilm was fixed in methanol for 30 min, and dried. Stain with 0.4% crystal violet for 30 min, wash with water, and 33% acetic acid was added to dissolve the stain. The absorbance was measured with a microplate reader at a wavelength of 595 nm to calculate the rate of biofilm destruction.

The formula is as follows:

$$Biofilm\ inhibition\ (\%) = 1 - (ODe - ODo)/(ODp - ODo) \times 100\%$$

ODe: the OD value of the compound sample set;

ODo: the OD value of the blank group;

ODp: OD value of acetic acid.

1.1.6 Biofilm inhibition

Dilute the logarithmic phase bacteria with MHB broth to a turbidity meter reading of 0.5. Mix 200 μL of the bacterial solution with 2800 μL of MHB to obtain a bacterial concentration of approximately 10^7 CFU/mL. Add the bacterial solution to a 96-well plate, 100 μL per well, and incubate at 37°C for 24 hours. After cultivation, proceed with washing, fixing, staining, and reading, following the same steps as before.

The calculation formula is as follows:

$Antibiofilm\ activity\ (\%) = 1 - (ODe - ODo)/(ODp - ODo) \times 100\%$

ODe: the OD value of the compound sample set;

ODo: the OD value of the blank group;

ODp: OD value of acetic acid.

1.1.7 Confocal Laser Scanning Microscopy (CLSM) Imaging Biofilm Disruption

In a 24-well plate, add 400 μL of 10^7 CFU/mL bacterial dilution to each well, place a cut square cover slip in each well, and culture at 37°C for 24 hours to form a biofilm. After maturation, remove the culture medium and wash three times with pure water. Add 400 μL of compound solution and co-cultivate with bacteria at 37°C for 24 hours. Afterward, aspirate the compound, wash three times with pure water. Stain with LIVE/DEAD dye for 15 minutes, place the cover slip onto a slide, and observe and image the samples using a confocal laser scanning microscope.

1.1.8 Cytotoxicity assay

Prepare the MTT solution by dissolving 250 mg of MTT powder in 50 mL of PBS under light-protected conditions, filter sterilize, aliquot, and store at -20°C. Wash L929 cells with PBS, digest with trypsin, and then add serum-containing complete medium. Collect the cells, count them, and seed onto a 96-well plate, culture at 37°C with 5% CO_2 until attachment. Add different concentrations of the drug to the 96-well plate and culture for 24 hours at 37°C with 5% CO_2. Aspirate the liquid from the wells, add MTT solution, and incubate for 4 hours

at 37°C with 5% CO_2. After discarding the liquid from the wells, add DMSO to each well and mix well. Measure the OD value at 490 nm using a plate reader and calculate the cell survival rate.

$$Cell\ viability\ (\%) = (ODe - ODo)/(ODp - ODo) \times 100\%$$

ODe: the OD value of the compound sample set;

ODo: the OD value of the blank;

ODp: OD value of the culture solution.

1.1.9 Hemolytic assay

Dilute the compound by preparing a 1 mM stock solution and diluting it with saline to five times the target concentration, then take 100 μL for standby use. Collect mouse blood, wash with saline three times, and centrifuge at 5000 rpm for 5 minutes until the supernatant is nearly colorless, then dilute the red blood cells. Take 100 μL of the compound and add it to 400 μL of blood sample; take 100 μL of saline and add it to 400 μL of blood sample; take 100 μL of 1% TX-100 and add it to 400 μL of blood sample. Refrigerate at 4°C for 1 hour, centrifuge for 5 minutes, and take a photo. Transfer the supernatant to a 96-well plate, with at least three wells for each concentration and 100 μL per well, and measure the OD value at 540 nm.

The hemolysis rate was calculated as follows:

$$Hemlisis\ rate\ (\%) = (ODe - ODo)/(ODp - ODo) \times 100\%.$$

ODe: the OD value of the compound sample set;

ODo: the OD value of saline group;

ODp: OD value of TX-100 (1%).

1.1.10 In vivo antimicrobial assay

The experiment utilized BALB/c mice, measuring their weight daily and recording the healing of wounds to evaluate the therapeutic effect of the compound on PA infection. A wound model was created by anesthetizing the mice and shaving their hair, then disinfecting and making a 1-centimeter diameter wound on the back, which was coated with PA solution. The following day, after confirming the infection, was recorded as Day 0. The mice were divided into two groups: the treatment group applied the compound solution at a concentration of 30 μM onto the wound, administering the medication daily for the first three days, then every other day for a total of ten days. The control group treated the wound with PBS, while also recording the mice's weight and wound changes. After the final administration, healthy mice, PBS-treated mice, and compound-treated mice were selected for dissection. The heart, liver, spleen, lungs, and kidneys were removed, fixed with 4% formalin, sectioned, and then stained with HE to observe inflammation. The skin from the wound area was also excised and analyzed.

S2. ^{1}H NMR, ^{13}H NMR and HRMS analyses

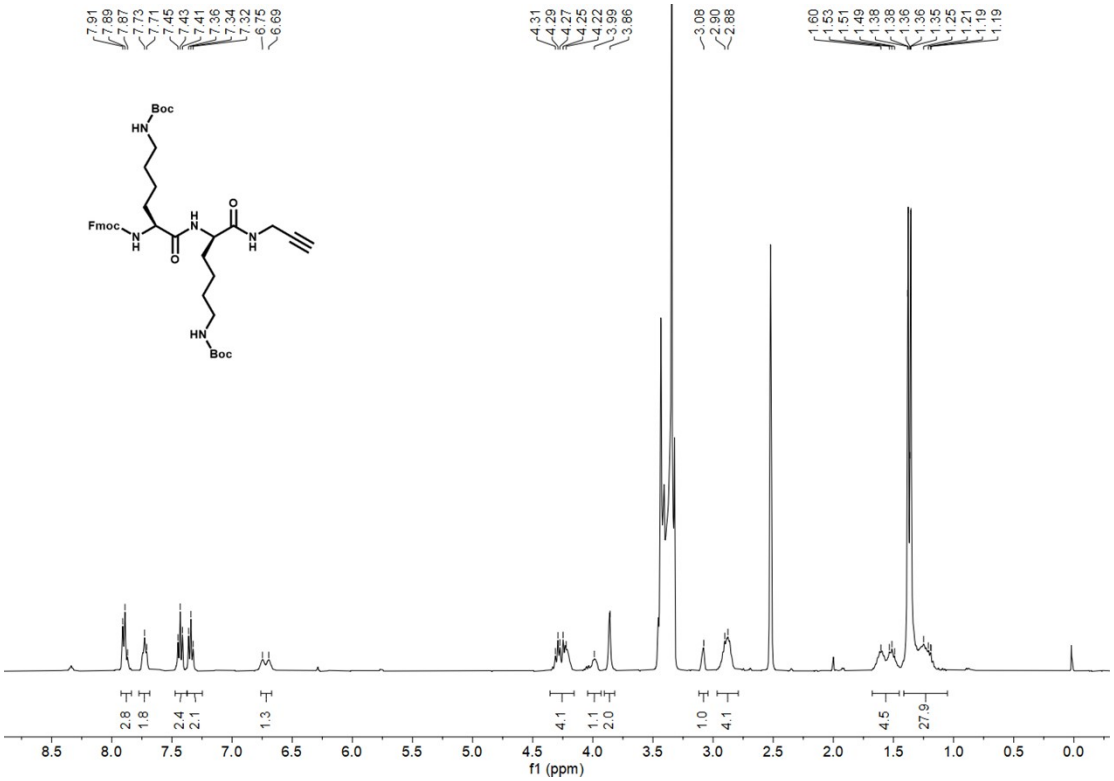


Fig. S1 ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **L-3**.

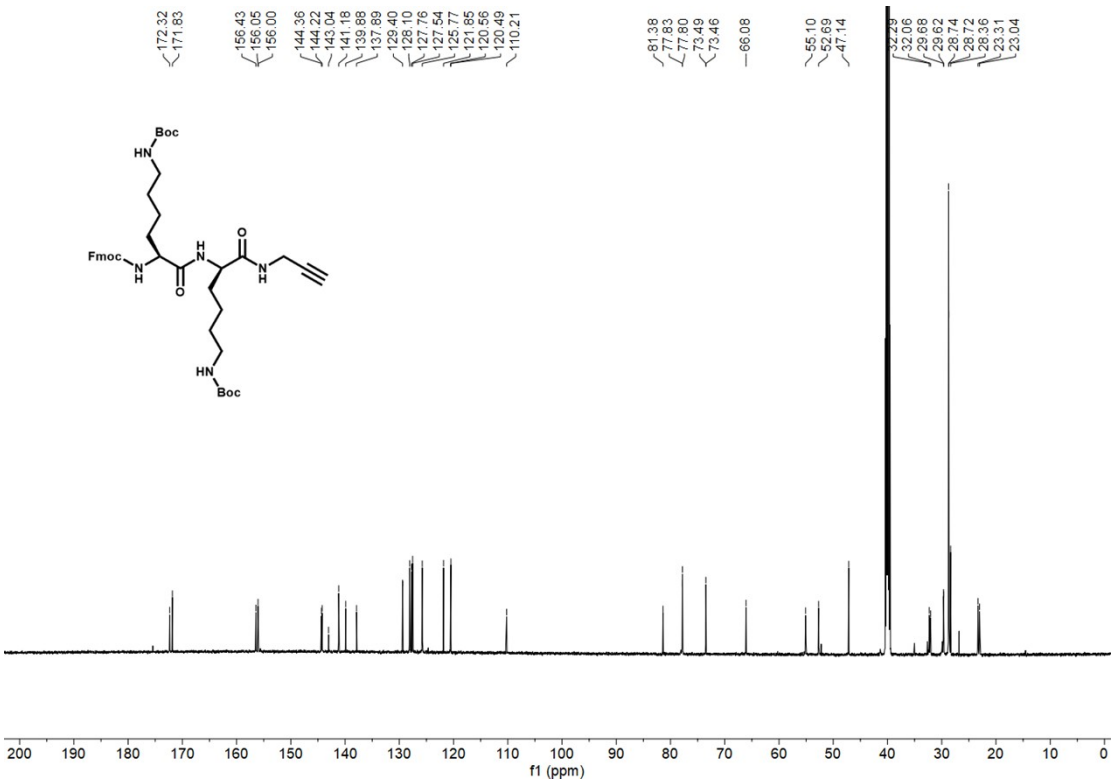
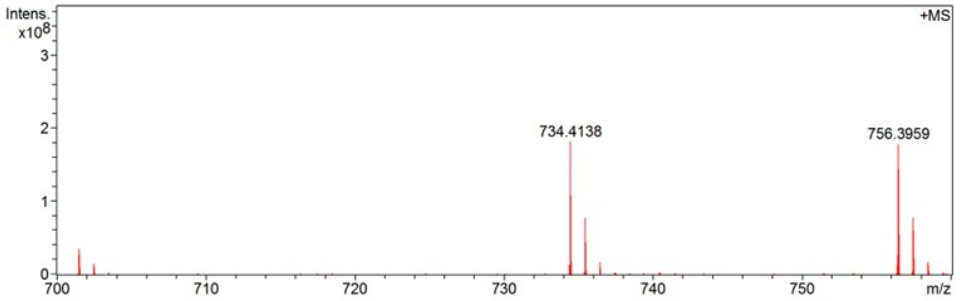


Fig. S2 ^{13}C NMR (150 MHz, DMSO-d_6) spectrum of compound **L-3**.



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¡¥ Conf	mSigma	Std I	Std Mean m/z	Std I VarNorm	Std m/z Diff	Std Comb Dev
734.4138	1	C 40 H 56 N 5 O 8	734.4123	-2.0	-2.0	15.5	ok	even	18.94	0.0279	0.0016	0.0098	0.0008	0.8427

Fig. S3 HRMS (MALDI-TOF) of compound **L-3**.

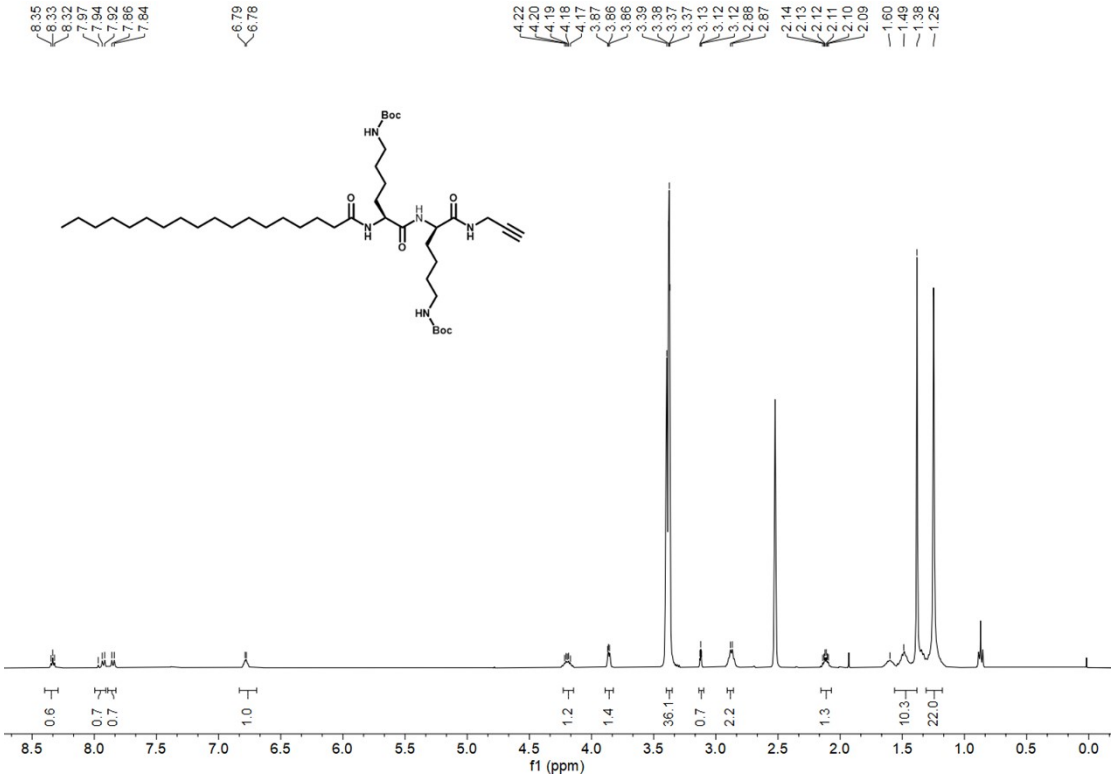


Fig. S4 ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **SA-L**.

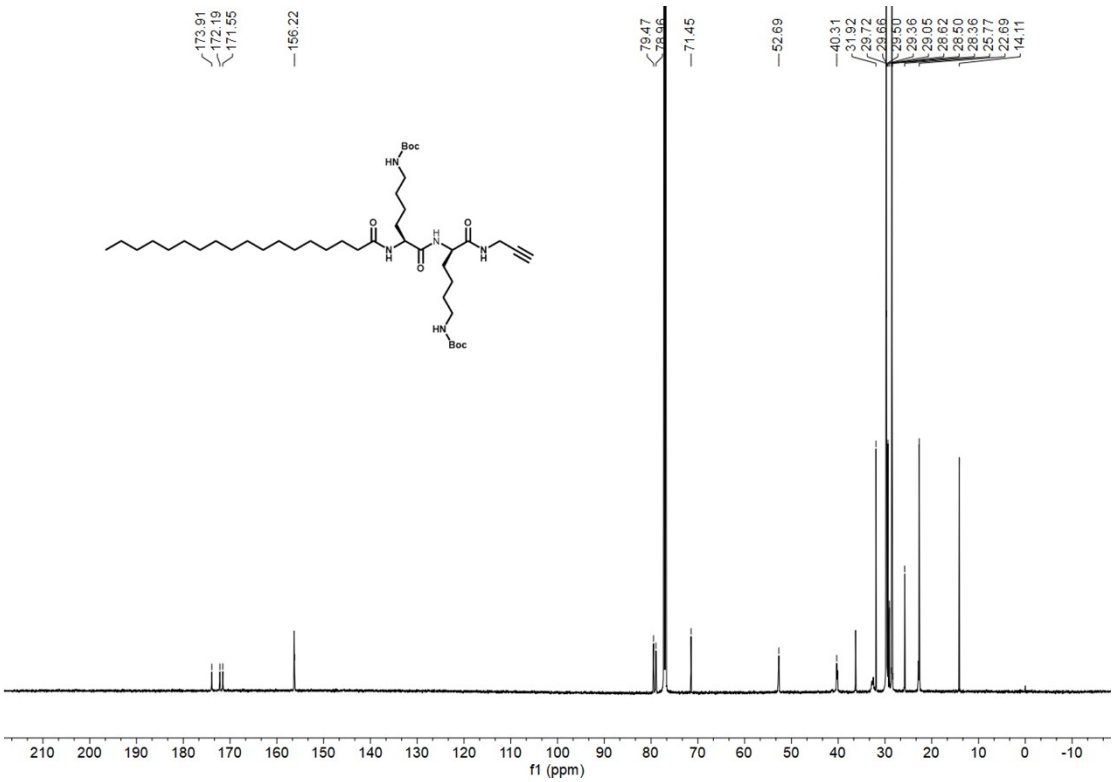
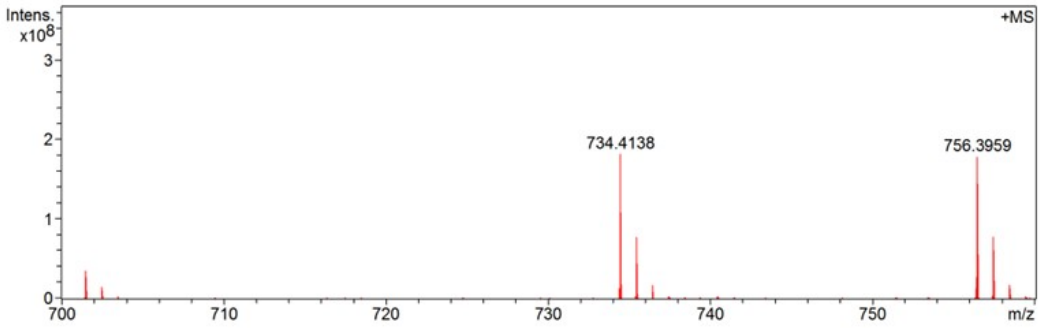


Fig. S5 ^{13}C NMR (150 MHz, $CDCl_3$) spectrum of compound **SA-L**.



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¡¥ Conf	mSigma	Std I	Std Mean m/z	Std I VarNorm	Std m/z Diff	Std Comb Dev
734.4138	1	C 40 H 56 N 5 O 8	734.4123	-2.0	-2.0	15.5	ok	even	18.94	0.0279	0.0016	0.0098	0.0008	0.8427

Fig. S6 HRMS (MALDI-TOF) of compound **SA-L**.

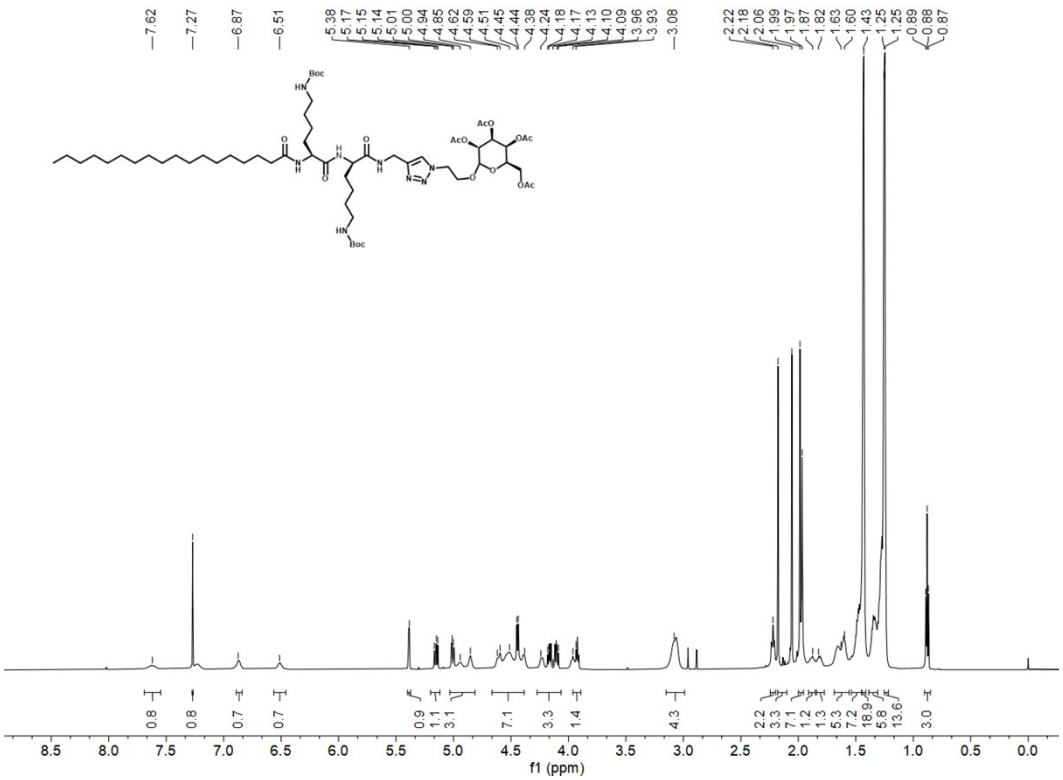


Fig. S7 ^{1}H NMR (600 MHz，$CDCl_3$) spectrum of compound **SA-L-AcGal**.

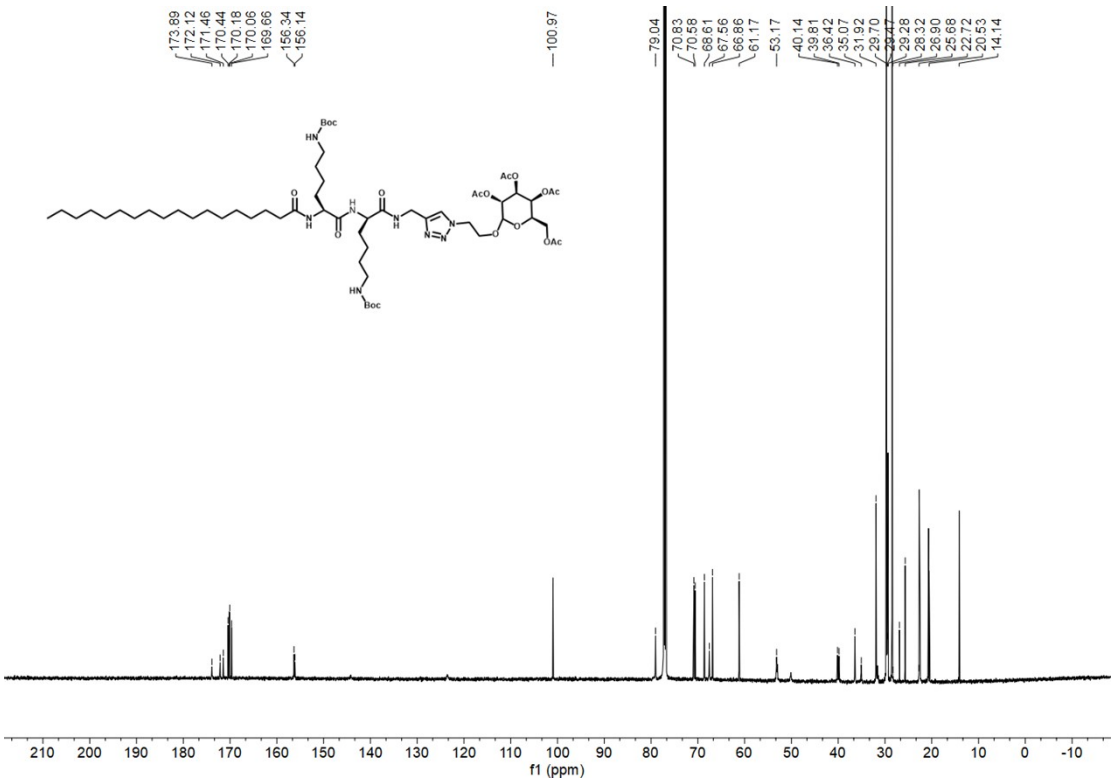


Fig. S8 ^{13}C NMR (150 MHz, $CDCl_3$) spectrum of compound **SA-L-AcGal**.

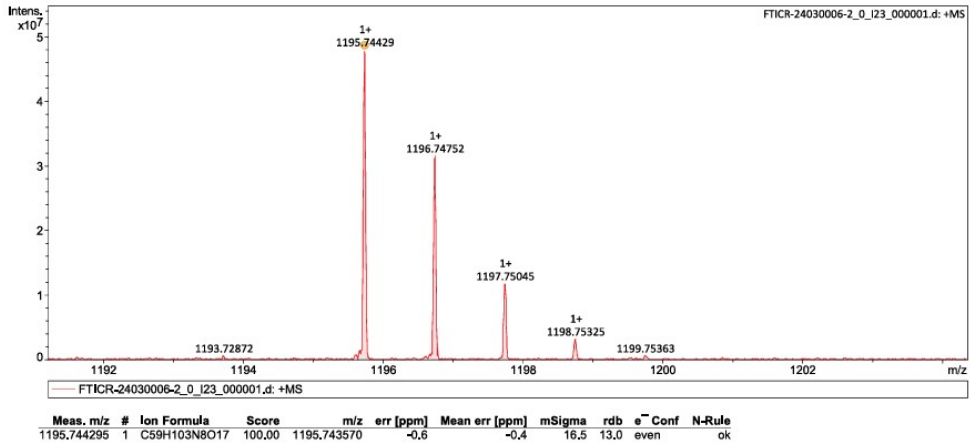


Fig. S9 HRMS (MALDI-TOF) of compound **SA-L-AcGal**.

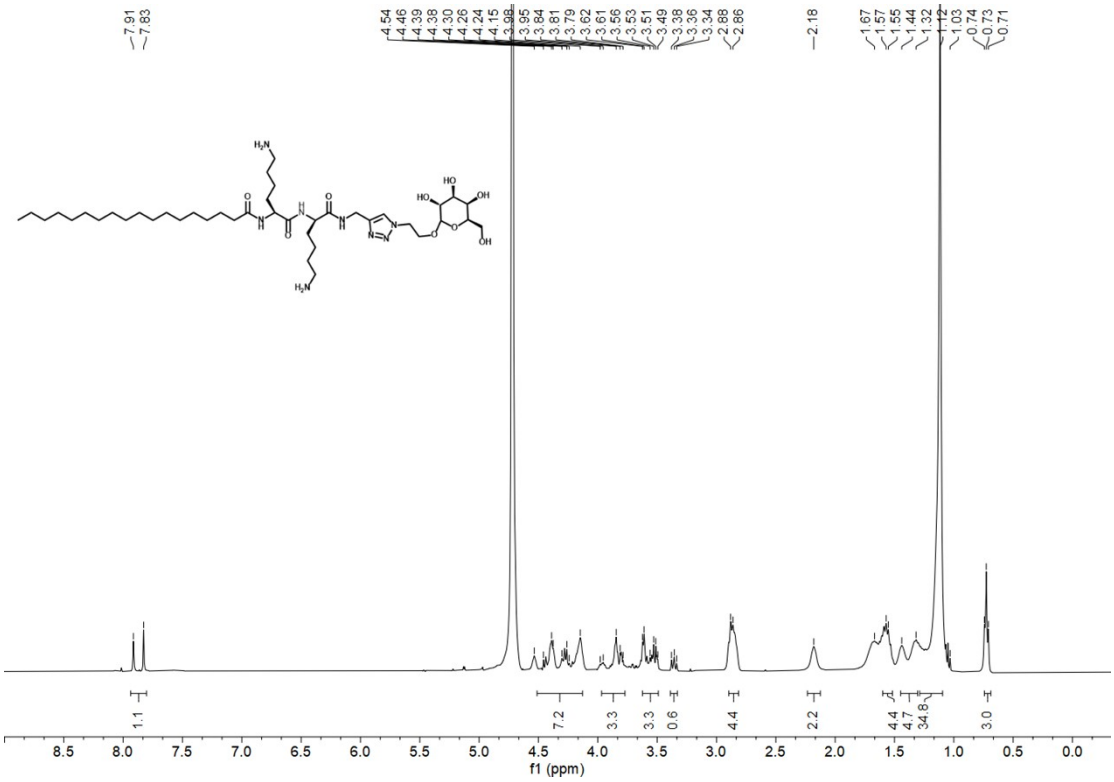


Fig. S10 ^{1}H NMR (600 MHz，D_2O) spectrum of compound **SA-L-Gal**.

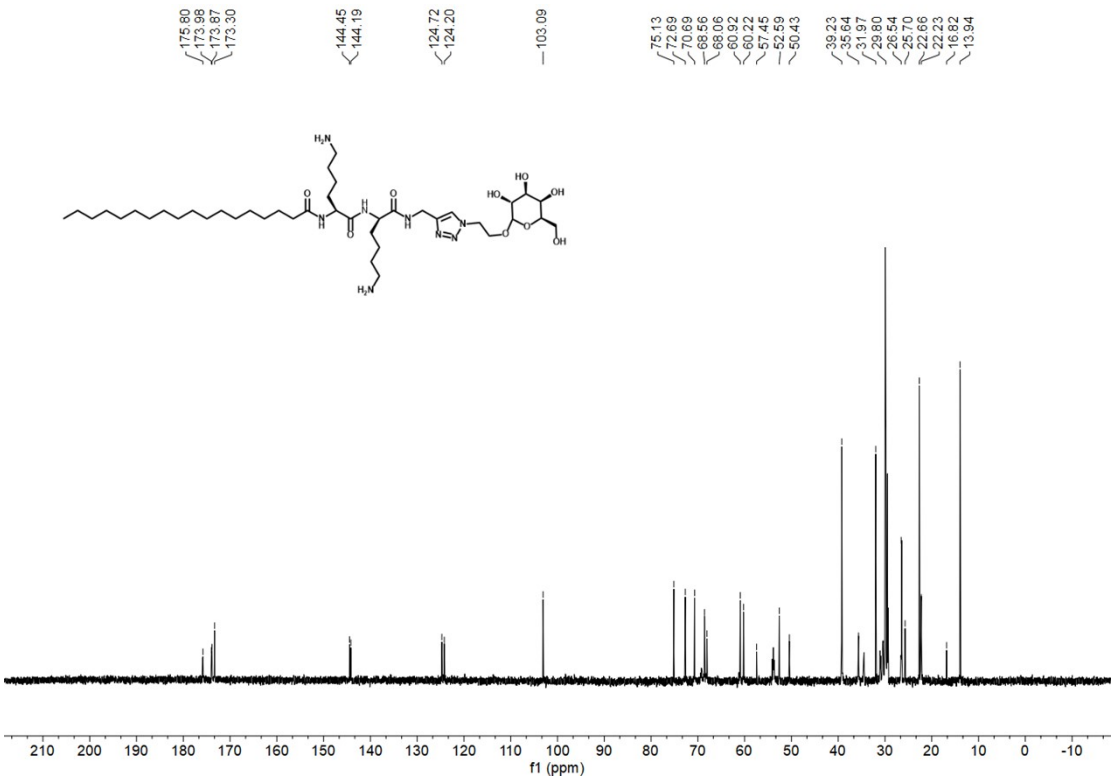


Fig. S11 ^{13}C NMR (150 MHz, D_2O) spectrum of compound **SA-L-Gal**.

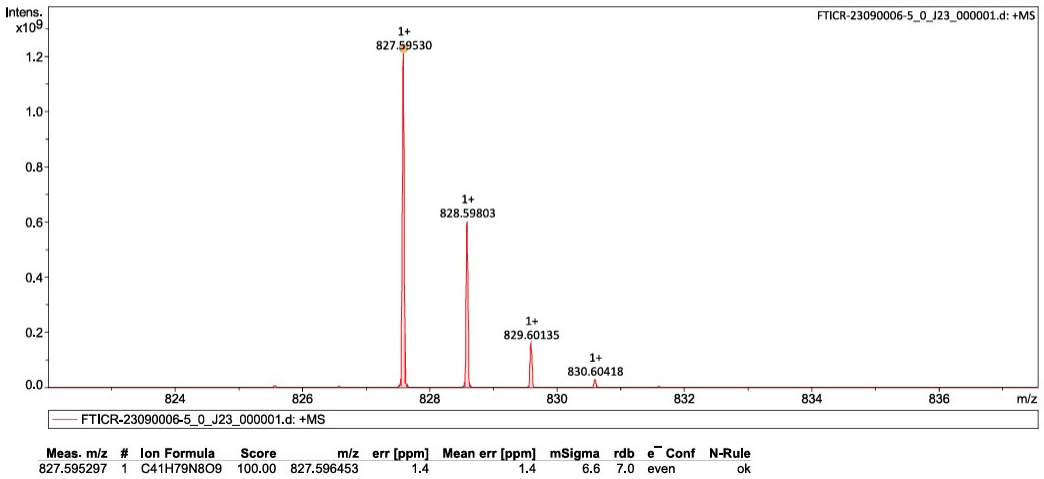


Fig. S12 HRMS (MALDI-TOF) of compound **SA-L-Gal**.

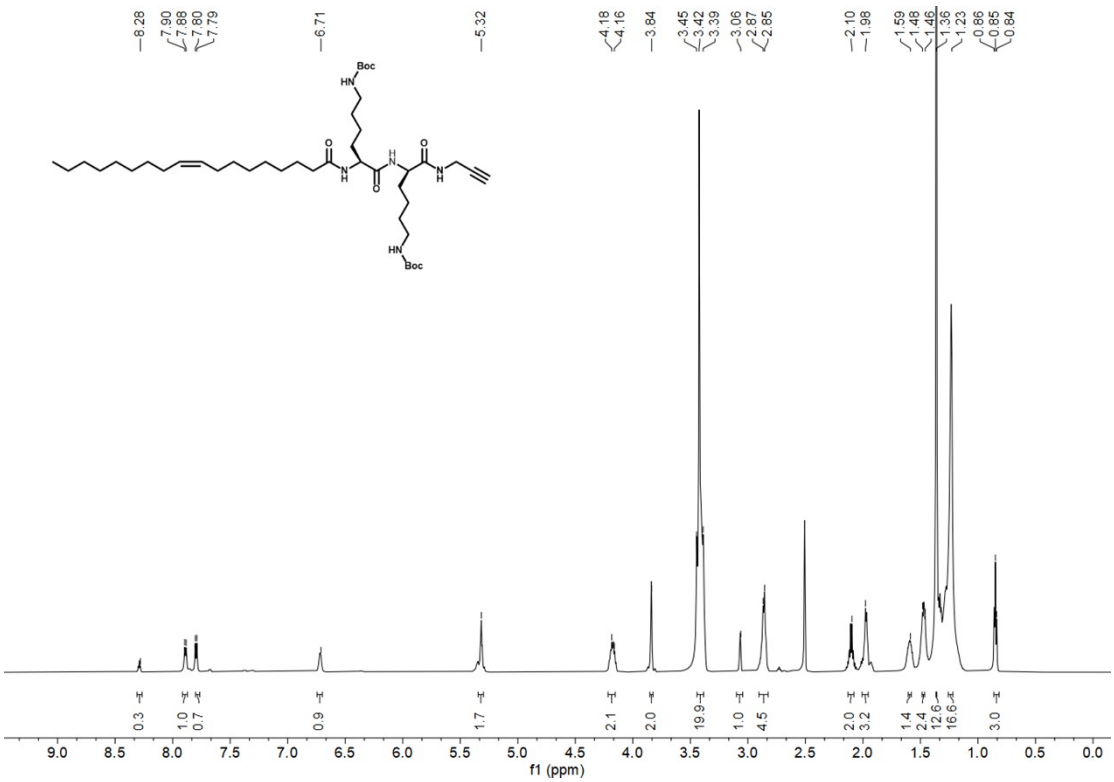


Fig. S13 ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **OA-L**.

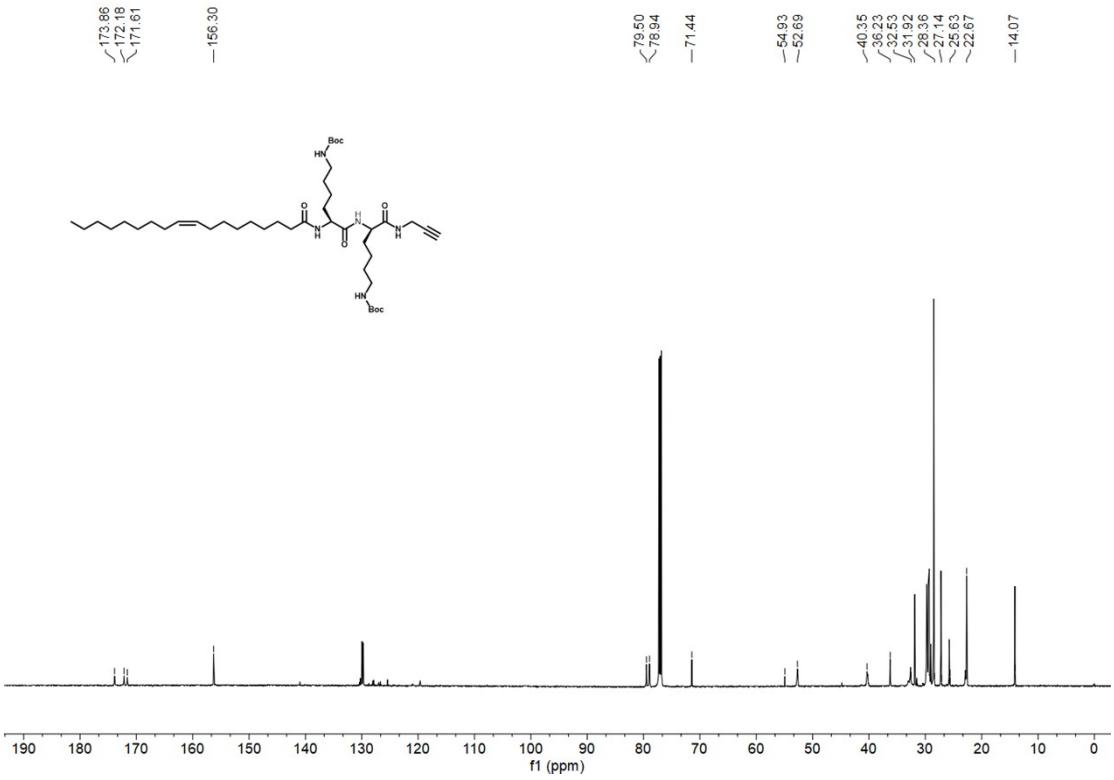
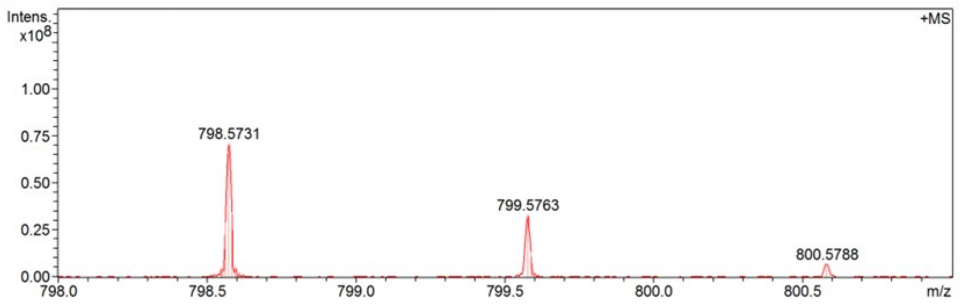


Fig. S14 ^{13}C NMR (150 MHz, $CDCl_3$) spectrum of compound **OA-L**.



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¡¥ Conf	mSigma	Std I	Std Mean m/z	Std I VarNorm	Std m/z Diff	Std Comb Dev
798.5731	1	C 43 H 77 N 5 Na O 7	798.5715	-1.9	-1.8	7.5	ok	even	23.92	0.0324	0.0015	0.0158	0.0006	0.8427
802.6743														

Fig. S15 HRMS (MALDI-TOF) of compound **OA-L**.

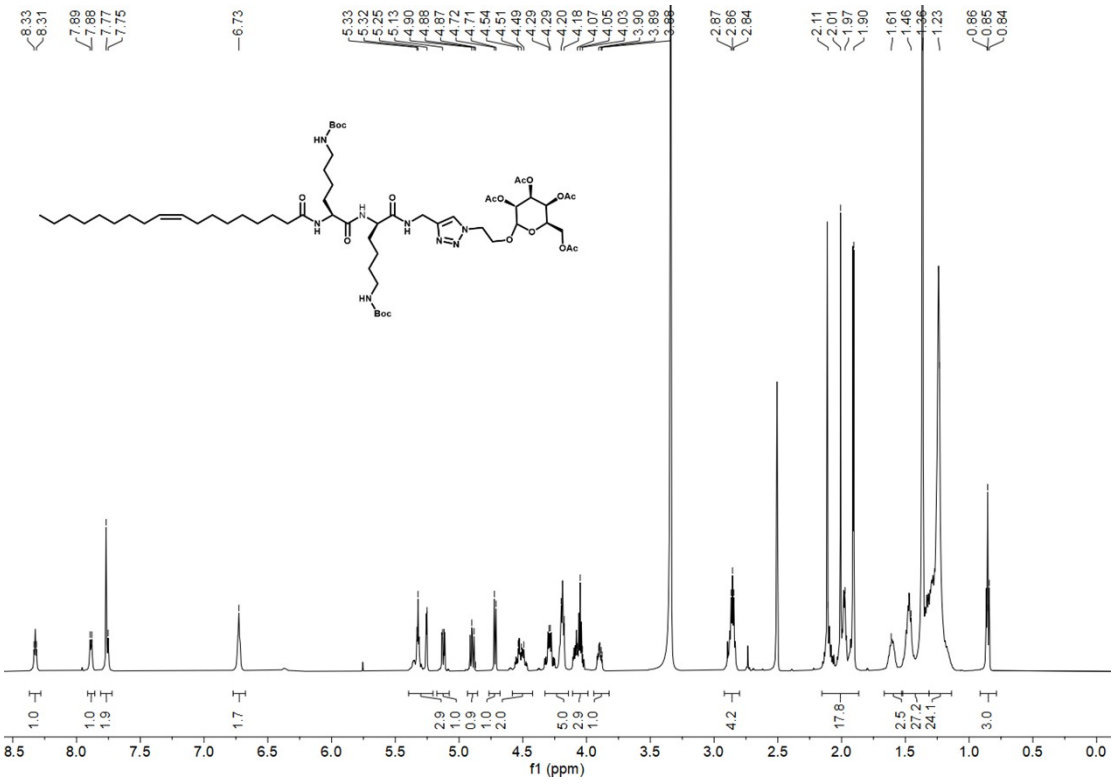


Fig. S16 ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **OA-L-AcGal**.

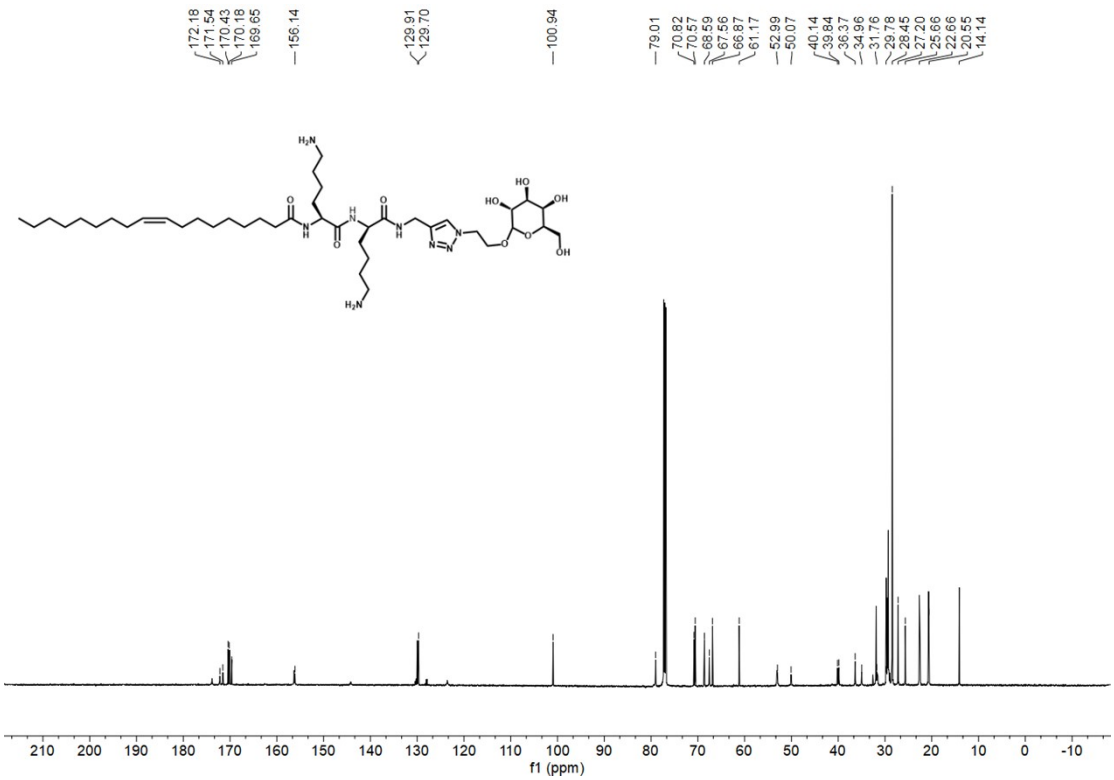


Fig. S17 ^{13}C NMR (150 MHz, DMSO-d_6) spectrum of compound **OA-L-AcGal**.

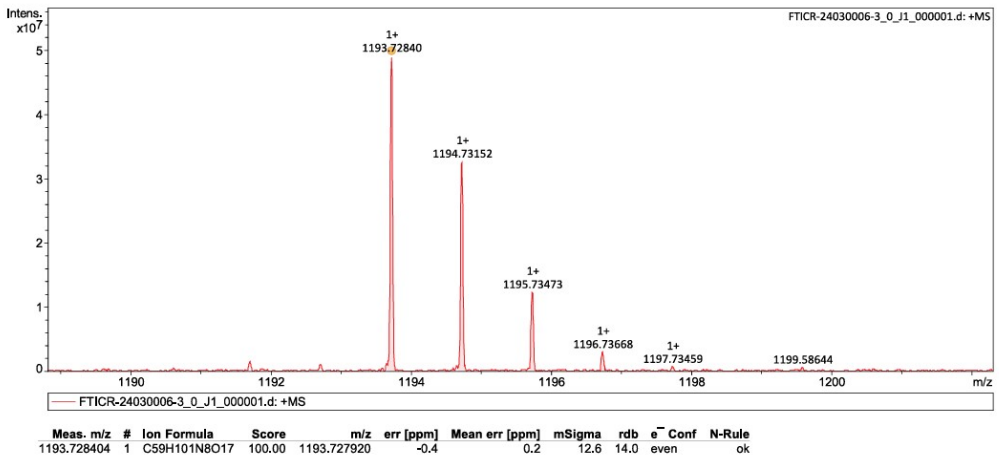


Fig. S18 HRMS (MALDI-TOF) of **OA-L-AcGal**.

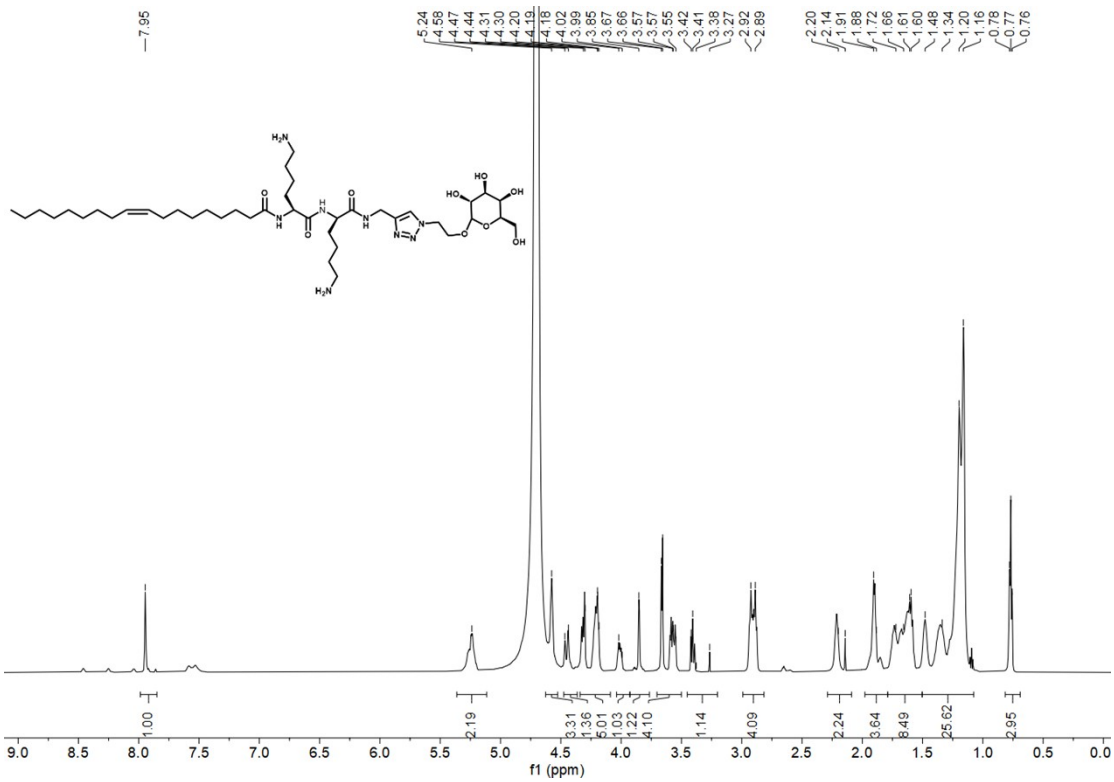


Fig. S19 ^{1}H NMR (600 MHz, D_2O) spectrum of compound **OA-L-Gal**.

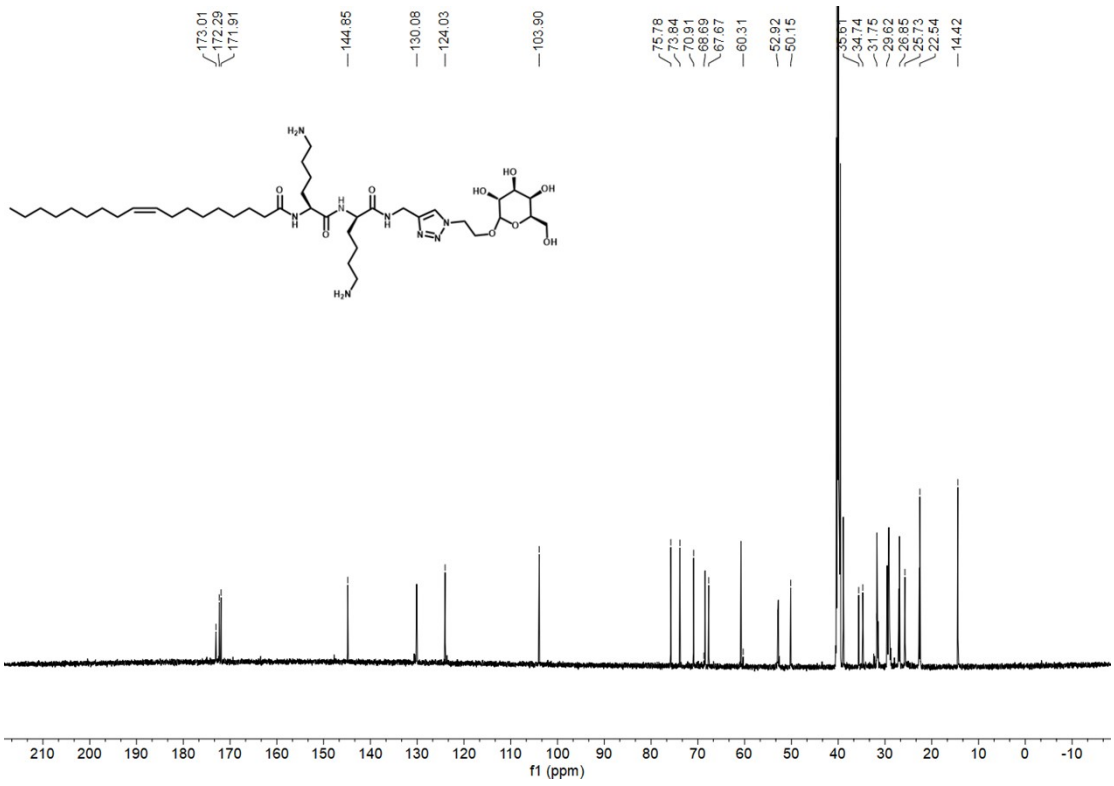
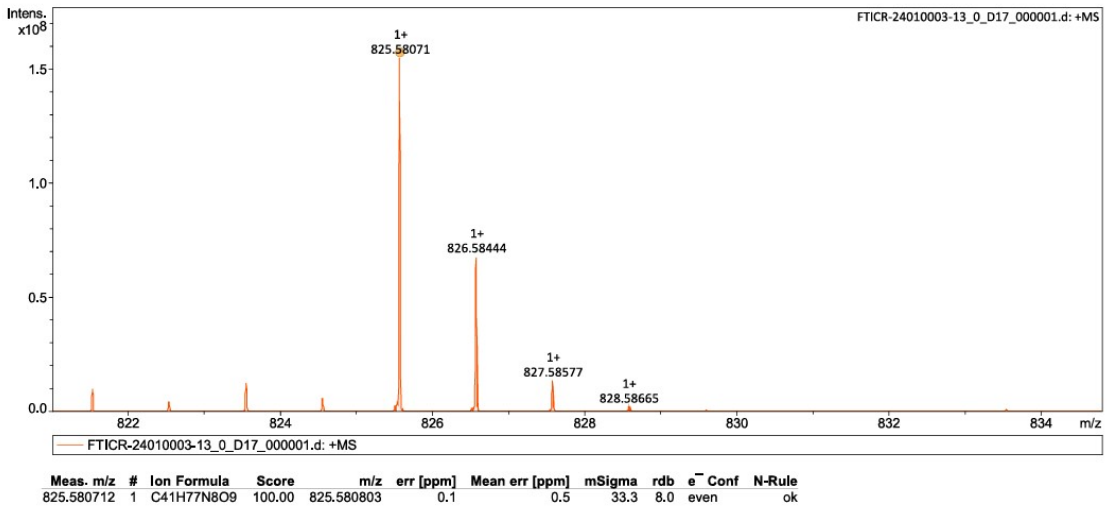


Fig. S20 ^{13}C NMR (150 MHz, DMSO-d_6) spectrum of compound **OA-L-Gal**.



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
825.580712	1	C41H77N8O9	100.00	825.580803	0.1	0.5	33.3	8.0	even	ok

Fig. S21 HRMS (MALDI-TOF) of compound **OA-L-Gal**.

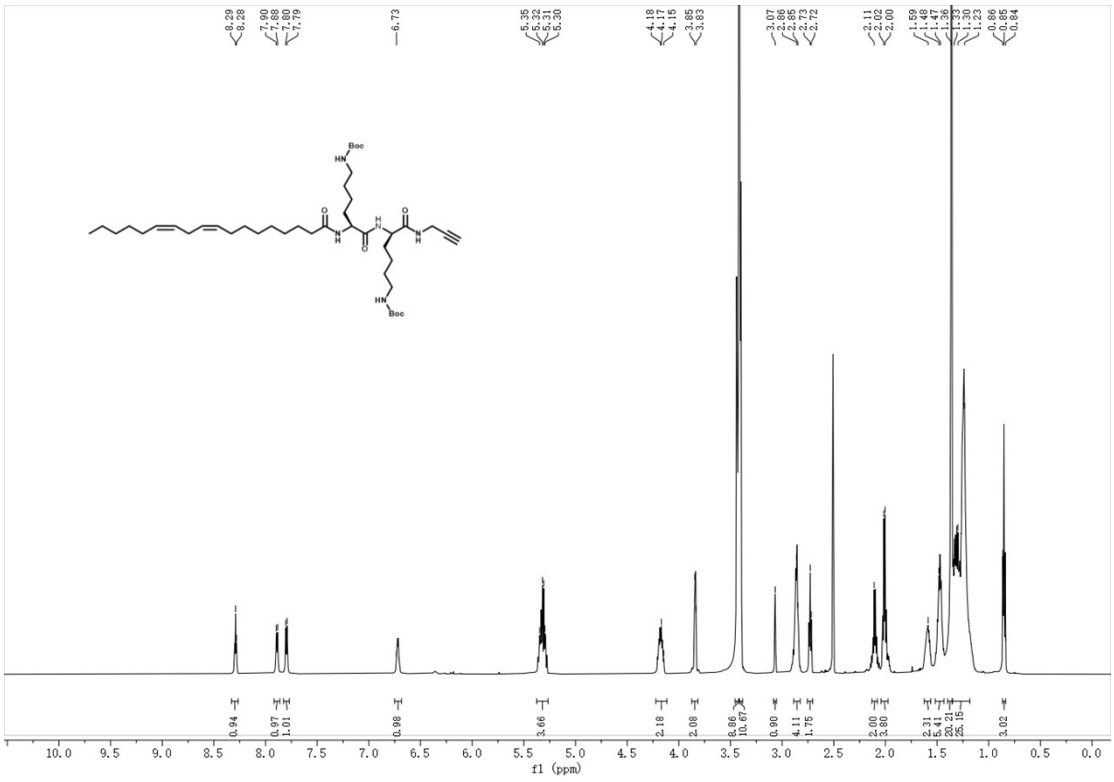


Fig. S22 ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **LOA-L**.

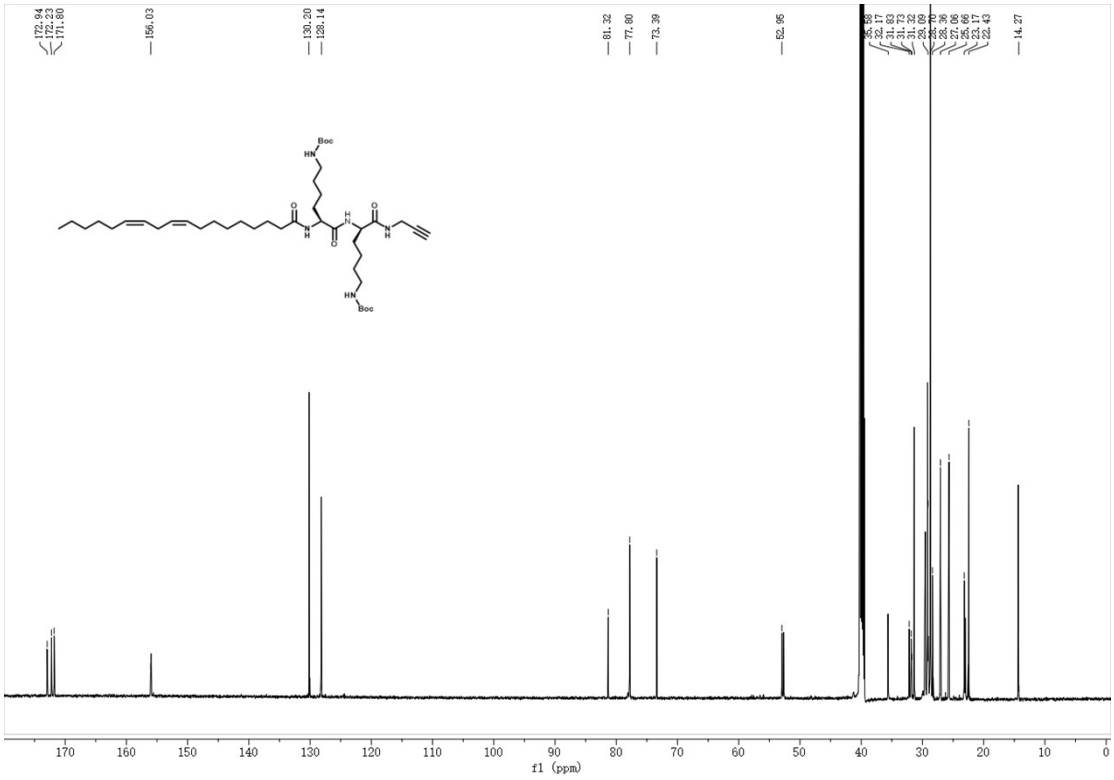
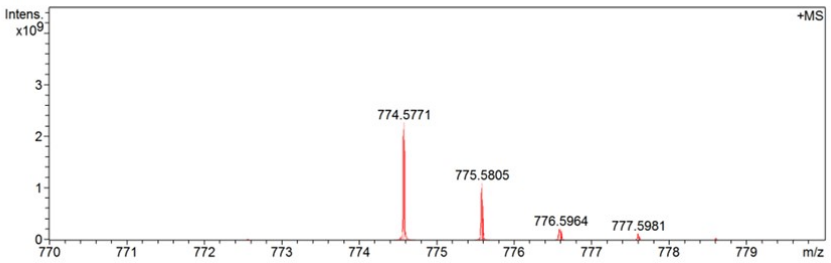


Fig. S23 ^{13}C NMR (150 MHz, DMSO-d_6) spectrum of compound **LOA-L**.



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¡¥ Conf	mSigma	Std I	Std Mean m/z	Std I VarNorm	Std m/z Diff	Std Comb Dev
774.5771	1	C 43 H 76 N 5 O 7	774.5739	-4.1	-3.7	8.5	ok	even	21.84	0.0337	0.0031	0.0142	0.0026	0.8427

Fig. S24 HRMS (MALDI-TOF) of compound **LOA-L**.

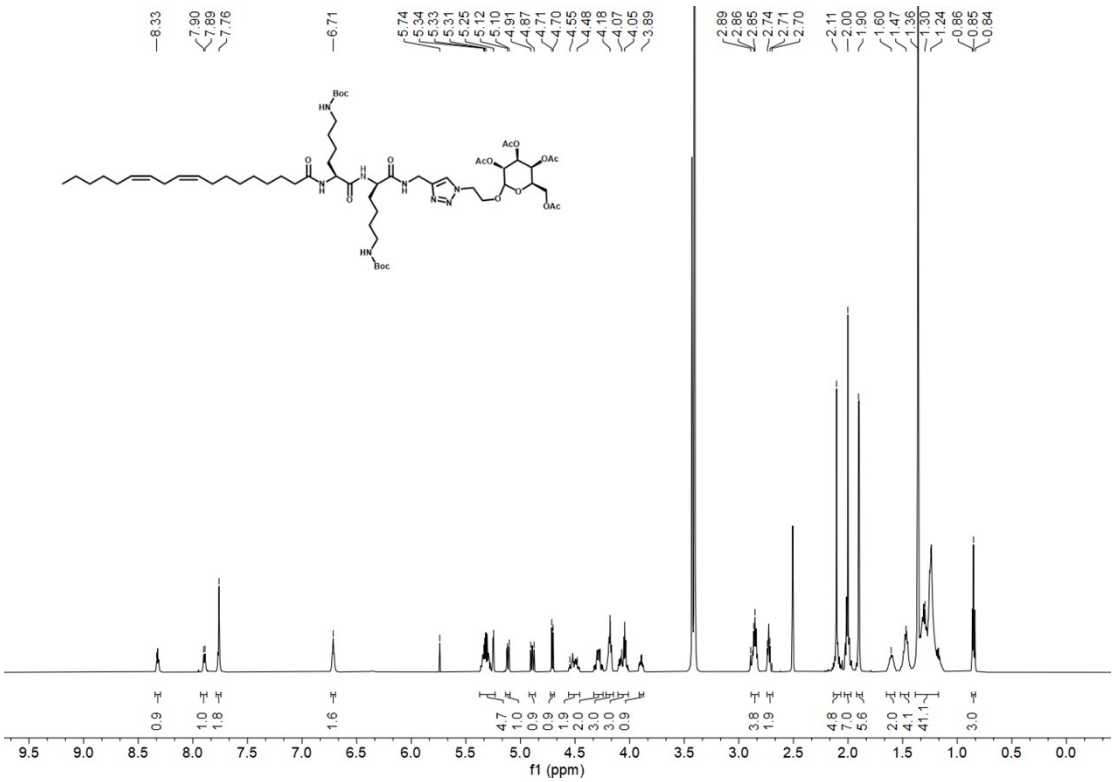


Fig. S25 ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **LOA-L-AcGal**.

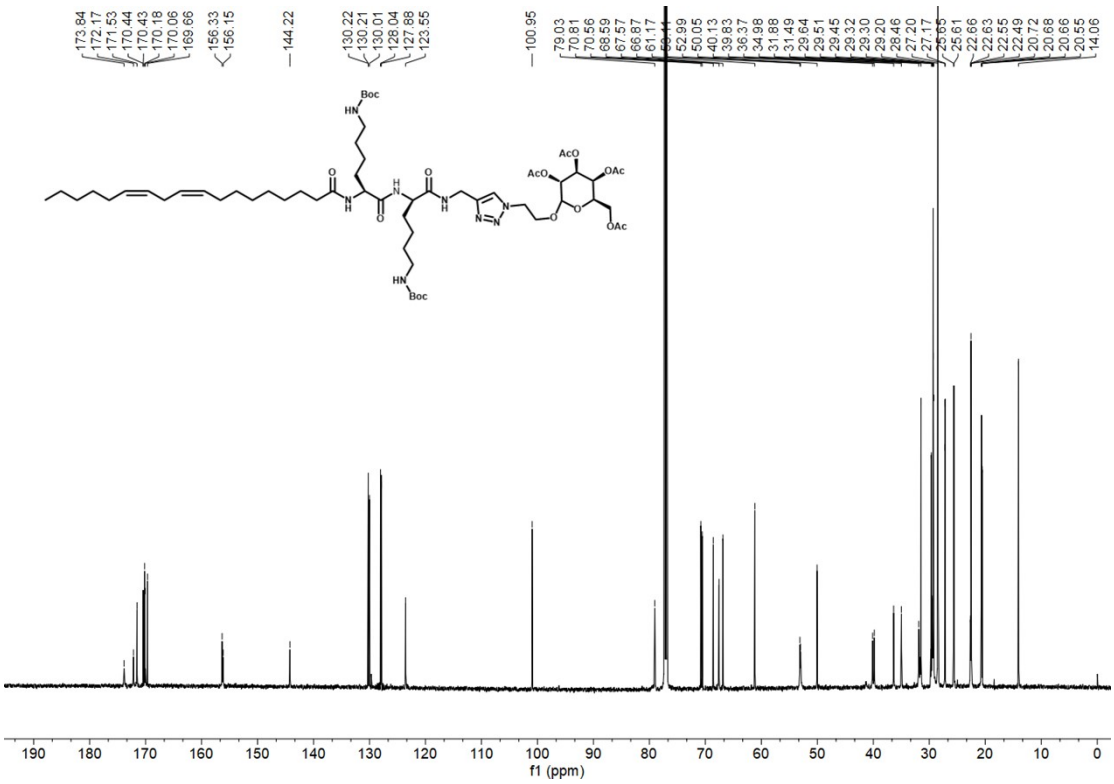
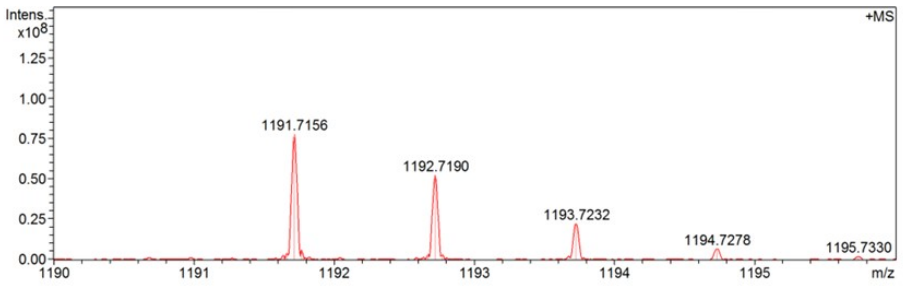


Fig. S26 ^{13}C NMR (150 MHz, $CDCl_3$) spectrum of compound **LOA-L-AcGal**.



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e¡¥ Conf	mSigma	Std I	Std Mean m/z	Std I VarNorm	Std m/z Diff	Std Comb Dev
1191.7156	1	C 59 H 99 N 8 O 17	1191.7123	-2.8	-3.1	14.5	ok	even	13.70	0.0184	0.0038	0.0069	0.0010	0.8427

Fig. S27 HRMS (MALDI-TOF) of compound **LOA-L-AcGal**.

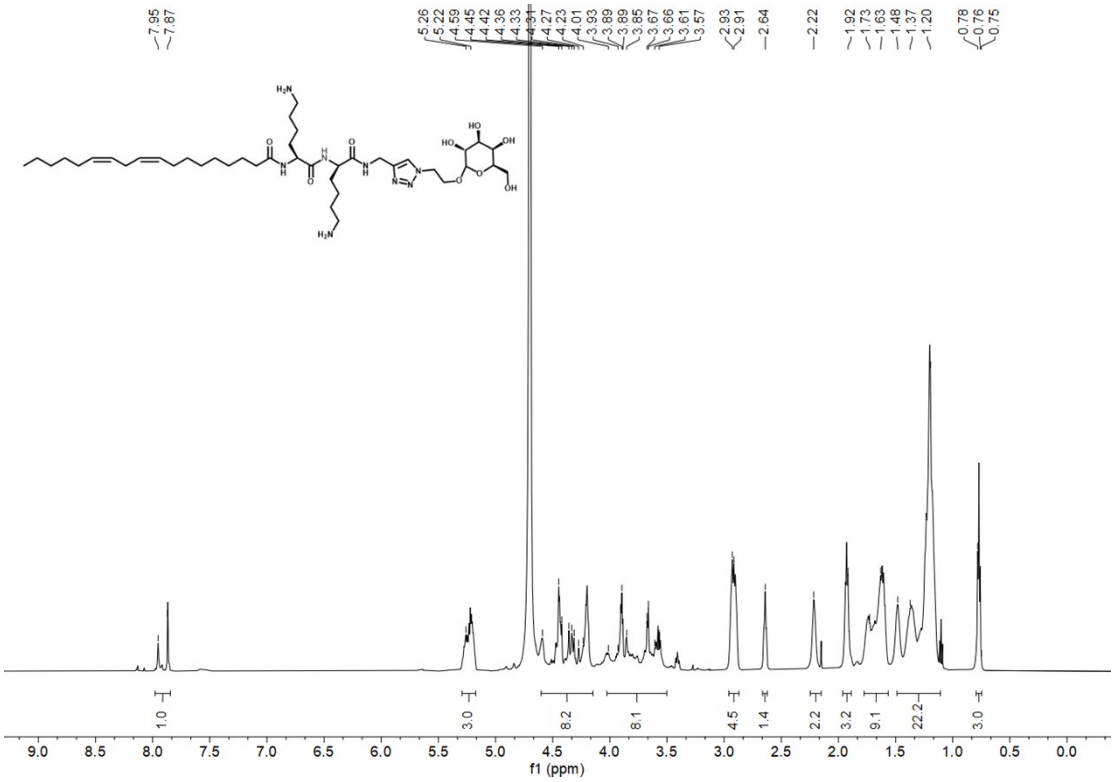


Fig. S28 ^{1}H NMR (600 MHz，D_2O) spectrum of compound **LOA-L-Gal**.

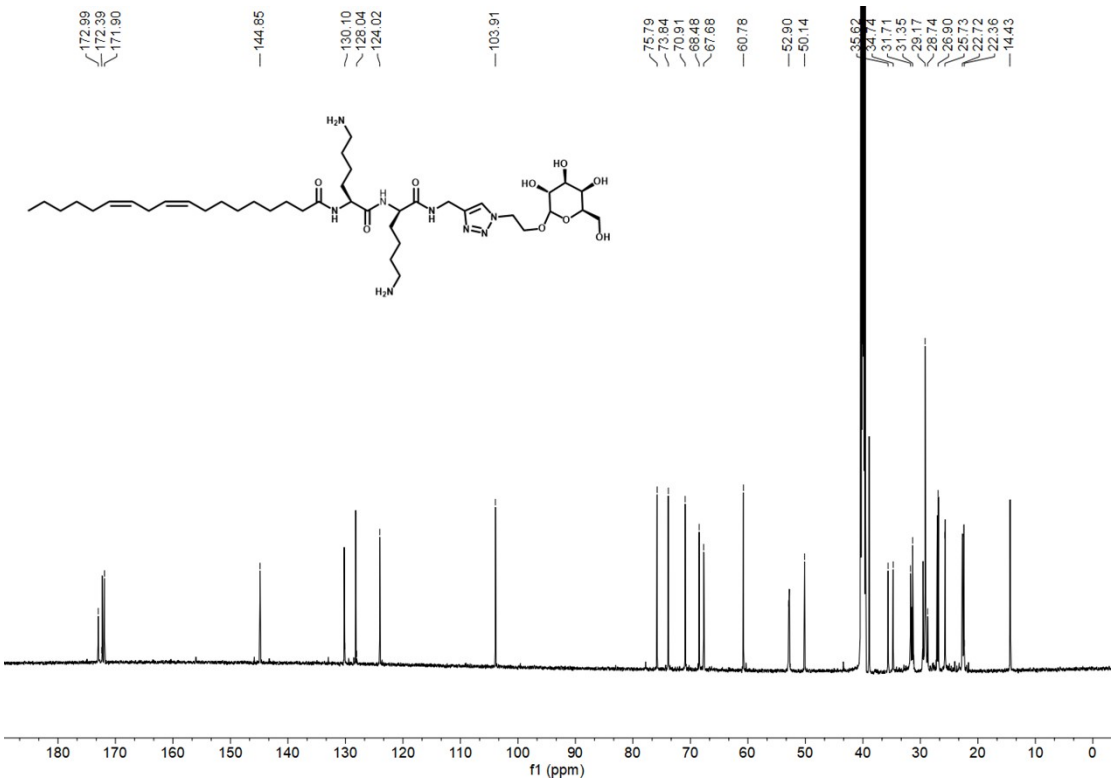
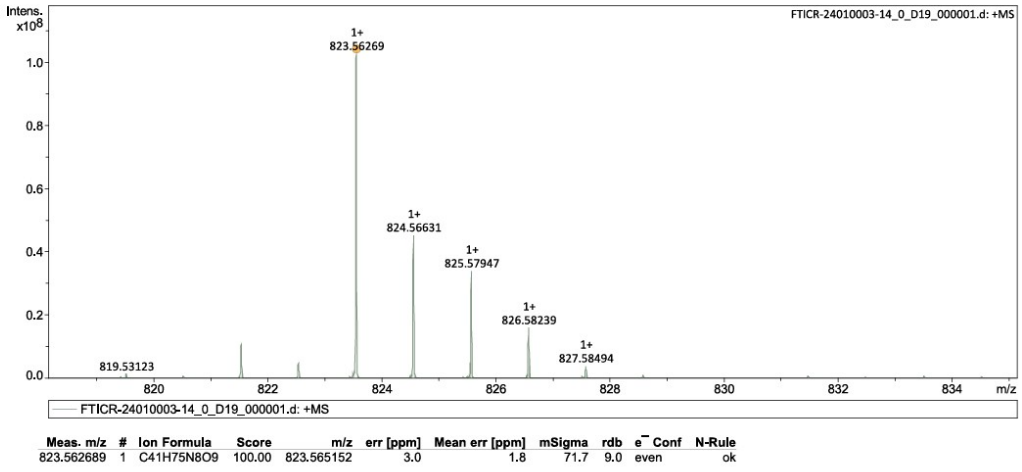


Fig. S29 ^{13}C NMR (150 MHz, DMSO-d_6) spectrum of compound **LOA-L-Gal**.



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
823.562689	1	C41H75N8O9	100.00	823.565152	3.0	1.8	71.7	9.0	even	ok

Fig. S30 HRMS (MALDI-TOF) of compound **LOA-L-Gal**.

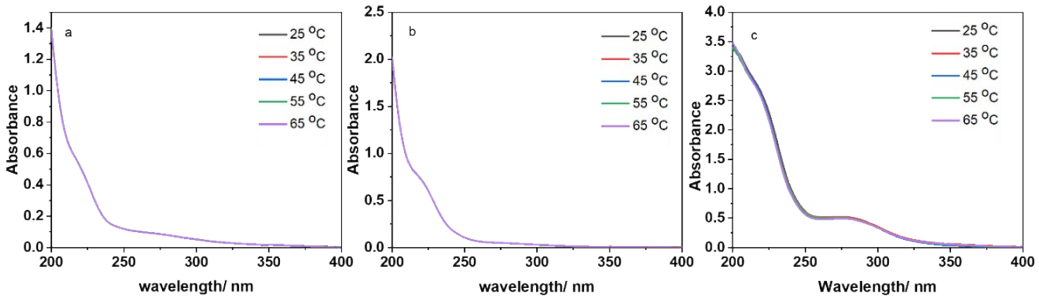


Fig. S31 Temperature-dependent UV-vis spectra of **SA-L-Gal**, **LOA-L-Gal**, **OA-L-Gal** in aqueous solution under the condition of 10 μM.

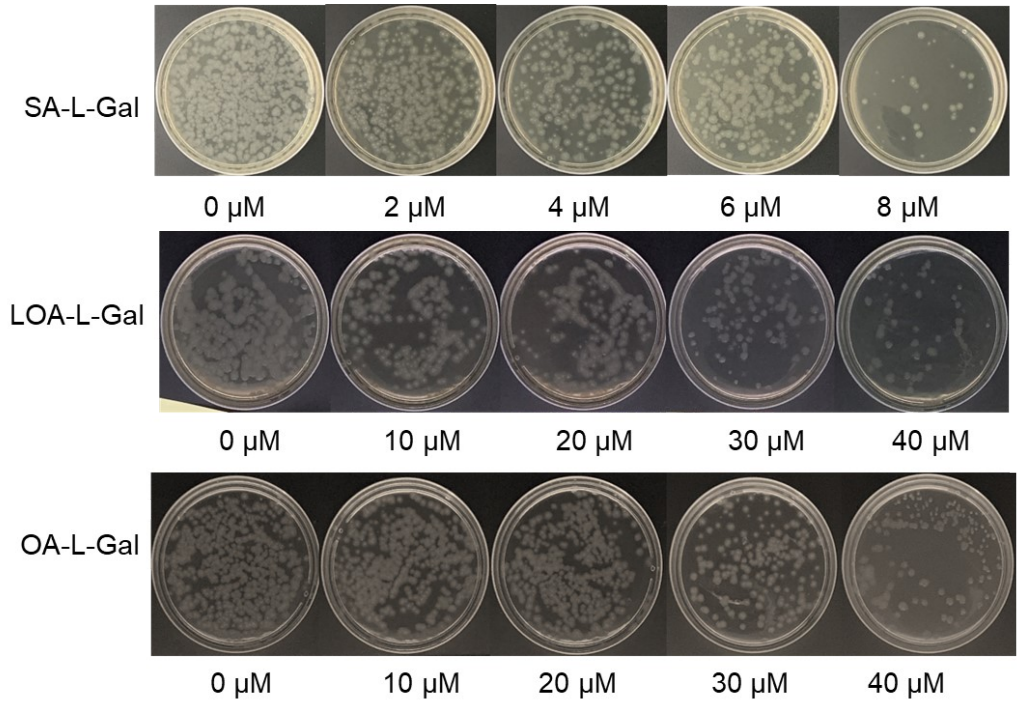


Fig. S32 The antimicrobial effect of compounds **SA-L-Gal**, **OA-L-Gal** and **LOA-L-Gal** against *P. aeruginosa* at different concentration gradients was investigated using the spread plate method.

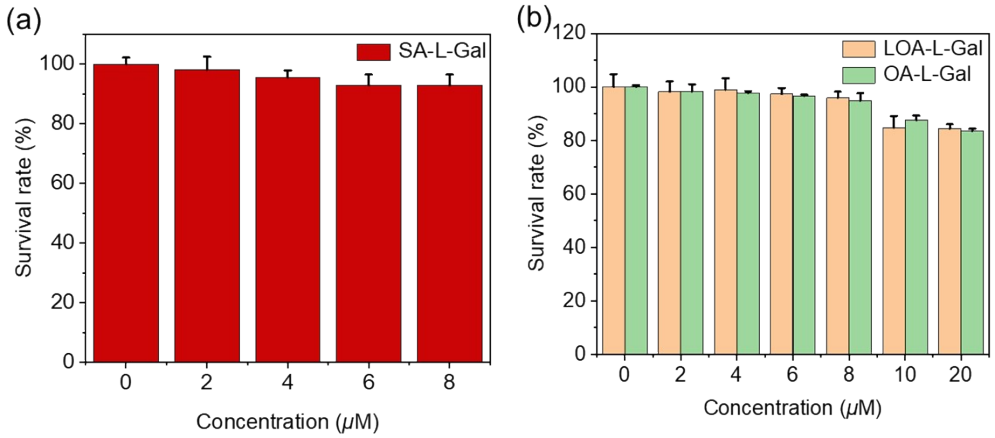


Fig. S33 The antibacterial effects of SA-L-Gal (a), OA-L-Gal and LOA-L-Gal (b) on S. aureus.

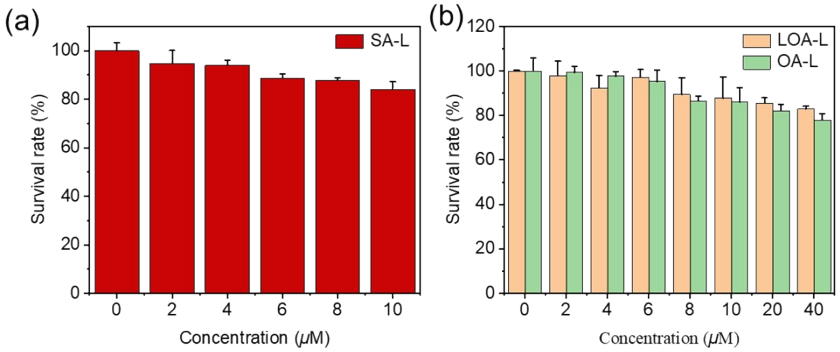


Fig. S34 Antibacterial effect of the disaccharide compounds SA-L(a), LOA-L and OA-L(b) against *P. aeruginosa*.

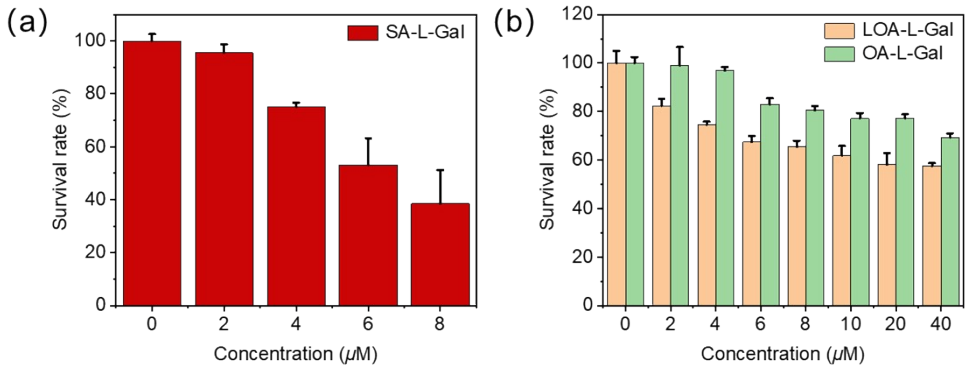


Fig. S35 Weakening effect of galactose on the antibacterial activity of compounds: the case of *P. aeruginosa*.

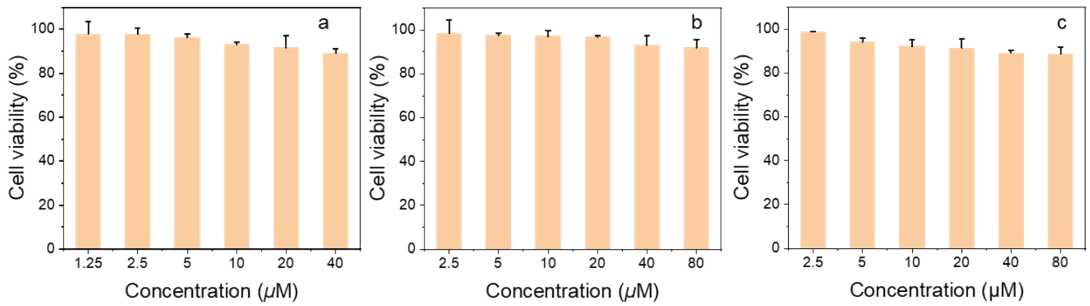


Fig. S36 Cytotoxicity of compounds **SA-L-Gal** (a), **OA-L-Gal** (b) and **LOA-L-Gal** (c) under different concentrations.

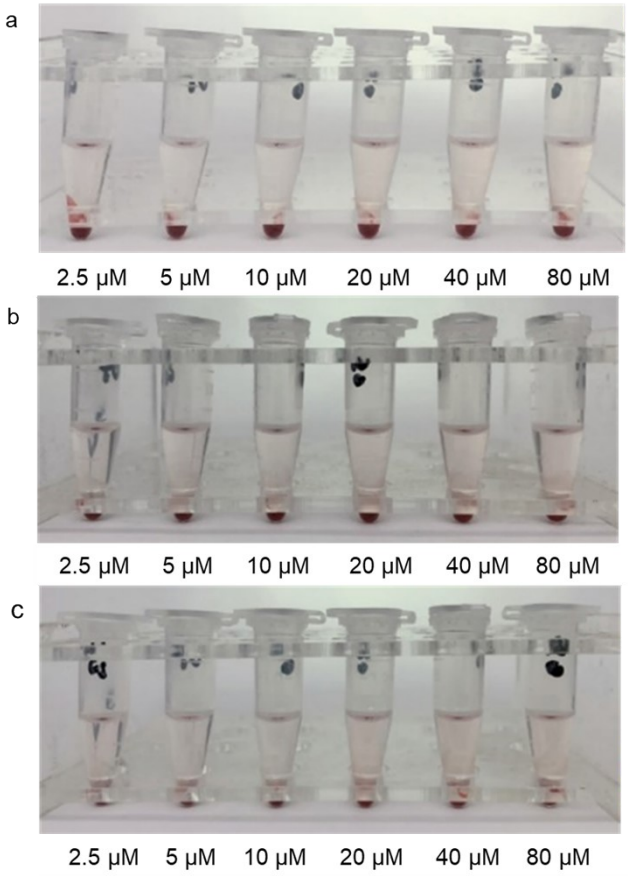


Fig. S37 Haemolytic effect of compounds **SA-L-Gal** (a), **OA-L-Gal** (b) and **LOA-L-Gal** (c) at the concentrations of 2.5-80 μM.

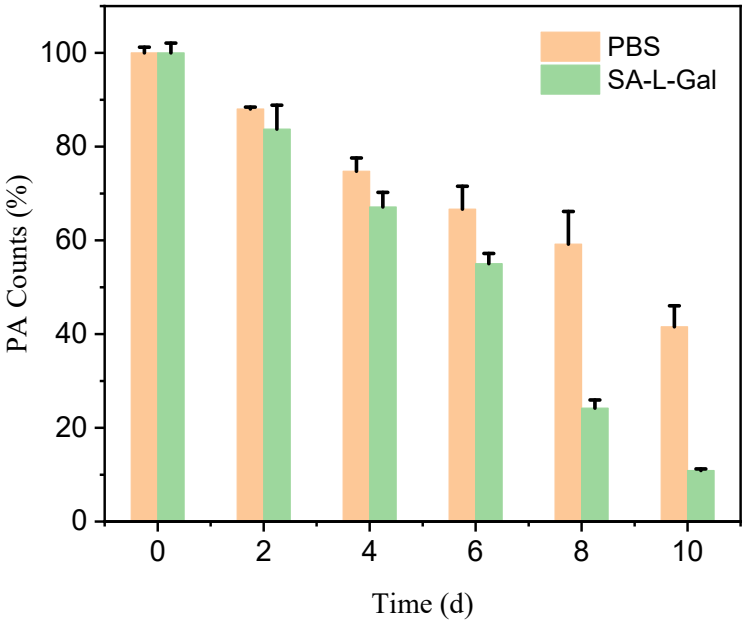


Fig. S38 Quantification of bacterial inhibition at the wound site by **SA-L-Gal**.

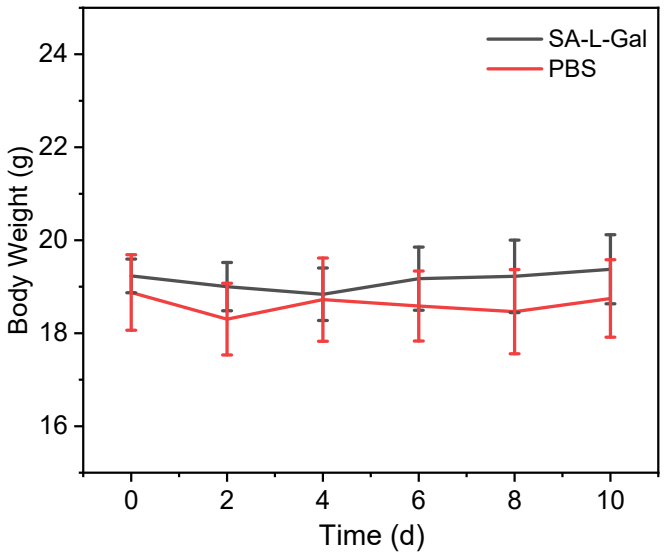


Fig. S39 Body weight changes in mice during wound infection treatment.

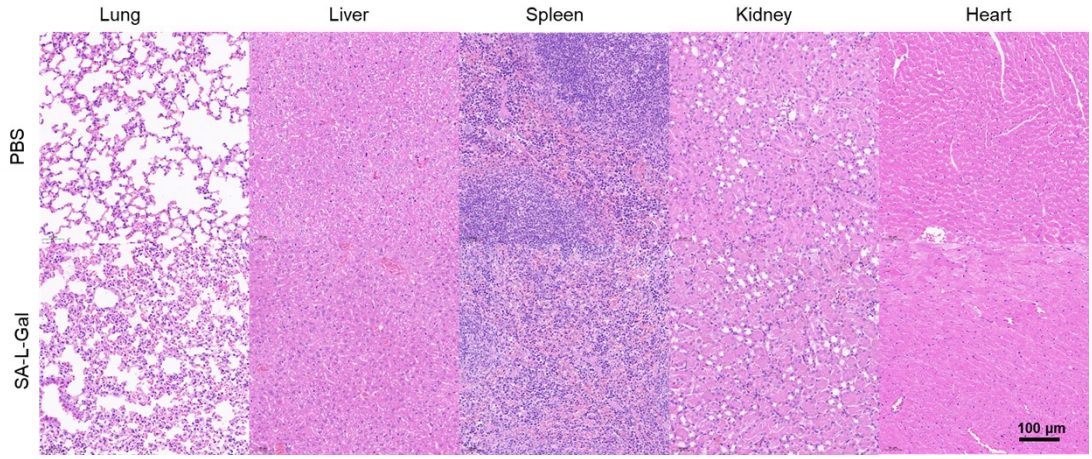


Fig. S40 H&E staining results of the major organs of the mice after treatment with **SA-L-Gal**, the scale bar indicates 100 μm.

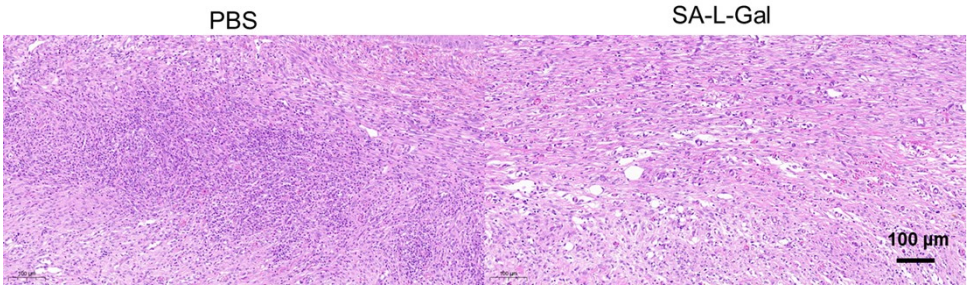


Fig. S41 H&E staining results of the major skins of the mice after treatment with **SA-L-Gal**, the scale bar indicates 50 μm.

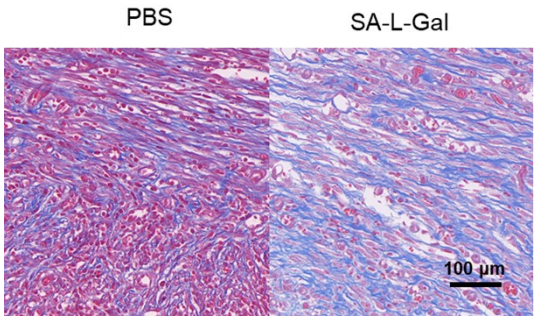


Fig. S42 Masson staining results of the skins of the mice after various treatment with **SA-L-Gal**, the scale bar indicates 100 μm.