Supporting Information for:

Iterative Glycosylation on a Single Residue of Mature Lasso Peptide

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Materials and Methods

General materials and methods. The *Streptomyces davaonensis* strain used in this work was purchased from the JCM (4913) strain collection. The primers were synthesized by General Biosystems Co., Ltd. Restriction endonucleases, and Ready-to-Use Seamless Cloning Kits were purchased from Sangon Biotech (Shanghai) Co., Ltd. Phanta Max Super-Fidelity DNA Polymerase was purchased from Nanjing Vazyme Biotech Co., Ltd. *Escherichia coli* DH5α, *E. coli* ET12567/pUZ8002, *S. lividans* Gx28 and *S. lividans* LJ018 were used for plasmid maintenance, protein overproduction, and conjugation, respectively. Chemical reagents were purchased from Sangon Biotech (Shanghai) Co., Ltd. DNA sequencing was performed by Xi'an Qingke Biotechnology Co., Ltd. All experiments were conducted at least three times. NMR data were collected using a Bruker AVANCE NEO 600 spectrometer with chemical shifts referenced to the solvent peak of DMSO-d_6 (Shanghai Haohong Scientific Co., Ltd.) at 2.50 ppm for ^{1}H NMR analysis. The MALDI-TOF MS data were recorded with a SHIMADZU MALDI-TOF mass spectrometer (MALDI-7090), using α-cyano-4-hydroxycinnamic acid as the matrix. LC-HRMS data were acquired using a ThermoScientific Q Exactive mass spectrometer equipped with a reversed phase column (Thermo Scientific, Acclaim 120 C18, 3 μm, 120Å, 3×150 mm). (Nanjing, Jiangsu, China).

Molecular biology techniques. The target DNA fragments were PCR-amplified using high fidelity Phanta Max Super-Fidelity DNA Polymerase with *Streptomyces davaonensis* genomic DNA (gDNA) as the template. The amplification products were validated through 1.0% agarose gel electrophoresis and subsequently purified using spin columns. The selected vectors were digested using selected restriction enzymes for 3 h in a 37°C water bath. Following digestion, the resulting linearized vectors and PCR products were assembled using the Ready-to-Use Seamless Cloning Kit. *E. coli* DH5α chemically competent cells were transformed with 5 μL of the assembled products through heat shock, and cells were plated on LB agar plates supplemented with appropriate antibiotics. The plates were then incubated overnight at 37 °C. Subsequently, several colonies were picked to inoculate separate 5 mL cultures of LB medium, which were grown at 37 °C for 16 h before plasmid extraction. The sequences of the cloned DNA in the resulting plasmids were confirmed by DNA sequencing. For reference, the protein sequences and IDs are provided in Table S5. The primers used in this study are detailed in Table S6, and the list of plasmids used can be found in Table S7.

Construction of Heterologous Expression Plasmids. The different regions of the *igt* BGC were amplified by PCR using primers of 4913-1.5-KasO-F1/4913-1.5-KasO-R1 and 4913-1.5-KasO-F3/4913-1.5-KasO-R3 with the gDNA of *S. davaonensis* as template, and 4913-1.5-KasO-F2/4913-1.5-KasO-R2 with the *ermEP*-containing vector as template. The resulting three PCR products were assembled with the

AflII/EcoRI-digested pSET-KasO vector using seamless cloning techniques to yield two plasmids, pSET-KasO-*igtABCD* and pSET-KasO-*igtABCDG*, which were sequenced to verify the authenticity of the sequences.

Construction of knockout Plasmids. The homologous arms flanking the knock out region of the *igt* BGC were amplified by PCR using primers of 4913-1.5-Blue-F1/4913-1.5-Blue-R1 and 4913-1.5-Blue-F2/4913-1.5-Blue-R2 with the gDNA of *S. davaonensis* as template. The resulting two PCR products were assembled with the HindIII-digested pZDBlue vectors using seamless cloning techniques to yield one plasmid, pZDBlue-*igtAC*, which was sequenced for use in knocking out *igtAC* genes through homologous double-crossover.

Protein expression and purification. The plasmids with constructs of interest were transformed into *S. lividans* Gx28 cells for protein expression. Cells were grown for 120 h on MS agar plates containing corresponding antibiotics at 30 °C. Single colonies were picked to inoculate 10 mL of TSB media, containing corresponding antibiotics and grown at 30 °C for 48 h. This culture was used to inoculate 100 mL of TSB containing appropriate antibiotics and grown for 72 h. Cells were harvested via centrifugation at 6,000 × g for 20 min and washed off the medium by two more centrifugations using deionized water, then resuspended in 30 mL suspension buffer [500 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol (*v/v*)]. Cells were lysed by sonication (1s on, 2s off) at 50% amplitude in ice water. In this process, the suspension in the tube is reversed every three minutes, and the whole process needs to be reversed 7-9 times. After the second lysis, an appropriate concentration of PMSF (0.1-1 mM) was added to the tube to inhibit the activity of proteases and protect microsomal proteins. Insoluble cell material was removed by centrifugation at 12,000 × g for 30 min at 4 °C. The resultant supernatant was centrifuged a second time using an ultracentrifuge at 100,000 × g for 1 h. Then resuspended in 1 mL membrane protein dispersion buffer [100 mM NaCl, 50 mM Tris pH 8.0, 5% glycerol (*v/v*)].

IgtA-related peptide expression and purification. A previously reported protocol was followed.[1,2] The plasmids of *MBP-igtA* were transformed into *E. coli* BL21(DE3) for the expression of modified peptides. Cells were grown for 24 h on Luria-Bertani (LB) agar plates containing corresponding antibiotics at 37 °C. Single colonies were picked to inoculate 10 mL of LB containing corresponding antibiotics and grown at 37 °C for 16-18 h. This culture was used to inoculate 1 L of LB containing appropriate antibiotics and grown to an optical density at 600 nm (OD_{600}) of 0.6-0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM, followed by an induction period at 37 °C for 3 h. Cells were harvested via centrifugation at 6,000 × g for 20 min and resuspended in 30 mL suspension buffer [500 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol (*v/v*)]. Cells were lysed by sonication (1s on, 2s off) at

50% amplitude in ice water. Insoluble cell material was removed by centrifugation at 12,000 × g for 30 min at 4 °C. The resultant supernatant was loaded onto a pre-equilibrated NiNTA-HisTalon μSphere column (Wuxi Tianyan Biotechnology Co., Ltd). The column was washed with 40 mL wash buffer containing 1 M NaCl, 20 mM Tris, 30 mM imidazole pH 8.0. Then the column was eluted with 40 mL elution buffer containing 1 M NaCl, 20 mM Tris, 250 mM imidazole pH 8.0 with 5 mL in each fraction. The resultant fractions were examined visually by Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. A buffer exchange with protein storage buffer [20 mM Tris, pH 8.0, 300 mM NaCl, 12% glycerol (*v/v*)] was performed prior to concentration. The resulting MBP-IgtA solution was treated with TEV for 5 hours in a 37 °C water bath to remove the MBP tag. The cleaved MBP was precipitated by adding acetonitrile with 0.1% formic acid in a 1:1 (*v/v*) ratio. Subsequently, the supernatant was obtained through centrifugation and dried by lyophilization. The samples were directly dissolved in 200 μL MeOH for LC-HRMS analysis. The IgtA was further purified using reversed-phase HPLC (Shimadzu, C18, 10 mm × 250 mm, 5 μm; 5%-100% ACN in H_2O with 0.1% trifluoroacetic acid (TFA) for 45 min; 2 mL/min; t_R = 21.7 min for IgtA). Finally, 20.1 mg of IgtA was obtained from 8 L cultures.

Cultivation of *S. davaonensis*. The freeze-dried powder of the strain was inoculated into TSB media, which was then cultivated for approximately 3 days at 220 rpm and 30 °C. Subsequently, the resulting seed cultures were evenly distributed to inoculate six different freshly prepared media, as follows:

1. MB (10 g/L glucose, 2 g/L tryptone, 2 g/L yeast extract, 4 g/L NaCl, 3 g/L $CaCO_3$, 0.244 g/L $MgSO_4$, 0.5 g/L K_2HPO_4, 5 g/L starch soluble, pH 7.3).

2. ISP2 (4g/L yeast extract, 10 g/L malt extract, 4g/L dextrose, pH 7.3).

3. PTM (40 g/L dextrin, 40 g/L lactose, 5 g/L yeast extract, 3 g/L $CaCO_3$, pH 7.3).

4. MD (10 g/L glucose, 15 g/L tryptone, 5 g/L yeast extract, 10 g/L glycerol, 3 g/L $CaCO_3$, 10 g/L starch soluble, pH 7.3).

5. YEME (3g/L yeast extract, 3 g/L malt extract, 5g/L peptone, 10g/L glucose, 340g/L source, pH 7.3)

6. TSB media (17 g/L tryptone, 3 g/L peptone, 2.5 g/L glucose, 5 g/L NaCl, 2.5 g/L K_2HPO_4, pH 7.3)

Following 7 days of cultivation, the cultures were harvested by centrifugation. The supernatants were extracted using *n*-butanol (*v/v*, 1:1.2) and the cells were soaked using MeOH. The resultant extracts were dried and re-dissolved in MeOH for analysis using MALDI-TOF MS and LC-HRMS.

Lasso peptides expression and purification. *S. davaonensis* strain was inoculated on MS agar plate and grown at 30 °C for 120 h. Colonies were picked to inoculate 20 mL of TSB and grow at 30 °C for 3 days. The resultant culture was then used to inoculate 150 mL of TSB, which were agitated at 220 rpm and 30 °C for approximately 3 days. The obtained seed cultures were then evenly used to inoculate freshly prepared PTM media with total volume of 35 L (100 × 350 mL). The incubation lasted for 7 days at 220 rpm and 30 °C. Cells were harvested via centrifugation at 6,000 × g for 20 min and resuspended in 10 L MeOH. The supernatant was extracted five times using n-butanol in a 1:1.2 (*v*/*v*) ratio, and the resulting solution was concentrated under reduced pressure, approximately, resulting in 200 g of extract. This extract was then subjected to a column filled with HP20 resins and eluted using a gradient of H_2O/MeOH (90/10, 70/30, 50/50, 20/80, 0/100, *v*/*v*) to yield five fractions, R1-R5, which were analyzed by LC-HRMS. Fraction R4 was relatively pure and have a high lasso content, which gave a 6.7 g sample. This sample underwent further purification using a silica gel column eluted with a CH_2Cl_2/MeOH/H_2O mixture (100/0/0, 80/20/0, 75/25/0, 67/33/0, 50/50/0, 33/67/0, 25/75/0, 20/80/0, 0/100/0, 0/90/10, 0/80/20, 0/70/30, 0/60/40, 0/50/50, 0/40/60, 0/30/70, 0/20/80, 0/10/90, 0/0/100) to yield nineteen fractions, G1-G19. Lasso peptides were detected in fractions G9-G19, which were combined and concentrated under reduced pressure. The sample underwent further purification using a column filled with LH-20 Sephadex. The column was eluted using MeOH, and the elution fractions containing target lasso peptides were combined and concentrated under reduced pressure. The resulting sample was finally purified by reversed-phase HPLC twice equipped with a C18 reversed-phase column. First, the elution program was 15%-100% ACN in H_2O with 0.1% formic acid for 21 min (Shimadzu, C18, 10 mm × 250 mm, 5 μm; 15% keeping for 5 min, 15%-35% for 5 min, 35%-40% for 10 min then go to 100%, 3 mL/min. t_R = 13-19 min for lasso peptides). Second, the elution program was 10%-100% ACN in H_2O with 0.1% formic acid for 40 min (Shimadzu, C18, 4.66 mm × 250 mm, 5 μm; 10%-20% for 5 min, 20% keeping for 30 min, 20%-100% for 5 min, 1 mL/min). Compound **3** and **1** were eluted at 16.6 and 17.8 min with the yield of 14.5 and 20.2 mg from 35 L cultures, respectively.

In vitro enzymatic activity assay of IgtG. The complete reactions were performed in a 50 μL reaction system, which included approximately 50-60 μg IgtG microsome,[3] 5 mM $CaCl_2$ (China National Medicines Corporation Ltd.), 1 mM TECP (Shanghai Macklin Biochemical Co., Ltd.), 5 mM NaCl (Shanghai Hushi Laboratory Equipment Co., Ltd.), 5 mM sugar donor (Shanghai Yuanye Bio-Technology Co., Ltd.), 100 μM lasso peptide **1** or MBP-IgtA peptide that had undergone TEV cleavage at 30 °C in a water bath for 16 h, pH 7.5-8.0. All the reactions were terminated by adding equal volume of acetonitrile with 0.1% formic acid. Subsequently, the mixture was centrifugated, and the supernatant was dried by lyophilization and dissolved in 50 μL MeOH for LC-HRMS analysis.

LC-HRMS data acquisition. MBP-IgtA samples that had undergone TEV cleavage were subjected to LC-HRMS analysis using a ThermoFisher Scientific Q Exactive mass spectrometer. Separation was performed on an Acclaim™ 120 C18 3μm 120Å (3*150mm) column running at 0.3 mL/min. Mobile phases were solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol). The following gradient was used unless specified otherwise: 5-100% B over 20 min. The mass spectrometry instrument was configured to operate in positive mode, with a mass range of 400-6000 m/z and the following parameters: sheath gas flow rate of 35 mL/min, auxiliary gas flow rate of 10 mL/min, sweep gas flow rate of 1 mL/min, spray voltage of 4.00 kV, capillary temperature of 320 °C, and auxiliary gas heater temperature of 30 °C. The subjection processes of lasso peptides were like those used for peptide that had undergone TEV cleavage. Notably, the following gradient was used for lasso peptides unless specified otherwise: 5-100% B over 10 min. The mass spectrometry instrument was configured to operate in positive mode, with a mass range of 200-3000 m/z. For MS/MS analysis, the instrument was set to run Auto MS^2 mode and a collision energy of 20-30 kV was used for fragmentation.

Heterologous Expression of *igt* BGC. The introduction of each recombinant plasmid into model *Streptomyces* was carried out by *E. coli* ET12567-*Streptomyces* conjugation, which resulted in the corresponding recombinant strains.[4] Cells were grown for 7 days on MS agar plates containing corresponding antibiotics at 30 °C. Single colonies were picked to inoculate 10 mL of TSB containing corresponding antibiotics and grown at 30 °C for 2 or 3 days.

The cultivation of GX28/LJ1018-*igtABCD* and GX28/LJ1018-*igtABCDG* strain seed cultures was carried out in TSB media containing 10 μg/mL of apramycin and 25 μg/mL nalidixic acid with a total volume of 10 mL. The cultures were agitated at 220 rpm and 30 °C for approximately 3 days. The obtained seed cultures were then evenly used to inoculate freshly prepared PTM media with volume of 50 mL. The incubation lasted for 7 days at 220 rpm and 30 °C. After cultivation, the cultures were harvested via centrifugation at 6000 rpm for 20 min, and the supernatant was extracted with an equal volume of n-butanol. The resultant extracts were dried and re-dissolved in MeOH for analysis using LC-HRMS.

Knockout of *igtAC*. The introduction of recombinant plasmid into *S. davaonensis* was carried out by *E. coli* ET12567-*Streptomyces* conjugation, which resulted in the corresponding recombinant strains.[4] Cells were grown for 7 days on MS agar plates containing corresponding antibiotics at 30 °C. Single colonies were picked to 10 μg/mL apramycin and 25 μg/mL nalidixic acid MS agar plates grown for 7 days. Single colonies with blue color were selected to be passed to MS agar plates without antibiotics, which were repeated twice. The same colonies were then passed to MS agar plates with and without antibiotics.[5]

Approximately nine consecutive passages resulted in a resistance-deficient single colonies. The deletion of the target genes was verified by PCR.

The cultivation of knockout strain seed cultures was carried out in TSB media containing 10 μg/mL of apramycin and 25 μg/mL nalidixic acid with a total volume of 10 mL. The cultures were agitated at 220 rpm and 30 °C for approximately 3 days. The obtained seed cultures were then evenly used to inoculate freshly prepared six media (PTM, ISP2, MB, MD, YEME and TSB) with volume of 50 mL. The incubation lasted for 7 days at 220 rpm and 30 °C. After cultivation, the cultures were harvested via centrifugation at 6000 rpm for 20 min, and the supernatant was extracted with an equal volume of n-butanol. The resultant extracts were dried and re-dissolved in MeOH for analysis using LC-HRMS.

NMR data acquisition. Due to the low solubility of lasso peptides in water or methanol, all obtained pure compounds were dissolved in 150 μL of DMSO-d_6 and transferred into heavy wall NMR tubes. All NMR spectra were acquired using a Bruker AVANCE NEO 600 spectrometer. For lasso peptide **1**, ^{1}H and ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC experiments were collected using 16, 16, 28, 128, 16, and 64 scans, respectively. For diglycosylated lasso peptide **3**, ^{1}H, ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC spectra were acquired using 16, 128, 256, 256, 128, and 256 scans, respectively.

Characterization of the glycosyl groups in 3. To determine the type and absolute configuration of the sugar residues, we designed this experiment in this study.[6]

1. D-Derivative. D-(+)-Glucose (Sigma-Aldrich) (10 mg) and L-cysteine methyl ester hydrochloride (Energy Chemical) (15 mg) were dissolved in pyridine (Rionlon Bohua (Tianjin) Pharmaceutical & Chemical Co., Ltd.) (0.5 mL) and heated at 60°C for 60min, and then o-Tolyl isothiocyanate (Aladdin Holdings Group Co., Ltd) (15 μL) was added to the mixture and heated at 60°C for 60min. The reaction mixture was extracted using hexane, and then analyzed by LC-HRMS. The following gradient was used: 45% B over 40 min. The product was eluted at 18.12 min.

2. L-Derivative. L-(-)-Glucose (Energy Chemical) was reacted in the manner like described for the D-enantiomer. The reaction mixture was extracted using hexane then analyzed by LC-HRMS. The product was eluted at 16.88 min.

3. Lasso peptide **3** purified by HPLC (1 mg) were hydrolyzed by heating in 0.5 M HCl (2mL). After drying by lyophilization the residue was dissolved in pyridine (0.5 mL) containing L-cysteine methyl ester hydrochloride (1.2 mg), which was heated at 60°C for 1h. A 10 µL solution of o-Tolyl isothiocyanate (0.5

mg) in pyridine was added to the mixture, which was heated at 60 °C for 1h. The reaction mixture after drying by lyophilization was extracted using hexane, which was then analyzed by LC-HRMS. The peaks at 16.88 and 18.12 min corresponded to derivatives of L-glucose and D-glucose.

Characterization of the 1,3-glycosidic linkage between the two glycosyl groups in 3 through methylation. A previous protocol was followed with details described.[7]

1. During the reaction, a nitrogen-protected environment was maintained. Lasso peptide **3** (2 mg) was completely dissolved in 0.3 mL of DMF (Energy Chemical). Subsequently, 1.6 mg of NaH (Energy Chemical) was added, and the mixture was stirred on ice until equilibrium was achieved. CH_3I (30 μL; Chengdu Chorn Chemicals Co., Ltd.) was then added dropwise to the reaction mixture under continuous stirring. The reaction was maintained at 0 °C for 30 min, followed by stirring at room temperature for an additional 30 min. The reaction was quenched by the gradual addition of ice-cold water. The resulting mixture was lyophilized and extracted with ethyl acetate (Rionlon Bohua (Tianjin) Pharmaceutical & Chemical Co., Ltd.). The final product was dissolved in 50 μL of MeOH for LC-HRMS analysis.

2. The sample was hydrolyzed by the addition of 1 mL of 2 M trifluoroacetic acid (TFA) and incubated at 120 °C for 2 hours. The hydrolyzed mixture was subsequently dried by lyophilization.

3. The lyophilized hydrolyzed sample was dissolved in 1 mL of freshly prepared $NaBD_4$ (10 mg/mL; Shanghai Aladdin Biochemical Technology Co., Ltd.), followed by the addition of 10 μL of ammonia solution. The mixture was stirred at 25 °C for 2 hours. The reaction was subsequently quenched with 4 mM acetic acid solution (Rionlon Bohua (Tianjin) Pharmaceutical & Chemical Co., Ltd.), and the pH was adjusted to 7.0. A MeOH solution containing 0.1% hydrochloric acid was then added, and boron was removed by rotary evaporation.

4. The dried samples obtained from the previous steps were reacted with acetic anhydride in pyridine at 120 °C for 30 min. Following the reaction, the samples were extracted with dichloromethane. The final product was dissolved in MeOH and subjected to gas chromatography–mass spectrometry (GC-MS) analysis.

The samples were analyzed using an Agilent 8890-5977B Series GC-MS system (Agilent Technologies, Inc.). Separation was conducted on an Agilent 19091S-433UI capillary column (30 m × 250 μm × 0.25 μm) with a total flow rate of 24 mL/min. Nitrogen (N_2) was used as the carrier gas at a flow rate of 1 mL/min. The temperature program ranged from -60 °C to 325 °C (maximum 350 °C), with an initial temperature of 60 °C and a heating rate of 30 °C/min. The auxiliary gas heater was set to 300 °C.

The mass spectrometer operated in positive ion mode over a mass range of 40–400 m/z. The ion source temperature was set to 230 °C (maximum 250 °C), and the quadrupole temperature was maintained at 150 °C (maximum 200 °C). Additional parameters included a gasket blowdown flow rate of 3 mL/min, a capillary temperature of 310 °C, an injection volume of 1 μL, and an electron ionization energy of 70 eV.

Antibacterial and cytotoxic activity evaluation of 1 and 3. The antibacterial activity of **1** and **3** were assessed using the disc diffusion method, following our published protocols.[8] A panel of bacterial strains were tested, including *E. coli* K12, *E. coli* MG1655, *E. coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* Newman, *Staphylococcus aureus* ATCC 29213, *Staphylococcus cohnii* DKG4, *Staphylococcus simulans* AKA1, *Enterococcus faecalis* ATCC 29212, *Enterococcus gallinarum* 5F52C, *Bacillus subtilis*, *Bacillus thuringiensis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Clostridium perfringens* FSKP20, *Salmonella typhimurium* SH138, *Salmonella heidelberg* SH36, and *Proteus mirabilis* SG0508. Additionally, the compounds were evaluated for cytotoxicity against HepG2, LoVo, and RAW264.7 macrophage cells using the CCK8 assay. No activity was observed at concentrations up to 50 μM.

Bioinformatics. The SSN of IgtG was generated using "Sequence BLAST" option in EFI-EST Tools,[9-12] with the IgtG sequence as the query. Sequences with 100% identity were conflated into a single node, and other parameters were kept as default. The SSN was visualized using the organic layout within Cytoscape.[13] A 72% sequence identity threshold was applied to separate the clusters, and the resulting SSN was subjected to EFI-GNT analysis. The resulting GNNs and GNDs revealed the presence of SirG-encoding BGC from *S. iranensis*. The SirD sequence was then used to run blastP to retrieve other GT-encoding graspetide BGCs. Sequences of the representative IgtG-like and all known RiPP GTs were collected and organized into FASTA files. These sequences were input into Mega11[14] for sequence alignment using ClustalW[15] and Maximum likelihood (ML) tree analysis.

Table S1: ^{1}H- and ^{13}C-NMR chemical shift assignment of 1. The ^{13}C-NMR chemical shifts were determined from the analysis of ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectra. Chemical shifts labeled with the same superscript (a, b, c, or d) are interchangeable.

Residue	Position	δ_H (ppm)	δ_C	Residue	Position	δ_H (ppm)	δ_C
Leu1	NH	8.36		**Leu10**	NH	8.77	
	1	3.54	58.5		1	4.47	57.2
	2	1.29, 0.88	26.0		2	1.24, 1.36	[c]29.4
	3	2.38	32.7		3	1.58	24.2
	4	0.79	11.1		4	0.76	[d]23.5
	5	1.19	19.2		5	0.74	[d]23.8
Leu2	NH	7.64		**Ile11**	NH	7.99	
	1	4.32	50.3		1	4.18	[b]54.2
	2	1.71, 1.39	39.5		2	1.71	24.1
	3	1.52	n.d.		3	1.50, 1.60	43.7
	4	0.79	21.0		4	0.75	21.7
	5	0.86	[a]14.4		5	0.86	[a]23.9
Gly3	NH	8.35		**Leu12**	NH	6.63	
	1	3.29, 4.01	45.3		1	4.68	n.d.
Arg4	NH	7.99			2	0.89	n.d.
	1	4.19	[b]58.1		3	1.30	30.3
	2	1.46, 1.98	27.1.		4	0.52	24.0
	3	1.21, 1.36	[c]23.4		5	0.52	21.1
	4	3.01	37.4	**Ser13**	NH	8.77	
	NH_2	8.14, 7.79			1	4.45	51.5
Ser5	NH	7.83			2	3.89	62.0
	1	4.49	54.3	**Lys14**	NH	8.38	
	2	3.50, 3.63	61.7		1	3.62	51.7
Gly6	NH	7.36			2	1.57, 1.43	42.7
	1	3.49, 3.94	44.9		3	0.90.1.26	26.1
Asn7	NH	8.06			4	1.75, 1.89	29.8
	1	4.76	50.5		5	3.43	45.2
	2	2.76,2.32	38.3		NH_2	8.35	
	NH_2	6.80		**Asn15**	NH	7.61	
Asp8	NH	7.54			1	4.14	51.8
	1	4.54	48.6		2	2.36,2.47	40.3
	2	2.78, 3.79	36.3		NH_2	6.73	
Arg9	NH	8.46					
	1	3.77	57.3				
	2	1.65	28.4				
	3	1.46	27.1				
	4	2.97	39.4				
	NH_2	6.25					

Table S2: ^{1}H-^{1}H NMR NOESY correlations of 1.

Residue	Protons with correlations
Leu1	
NH	L1-H1, L1-H2, L1-H3, L1-H4, L1-H5, L2-NH, L2-H2, R9-H1, L12-NH.
2	L1-NH, L1-H1, L1-H3, L1-H4.
3	L1-NH, L1-H1, L1-H2, L1-H4, L1-H5.
4	L1-NH, L1-H1, L1-H2, L1-H3, L1-H5.
5	L1-NH, L1-H1, L1-H2, L1-H3, L1-H4.
Leu2	
NH	L2-H1, L2-H2, L2-H4, L2-H5, L1-H1, L1-NH, S13-NH, L12-NH.
1	L2-NH, L2-H2, L2-H4, L2-H5, L1-NH, G3-NH, L12-NH.
2	L2-NH, L2-H1, L2-H4, L2-H5.
3	n. d.
4	L2-NH, L2-H1, L2-H2.
5	L2-NH, L2-H1, L2-H2.
Gly3	
NH	G3-H1, L2-H1, R4-H1.
1	G3-NH.
Arg4	
NH	R4-H1, R4-H3, R4-H4, G3-NH, S5-H2.
1	R4-NH, R4-H3, G3-NH, S5-NH.
2	R4-H1, R4-H2, R4-H4.
3	R4-H1, R4-H4.
4	R4-H2, R4-H3.
NH2	R4-H2, R4-H3, R4-H4.
Ser5	
NH	S5-H1, S5-H2, R4-H1.
1	S5-H2, G6-H1, G6-NH.
2	S5-NH, S5-H1, G6-NH, R4-NH.
Gly6	
NH	G6-H1, S5-H1, S5-H1.
1	G6-NH, S5-H1, N7-NH, N7-H1.
Asn7	
NH	N7-H1, N7-H2, G6-H1.
1	N7-NH, N7-NH2, N7-H2, G6-H1, L12-NH, D8-NH, D8-H1.
2	N7-NH, N7-H1, N7-NH2, D8-NH, D8-H1.
NH2	N7-H2.
Asp8	
NH	N7-H1, N7-H2.
1	D8-H2, R9-NH, L12-NH.
2	D8-H1, N7-NH, R9-NH.
Arg9	
NH	R9-H1, R9-H2, R9-H3, R9-H4, L2-NH, L2-H5, D8-H1, D8-H2, L10-H1, L10-H5.
1	R9-NH, R9-NH2, R9-H2, R9-H3, R9-H4, L1-NH, L2-NH, L2-H1, L2-H5, L10-NH, L10-H5, L12-NH.
2	R9-NH2, R9-H1, R9-H4.
3	R9-NH2, R9-H1, R9-H4.

4	R9-NH, R9-NH2, R9-H1, R9-H2, R9-H3, L2-NH, L2-H1, L2-H5, D8-H1, D8-H2, L10-H5, I11-H1.
NH2	R9-H2, R9-H3, R9-H4, L2-H5, L10-H5.
Leu10	
NH	L10-H1, L10-H2, L10-H3, I11-H1, G6-H1, N7-NH.
1	L10-NH, L10-H2, L10-H3, L10-H4, L10-H5.
2	L10-H1, L10-H3.
3	L10-NH, L10-H1.
4	L10-H1, R9-H1, R9-H4.
5	L10-H1, R9-H1, R9-H4.
Ile11	
NH	I11-H1, I11-H2, I11-H3, I11-H4, I11-H5.
1	I11-H2, I11-H3, I11-H4, I11-H5, L2-NH, L12-NH, L12-H4.
2	I11-H1, I11-H4, I11-H5.
3	I11-H1, I11-H4, I11-H5.
4	I11-H1, I11-H2, I11-H3.
5	I11-H1, I11-H2, I11-H3.
Leu12	
NH	L12-H1, L12-H2, L12-H3, L12-H4, L12-H5, L1-NH, L1-H1, L1-H3, L1-H5, L2-NH, L2-H1, L2-H2, N7-H1, D8-H1, R9-H1, I11-H1, S13-NH.
1	L12-NH, L12-H3, L12-H4, L12-H5, L2-NH, S14-NH.
2	L12-NH, L12-H4, L12-H5, S13-NH, S13-H1,
3	L12-NH, L12-H4, L12-H5, L2-H1, I11-H1, S14-NH.
4	L12-NH, L12-H1, L12-H2, L12-H3, L1-NH, L1-H1, L2-NH, L2-H1, I11-H1. I11-H3, D8-H1, S13-NH, S13-H1.
5	L12-NH, L12-H1, L12-H2, L12-H3, L1-NH, L1-H1, L2-NH, L2-H1, I11-H1. I11-H3, D8-H1, S13-NH, S13-H1.
Ser13	
NH	S13-H1, S13-H2, L12-NH, L12-H1, L12-H4, L12-H5, L2-NH,
1	S13-NH, S13-H2, L12-H4, L12-H5, K14-NH, K14-H5, N15-NH.
2	S13-NH, S13-H1, L12-H4, L12-H5, K14-H2, K14-H3, N15-NH.
Lys14	
NH	K14-H2, K14-H3, K14-H4, S13-H1.
1	K14-H3, K14-H4, N15-NH.
2	K14-NH, S13-NH, S13-H1, N15-NH.
3	n. d.
4	K14-NH, S13-NH, S13-H1, N15-NH.
5	n. d.
NH2	n. d.
Asn15	
NH	N15-H1, N15-H2, S13-H2.
1	N15-NH, N15-NH2. N15-H2.
2	N15-NH, N15-NH2. N15-H1.
NH2	N15-H1, N15-H2.

Table S3: ^{1}H- and ^{13}C-NMR chemical shift assignment of 3. The ^{13}C-NMR chemical shifts were determined from the analysis of ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectra. Chemical shifts labeled with the same superscript (a, b, c, or d) are interchangeable.

Residue	Position	(ppm)	δ_C	Residue	Position	(ppm)	δ_C
Leu1	NH	8.35			3	1.58	24.2
	1	3.51	n.d.		4	0.76	[c]23.5
	2	1.26, 0.88	26.0		5	0.74	[c]23.8
	3	2.36	32.6	**Ile11**	NH	8.24	
	4	0.79	11.1		1	4.14	54.2
	5	1.19	19.2		2	1.59	n.d.
Leu2	NH	7.62			3	1.24, 1.35	[b]29.3
	1	4.32	50.4		4	0.82	[a]23.0
	2	1.68, 1.39	44.3		5	0.74	22.5
	3	1.52	n.d.	**Leu12**	NH	6.65	
	4	0.76	21.0		1	4.68	n.d.
	5	0.84	[a]14.4		2	0.86	n.d.
Gly3	NH	8.37			3	1.31	30.2
	1	4.03, 3.30	n.d.		4	0.51	[d]21.0
Arg4	NH	7.99			5	0.51	[d]24.0
	1	4.21	55.5	**Ser13**	NH	7.91	
	2	1.45, 1.98	27.1		1	4.20	n.d.
	3	1.21, 1.35	[b]23.7		2	3.51	70.3
	4	3.00, 3.16	38.8	**Lys14**	NH	8.38	
	NH_2	8.13, 7.81			1	4.05	49.5
Ser5	NH	8.55			2	1.44, 1.56	n.d.
	1	3.94	49.5		3	0.85, 1.25	22.7
	2	3.35, 3.59	61.7		4	1.74, 1.86	n.d.
Gly6	NH	7.35			5	3.43	n.d.
	1	3.94, 3.51	n.d.		NH_2	8.00	
Asn7	NH	8.01		**Asn15**	NH	7.61	
	1	4.76	50.4		1	4.14	51.8
	2	2.76,2.32	30.8		2	2.46, 3.13	44.2
	NH_2	6.81		**Glucose1**	1	4.97	102.5
Asp8	NH	6.26			2	3.97	64.0
	1	4.59	48.5		3	3.31	82.5
	2	3.21, 3.35	45.4		4	4.07	n.d.
Arg9	NH	8.43			5	3.68	70.2
	1	3.79	57.3		6	4.20, 4.28	64.0
	2	1.63	28.7	**Glucose1**	1	5.01	101.9
	3	1.47	27.5		2	3.94	66.2
	4	2.97	40.2		3	3.31	n.d.
	NH_2	6.22			4	3.49	60.9
Leu10	NH	8.72			5	3.75	72.6
	1	4.44	58.7		6	3.30,3.37	63.5
	2	1.35, 1.24	29.4				

Table S4: ^{1}H-^{1}H NMR NOESY correlations of 3.

Residue	Protons with correlations
Leu1	
NH	L1-H2, L1-H3, L1-H4, L2-NH, L2-H1, D8-H1, R9-H1, L10-H1, I11-H1, L12-NH.
1	L1-H3, L1-H4.
2	n. d.
3	n. d.
4	n. d.
5	n. d.
Leu2	
NH	L2-H1, L2-H2, L2-H3, L2-H4, L2-H5, L1-NH, G3-NH, R9-H1, L10-H1, I11-H1, S13-NH.
1	L2-NH, L2-H2, L2-H4, L2-H5, L1-NH, G3-NH.
2	L2-NH, L2-H1, L2-H4, L2-H5.
3	n. d.
4	L2-NH, L2-H1, L2-H2.
5	L2-NH, L2-H1, L2-H2.
Gly3	
NH	L2-H1.
1	n. d.
Arg4	
NH	R4-H2, R4-H3.
1	R4-NH, R4-NH2, R4-H3, S5-NH.
2	n. d.
3	n. d.
4	n. d.
NH2	R4-H1, R4-H2, R4-H3.
Ser5	
NH	R4-H1.
1	n. d.
2	n. d.
Gly6	
NH	n. d.
1	N7-NH.
Asn7	n. d.
NH	N7-H1, N7-H2, G6-H1.
1	N7-NH, N7-NH2, N7-H2.
2	
NH2	N7-H1, 7-H2.
Asp8	
NH	R9-H1.
1	R9-NH.
2	L1-NH, L10-NH, I11-NH.
Arg9	
NH	R9-H1, R9-H2, R9-H3, R9-H4, L2-NH, D8-H1, D8-H2, L10-H1.
1	R9-NH, R9-H2, R9-H3, R9-H4, L1-NH, L2-NH, L10-H1.
2	R9-NH2, R9-H1, R9-H4.

3	R9-NH2, R9-H1, R9-H4.
4	R9-NH2, R9-H2, R9-H3.
NH2	R9-H2, R9-H3, R9-H4.
Leu10	
NH	L10-H1, L10-H2, L10-H3, D8-H1.
1	L10-NH, L10-H2, L10-H3, L10-H4, L10-H5, L1-NH, L2-NH, R9-H1.
2	n. d.
3	n. d.
4	n. d.
5	n. d.
Ile11	
NH	I11-H1, I11-H2.
1	I11-NH, I11-H2, I11-H3, I11-H4, I11-H5, L1-NH, L2-NH, L12-NH.
2	n. d.
3	n. d.
4	n. d.
5	n. d.
Leu12	
NH	L12-H1, L12-H2, L12-H3, L12-H4, L12-H5, L1-NH, I11-H1, S13-H1.
1	n. d.
2	n. d.
3	n. d.
4	n. d.
5	n. d.
Ser13	
NH	S13-H1, L1-H2, G1-H1'.
1	S13-NH.
2	
Lys14	
NH	K14-H1, K14-H2, N15-H1, G1-H1'.
1	n. d.
2	n. d.
3	n. d.
4	n. d.
5	n. d.
NH2	n. d.
Asn15	
NH	N15-H1, N15-H2.
1	N15-NH, N15-NH2. N15-H2, L14-NH.
2	N15-NH, N15-NH2. N15-H1.
NH2	N15-H1, N15-H2.
Glucose1	
1'	G1-H2, G1-H3, G1-H5, K14-H1, S13-H1.
2'	G1-H2, G1-H3.
3'	G1-H1, G1-H2, G1-H5.
4'	G1-H5, G1-H6.
5'	G1-H1, G1-H4, G1-H6.
6'	G1-H4, G1-H5.
Glucose2	

1’	G2-H2, G2-H3, G2-H5, G1-H4.
2’	G2-H1, G2-H3,
3’	G2-H1, G2-H2,
4’	n. d.
5’	G2-H1.
6’	n. d.

Table S5: Sequences and IDs of proteins encoded by *igt* BGC.

Protein Name and ID	Primary sequences
IgtA CCK25647.1	MKKVYEAPTLVRLGSFRKQTGLLGRSGNDRLILSKN
IgtC CCK25646.1	MTELHESAAAGRGDAYCTVFPDCADAAAVARSFSRPGTQTLEHDSGRPWLIGRW RDDEIVQARAGSTVALALLGRSDVDAADLRRAAGRARNLSDIDTWARSLPGSFHL LACVDGRIHVRGTASGLRLVFHAEVDGVQVAATRADVLAAALGADPDPEQIAVR LLWPVPHPLYQAPMWRGITAVAPQDALELSADGHLARHTPWWTPPEPTVPLAMG APRVQEALVEAVAARTRQGGVVSCDLSGGLDSTSITFLAHRSPARVVASTWPGRD PADTDLYWAQQAARRLPGVEHVIWDAEDSPLVYTDLLDIDDLLDEPTIGVMDRSR VLHHLPALAEHGSRLHLTGIGGDHVAWCSEAYYHRMARTRPLFALRQLRGFKAL WQWPLGGMLRALADGRSYGTWLADAEHRMRDPLPDTVSAGLGWGMSPRLFAW VTPEAEDLAGAALRQAAERARPLHRDRGLHTDLEQIRSCSRIIRQWDHMAARTGV PMASPFLDDRVIEACLAVRPSERVSPWRYKPLLTAAMHGIVPEPSLRRSNKAAASM DAADGLRRHRGDLMALWEDSHLARMGLVDADMLRRLAQRPATPELRDAVLYSTI AAEVWLRSLSRVAHGPSSGLH
IgtB1 CCK25645.1	MALRFGADVSTAATDYGTVLLDQRTGQYWELNPTATLVIETLMAGGDEEAATTA LMREYDVPQSQARNDVNTLVQDLRASGLAT
IgtB2 CCK25644.1	VTTPSAIERAHDVPFGHRLAARAVFLPAVALSLLPPRHLRRFLELLRRGAAPADEER AKRARDAMCAVSLRCAGPKGCLPRSLGAALVCRLGGTWPTWCTGVRVVPPFTAH AWIEVAGHPVDEGVSDAYFARLIAVEPLSGSRRQ
IgtD CCK25643.1	VTARHKRGPGESGTARSSVAAVYGLTAGHRTAIAAATAFTLVASGLGLAQPLVAK EVVDASTGGGPVGLSLLLLGALFTAEAGTGALGRYLLERTGEGVVRQLRHGLVNR LLRLEMREYGRHRGGDLTARVTADTTLLREVVSQALVDLVTGALAAAGALVLMA WLDPLLLLVVTVTVAAAAAVVTSLLARIRAASETMQGAVGAISADLERALGALA MVRVHRAEDREAARIGARVDEARDAGVRTAKYAAVMSSAVELAVQGSFLLVLVI GGLRVGSGGDRSLGDLVAFLLYASYLVLPLSSVFRAIGLIQRGAGAQQRVEEALAL PVEPTTAEPAPPSPRTVSADAPALLLSDVHFSYRPGHPVLNGVSLSVPHRSQVALVG PSGAGKSTIFALIARFYEPDSGTLHFEGRPATSLTRPTCRERIAVVDQNNSVVHGTL RDNITYGVPDATDADVDRVVRLTRLETVVERLPGGLHGTVGEHGANLSGGERQR VALARALLTRPRLLLLDEPTSHLDAANETALTAALKEISRTCAVLVIAHRLSTVQHS DHINVLDHGRVIASGRHEELLAISTAYRTLAKGQTLRTTAASENALPTGAHSTQ
IgtG CCK25639.1	LSRSARRRHARSREPRGHWRLLVLTLPVVIVALLFEGWTAHEVDAAKSRIPCTEPV PEAVDKSGPVLRRIGGDHDVTSSAMPAGTVALTFDGGPDPVWTPRILDLLRRHHA HATFFVLGAQAARHPELIRRILAEGHEIGSHTYTGADLGSSSRVRTAMELTLTQKTL AGSAGIRTSLLRMPMTTEVDTLCGAEWTAARHAAADGYVLVAADRPDRDPAHG MVRQFSQTDLAYREAKDLLGNPHAKRFTTVTAALGMPSADTQVSTAERWQGRAL NWTTTAGHTFTHTMNWVMLALGVLGVLRLLMLTVFARAHVRRLTRFRPGSPWL REVQAPVTVLVPAYNEEAGIESTIHSLLASTHPYLEIVVIDDGSTDGTADLATWIDD PRVRVIRQPNAGKAAALNTGLAHASYDIVVMVDADTVFEPDALYRLIQPLAHPAV GAVSGNTKVGNRRGLLGRWQHLEYVFGFNLDRRMFEVLECMPTVPGAIGAFRRD ALLGVGGVSEDTLAEDTDLTMALWRAGWRVVYEESAIAWTEVPTSLRQLWRQR YRWCYGTLQAMWKHRGAVLEVGSAGRFARRGLSYLAIFQVVLPLIAPVVDLFLL YGVLFSDLRQSLGIWLTFLVLQLLCAGYALRLDGERLRTLWSMPFQLFVYRQLMY LVVIQSVFALLVGTRLKWHRMQRAGTAATEQLRQPVTARELSSN

Table S6: Oligonucleotide primers used in this study. Nucleotide sequences are given in the 5' to 3' direction. F, forward primer; R, reverse primer.

Primer name	Oligonucleotide sequence
4913-1.5- KasO-F1	CAGCGTGCAGGACTGGGGGAGTTCcgttgaggaggaattcttcatgaag
4913-1.5- KasO-R1	CACAGGAAACAGCTATGACATGATTACGttaacagctacgcctcgttcactgagtc
4913-1.5- KasO-F2	gactcagtgaacgaggcgtagctgtTGACGGCTGGCGAGAGGTGCG
4913-1.5- KasO-R2	cgggtgacgttaATCCTACCAACCGGCACGATTGTG
4913-1.5- KasO-F3	GGTTGGTAGGATtaacgtcacccgggggagtgtggttt
4913-1.5- KasO-R3	CACAGGAAACAGCTATGACATGATTACGttcagttcgacgacaactcccgtgcg
4913-1.5-Blue-F1	GTACCCACGATATCCACCTCGAGCACAgccaagacgttcttcaccgagatc
4913-1.5-Blue-R1	tcgcttgttcggCTTTCGACAATCGACTGTCACAGCTTC
4913-1.5-Blue-F2	CGATTGTCGAAAGccgaacaagcgaaaggaattccatg
4913-1.5-Blue-R2	CATCATCAGTGGTGGTGGTGGTGAgagcccagcaggagttccctgatc
4913-1.5-kaso-R4	CACAGGAAACAGCTATGACATGATTACGttaaccggcgagggtcttctgggtgag
4913-1.5-kaso-F4	ctcacccagaagaccctcgccggttc
4913-1.5GT-D418A-R	ggtgtcggcTGcgaccatcaccacgatgtcgtac
4913-1.5GT-D418A-F	gtgatggtcgCAgccgacaccgtcttcgaacc
4913-1.5GT-D420A-R	gaagacggtTGcggcgtcgaccatcaccacgatg
4913-1.5GT-D420A-F	gtcgacgccgCAaccgtcttcgaacccgacgccctgtatc
4913-1.5GT-2DA-R	gaagacggtTGcggcTGcgaccatcaccacgatgtcgtac
4913-1.5GT-2DA-F	gtgatggtcgCAgccgCAaccgtcttcgaacccgacgccctgtatc
4913-1.5C-KasO-F1	CAGCGTGCAGGACTGGGGGAGTTaactgactcggcggagcctgatc
1.5bGT-KasO-F	CAGCGTGCAGGACTGGGGGAGTTCtaacgtcacccgggggagtgtggttt

Table S7: Plasmids used in this study. Genes, vectors, cloning sites, and use of each plasmid were listed.

Gene	Vector	Cloning site	Use
Heterologous expression experiments			
igtABCD	pSET-KasO	AflII/EcoRI	Heterologous expression
igtABCDGT	pSET-KasO	AflII/EcoRI	Heterologous expression
Knockout experiments			
PZDBlue-igtAC	pZDBlue	HindIII	Knockout *igtAC*
Protein overproduction experiments			
igtA	pET His6 MBP TEV LIC	SspI	Precursor peptide co-expression
igtG	pSET-KasO	AflII/EcoRI	membrane protein expression

Table S8: Information of graspetide BGCs encoding IgtG-like GTs. PIMT, protein L-isoaspartyl methyltransferase.

Source strain	Precursor peptide	ATP-grasp ligase	PIMT	FAD binding monooxygenase	Methyltransferase	GT
S. iranensis	WP_044569794.1	WP_044569793.1, WP_044569796.1	WP_052701356.1	WP_044569800.1		WP_044569802.1
S. yogyakartensis	WP_198280035.1	WP_344316072.1, WP_198280034.1	WP_309142863.1	WP_198280032.1	WP_198280031.1	WP_198280030.1
S. sp. MnatMP-M27	WP_093702825.1	WP_093702824.1, WP_093702826.1	WP_256116303.1	WP_093702822.1	WP_093702820.1	WP_141729725.1
S. sp. PSAA01	WP_237510942.1	WP_093702826.1, WP_237510943.1	WP_237510940.1	WP_237510939.1		WP_237510937.1
S. sp. 5-10	WP_191065467.1	WP_191065466.1, WP_223863891.1	WP_317985806.1	WP_191065464.1	WP_191065463.1	WP_191065462.1
S. niphimycinicus	WP_216340605.1	WP_216340604.1, WP_216340606.1	WP_253208783.1	WP_216340608.1	WP_216340610.1	WP_216340611.1
S. solisilvae-BGC1	WP_102937250.1	WP_383139007.1, WP_383139009.1	WP_383139013.1	WP_383139013.1	WP_174882750.1	WP_383139019.1
S. solisilvae-BGC2	WP_147968509.1	WP_069866715.1, WP_383167271.1	WP_231981287.1	WP_383167264.1	WP_383167259.1	WP_383167256.1
S. sp. DR7-3	WP_102937250.1	WP_251771628.1. WP_251771629.1	WP_251771630.1	WP_251771632.1	WP_251771634.1	WP_251771635.1
S. malaysiensis-BGC1	WP_147968509.1	WP_147968510.1, WP_347231720.1	WP_232637830.1	WP_147968512.1	WP_147968514.1	WP_147968515.1
S. sp. 8ZJF_21	WP_093702825.1	WP_232637828.1, WP_232637829.1	WP_232637830.1	WP_232637831.1	WP_232637833.1	WP_232637834.1
S. samsunensis	WP_102937250.1	WP_174882746.1, WP_174882747.1	WP_232637830.1	WP_174882748.1	WP_174882750.1	WP_174882751.1
S. malaysiensis-BGC2	WP_102937250.1	WP_102937249.1. WP_102937251.1	WP_232637830.1	WP_102937252.1	WP_102937254.1	WP_102937255.1
S. sp. NBRC 110028	WP_055547559.1	WP_055547557.1. WP_055547561.1	WP_055547551.1, WP_055547551.1			
S. malaysiensis-BGC3	WP_102937250.1	WP_251771628.1, WP_347235296.1	WP_232637830.1	WP_347235298.1	WP_347235300.1	WP_347235301.1
S. solisilvae-BGC3	WP_363326815.1	WP_363326814.1, WP_363326816.1	WP_232564720.1	WP_363326817.1	WP_363326819.1	WP_363326820.1
S. solisilvae-BGC4	WP_093702825.1	WP_383124950.1, WP_174882746.1	WP_383124948.1	WP_383124946.1	WP_383124943.1	WP_383124941.1
S. solisilvae-BGC5	WP_093702825.1	WP_383153908.1, WP_383153910.1	WP_383124948.1	WP_383153915.1	WP_383153920.1	WP_383153922.1
S. solisilvae-BGC6	WP_392985787.1, WP_392985812.1	WP_257633098.1, WP_392985788.1, WP_392985790.1	WP_392985785.1	WP_392985782.1	WP_392985778.1	WP_392985777.1
S. sp. M56		AUA11128.1	AUA11130.1	AUA11132.1	AUA11134.1	AUA11135.1
S. malaysiensis-BGC4	MFD0652562.1	MFD0652561.1, MFD0652563.1	MFD0652560.1	MFD0652558.1	ACFQ2Y_32490	MFD0652557.1
S. sp. SID8382		WP_100806112.1	WP_231981287.1	WP_100806115.1	WP_100806117.1	WP_100806118.1
S. sp. HNM0561	WP_102937250.1	WP_206333342.1, WP_206333343.1	WP_232564720.1	WP_206333341.1	WP_206333339.1	WP_232564719.1
S.sp. NPDC056697	WP_383139409.1	WP_383139412.1, WP_383139404.1	WP_383139415.1	WP_383139418.1	WP_383139421.1	WP_383139424.1

Table S9: Sequences of representative IgtG-like and know RiPP GTs for ML tree construction.

>IgtG

MSRSARRRHARSREPRGHWRLLVLTLPVVIVALLFEGWTAHEVDAAKSRIPCTEPVPEAVDKSG
PVLRRIGGDHDVTSSAMPAGTVALTFDGGPDPVWTPRILDLLRRHHAHATFFVLGAQAARHPEL
IRRILAEGHEIGSHTYTGADLGSSSRVRTAMELTLTQKTLAGSAGIRTSLLRMPMTTEVDTLCGAE
WTAARHAAADGYVLVAADRPDRDPAHGMVRQFSQTDLAYREAKDLLGNPHAKRFTTVTAAL
GMPSADTQVSTAERWQGRALNWTTTAGHTFTHTMNWVMLALGVLGVLRLLMLTVFARAHVR
RLTRFRPGSPWLREVQAPVTVLVPAYNEEAGIESTIHSLLASTHPYLEIVVIDDGSTDGTADLATW
IDDPRVRVIRQPNAGKAAALNTGLAHASYDIVVMVDADTVFEPDALYRLIQPLAHPAVGAVSGN
TKVGNRRGLLGRWQHLEYVFGFNLDRRMFEVLECMPTVPGAIGAFRRDALLGVGGVSEDTLAE
DTDLTMALWRAGWRVVYEESAIAWTEVPTSLRQLWRQRYRWCYGTLQAMWKHRGAVLEVG
SAGRFARRGLSYLAIFQVVLPLIAPVVDLFLLYGVLFSDLRQSLGIWLTFLVLQLLCAGYALRLD
GERLRTLWSMPFQLFVYRQLMYLVVIQSVFALLVGTRLKWHRMQRAGTAATEQLRQPVTAREL
SSN

>SirG

MFGLLMFQGWTNHEVDAAKARRPCTSPVPRALADGGPVVQINGGRVRTVGMPAGTVALTYDG
GPDPVQTPRLLDLLRRYDARATFFVSGAKAAQYPGLVRRIRAEGHEIGSNTYTGADMGTASSSR
SRMELSLTESALAGSVGVQPRLLRLPLTTDVDTLCGDEWQAARRVAAEGYALVAADRSGTKPS
QGMIRQLSQTDTAYQETERLLKDPRAKKFTTVTGGLGVPPVDVPVSGLERWEGKALIWVAAIGR
GFVSTMAWVLGIAGALGVLRLLMLVVFARTHVRRLTRFRPGKQVNEPVTVLIPAYNEEAGIEAT
IRSLLASTHQRLQVVVIDDGSTDRTADIAASVRDRRVLVVRQPNAGKAAALNTGLAHTKHDIVV
MVDADTVFEPDAIHQLIQPLAHPAIGAVSGNTKVGNRRSLLGRWQHLEYVFGFNLDRRMFEVLE
CMPTVPGAIGAFRRDAVMGVGGVSEDTLAEDTDLTMALWRAGWRVVYEETAVAWTEVPTSLR
QLWRQRYRWGYGTLQAMWKHRRAMTSLGSVGRFGRRGLSYLTLFQVLLPLLAPVIDLFALYG
ALFLDPAEAAGVWFGYLTIQVVCAGYALRLDGERMRALWSLPFQLFVYRQLMYLVVIQSVVAL
LLGTRLRWHRIQRSGTAAQALGEAPGHRSLTSR

>soSirG

MFEGNRSKKRQEPSHLTRGGRHKRSRDPRAHWLLLLVLPAMFGLLMFQGWTDHEVDAAKTR
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FFVSGAKAAQHPELVRRIRAEGHEIGSNTYTGADMGTASPTRSRMELSLTESALAGSVGVQPRLL
RLPLTTDVDTLCGDEWQAARRVAAEGYALVAADRSGRKPSQGVVRQFSQTDTAYQETEKLLK
DPRARKFTTVTGGLGVPPVDVPVSGLERWEGKALIWVAAIGRGFVSTMVWVLGIAGALGVLRL
LMLVLFARAHVRRLTRFRPGAPWLRQVNEPVTVLIPAYNEEAGIEATIRSLLASTHQRLQVVVID
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AVQTACAGYALRLDGERIRALWSLPFQLFVYRQLMYLVVIQSVVALLLGTRLRWHRGRRSGTA
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>maSirG

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>yoSirG

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TFFVSGAKAAQYPDLVRRIRAEGHEIGSNTYTGADMGTASSSRSRMELSLTESALAGSVGVQPRL
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KHRRAVISLGSVGRFGRRGLSYLTLFQVLLPLLAPVIDLFALYGALFLDPAEAAGVWFGYLTVQV
VCAGYALRLDGERMRALWSLPFQLFVYRQLMYLVVIQSVVALLLGTRLRWHRIQRSGTAAQAL
GEAPAHRSLTSR

>saSirG

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RLPLTTDVDTLCGDEWQAARRVAAEGYALVAADRSGRKPSQGVVRQFSQTDTAYQETEKLLK
DPRARKFTTVTGGLGVPPVDVPVSGLERWEGKALIWVAAIGRGFVSTMVWVLGIAGALGVLRL
LMLVLFARAHVRRLTRFRPGAPWLRQVNEPVTVLIPAYNEEAGIEATIRSLLASTHQRLQVVVID
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AAQTLGEAPAYRSLTSR

>Thus

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QSTEARNKFLRRVKTINTHISKKI

>SunS

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YFRSAILLVDMQNKLTSLSNMIDEALTDERYSAINTTKDHFKRILISLNIQLENWERVKEISGEIKN
DNMKKEIKQYLANSLHNIEHVLKGIEV

>GccA

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GDELIVLDTGSTDETVHLVKKNMPQAKISVTNWKNDFSEVRNKALKLASKDWVFYVDSDEWL
DVDDGAQLKKILFKVQAKNFKFVINPTFSDHSGQIYQTVGRIFPKKSSFHYYAKIHEEVRKEDQK
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>PerS4

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RFRAQAPAPPPVTGEGTAEAMVAHLFGTCSVDMAAAAVALAADWRPDCIVHSALDGAGPMAA
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VAAR

>SrGT822

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PLGTLPPNVRPLPWVPLAQLLDTADAVVHHGGSGTMLTAAARGVPQLILPQGADHFINVDAATG
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>Af-GT3

MRVLFVTTPVIAHTYPLVTTAWALRAAGHDVLVATCGATAPRVARAGLAVTDTAPGLELGDVF
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QIRETYAGAGVAADFAAGIDVAPDSMRVGERLGWSARYVPFNEGSVLPAWVREPAPRRRVLVT
LGTTVPSMVGLARLTPLWKTIDAAADVDAEFVIALGDIDTTELGELPPNVRVVPGWLPLIALLRT
CDAAVHHGGSGTLMAVLDAGLPQLVLPQGADQFANAEAVRKRGVGLVRTPDGLSAAAITELLG
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>AplG

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QIPERLGVPGVLTVLYPFYVPSRHYPSTLLGSLGTAPRTLNRLSHALARRRRPAPEVAAAAAAWR
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LTDFLAAGPPPLAVGFGSLVGTDPKAAGRHVAAAIRATGHRAVVVTGWGGISIPDPPPEILVTSD
VPYEWLLPRARLAVHAGGTGTLHTATAAGLPQVACPFHREQAQWSRRLHRLGVAPAPLHQRDL
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>Cao8

MNGNTPDTRSPGTEPEYVTLRAARRTAGKSRIDVICPTHNRSTRIRPTLDSVLAQSVGDWRLVVV
SDGSTDDTEDVVRAYDDPRIALLRCPPHGHPGGPRNIGLHHAGAPFAAYIDHDDTWRPDHLAVL
LEMLESGAQAAATGCRRLTPEGGVEDVGAVDMVWHPEIQALAGLNEPSRVGHVRELVPRVGG
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EAVVERLREPRTARRMRDGARADLAAWHTRLAATPDYVVPRGHTVAALRDACLERVARMEN
QHGYQSVFTGPKGDGWALFLTLWCTQPAHASRVSGLLARRDVRQRAVLAELVEECERHAGRG
SGHRVGS

>Cao16

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TYIDHEGRETGRTEPADMVWHPDLQAVYALFEPARVGHVRGVVESVGGWTTDTAGFEDWDL
WWRLGEAGHAFQPVLERTAVIYRGSDTRTESVRARYAIPVGRTDSEDAARGCLDALADDGTRD
RLAALYAADFADWWDALARDDRFRTAPGTARAEVLGALRERSDGARTHVFTQLRHARRRDGH
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>Cao24

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RGPVRLGQEHLTLATHSARLKRVLRSNYPRLDAVTTVTEADARDYRKHMWLPGVHVQSLPNSV
PEPAVSPADGSGKWVVAAGRLAPAKRYDVLVRAFAEVVSERPDWGLRIYGHGREEGKLRKLID
ELGLYNHVFLMGSANPIEAEWVKGAICAVTSSLESFGMTIVEAMRCGLPVVATDCPHGPKEIIRD
GEDGLLVPTGDVRAISGALLRLIDDEGLRTRMGKAALTASARFDPAEVAARYEELLTSLVNHRP
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>PsmN

MYTGCNLDLSHLPKELKLLLEIIKKEDKEIQEIPGDWFINIDWNKFLKLALHHRMYAFIYPKMKSI
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TSGDLDALVPIEDLGKVNELLVKNGYVKEDDFPTVMNEWKWRRHHTTYMHPISKVKLEIHWRL
HPGPGKEPRFDELWGRKRTSPVTNYPVYFLGREDLFMFLVTHGTRHGWSRLRWLTDIDRMVRQ
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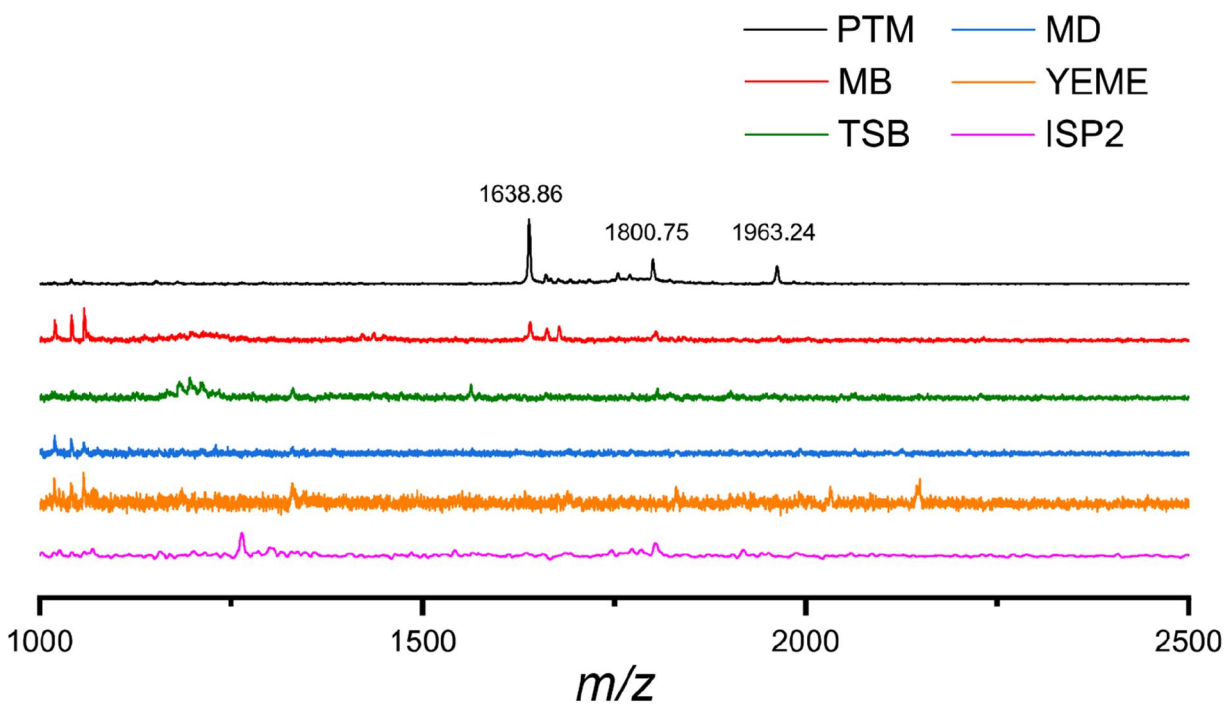


Figure S1. MALDI-TOF MS analysis of *S. davaonensis* JCM 4913 extracts using indicated media.

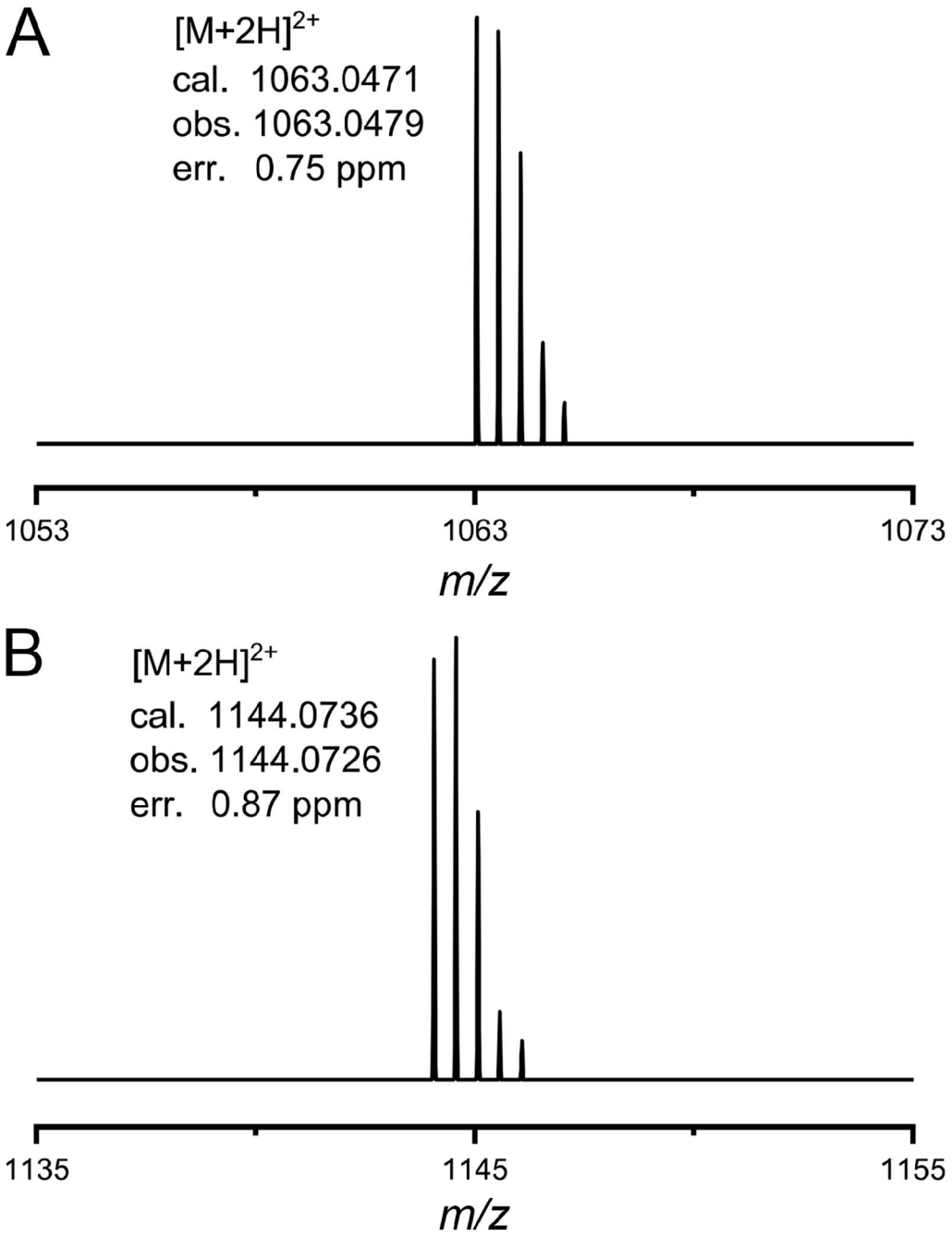
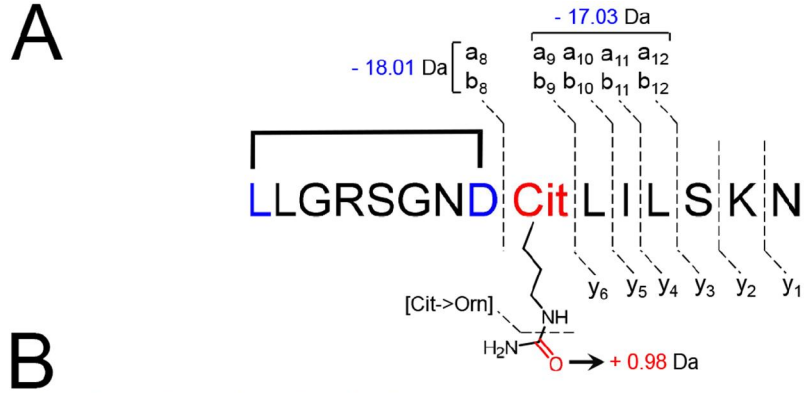
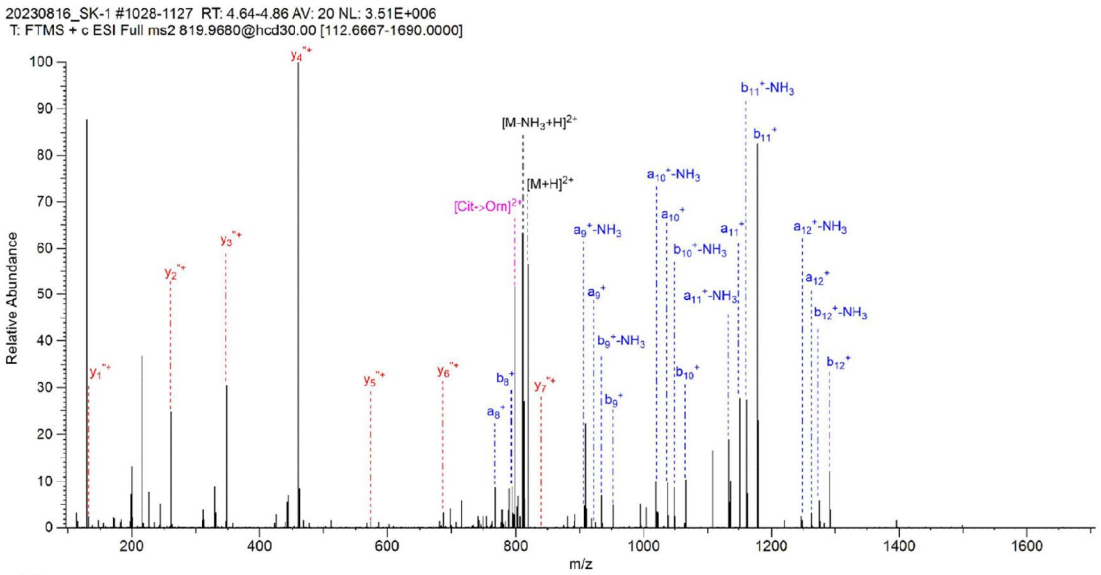


Figure S2. HRMS spectra of lasso peptides with three (A) and four (B) glycosyl groups from *S. davaonensis* JCM 4913.

A



B

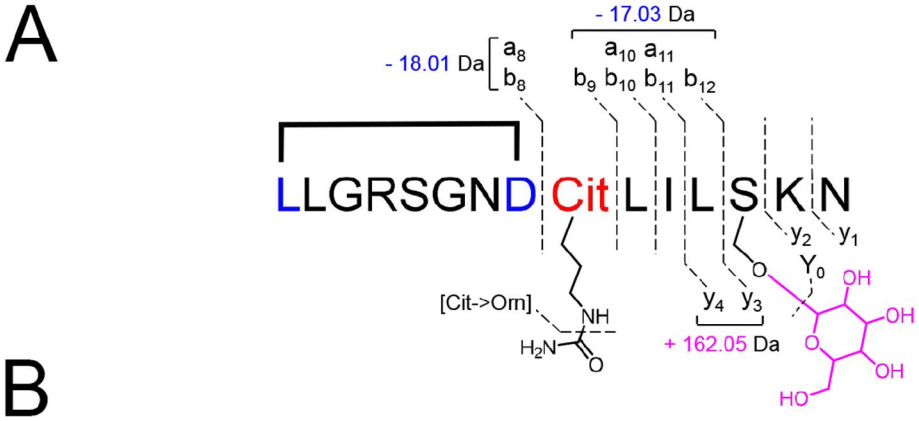


C

number	Cal. (Da)	Obs. (Da)	Error (ppm)	number	Cal. (Da)	Obs. (Da)	Error (ppm)
b_8^+	795.4108	795.4111	0.4023	y_1^+	133.0608	133.0605	-1.9540
a_8^+	767.4159	767.4159	0.0391	y_2^+	261.1557	261.1554	-1.2253
b_9^+	952.4959	952.4960	0.1260	y_3^+	348.1878	348.1876	-0.5744
b_9^+-NH_3	935.4694	935.4674	-2.0845	y_4^+	461.2718	461.2713	-1.1924
a_9^+	924.5010	924.5018	0.9194	y_5^+	574.3559	574.3550	-1.6366
a_9^+-NH_3	907.4744	907.4739	-0.5840	y_6^+	687.4400	687.4383	-2.4584
b_{10}^+	1065.5800	1065.5789	-1.0229	y_7^+	844.5251	844.5255	0.5447
b_{10}^+-NH_3	1048.5534	1048.5522	-1.1349	$[Cit->Orn]^{2+}$	798.4650	798.4638	-1.5780
a_{10}^+	1037.5851	1037.5818	-3.1226	$[M+H]^{2+}$	819.9679	819.9667	-1.5123
a_{10}^+-NH_3	1020.5585	1020.5566	-1.8225	$[M+H-NH_3]^{2+}$	811.4547	811.4537	-1.1584
b_{11}^+	1178.6640	1178.6618	-1.9005				
b_{11}^+-NH_3	1161.6375	1161.6357	-1.4979				
a_{11}^+	1150.6691	1150.6672	-1.6425				
a_{11}^+-NH_3	1133.6426	1133.6419	-0.6351				
b_{12}^+	1291.7481	1291.7457	-1.8270				
b_{12}^+-NH_3	1274.7167	1274.7216	3.8205				
a_{12}^+	1263.7532	1263.7494	-3.0307				
a_{12}^+-NH_3	1246.7266	1246.7242	-1.9411				

Figure S3. HRMS/MS analysis of 1. (A) Annotation of fragment ions of **1**. (B) HRMS/MS spectrum of **1**. (C) Detailed information of identified fragment ions.

A



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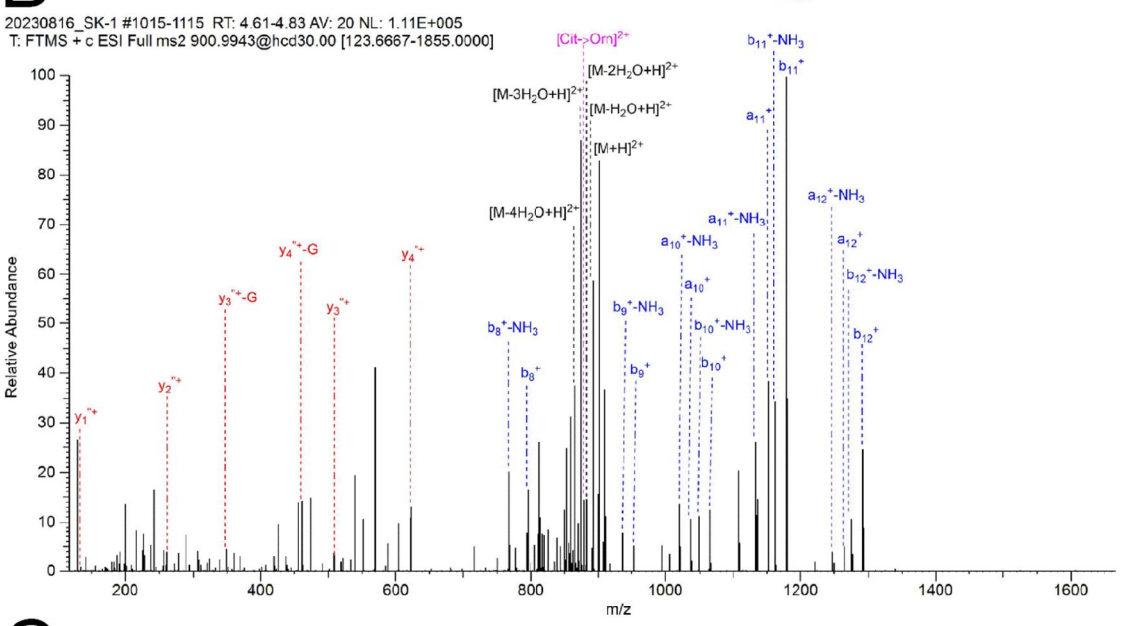
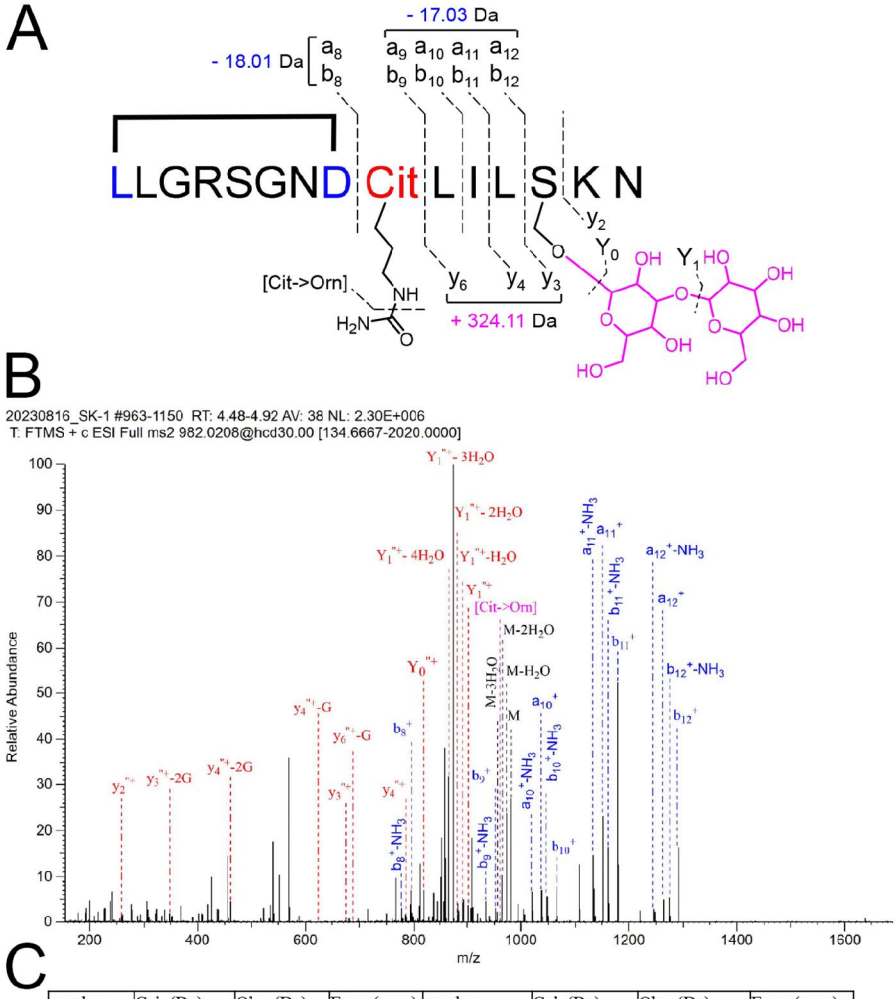


C

number	Cal. (Da)	Obs. (Da)	Error (ppm)	number	Cal. (Da)	Obs. (Da)	Error (ppm)
b_8^+	795.4108	795.4148	5.0414	y_1^+	133.0608	133.0607	-0.9018
a_8^+	767.4159	767.4159	0.0521	y_2^+	261.1557	261.1556	-0.4978
b_9^+	952.4959	952.4954	-0.5144	y_3^+	510.2406	510.2407	0.2940
b_9^+-NH_3	935.4694	935.4675	-2.0097	y_3^+-G	348.1878	348.1875	-0.6893
b_{10}^+	1065.5800	1065.5787	-1.2012	y_4^+	623.3247	623.3248	0.1925
b_{10}^+-NH_3	1048.5534	1048.5560	2.4605	y_4^+-G	461.2718	461.2713	-1.1490
a_{10}^+	1037.5851	1037.5862	1.0987	$[Cit->Orn]^{2+}$	879.4914	879.4877	-4.2979
a_{10}^+-NH_3	1020.5585	1020.5565	-1.9597	$[M+H]^{2+}$	900.9943	900.9923	-2.2975
b_{11}^+	1178.6640	1178.6626	-1.1793	$[M+H-H_2O]^{2+}$	891.9891	891.9866	-2.7691
b_{11}^+-NH_3	1161.6375	1161.6361	-1.2224	$[M+H-2H_2O]^{2+}$	882.9838	882.9819	-2.1518
a_{11}^+	1150.6691	1150.6670	-1.8859	$[M+H-3H_2O]^{2+}$	873.9785	873.9778	-0.7552
a_{11}^+-NH_3	1133.6426	1133.6399	-2.3993	$[M+H-4H_2O]^{2+}$	864.9732	864.9735	0.2775
b_{12}^+	1291.7481	1291.7473	-0.6580	Y_0^{2+}	819.9679	819.9687	0.9269
b_{12}^+-NH_3	1274.7167	1274.7195	2.2358	$[Y_0-NH_3]^{2+}$	811.4547	811.4552	0.6285

Figure S4. HRMS/MS analysis of 2. (A) Annotation of fragment ions of **2**. (B) HRMS/MS spectrum of **2**. (C) Detailed information of identified fragment ions.



number	Cal. (Da)	Obs. (Da)	Error (ppm)	number	Cal. (Da)	Obs. (Da)	Error (ppm)
b_8^+	795.4108	795.4153	5.7329	y_2^+	261.15573	261.15627	2.067732
b_8^+-NH_3	778.3842	778.3858	2.0170	y_3^+	672.29341	672.29222	-1.77006
a_8^+	767.4159	767.4159	0.0521	y_4^+	785.37747	785.37415	-4.227267
a_8^+-NH_3	750.3893	750.3886	-0.9328	y_3^+-2G	348.18776	348.18825	1.407287
b_9^+	952.4959	952.4991	3.3386	y_4^+-2G	461.27182	461.27119	-1.365789
b_9^+-NH_3	935.4694	935.4639	-5.7939	y_4^+-G	623.32465	623.32153	-5.005417
a_9^+	924.5010	924.5007	-0.3461	y_6^+-2G	687.43995	687.44012	0.247294
a_9^+-NH_3	907.4744	907.4749	0.4959	M	982.02075	982.01921	-1.568195
b_{10}^+	1065.5800	1065.5779	-1.9801	M-H_2O	973.01547	973.01468	-0.811909
b_{10}^+-NH_3	1048.5534	1048.5515	-1.7929	M-$2H_2O$	964.01019	964.00913	-1.099573
a_{10}^+	1037.5851	1037.5832	-1.8023	M-$3H_2O$	955.0049	955.00351	-1.45549
a_{10}^+-NH_3	1020.5585	1020.5570	-1.4404	Y_1^+	900.99434	900.99276	-1.753618
b_{11}^+	1178.6640	1178.6625	-1.3150	Y_1^+-H_2O	891.98906	891.98708	-2.219758
b_{11}^+-NH_3	1161.6375	1161.6364	-0.9469	Y_1^+-$2H_2O$	882.98377	882.9828	-1.098548
a_{11}^+	1150.6691	1150.6681	-0.8951	Y_1^+-$3H_2O$	873.97849	873.97756	-1.064099
a_{11}^+-NH_3	1133.6426	1133.6407	-1.6143	Y_1^+-$4H_2O$	864.97321	864.97309	-0.138733
b_{12}^+	1291.7481	1291.7470	-0.8206	Y_0^+	819.96793	819.96506	-3.500137
b_{12}^+-NH_3	1274.7167	1274.7198	2.4162	[Cit->Orn]	960.51784	960.52228	4.622507
a_{12}^+	1263.7532	1263.7508	-1.8754				
a_{12}^+-NH_3	1246.7266	1246.7230	-2.8795				

Figure S5. HRMS/MS analysis of 3. (A) Annotation of fragment ions of **2**. (B) HRMS/MS spectrum of **3**. (C) Detailed information of identified fragment ions.

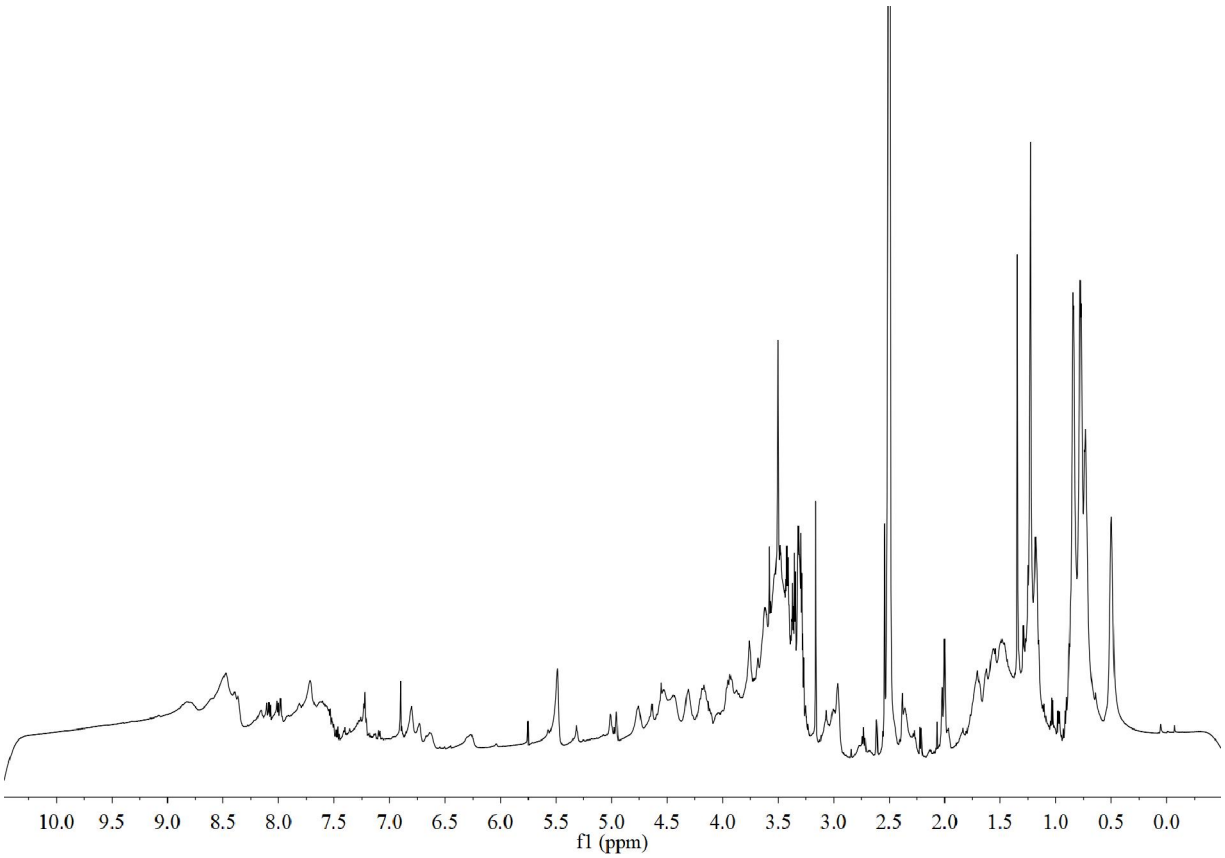


Figure S6. ^{1}H NMR spectrum of 1 in DMSO-d_6.

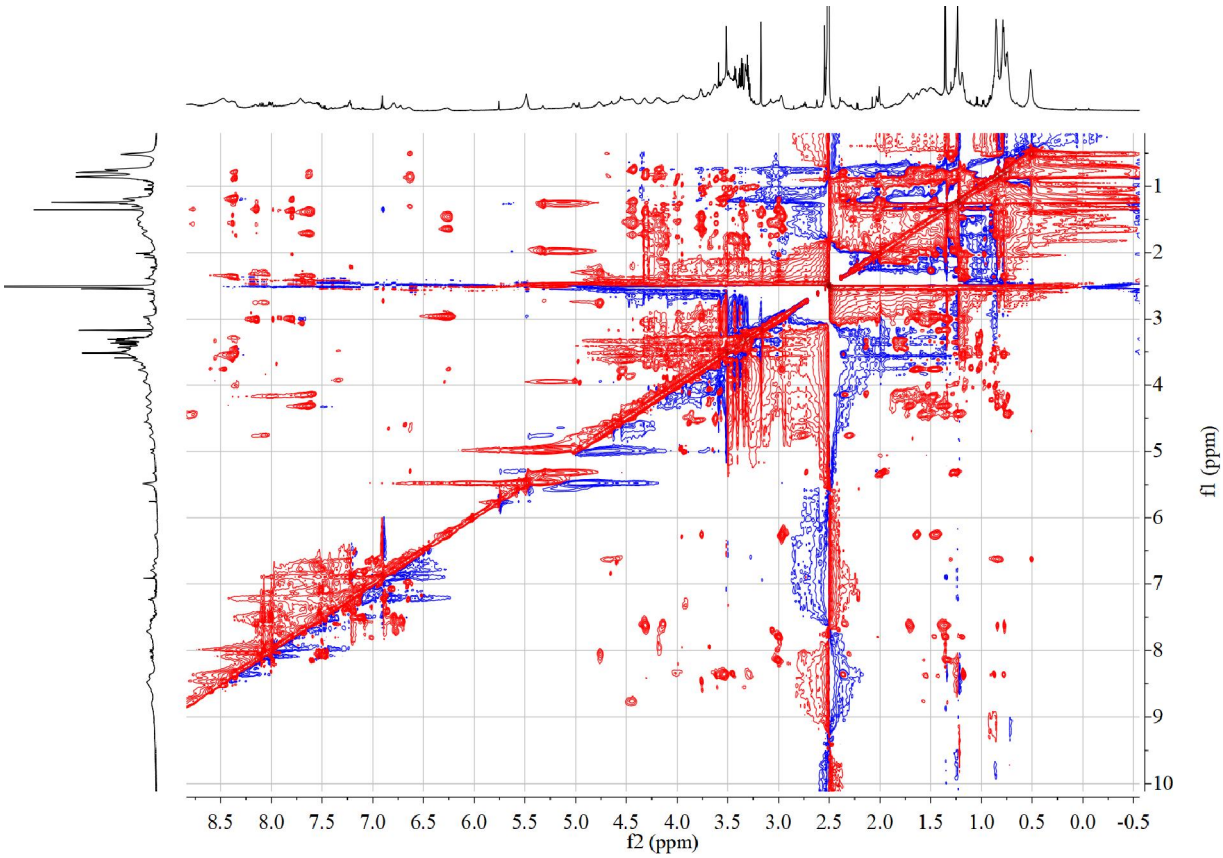


Figure S7. ^{1}H-^{1}H TOCSY NMR spectrum of 1 in DMSO-d_6.

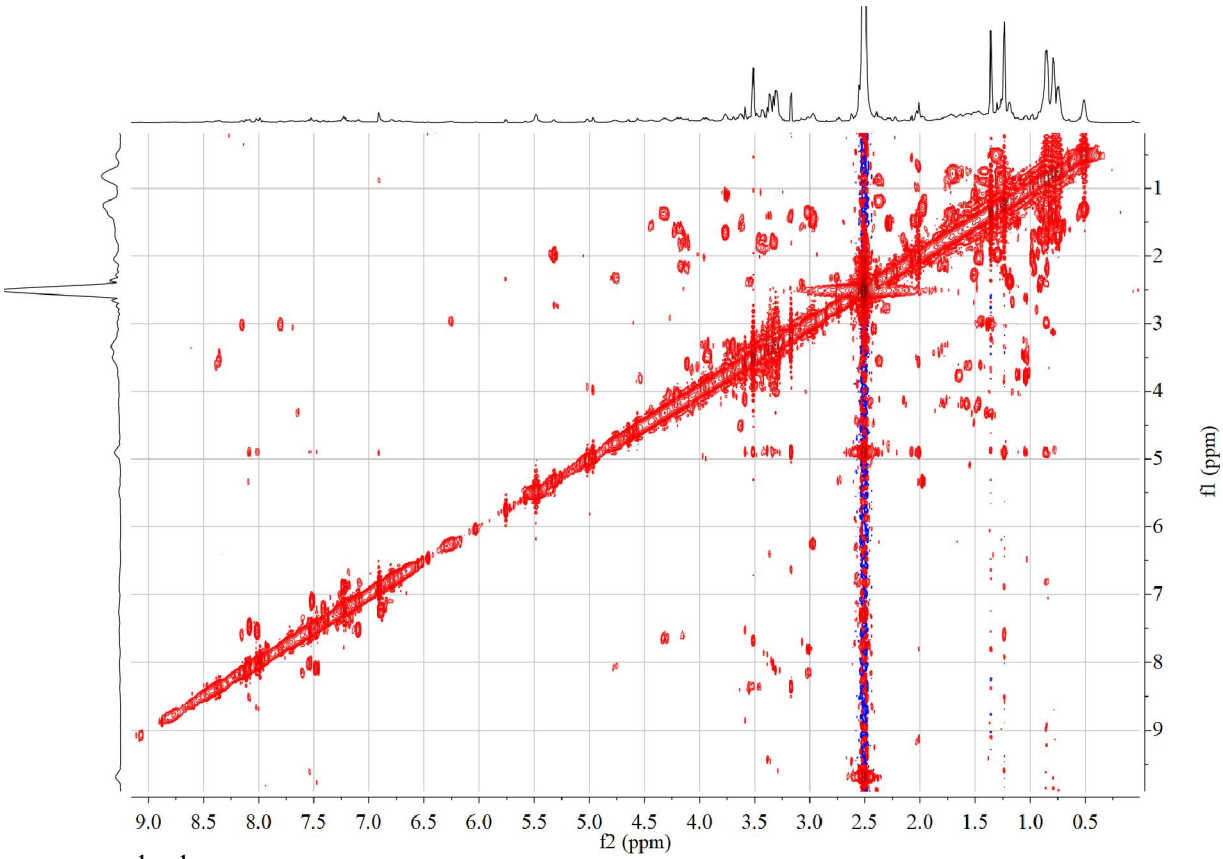


Figure S8. ^{1}H-^{1}H COSY NMR spectrum of 1 in DMSO-d_6.

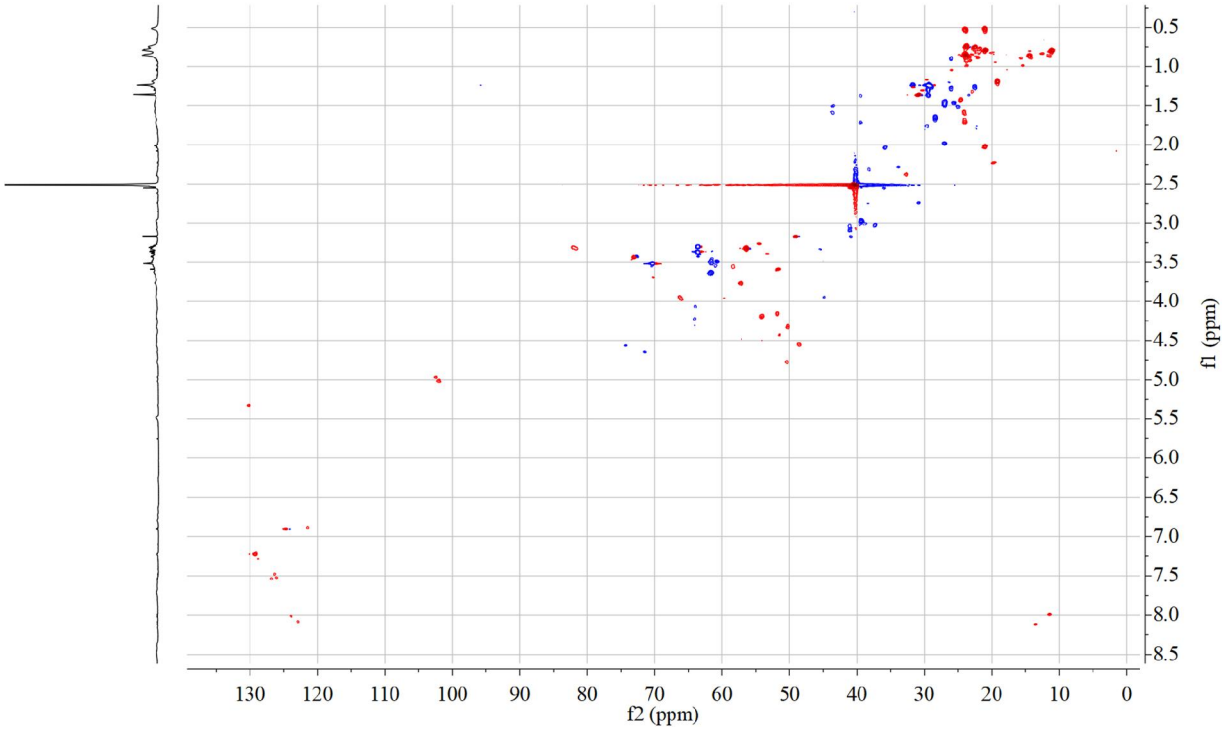


Figure S9. ^{1}H-^{13}C HSQC NMR spectrum of 1 in DMSO-*d*$_6$.

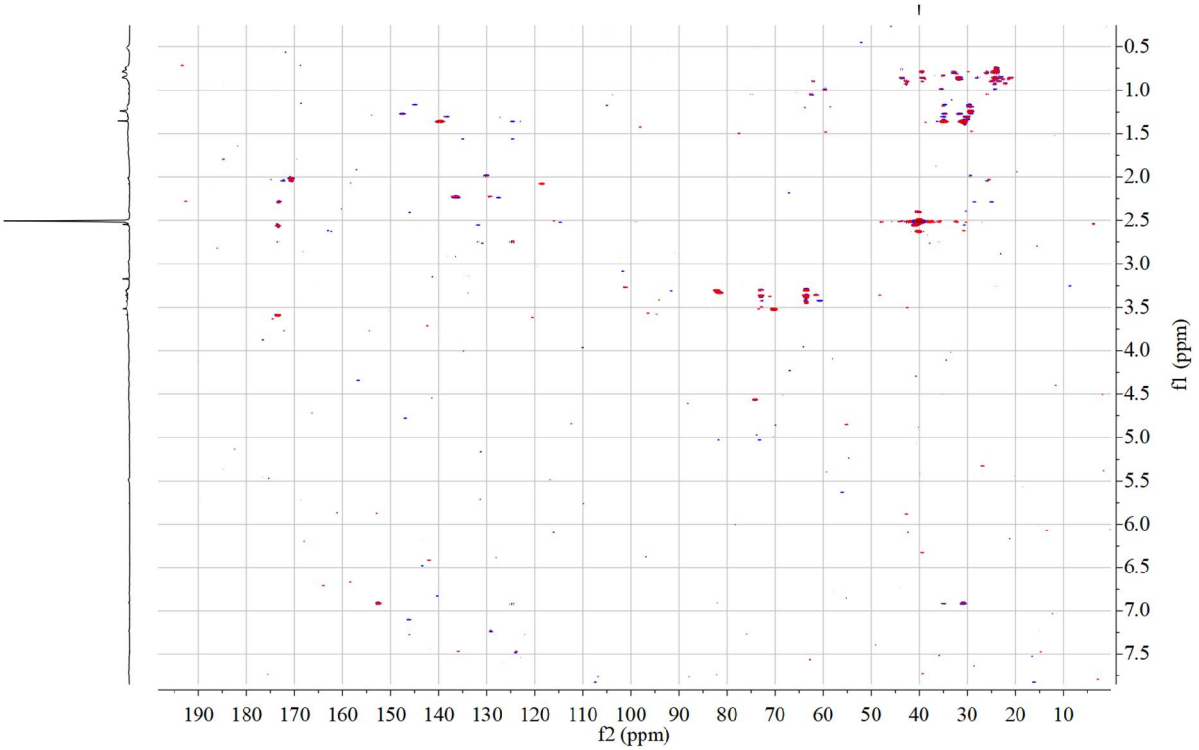


Figure S10. ^{1}H-^{13}C HMBC NMR spectrum of 1 in DMSO-d_6.

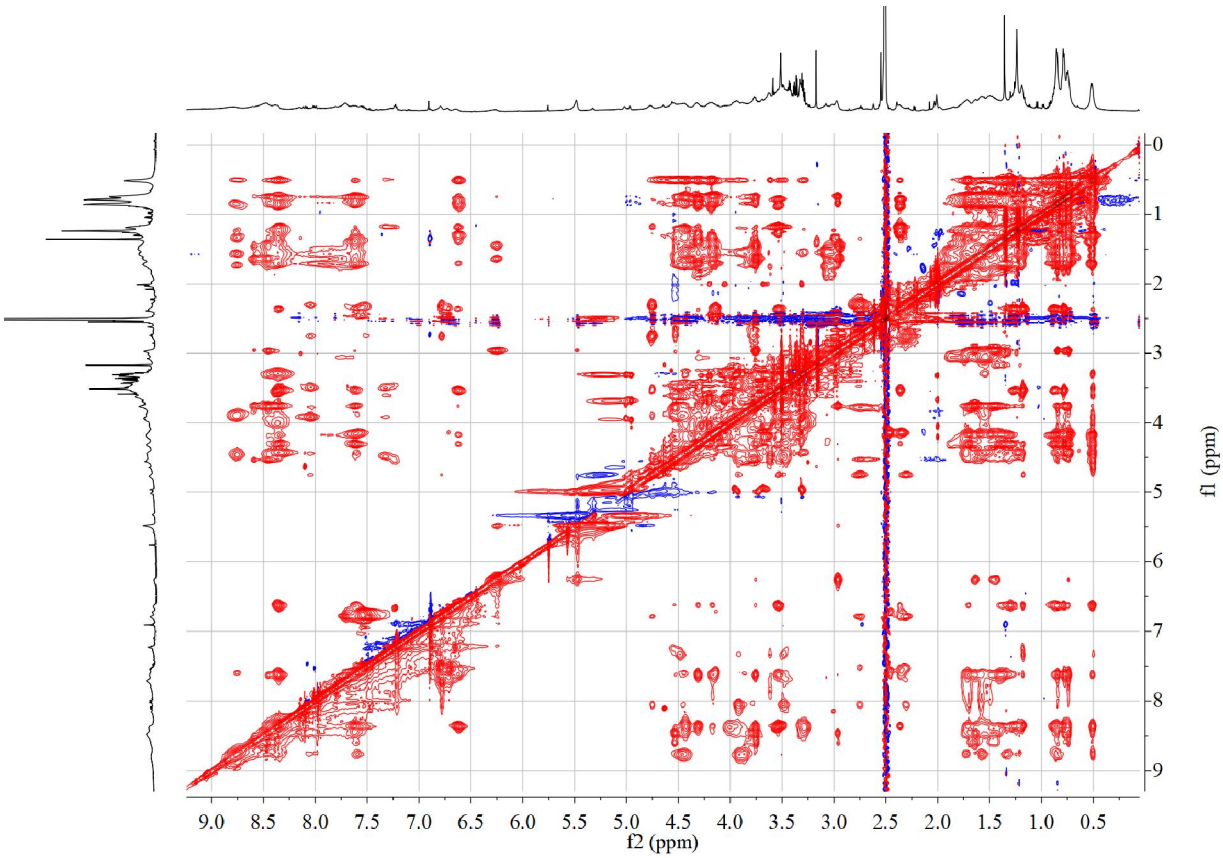


Figure S11. ^{1}H-^{1}H NOESY NMR spectrum of 1 in DMSO-d_6.

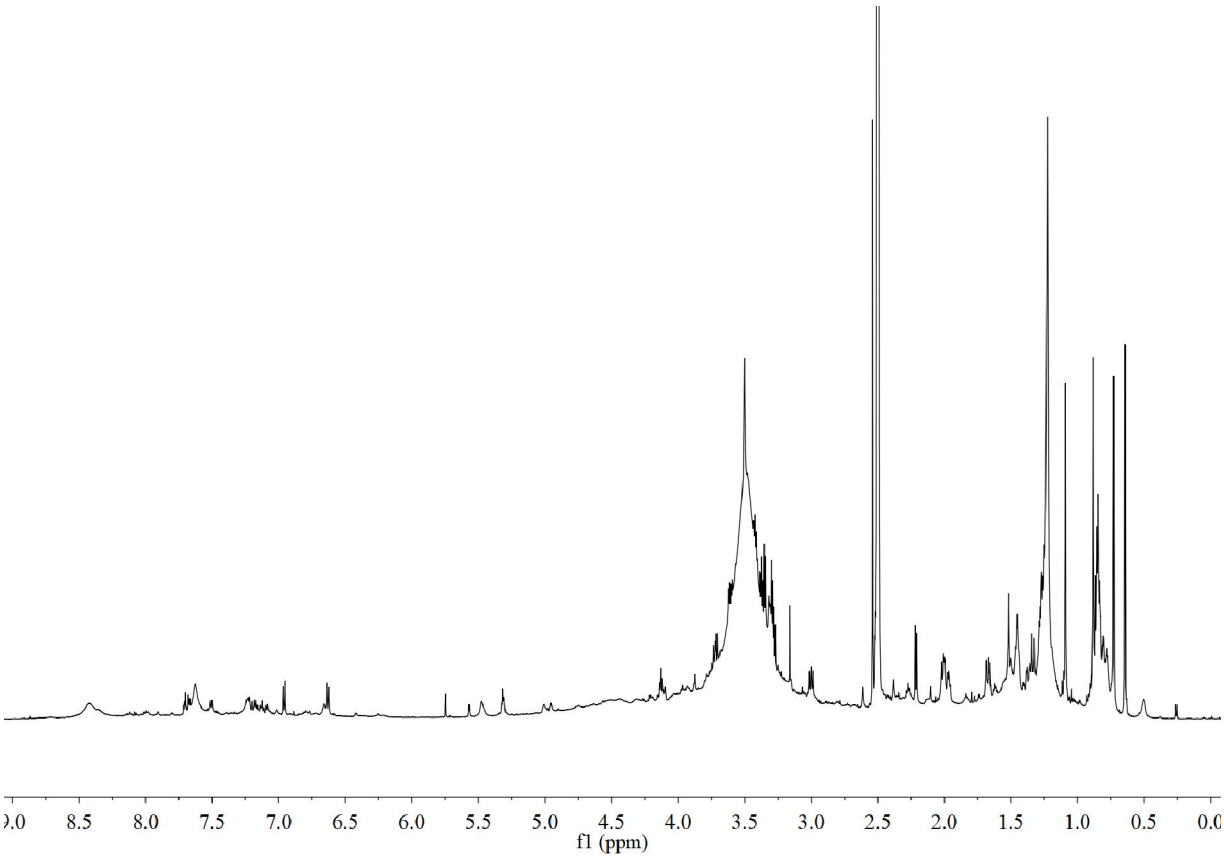


Figure S12. ^{1}H NMR spectrum of 3 in DMSO-d_6.

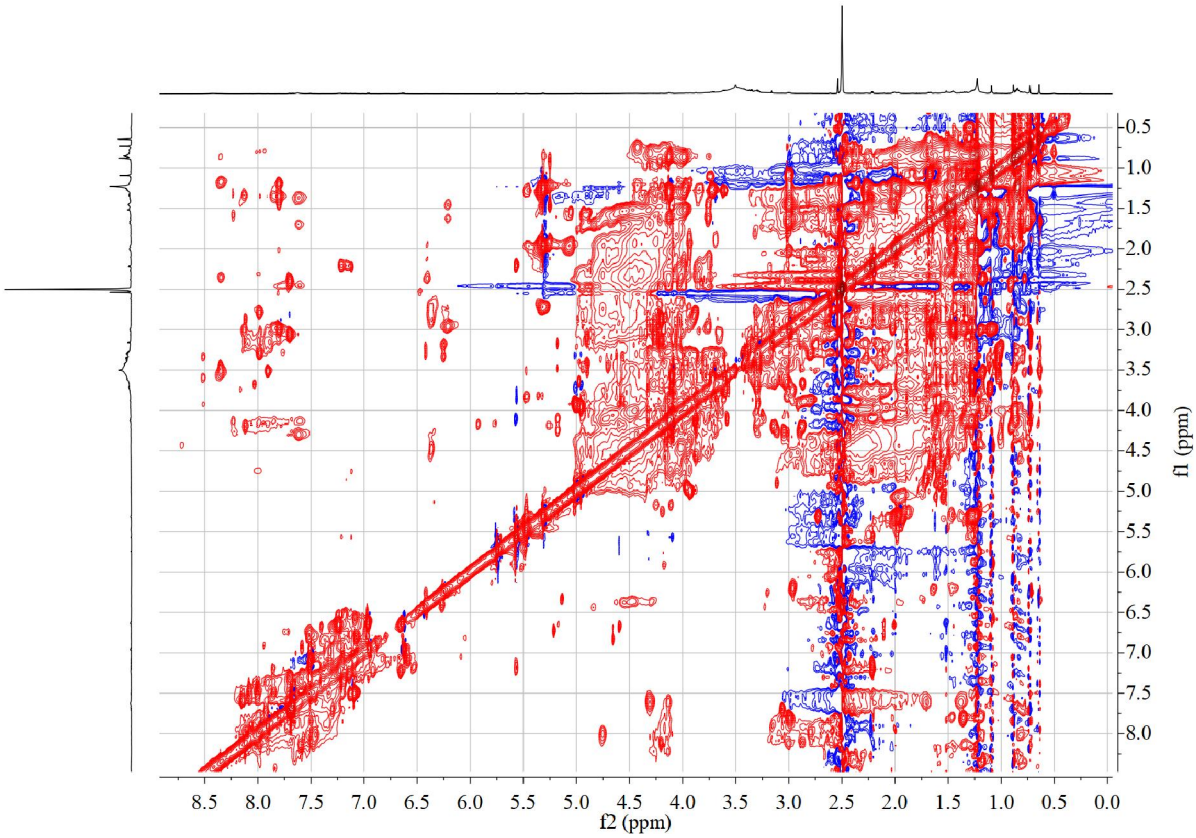


Figure S13. ^{1}H-^{1}H TOCSY NMR spectrum of 3 in DMSO-d_6.

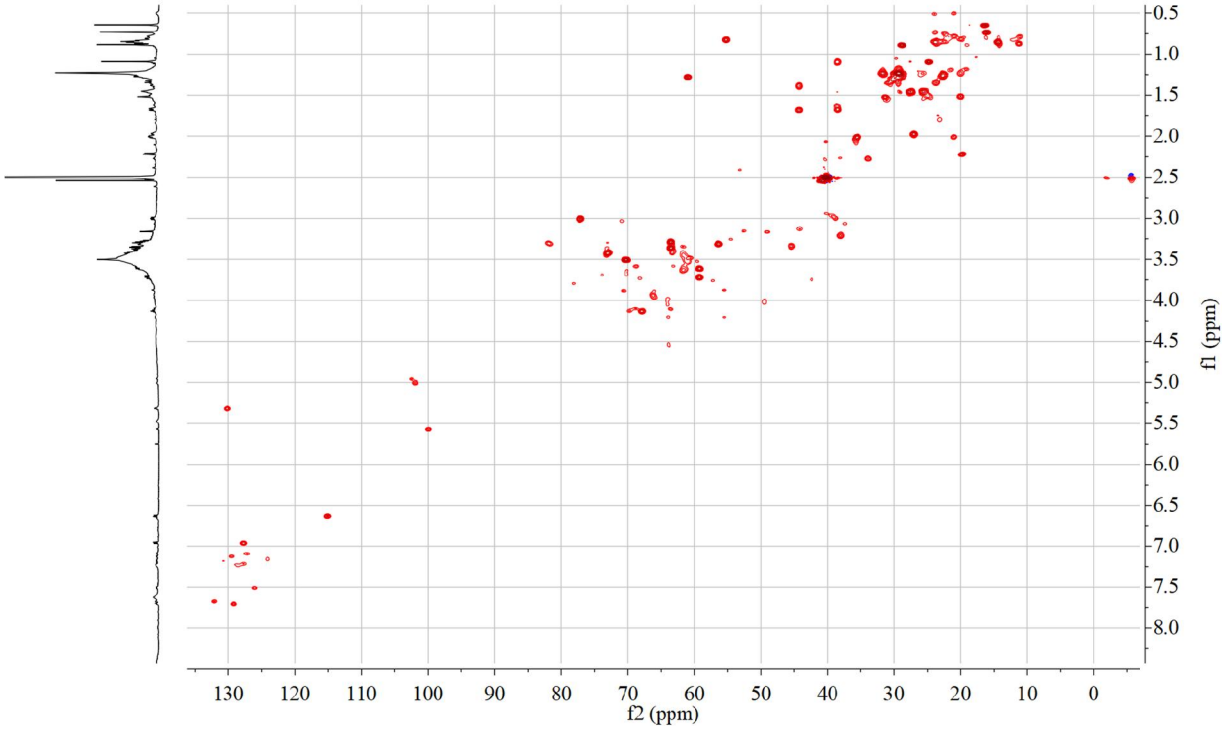


Figure S14. ^{1}H-^{13}C HSQC NMR spectrum of 3 in DMSO-d_6.

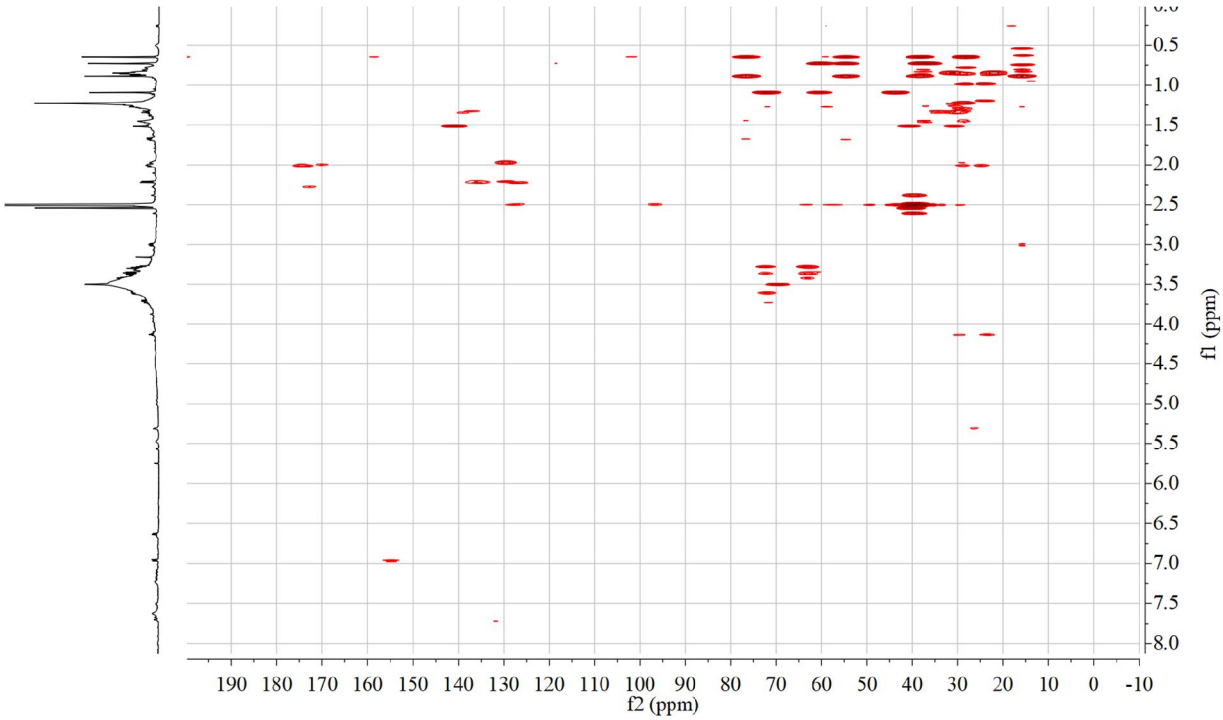


Figure S15. ^{1}H-^{13}C HMBC NMR spectrum of 3 in DMSO-d_6.

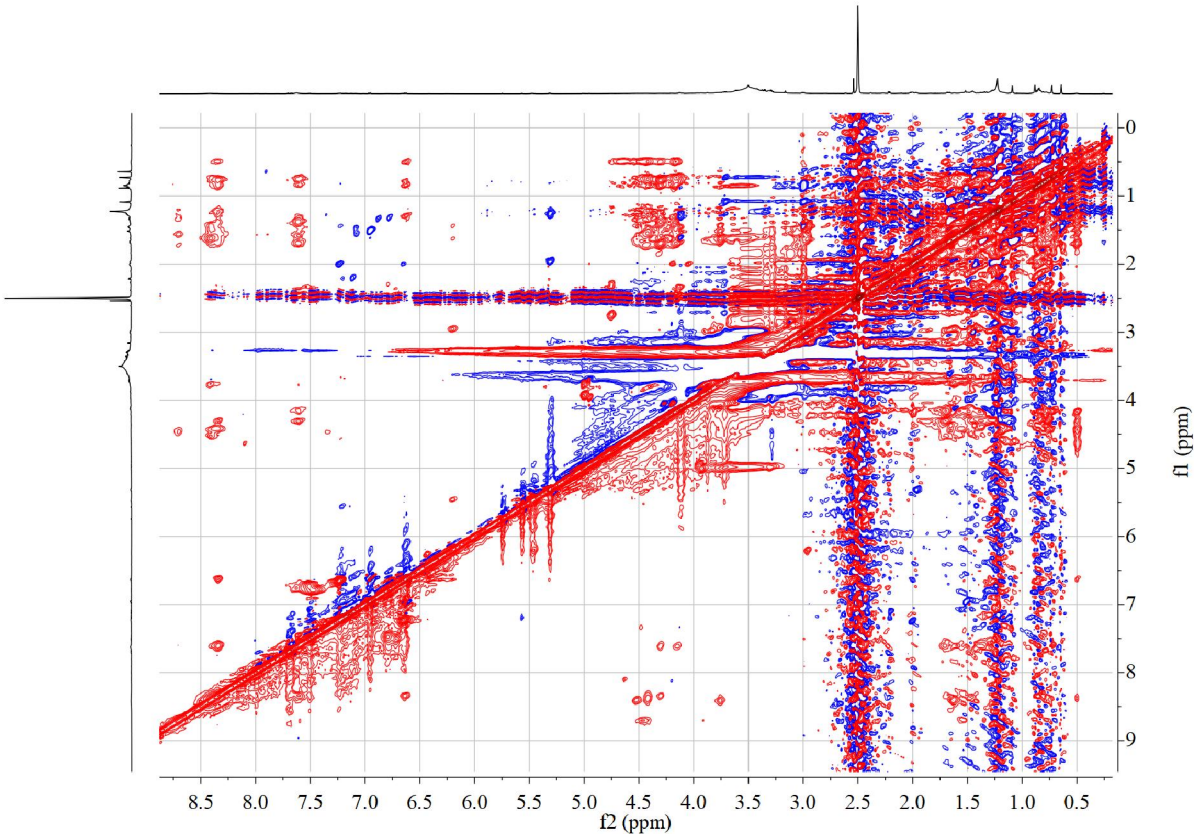


Figure S16. ^{1}H-^{1}H NOESY NMR spectrum of 3 in DMSO-d_6.

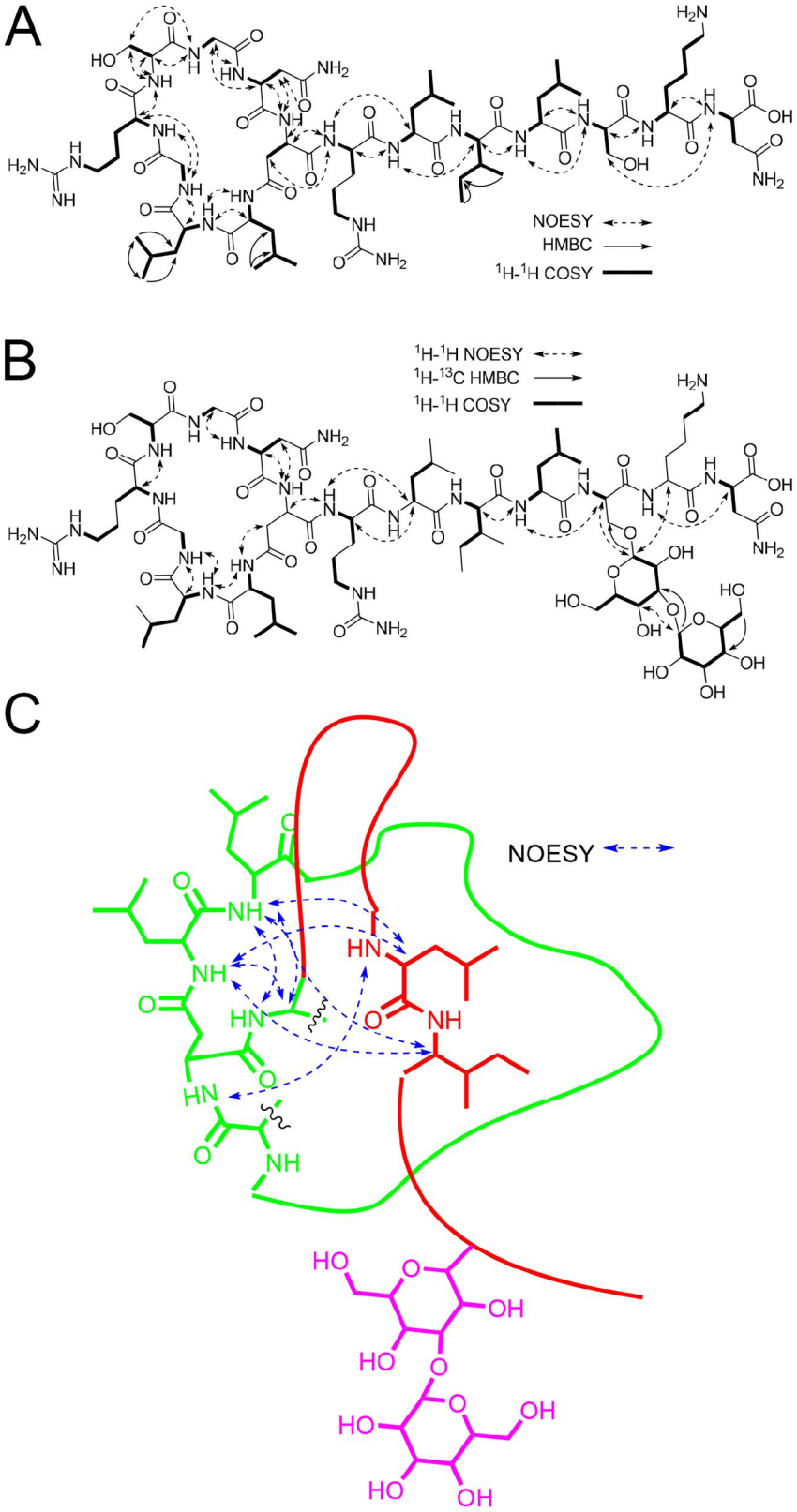


Figure S17. The key 2D NMR correlations of 1 (A) and 3 (B,C).

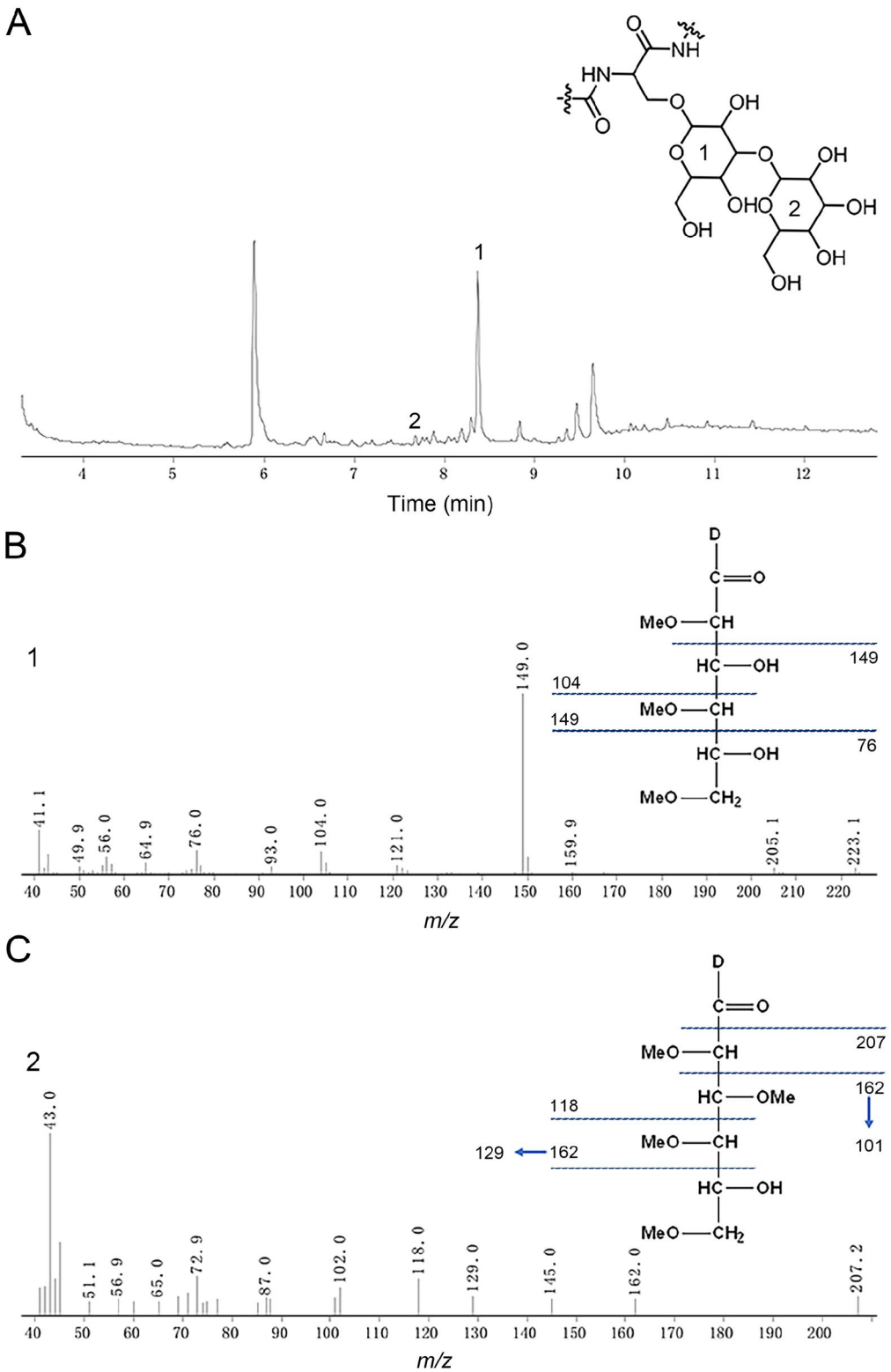


Figure S18. GCMS analysis of the derivatized glycosyl groups of 3. (A) TIC of GCMS analysis. The two derivatized glycosyl groups are indicated. The MS spectra of the derivatized glycosyl group 1 (B) and 2 (C).

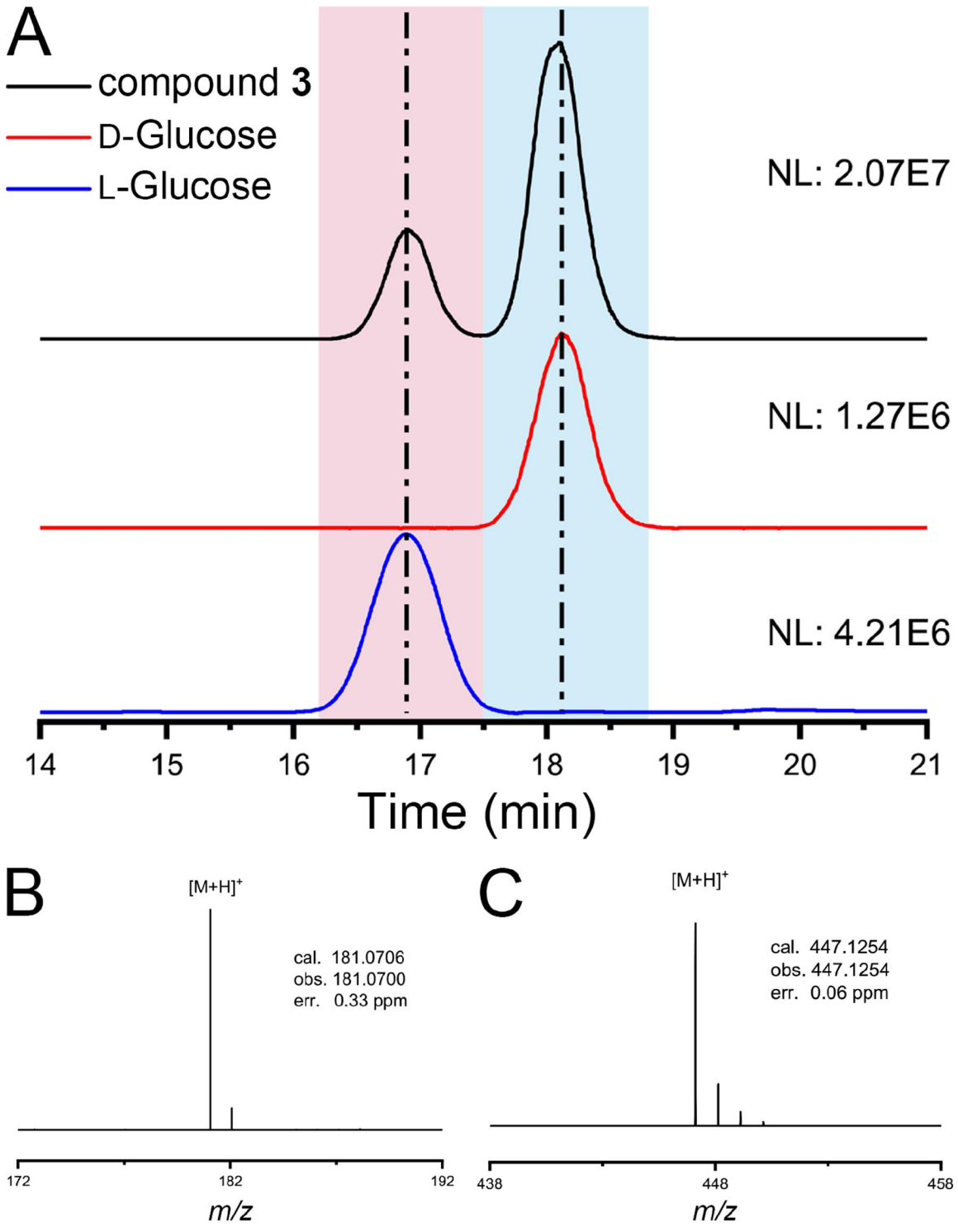


Figure S19. Characterization of the glycosyl groups of 3. (A) LC-HRMS analysis of the derivatization product of glucose from the acid hydrolysate of compound **3** in comparison with derivatization products of standard L- and D-glucoses. $[M+H]^+$ 447.1254 corresponding to the derivatization product of glucose was applied to the EIC. HRMS spectra of glucose (B) and derivatization product of glucose (C) from the hydrolysate of **3**.

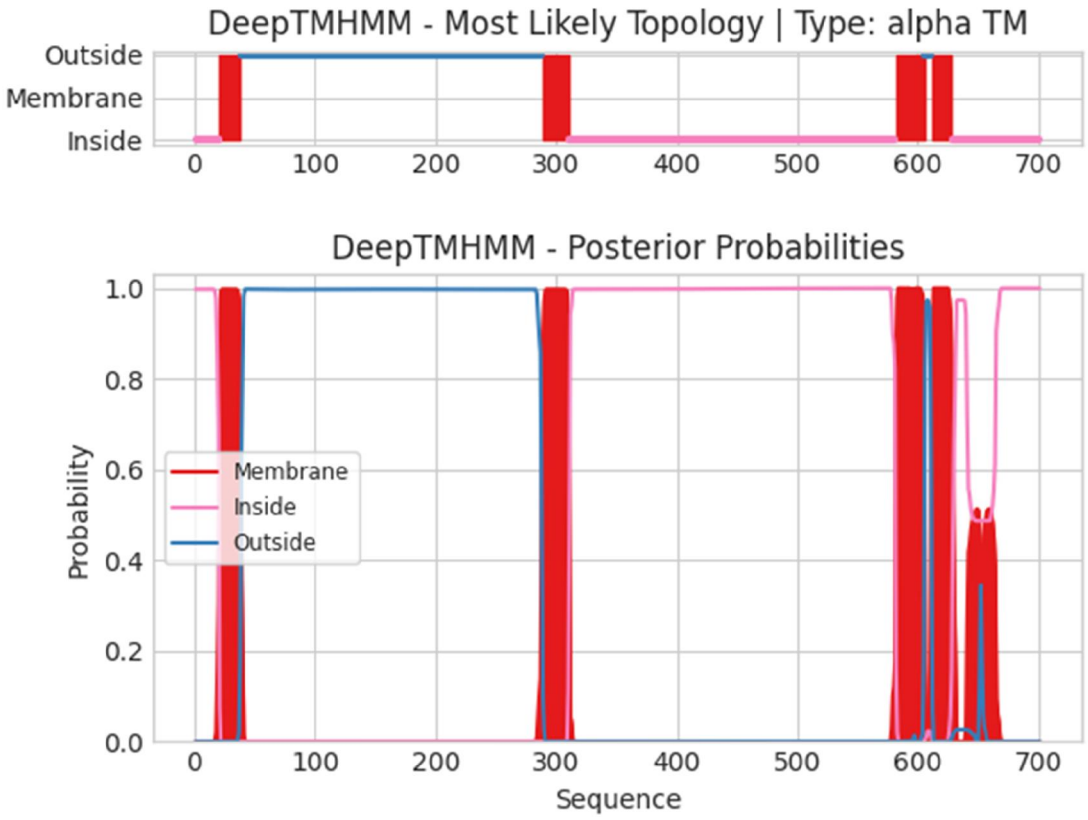


Figure S20. Transmembrane topology prediction of IgtG using the DeepTMHMM[16] online server.

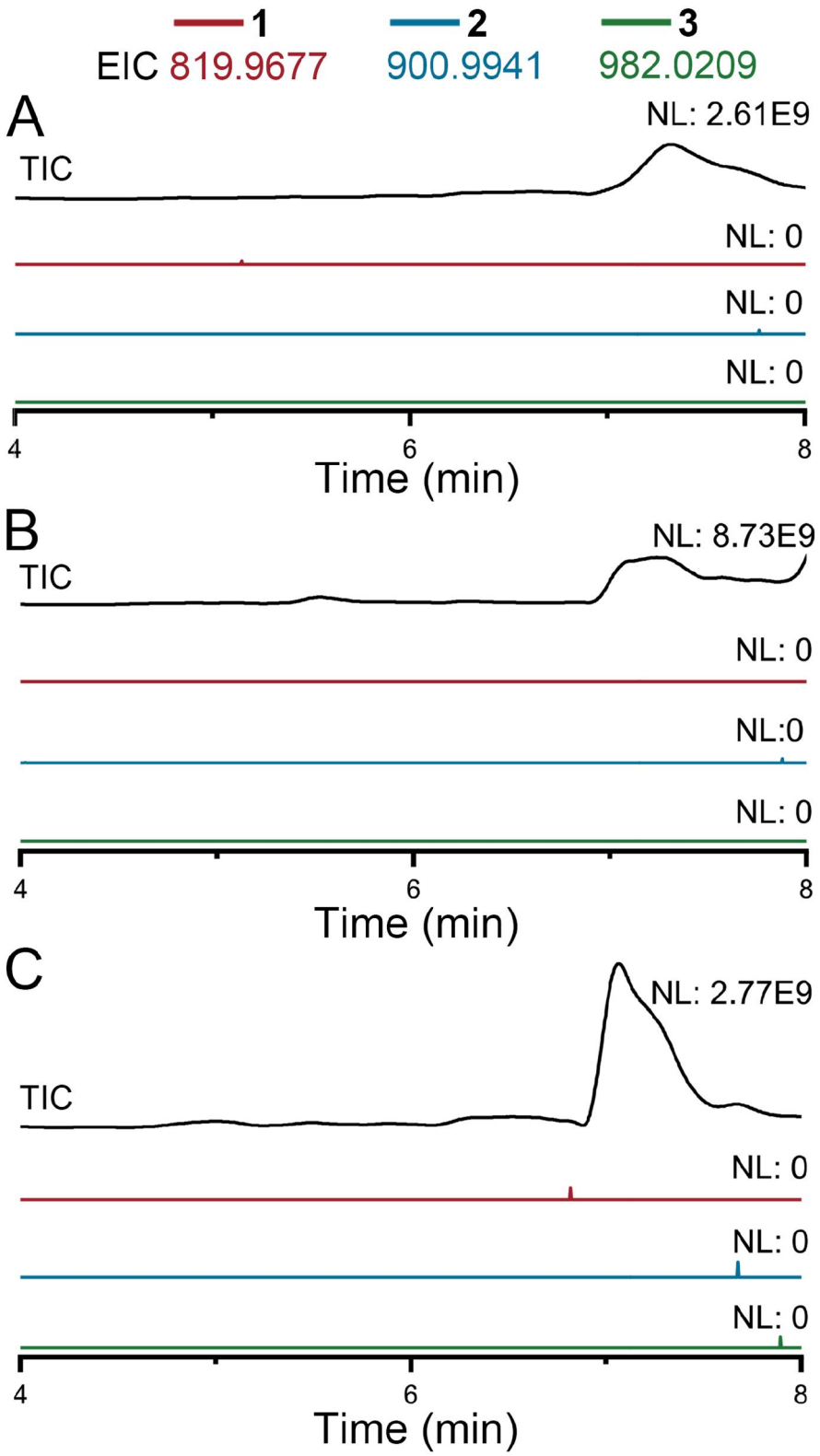


Figure S21. LC-HRMS analysis of *S. davaonensis* JCM 4913 extracts after gene knock out. The results of three independent knock out colonies were shown.

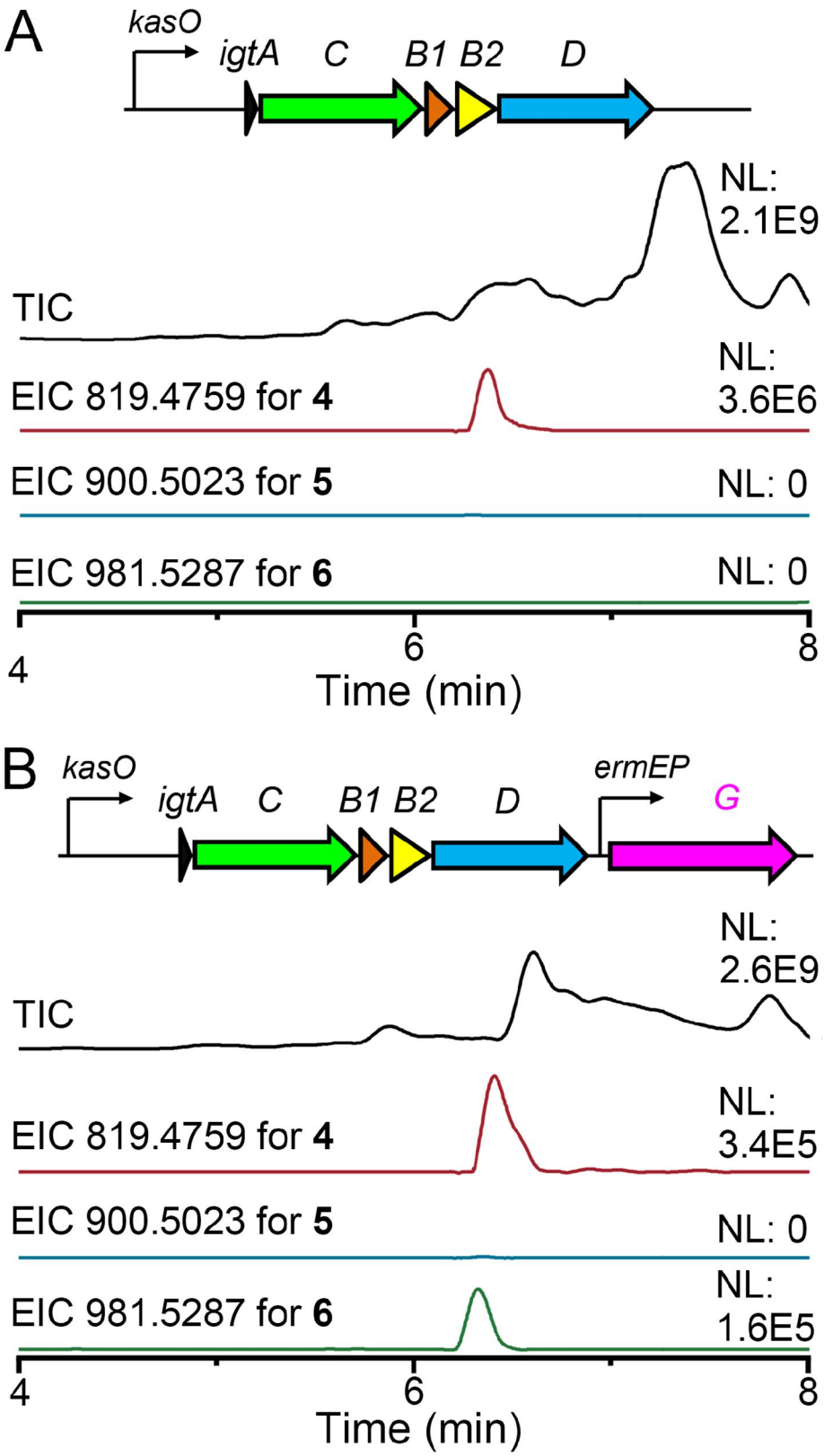


Figure S22. Heterologous expression of *igtABCD* (A) and *igtABCDG* (B) in *S. lividans* GX28.

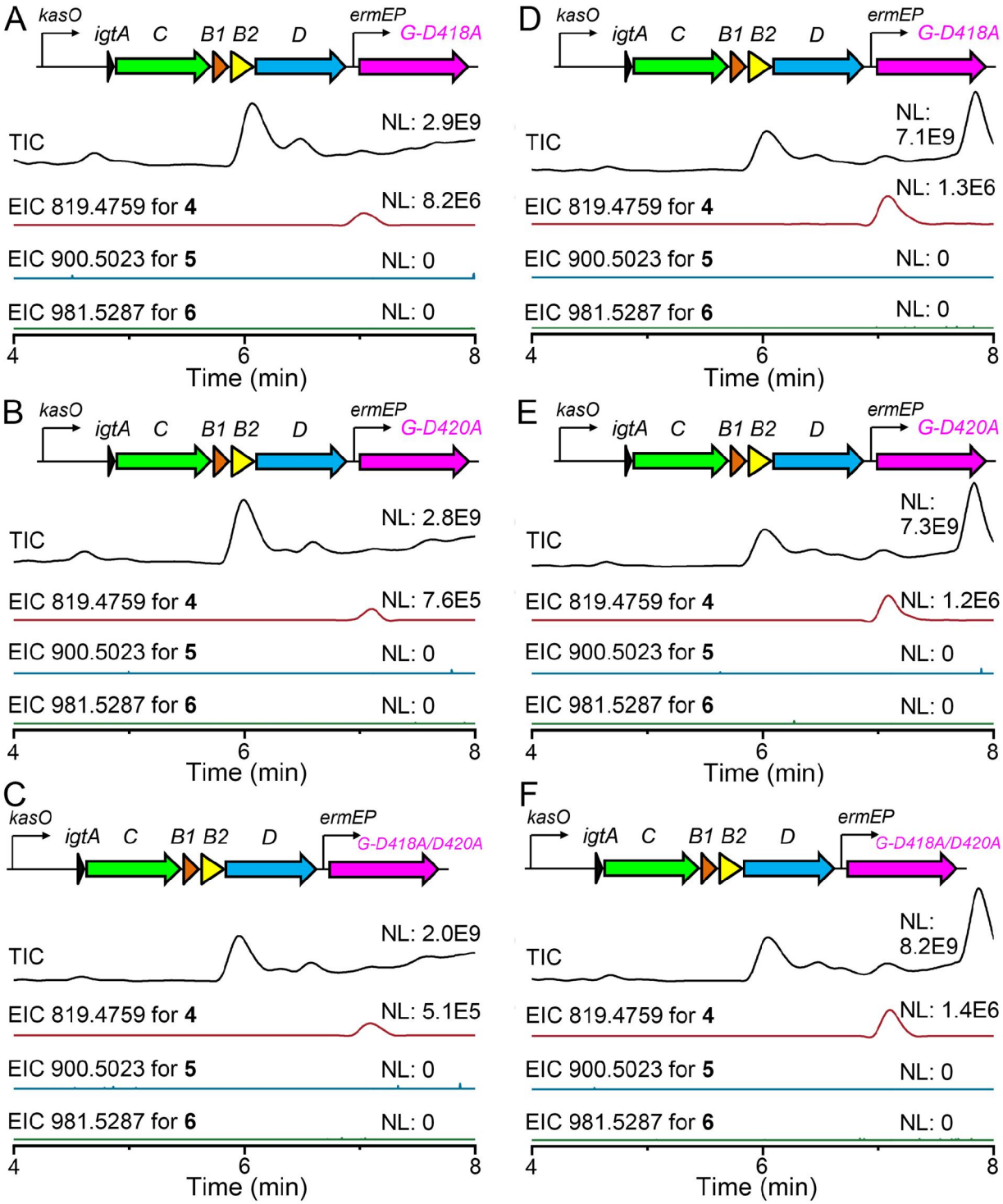


Figure S23. Heterologous expression of *igtABCDG* with IgtG mutant in LJ1018 (A-C) and GX28 (D-F). The information of the IgtG mutant is indicated above the *igtG* gene.

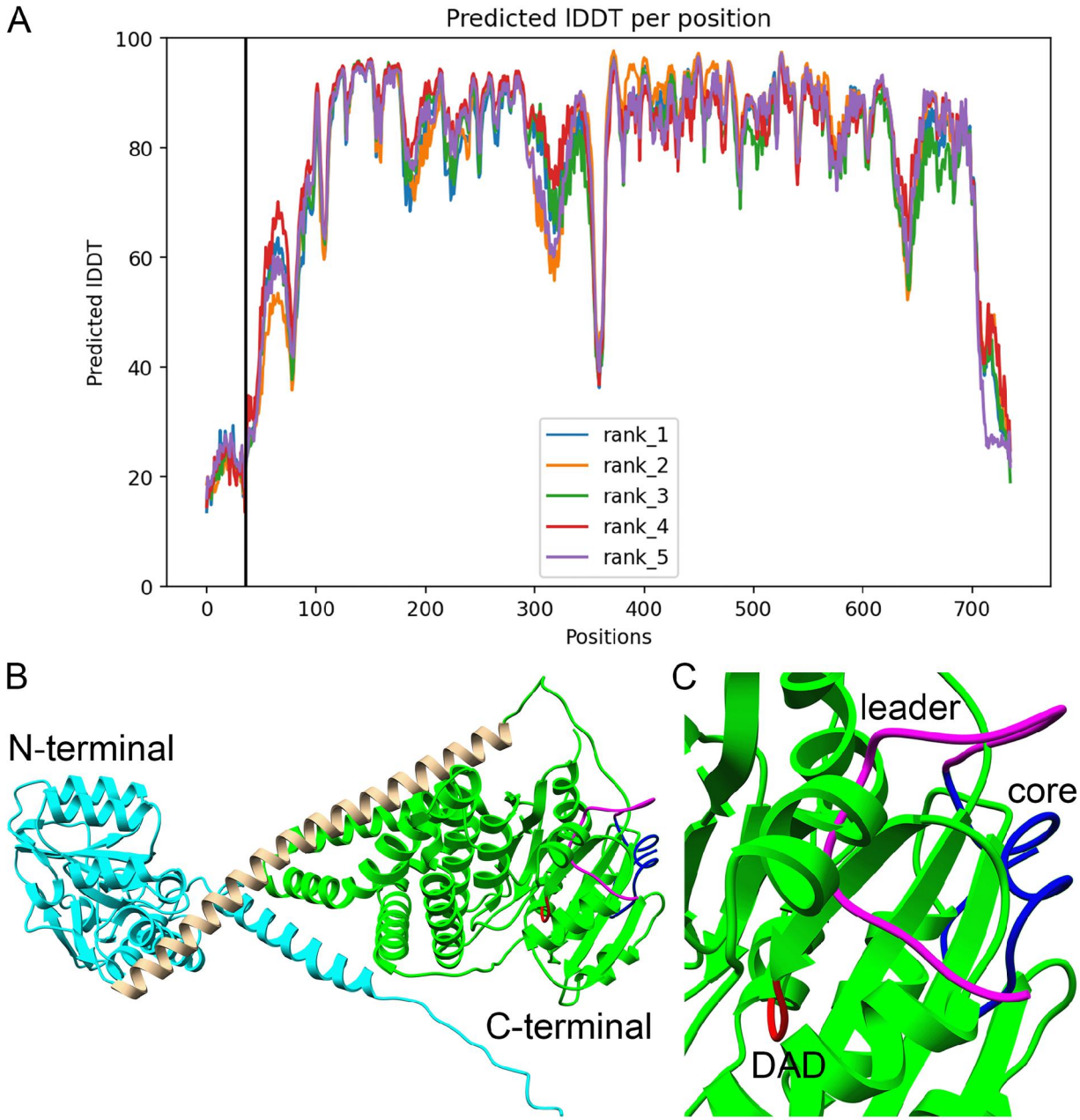


Figure S24. AlphaFold-Multimer-predicted structure of the IgtG-IgtA complex. (A) The predicted local distance difference test (pLDDT) plot of IgtGA multimer. (B) The predicted multimer structure of IgtGA. The N- and C-terminal domains and the middle transmembrane helix of IgtG are shown in cyan, tan, and green, respectively. The DAD motif of IgtG and the leader and core region of IgtA are shown in red, purple, and blue, respectively. (C) The enlarged view of the IgtG DAD motif and IgtA.

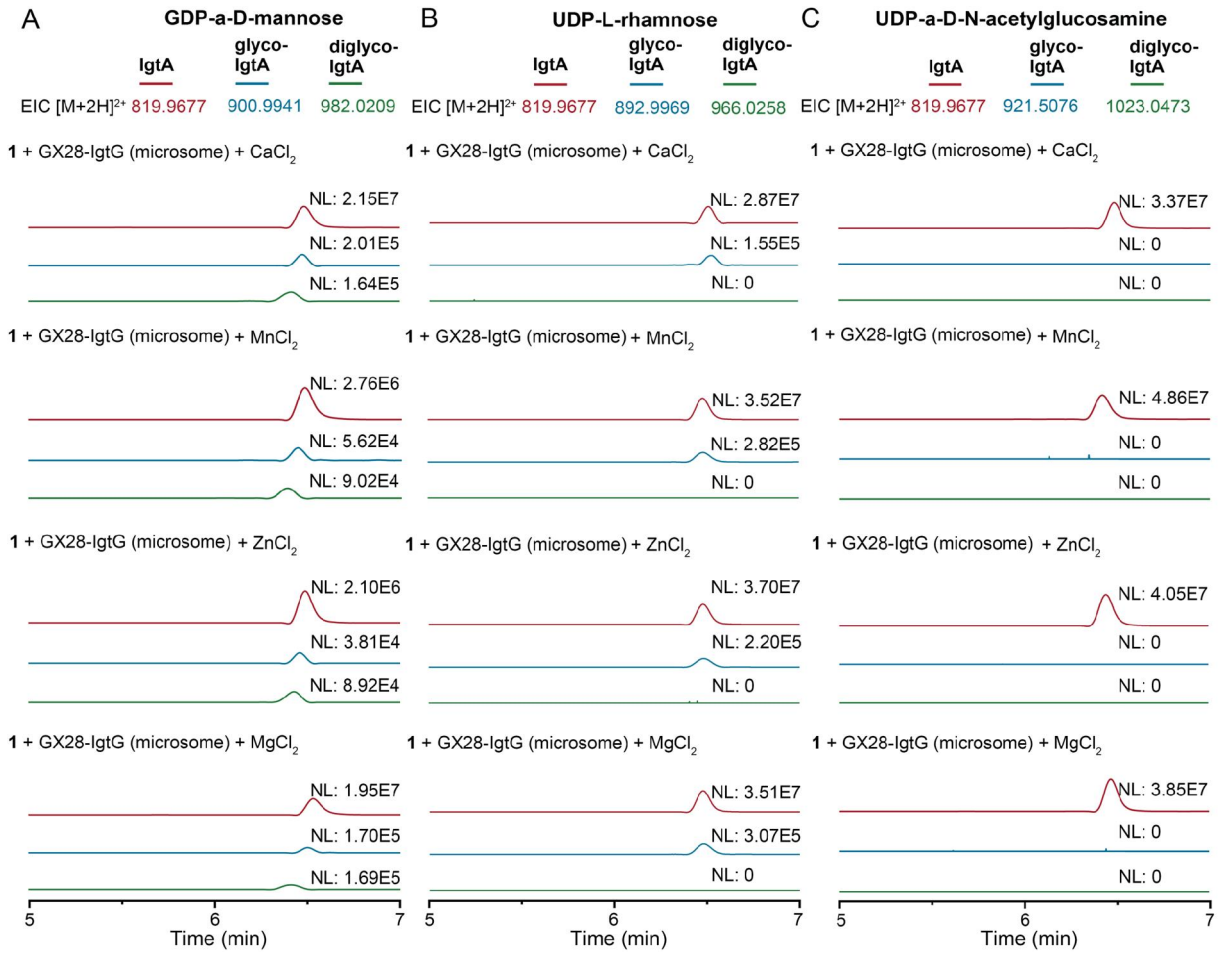


Figure S25. In vitro reconstitution of IgtG using various sugar donors and metal ions. LC-HRMS analysis of IgtG reactions using lasso peptide **1** as substrate together with different sugar donors and metal ions as indicated.

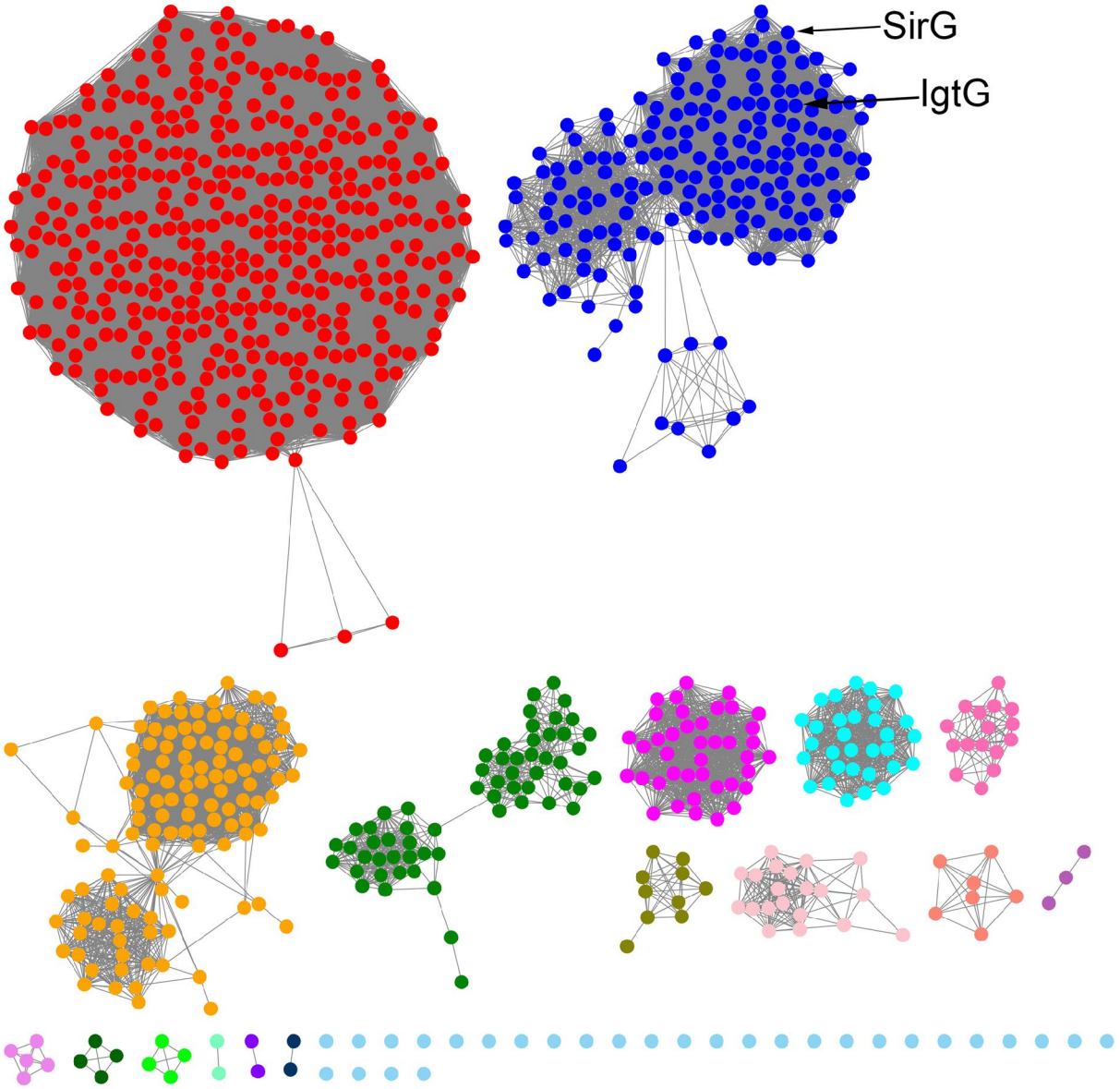


Figure S26. The SSN of IgtG. The clusters are colored based on their size.

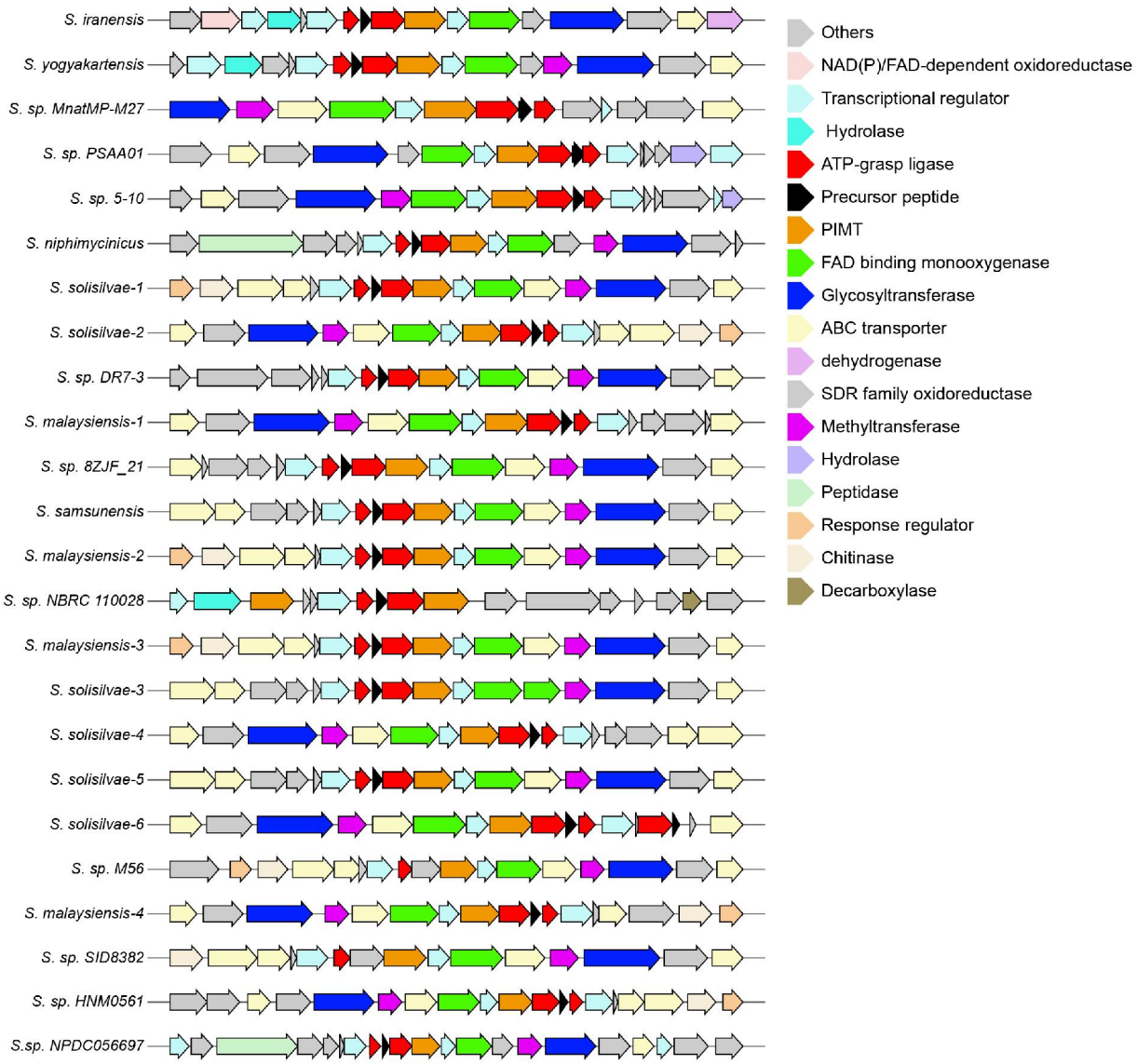


Figure S27. Organization of *sir*-like graspetide BGCs. The information of these BGCs is listed in Table S8.

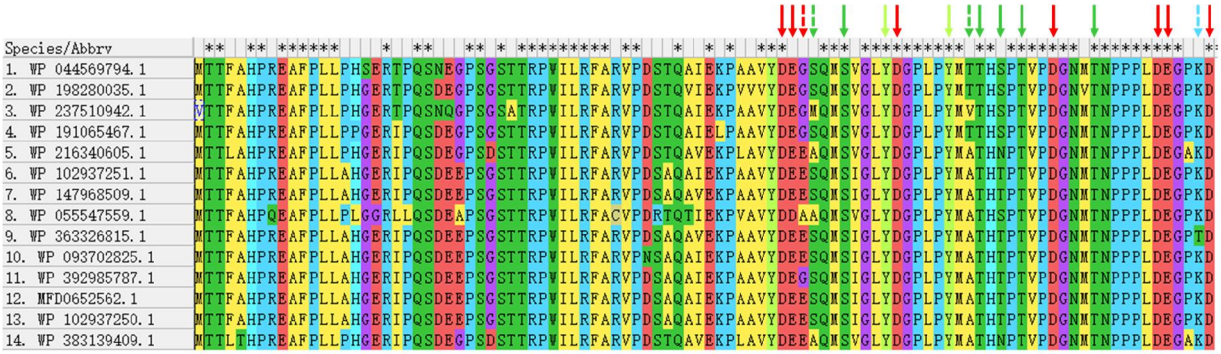


Figure S28. Sequence alignment of 14 non-redundant SirAs. Residues are shaded in different colors based on their polarity. Arrows indicate potential PTM sites, with the color code matching that of the corresponding residues.

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