Supporting Information

TABLE OF CONTENTS ***Page***

Materials and Methods

Chemicals, strains, medium and general DNA techniques

Escherichia coli JM109, restriction enzymes, and DNA modifying enzymes were purchased from Takara Bio Inc. Media, growth conditions, and general recombinant DNA techniques for *E. coli* were described by Sambrook and colleagues[1]. *Streptomyces avermitilis* ATCC 31267 and *Streptomyces coelicolor* A3(2) ATCC BAA-471 were obtained from the American Type Culture Collection (ATCC). *S. griseus* IFO13350 was obtained from the Institute of Fermentation, Osaka (IFO). *Saccharopolyspora erythraea* NRRL 3887 was obtained from the Agricultural Research Service Culture Collection (NRRL).

Composition of medium for *Sphaerisporangium* sp. SANK 60911 were as follows; YMA: 0.4% glucose, 0.4% yeast extract, 1% malt extract and 2% agar; CNZ4: 1% glucose, 1% soybean meal, 4% soluble starch, 0.25% corn steep liquor, 0.45% yeast extract, 0.0001% $CoCl_2 \cdot 6H_2O$, 0.05% KH_2PO_4, 0.001% $ZnSO_4 \cdot 7H_2O$, 0.005% $Mg_3(PO_4)_2 \cdot 8H_2O$, 0.0001% $NiSO_4 \cdot 6H_2O$ and 0.005% CB-442; AP-1: 5% glucose, 1% soybean meal, 0.4% polypepton, 0.4% meat extract, 0.1% yeast extract, 0.5% $CaCO_3$, 0.25% NaCl and 0.005% CB-442; 172F: 1% glucose, 1% soluble starch, 0.5% casitone, 0.5% yeast extract, 8 mM $Ca(NO_3)_2 \cdot 4H_2O$, 10 mM $MgSO_4 \cdot 7H_2O$.

PCR based gene-targeting

Actinomycetes were cultivated on a YMA slant, and the crude genomic DNA for the PCR template was extracted with InstaGene Matrix™ (Bio-Rad). PCR was performed on a GeneAmp PCR system 9700 (Perkin-Elmer/ABI, Foster City, CA) using GoTaq® Master Mix (Promega). The 12.5 μl reaction mixture was composed of 2 μl template DNA, 5 pmol of forward and reverse primers (Table 1), 0.5 M betaine and 1 X GoTaq® Green Master Mix. The conditions were as follows: 95 °C for 3 min; the amplification, 35 cycles of [94 °C for 30 sec, 45 °C for 30 sec and 72 °C for 60 sec]; 72 °C for 7 min. The amplified DNA fragments were purified using a QIAquick® PCR purification kit (Qiagen), and the DNA sequencing were performed on an ABI PRISM® 3700 DNA Analyzer.

Cloning and expression of *sphJ*

The native and *E. coli* codon optimized genes for *sphJ* were synthesized by GenScript. The gene constructs were amplified by PCR using Expand Long Template PCR System from Roche (Indianapolis, IN) with supplied Buffer 2, 200 μM dNTPs, 5% DMSO, 10 ng DNA template, 5 U DNA polymerase, and 200 nM each of the following primer pairs: *sphJnat* (forward) 5'-GGTATTGAGGGTCGCATGAGCTGGGAATCACCGG -3' / (reverse) 5'-AGAGGAGAGTTAGAGCC TCAGGAGTGCTGCAGGTGC-3'; and *sphJopt* (forward) 5'-GGTATTGAGGGTCGCATGTCTTGGGAAAGCCCGG-3' / (reverse) 5'-AGAGGAGAGTTAGAGCCTCAGGAATGTTGCAGATGGC-3'. The PCR program included an initial hold at 94 °C for 2 min, followed by 30 cycles of [94 °C for 10 sec, 56 °C for 15 sec, and 68 °C for 90 sec]. The gel-purified PCR product was inserted into pET-30 Xa/LIC using ligation-independent cloning as described in the provided protocol to yield pET30-*sphJnat* and pET30-*sphJopt*. The genes were sequenced to confirm PCR fidelity.The sequence of the optimized gene (*sphJopt*) has been deposited in GenBank under accession number AB830104.

Site-directed mutagenesis of *sphJ*

A K248A point mutation of SphJ was generated by PCR amplification using pET30-*sphJopt* as a template and the Expand Long Template PCR system (Roche Applied Science). Reactions were performed using the manufacturer's provided Buffer 2 with 5% DMSO, primers of 5'-GGCGGTTCGACCCATGCAACGTTTCCGGGCCCG-3' and the reverse complement (the engineered Ala codon is underlined), and a PCR program consisting of an initial hold at 94 °C for 2 min followed by 18 cycles of [94 °C for 10 sec, 56 °C for 20 sec, and 68 °C for 7.5 min]. The template DNA was digested with 10 units of *Dpn*I (New England Biolabs) for 1 h at 37 °C and transformed into *E. coli* NovaBlue competent cells. The introduction of the correct point mutation and the sequence of the entire gene including 200 bp upstream and downstream were confirmed by DNA sequencing to yield pET30-*sphJopt*(K248A).

Production of SphJ proteins

Plasmids were introduced into *E. coli* BL21(DE3) cells, and the transformed strains were grown in LB supplemented with 30 μg/mL kanamycin. Following inoculation of 500 mL of LB with 30 μg/mL kanamycin, the cultures were grown at 18 °C until the cell density reached an OD_{600} ~ 0.5 when expression was induced with 0.1 mM IPTG. Cells were harvested after an overnight incubation at 18 °C and lysed in 100 mM Tris-HCl (pH 8) and 300 mM KCl using a French Press with one pass at 16000 psi. Following centrifugation the protein was purified using affinity chromatography with Ni-NTA agarose from Qiagen (Valencia, CA), and the recombinant proteins were desalted into 50 mM phosphate (pH 7.5), 100 mM KCl, and 5% glycerol using a PD-10 desalting column (GE Healthcare). The partially purified protein was concentrated using an Amicon Ultra 10000 MWCO centrifugal filter (Millipore) and stored as a glycerol stock (40%) at -20 °C or dialyzed against 20 mM N-methylmorpholine pH 7.6 (Buffer A) overnight for further purification. Anion exchange chromatography was performed with a BioLogic DuoFlow (Bio-Rad) using a UNO Q1 column. A series of linear gradients were developed from Buffer A to Buffer A containing 1 M NaCl (Buffer B) in the following manner (time range and linear increase to % B): 0-6 min, 0%; 6-16 min, 50%; 16-20 min, 100%; 20-24, 100%; and 24-25 min, 0%. The flow rate was kept constant at 1 mL/min and 1 mL fractions were collected for activity analysis. Protein purity was assessed by 15% acrylamide SDS-PAGE; His_6-tagged proteins were utilized without further modifications. Protein concentration was determined using Bradford protein assay.

Characterization of SphJ

HPLC and UV/Vis spectroscopic analysis of SphJ activity along with structural characterization of the SphJ product were performed as previously described using LipK as a positive control[2]. Mass analysis of the SphJ product yielded $(M + H)^+$ ions at *m/z* = 318.1 and 340.1, consistent with the molecular formula $C_{11}H_{13}N_3O_8$ of **10** and the Na salt (expected *m/z* = 318.1 and 340.1, respectively). MS analysis of the phosgene-modified product of SphJ yielded an $(M - H)^-$ ion at *m/z* = 341.8, consistent with the molecular formula $C_{12}H_{13}N_3O_9$ of phosgene-modified **10** (expected *m/z* = 342.1).

Sequence analysis of *sphJ* region

For constructing the genomic library, *Sphaerisporangium* sp. SANK 60911 genomic DNA was partially digested with *Sau*3AI to give 30-50 kb DNA fragments. These fragments were dephosphorylated with bacterial alkaline phosphatase (BAP) and ligated into *Bam*HI-digested cosmid vector SuperCos1 (Stratagene), which was dephosphorylated by BAP after *Xba*I digestion. The ligation products were packaged with Gigapack III Gold packaging extract (Stratagene) as described by the manufacturer and the resulting recombinant phage was used to transfect *E. coli* XL-1 Blue MR. Approximately 8,000 colonies from the obtained genomic library were screened by colony hybridization using digoxigenin (DIG)-labeled serine hydroxymethyltransferase obtained by degenerate PCR from *Sphaerisporangium* sp. SANK 60911. Hybridization was carried out using DIG easy hyb (Roche) at 42 °C and the resulting filter was washed under high stringency conditions (0.1 X SSC including 0.1% SDS, 68 °C). Detection was performed using CDP-Star (Roche) according to the manufacturer's procedures. The acquired three positive cosmids, S-1, S-2 and S-3, were isolated and the terminal sequences of the cosmid insert were obtained. Finally, the sequences were used as probes (Fig. S6) for cosmid screening to obtain 14 cosmids, A-2, A-3, A-4, A-5, A-6, A-7, P-2, P-3, P-4, P-5, P-6, P-7, P-8 and P-9.

Four cosmids, S-1, S-3, A-4 and P-2 were selected for sequence analysis using a Roche GS FLX system (Operon Biotechnologies) and for completion of the sequence, shotgun sequencing of the cosmid S-1 was conducted with an ABI PRISM® 3700 DNA Analyzer. The cosmid DNA was sheared using a Nebulizer Kit (Invitrogen) according to the manufacturer's procedures, and the treated DNA was analyzed by agarose gel electrophoresis. The DNA fragments from 1 to 3 kb were purified using a QIAquick® PCR purification kit (QIAGEN). The recovered DNA fragments were cloned into pHSG398 (Takara Bio) and transformed into *E. coli* JM109. Approximately 400 plasmids extracted using R.E.A.L. ® Prep 96 (QIAGEN) were sequenced and the sequence data was assembled using ATGC (GENETYX). Database comparison study for sequence homology was performed with BLAST search tools using the National Center for Biotechnology Information. The sequence of the cloned region has been deposited in GenBank under accession number AB830104.

Reverse transcriptase-PCR (RT-PCR) analysis

A loopful of mycelia of cultured *Sphaerisporangium* sp. SANK 60911 was inoculated into a 100-mL Erlenmeyer flask containing 20 mL AP-1 medium and cultured by shaking (310 rpm) at 28 °C for 7 days. A 1 mL aliquot was transferred into a 100-mL Erlenmeyer flask containing 20 mL of culture medium or a YMA plate, and cultivation was carried out for 6 or 14 days. The mycelia were harvested from the culture by centrifugation at 10,000 rpm or scraping off from the plates and were treated overnight with RNAlater (Ambion) at 4 °C. Each of the total RNA was isolated from the treated mycelia using RNAqueous (Ambion) according to the manufacturer's instruction. The isolated total RNA was treated with DNase I to digest any contaminating genomic DNA. RT-PCR amplification for expression analysis was carried out on a GeneAmp PCR system 9700 (Perkin-Elmer/ABI) using a TaKaRa One Step RNA PCR kit (AMV) (Takara Bio) using 30 cycles. 16S rRNA was used as an internal control.

Isolation and structural analysis of 11

Progress of the fermentation and purification of **11** was monitored by HPLC performed on an HP1100 system (Agilent) with a Unison UK-C_{18} column (4.6 ϕ x 75 mm, Imtakt Corp.) at a flow late of 1.0 ml/min. The effluent was monitored by UV absorption at 260 nm. The chromatography was conducted with a linear gradient from 5% to 90% acetonitrile in water containing 10 mM $HCOONH_4$–HCOOH buffer (pH 3.5) for 15 min.

In order to isolate **11**-**14**, the producing organism *Sphaerisporangium* sp. SANK 60911 was grown on a YMA plate for over 2 weeks. Harvested solid culture (2 L) was crushed into small pieces and dipped into an equal volume of 80% acetone in water and the mixture was stirred for 12 h. The extract was filtered with an addition of Celite 545 (KANTO CHEMICAL CO., INC.) as an adjunct. The filtrate was concentrated *in vacuo* and freeze-dried to yield a crude oil that was dissolved into DMSO (10 ml) following by the addition of 5 g of Cosmosil 140C_{18}-OPN (Nacalai Tesque, Inc.). The mixture was freeze-dried and then applied to a Cosmosil 140C_{18}-OPN column (40 mL). After washing the column with water, chromatography was performed by stepwise elution using 10%, 30%, 40%, 55%, 60% and 70% acetonitrile in water containing 0.1% HCOOH (200 ml each). By HPLC analysis, **11**-**14** were detected in the 55% and 60% acetonitrile fractions. These fractions were combined and concentrated to dryness to yield 55 mg of crude powder. The obtained crude powder was dissolved in DMSO and subjected to a Unison US-C18 column (20 x 150 mm, Imtakt Corp.). The chromatography was performed with 55% acetonitrile in water containing 0.1% HCOOH at a flow rate of 20 mL/min with UV detection at 260 nm. As a result, **11** eluted at a retention time of 16.5 min. The obtained fraction was concentrated and freeze-dried to yield 6.5 mg of pure **11**.

The NMR spectra of **11**, **15**, and **16** were recorded at 300 K on an AVC 500 spectrometer (Bruker BioSpin) equipped with a cryogenic probe or a BBFO broadband probe operating at 500 MHz for ^1H or 125 MHz for ^{13}C. The samples for NMR characterization were dissolved in methanol-d_4, dimethylsulfoxide-d_6 or deuterium oxide. High-resolution ESI mass spectra

were recorded on an LTQ-Orbitrap XL (Thermo Fisher Scientific) and UPLC-Xevo Q-Tof (Waters). The optical rotation, UV spectra and IR spectra were measured with a DIP-370 (JASCO), a UV-265FW spectrometer (Shimadzu) and a FT/IR 6300 (Jasco), respectively.

Hydrolysis of 11

To a stirring solution of **11** in MeOH, 1N NaOH was added. The mixture was stirred for 2 h at room temperature, and the reaction was stopped by adding 1N HCl to pH 3. The acyl unit of sphaerimicin A was extracted with EtOAc and concentrated by evaporation. The crude product was dissolved in DMSO and the resultant was subjected to a Unison UK-C18 column (6 ϕ x 75 mm, Imtakt Corp.). The chromatography was performed with 90% acetonitrile in water containing 0.1% HCOOH at a flow rate of 1.5 ml/min with UV detection at 230 nm. As a result, **16** eluted at a retention time of 3.1 min.

The remaining water layer following EtOAc extraction was adjusted to pH 7.0 and freeze-dried. The crude product was dissolved in water and subjected to a TSKgel Amide-80 column (4.6 ϕ x 150 mm, TOSOH). The chromatography was conducted with a linear gradient from 90% to 40% acetonitrile in water containing 10 mM $HCOONH_4$–HCOOH buffer (pH 3.5) for 15 min at a flow rate of 1.0 mL/min with UV detection at 260 nm. As a result, **15** eluted at a retention time of 10.0 min.

Measurement of translocase I inhibitory activity

Translocase I inhibitory activity was measured using a 96-well microtitre plate based assay. The assay mixture composed of 100 mM Tris-HCl (pH 7.5), 50 mM KCl, 25 mM $MgCl_2$, 0.8% Triton X-100, 166 μM undecaprenylphosphate and 70 μM UDP-MurNAc-L-Ala-γ-D-Glu-*m*-DAP-[N^{ε}-dansyl]-D-Ala-D-Ala in 100 μl solution was prepared in each well. The reaction was initiated by the addition of recombinant translocase I (0.63–2.5 μg protein), and the enzyme activity was monitored by measuring the increase of fluorescence detected at 535 nm (excitation at 355 nm) after a 30 minute incubation at 30 °C. The recombinant translocase I was prepared as described previously[3].

Antimicrobial activities

The MICs were determined by a standard broth microdilution method[4].

Supporting Results

Table S1. Sequence comparison in % identity/% similarity among putative SHMT-like transaldolases.

	LipK[a]	Cpz14	LpmL	Mra14	Mur17	CapH	ORF14
LipK	-------	92/98	89/98	83/95	47/78	47/79	55/81
Cpz14	-------	-------	87/97	81/94	47/79	47/79	47/86
LpmL	-------	-------	-------	83/96	45/79	47/80	50/81
Mra14	-------	-------	-------	-------	47/78	47/79	46/80
Mur17	-------	-------	-------	-------	-------	52/81	53/81
CapH	-------	-------	-------	-------	-------	-------	84/98
ORF14	-------	-------	-------	-------	-------	-------	-------

[a]LipK is encoded within the biosynthetic gene cluster for **1**, Cpz14 for **2**, LpmL for **3**, Mra14 for **4**, Mur17 for **5**, CapH for **7**, and ORF14 for **6**.

Table S2. Sequence of the degenerate primers designed to specifically amplify genes encoding a SHMT-like L-Thr:uridine-5'-aldehyde transaldolase.

Name[a]	Sequence[b]
A-F1	TGAACCTCGTGCCNAGYGARAAC
A-F2	ACTACTACAACCGGTAYTTYTTYAAC
B-F1	AACTCCGGCGGCCAYTAYGCNAC
C-R1	AGATCACCCCCTTGTGNGGNCC
D-R1	CCAGCGTCTCSGCRAARTGRTG

[a]The conserved region used to design each primer is shown in Fig. S2 and S3.
[b]IUB symbol is used for mixed bases.

Table S3. Deduced function of ORFs—including those with homologs within the gene clusters of other nucleoside antibiotics inhibiting bacterial translocase I (**blue**)—in the putative biosynthetic gene cluster for sphaerimicin.

ORF	Length[a]	Putative Function	Homolog (Accession #)	I/S[b]
Orf(-6)	350	2-Nitropropane dioxygenase	Cwoe_3779 (YP_003395571.1)	62/69
Orf(-5)	243	Chitin-binding protein	Sros_3599 (ACZ86532.1)	61/73
Orf(-4)	212	Lipoprotein	Sros_1394 (ACZ84389.1)	60/74
Orf(-3)	452	Secreted protein	Sros_1396 (ACZ84839.1)	74/85
Orf(-2)	122	Glyoxalase	Micau_0048 (YP_003833193.1)	64/78
Orf(-1)	334	Hypothetical Protein	AciX9_0800 (YP_004216648.1)	32/47
SphA	376	Glycosyl transferase group 1	Amir_3661 (YP_003101391.1)	26/38
SphB	302	Arylsulfatase	BXY_43640 (CBK69251.1)	27/41
SphC	232	Phosphoesterase	Strop_4315 (YP_001161121.1)	42/53
SphD	1196	ABC transporter	KSE_26800 (YP_004904447.1)	46/63
SphE	324	Dioxygenase	LipL (BAJ05888.1)	39/49
SphF	446	Pyrimidine-nucleoside phosphorylase	LipP (BAJ05892.1)	49/61
SphG	433	Aminotransferase	LipO (BAJ05891.1)	42/52
SphH	384	Glycosyltransferase	LipN (BAJ05890.1)	41/51
SphI	205	Nucleotidyltransferase	LipM (BAJ05889.1)	32/45
SphJ	438	Serine hydroxymethyltransferase	LipK (BAJ05887.1)	51/64
SphK	181	β-Hydroxylase	LipG (BAJ05883.1)	47/67
SphL	439	Aminotransferase	LipJ (BAJ05886.1)	35/47
SphM	309	Sucraseferredoxin family protein	SaccyDRAFT_0025 (ZP_09744620.1)	35/50
SphN	678	Transketolase	HolfoDRAFT_1935 (ZP_09578138.1)	51/65
SphO	167	MmgE/PrpD family protein	Bphyt_6217 (YP_001889915.1)	29/48
SphP	314	Aldo/keto reductase	Tter_2442 (YP_003324158.1)	45/59
SphQ	439	Diaminopimelate decarboxylase	LYNGBM3L_49020 (ZP_08430161)	30/48
SphR	169	Hypothetical Protein	Pmar_PMAR025297 (XP_002774745.1)	41/57
SphS	309	F420-dependent oxidoreductase	Noca_2313 (YP_923505.1)	48/61
SphT	271	Nucleotidyltransferase	Mur29 (ADZ45341.1)	48/58
SphU	1559	Type I polyketide synthase	FrEUN1fDRAFT_7525 (ZP_06417827.1)	45/58
SphV	4961	Type I polyketide synthase	Herb (AEZ64505)	49/61
SphW	393	Non-ribosomal peptide synthethase	PacI (ADN26245.1)	28/43
Orf1	308	Hypothetical protein	Francci3_0158 (YP_479279.1)	49/63
Orf2	342	Hypothetical protein	Franean1_2552	42/51
Orf3	710	Hypothetical protein	AN0539.2 (XP_658143.1)	29/44
Orf4	157	Branched chain amino acid ABC transporter	RD1_3590 (YP_683755.1)	48/66

[a]Number of amino acid residues.
[b]Identity/similarity

Table S4. Physico-chemical properties of sphaerimicin A-D (**11**-**14**).

	Sphaerimicin A	Sphaerimicin B	Sphaerimicin C	Sphaerimicin D
Appearance	pale yellow solid	pale yellow solid	pale yellow solid	pale yellow solid
$[\alpha]_D^{30}$ (MeOH)	+35° (*c* 0.10)	ND	ND	ND
Molecular formula	$C_{44}H_{70}O_{18}N_4S$	$C_{44}H_{70}O_{21}N_4S_2$	$C_{41}H_{66}O_{18}N_4S$	$C_{41}H_{64}O_{18}N_4S$
HR-ESI MS (*m/z*)	for $C_{44}H_{69}O_{18}N_4S$	for $C_{44}H_{69}O_{21}N_4S_2$	for $C_{41}H_{65}O_{18}N_4S$	for $C_{41}H_{63}O_{18}N_4S$
Found $[M-H]^-$:	973.4316	1053.3901	933.4016	931.3861
Calcd.:	973.4333	1053.3901	933.4020	931.3864
UV λ_{max} nm (ε) (MeOH)	237 (21000), 261 (6100)	ND	ND	ND
IR ν_{max} (ATR)	3343, 3285, 3097, 2954, 2924, 2870, 1685, 1595, 1458, 1377, 1259, 1162, 1107, 1085, 1036	ND	ND	ND
Solubility	DMSO, MeOH	DMSO, MeOH	DMSO, MeOH	DMSO, MeOH

ND, not determined

Table S5. NMR spectral data of sphaerimicin A (**11**) in DMSO-*d*$_6$

Position	δ_C[a]	δ_H[a] (mult, *J* in Hz)	
2	150.3 (C)		
4	163.2 (C)		
5	101.2 (CH)	5.68 (1H, d, 8.1)	
6	139.9 (CH)	7.83 (1H, d, 8.1)	
1'	89.4 (CH)	5.54 (1H, br. d, 2.1)	
2'	72.9 (CH)	4.19 (1H, br. m)	
2'-OH		5.29 (1H, br.d, 3.9)	
3'	73.0 (CH)	4.33 (1H, br. t, 4.9)	
4'	81.2 (CH)	4.54 (1H, br. d, 7.5)	
5'	78.0 (CH)	3.81 (1H, o)	
6'	70.1 (CH)	3.27 (1H, o)	
7'	169.8 (C)		
1"	110.0 (CH)	5.18 (1H, s)	
2"	75.6 (CH)	3.93 (1H, br. t, 3.5)	
2"-OH		4.79 (1H, br. d, 3.7)	
3"	73.9 (CH)	4.09 (1H, o)	
4"	80.2 (CH, br.)	3.87 (1H, m)	
5"	46.8 (CH_2)	2.96 (1H, o)	3.07 (1H, m)
2"'	49.7 (CH_2, br.)	2.93 (1H, o)	3.24 (1H, o)
3"'	53.8 (CH)	3.37 (1H, o)	
4"'	67.5 (CH)	4.76 (1H, br. s)	
5"'	66.2 (CH)	3.64 (1H, br. s)	
6"'	51.1 (CH_2, br.)	2.47 (1H, o)	2.94 (1H, o)
1a	172.8 (C)		
2a	46.2 (CH)	2.48 (1H, o)	
3a	74.0 (CH)	4.06 (1H, o)	
3a-OH		5.17 (1H, br. d, 3.9)	
4a	127.5 (CH)	5.46 (1H, dd, 15.3, 7.4)	
5a	136.0 (CH)	6.16 (1H, d, 15.3)	
6a	131.2 (C)		
7a	139.2 (CH)	5.20 (1H, d, 9.5)	
8a	29.8 (CH)	2.57 (1H, m)	
9a	44.2 (CH_2)	0.98 (1H, o)	1.24 (1H, o)
10a	26.8 (CH)	1.52 (1H, m)	
11a	45.2 (CH_2)	0.85 (1H, o)	1.13 (1H, m)
12a	27.6 (CH)	1.36 (1H, m)	
13a	44.9 (CH_2)	0.85 (1H, o)	1.13 (1H, m)
14a	29.2 (CH)	1.46 (1H, m)	
15a	38.5 (CH_2)	0.98 (1H, o)	1.22 (1H, o)
16a	19.4 (CH_2)	1.21 (1H, o)	1.29 (1H, m)
17a	14.3 (CH_3)	0.84 (3H, t, 6.9)	
18a	13.2 (CH_3)	0.96 (3H, d, 7.1)	
19a	12.6 (CH_3)	1.69 (3H, s)	
20a	21.7 (CH_3)	0.91 (3H, d, 6.6)	
21a	20.6 (CH_3)	0.77 (3H, d, 6.6)	
22a	20.7 (CH_3)	0.81 (3H, d, 6.6)	
23a	20.2 (CH_3)	0.81 (3H, d, 6.6)	

[a]Chemical shifts are shown with reference to DMSO-*d6* as δ_H 2.50 and δ_C 39.5.
"o" indicates overlapping signals.

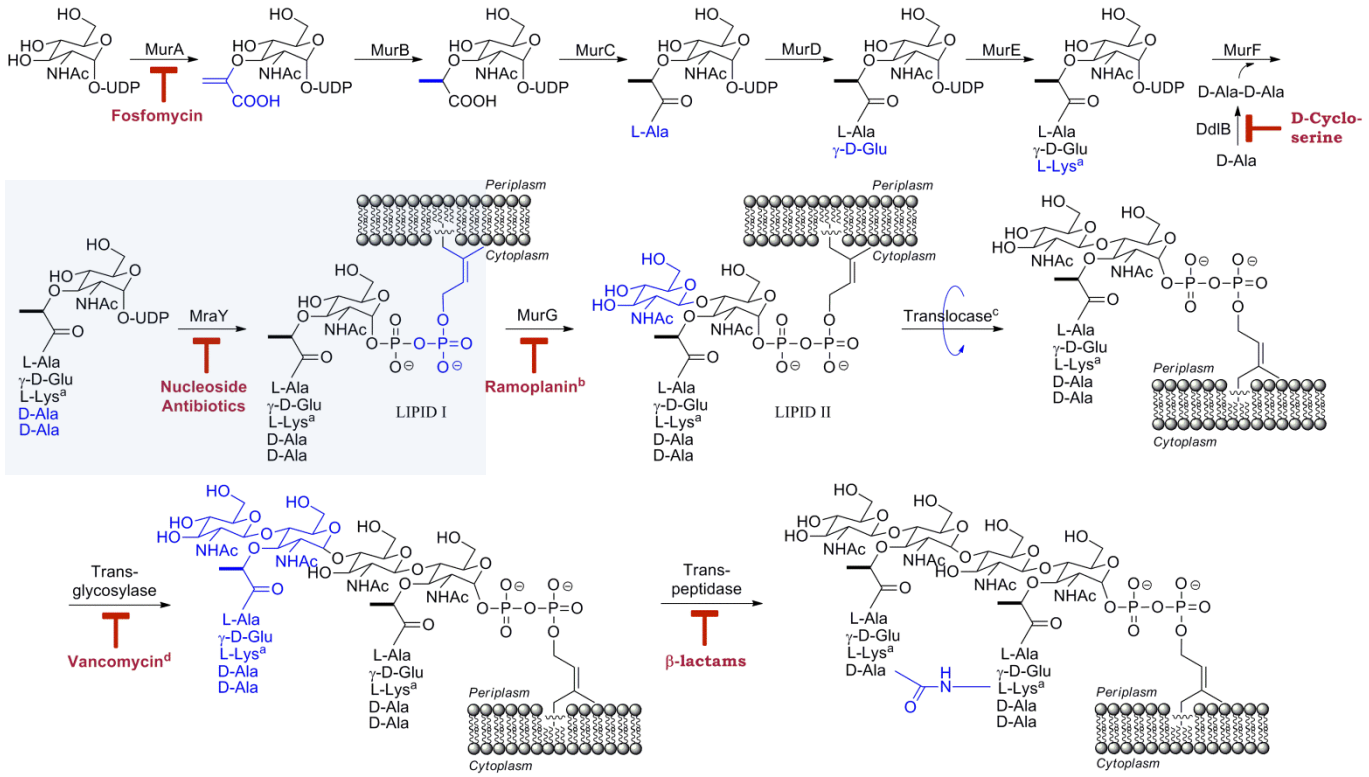


Figure S1. Schematic of the biosynthesis of bacterial peptidoglycan cell wall. The transformation catalyzed by each enzyme is highlighted in **blue**, and representative natural products that inhibit the specified enzyme are highlighted in **red**. Although the twelve shown enzymes are conserved in nearly all bacteria, several species-specific variations for this pathway are known. MraY, or bacterial translocase I, initiates the lipid cycle of cell wall biosynthesis. [a]Diaminopimelic acid or L-Lys is found at this position. [b]Ramoplanin also binds the product of MurG (Lipid II) and inhibits transglycosylases. [c]Glycine ligases can precede translocase activity to yield branched peptidoglycan cell walls. [d]Vancomycin inhibits the activity of transglycosylases by binding to the substrate, Lipid II.

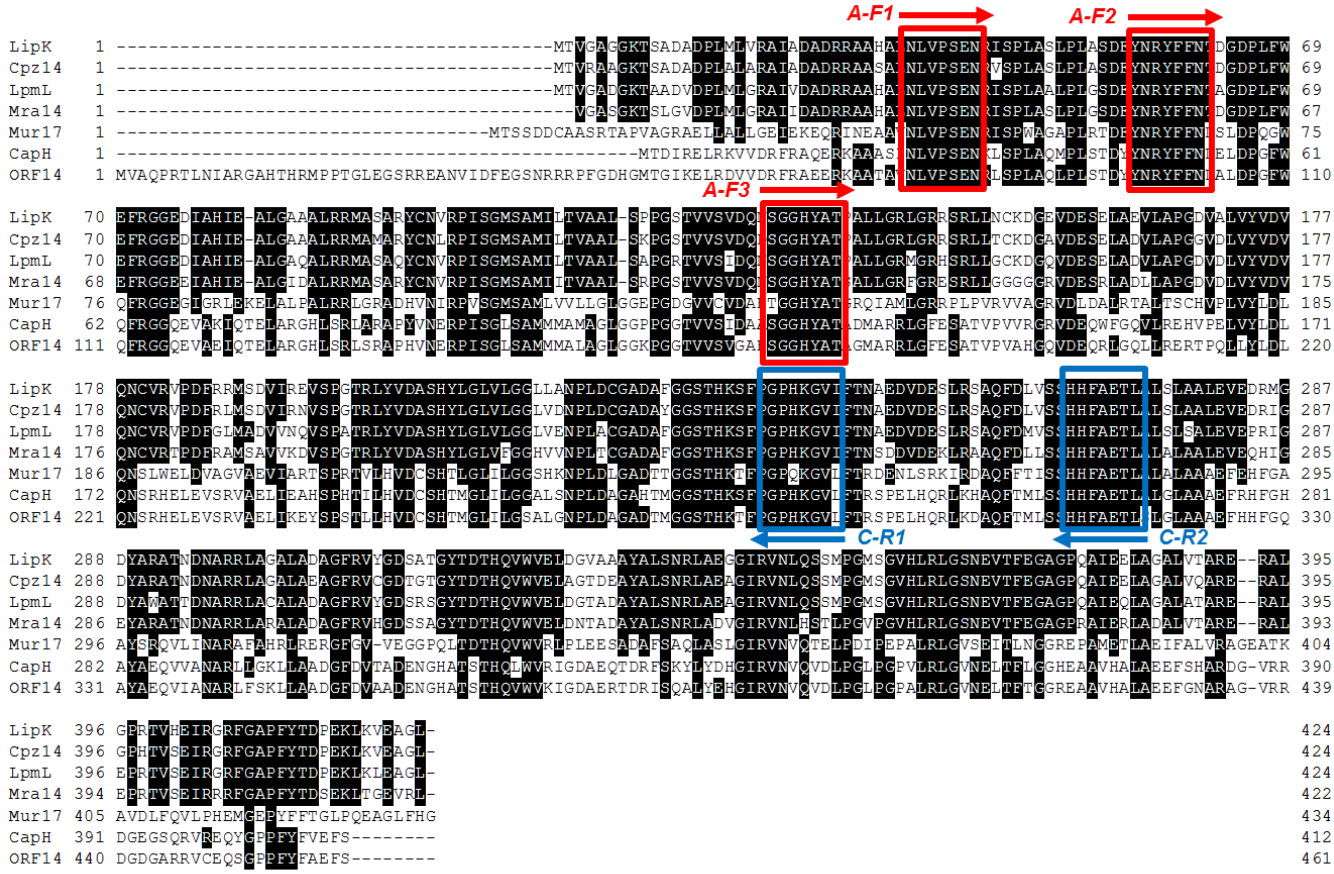


Figure S2. Sequence alignment of putative SHMT-like transaldolases. The alignment includes the enzymes involved in the biosynthesis of **10**-containing nucleoside antibiotics: LipK from the **1** gene cluster (Acc.# BAJ05887), Cpz14 from the **2** gene cluster (Acc.# ACQ63622), LpmL from the **3** gene cluster (Acc.# ADC96660), Mra14 from the **4** gene cluster (Acc.# AB746937), Mur17 from the **5** gene cluster (Acc.# ADZ45329); and enzymes involved in the biosynthesis of CarU-containing nucleoside antibiotics: CapH from the **7** gene cluster (Acc.# BAJ19052) and ORF14 from the **6** gene cluster (Acc.# BAI23322). Boxed are regions used to design the degenerate primers for specific amplification of the putative SHMTs.

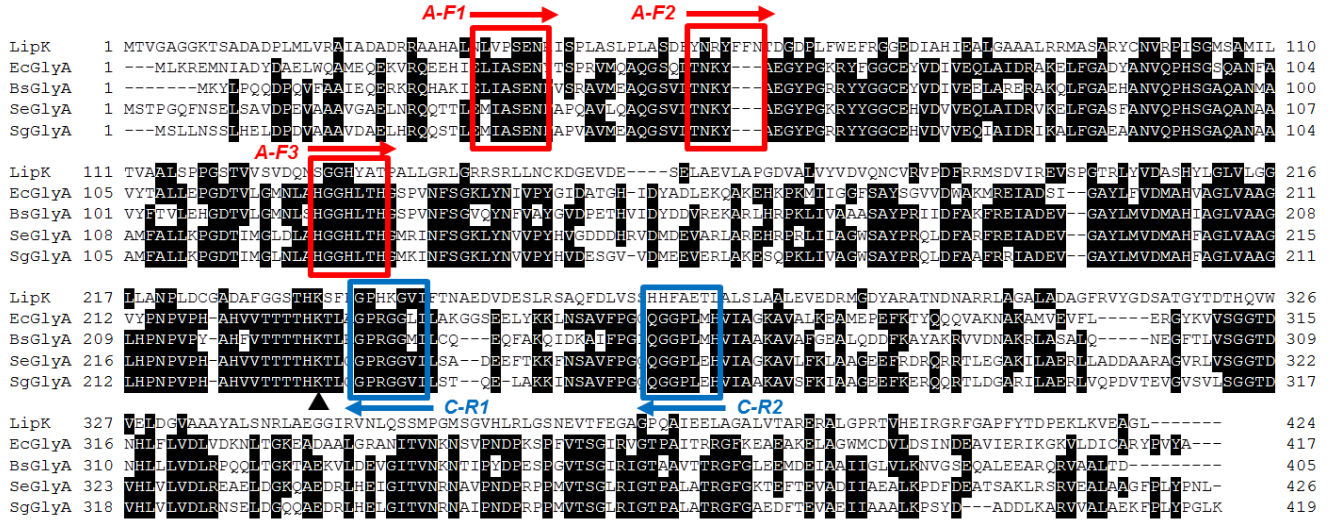


Figure S3. Sequence alignment of L-Thr:uridine-5'-aldehyde transaldolase LipK and serine hydroxymethyltransferases (SHMTs). Sequences include LipK from the **1** gene cluster (BAJ05887); authentic SHMTs with solved structures from *Escherichia coli* (EcGlyA, 1EQB_B) and *Bacillus stearothermophilus* (BsGlyA, 2VMV_A); and probable SHMTs identified from whole-genome sequencing of *Saccharopolyspora erythraea* (SeGlyA, YP_001107812) and *Streptomyces griseus* IFO 13350 (SgGlyA, YP_001823559). Boxed are regions used to design the degenerate primers for specific amplification of L-Thr:uridine-5'-aldehyde transaldolases. ▲ conserved Lys required for formation of the internal aldimine with pyridoxal-5'-phosphate.

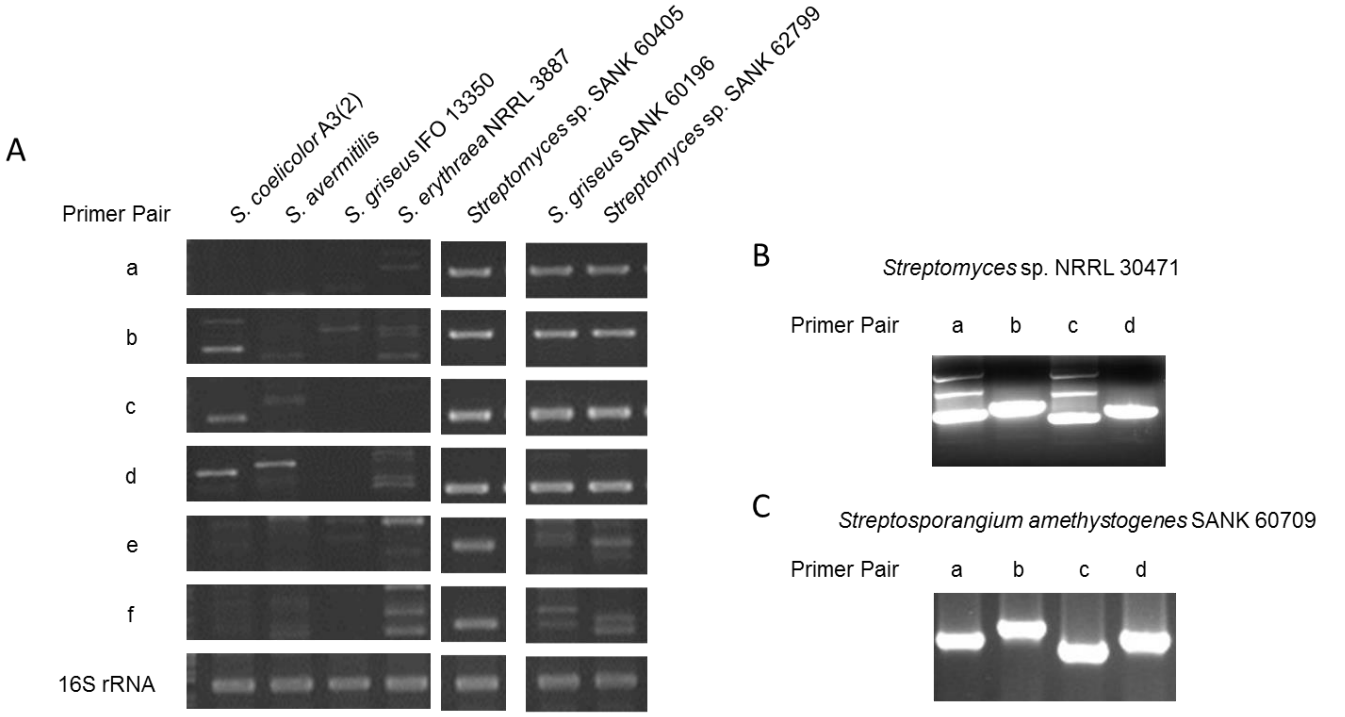


Figure S4. Validation of the constructed degenerate primers. The primer pairs utilized are as follows: a, A-F1/C-R1; b, A-F1/D-R1; c, A-F2/C-R1; d, A-F2/D-R1; e, B-F1/C-R1; and f, B-F1/D-R (see Table S2 for primer sequences). A) Genomic DNA templates used for PCR amplification include *Streptomyces coelicolor* A3(2), *Streptomyces avermitilis* ATCC 31267, *Streptomyces gieseus* IFO 13350 and *Saccharopolyspora erythraea* NRRL 3887 that are non-producers of high-carbon nucleoside antibiotics. *Streptomyces* sp. SANK 60405 is the producer of the **10**-containing nucleoside antibiotics **1**, and *Streptomyces griseus* SANK 60196 and *Streptomyces* sp. SANK 62799 are producers of CarU-containing nucleoside antibiotics, **6** and **7**, respectively. B) PCR analysis of the genomic DNA template from *Streptomyces* sp. NRRL 30471, producer of the **10**-containing nucleoside antibiotics **5**. C) PCR analysis of the genomic DNA template from *Streptosporangium amethystogenes* SANK 60709, producer of the **10**-containing nucleoside antibiotic **4**.

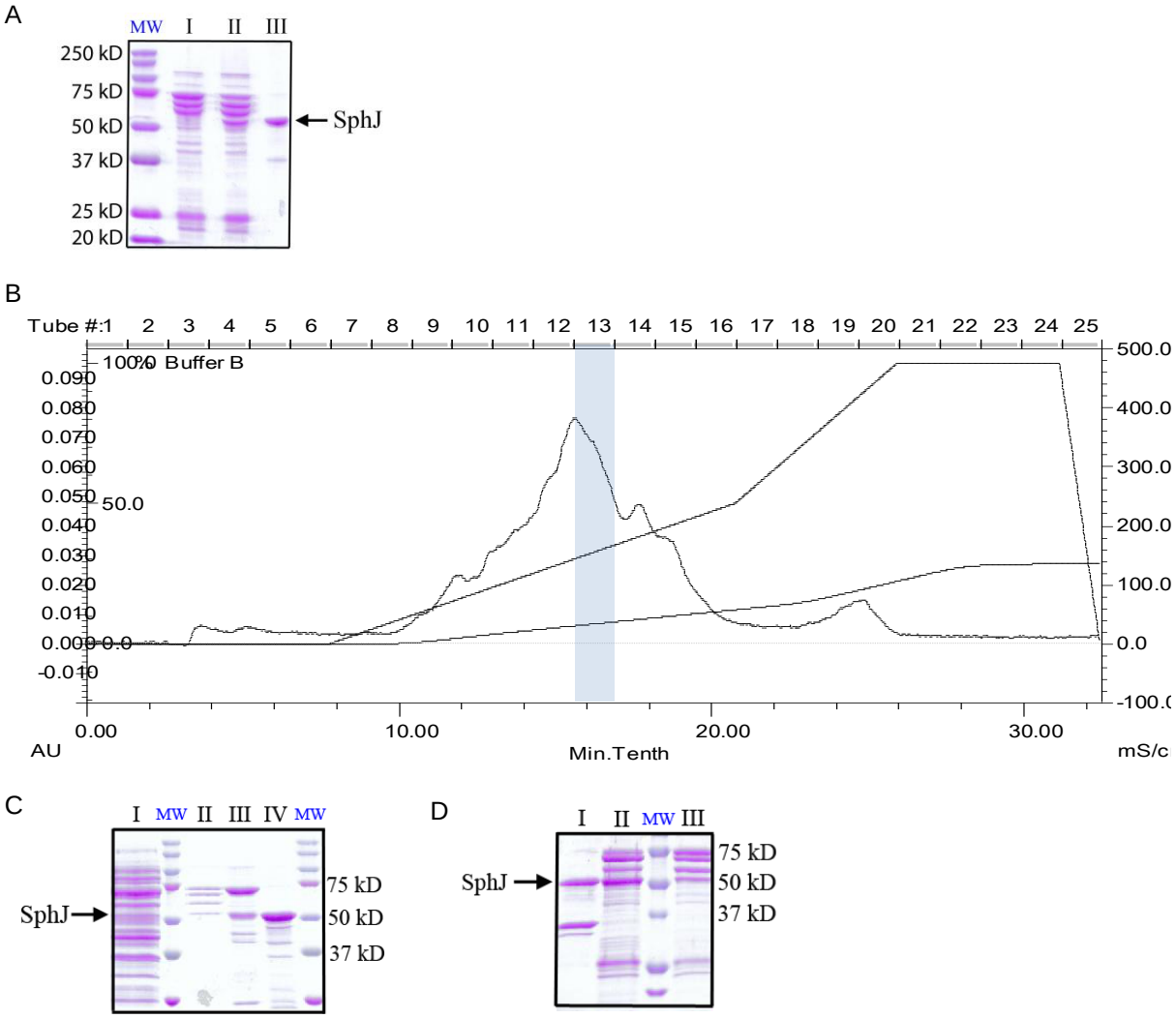


Figure S5. Production and partial purification of wild-type and mutant SphJ. (A) SDS-PAGE analysis of His_6-SphJ (expected MW of 52.4 kD) purified by IMAC from *E. coli* expressing the native gene (I), the codon optimized gene (II), and the insoluble debris upon expression of the native gene (III). (B) Anion-exchange chromatography elution profile with the fraction (tube #13) yielding the highest specific activity. (C) SDS-PAGE analysis of the purification steps of SphJ produced upon expression of the codon optimized gene including the cell free extract (I), protein isolated by IMAC (II), enriched SphJ from tube #13 (III), and insoluble debris upon expression of the codon optimized gene (IV). (D) SDS-PAGE analysis of insoluble debris upon expression of *sphJ(K248A)* (I), SphJ(K248A) purified by IMAC (II), and wild-type SphJ purified by IMAC (III). The engineered N-terminal His_6-tag contributes 5 kD to the predicted native molecular weight for each protein. MW, molecular weight standards.

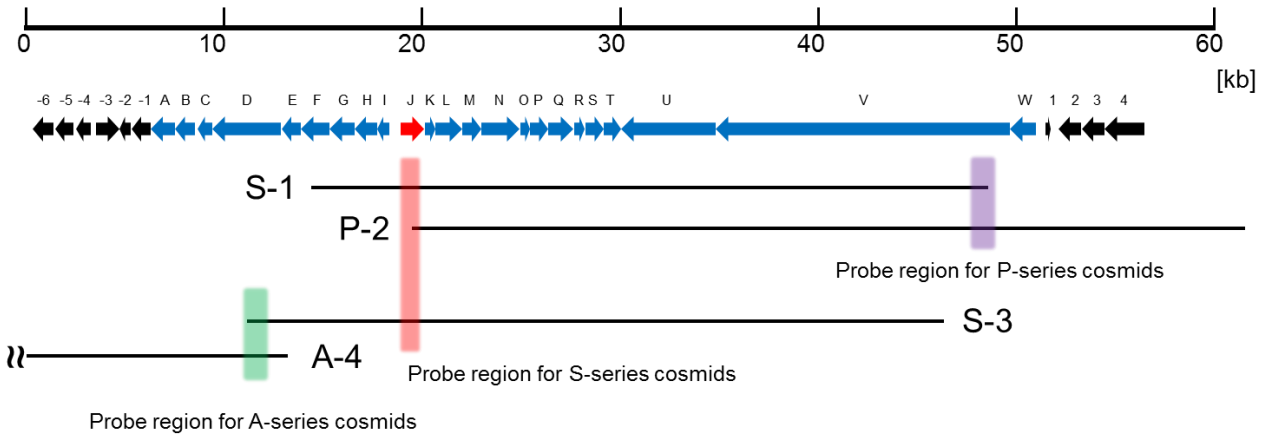


Figure S6. Genetic architecture of the sequenced chromosomal DNA. Four overlapping cosmids were identified by using probes for the transaldolase (pink), an ABC transporter (green) and a polyketide synthase (violet). These cosmids, covering 57-kb DNA, were sequenced and analyzed to reveal minimally 33 open reading frames (*orf*s). The putative biosynthetic gene cluster of **11** ranges from *sphA* through *sphW* (**blue**). The *orf* highlighted in **red** encodes the transaldolase that was initially used to identify the locus.

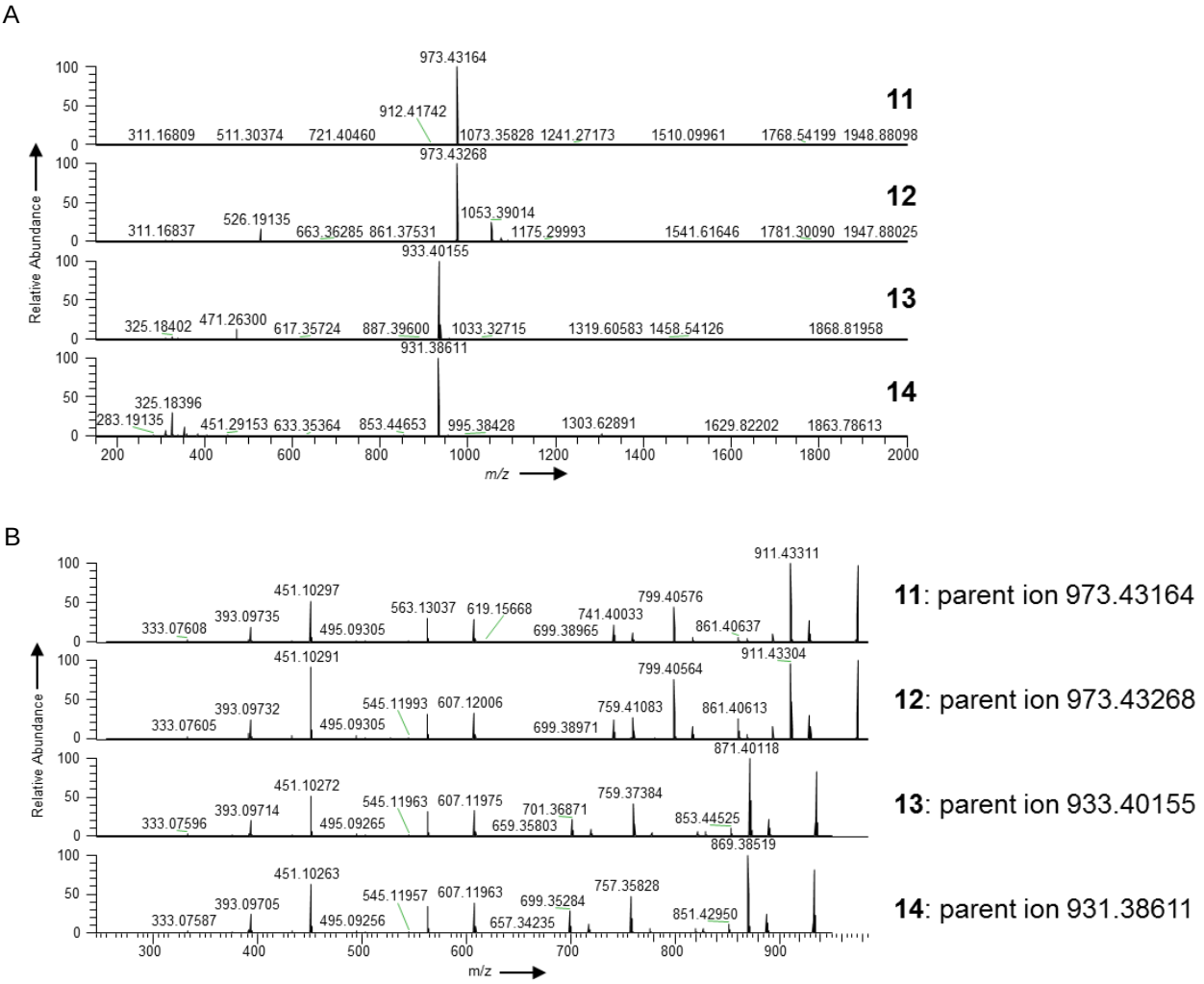


Figure S7. MS analyses of sphaerimicin A-D (**11**-**14**). A) FT-MS spectra (negative ion mode). B) CID spectra of indicated parent ions. The MS-MS fragmentation patterns along with preliminary ^{1}H NMR analysis are consistent with (*i*) the identity of **12** as a sulfated adduct of **11**, (*ii*) **13** containing a different acyl chain substituent relative to **11**, and (*iii*) **14** also containing a different acyl chain that is likely saturated at 4a-5a due to the loss of the vinyl proton signals observed in **11**. We are currently improving the yields and purification strategy in order to assign the structures, which will be reported in due course.

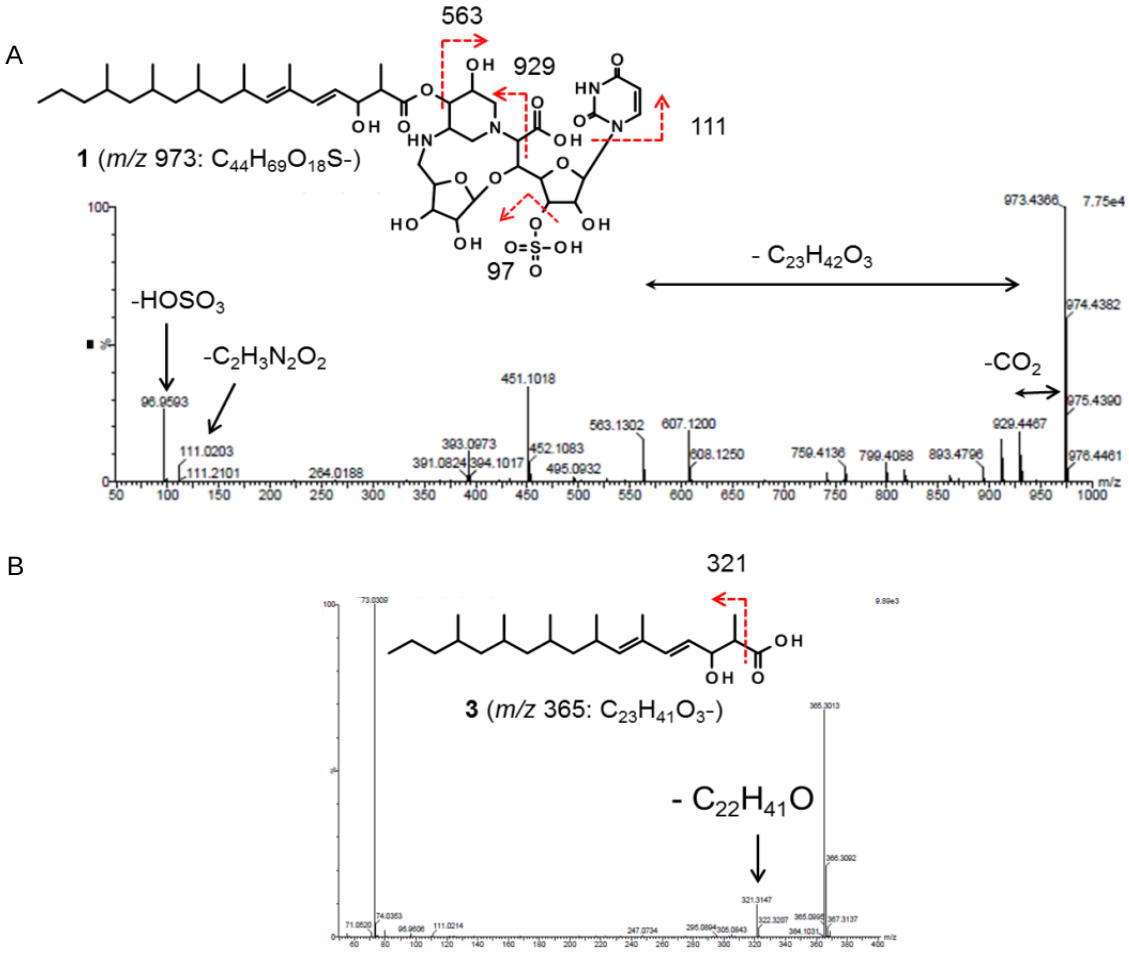


Figure S8. MS/MS analysis of A) **11** and B) **16**.

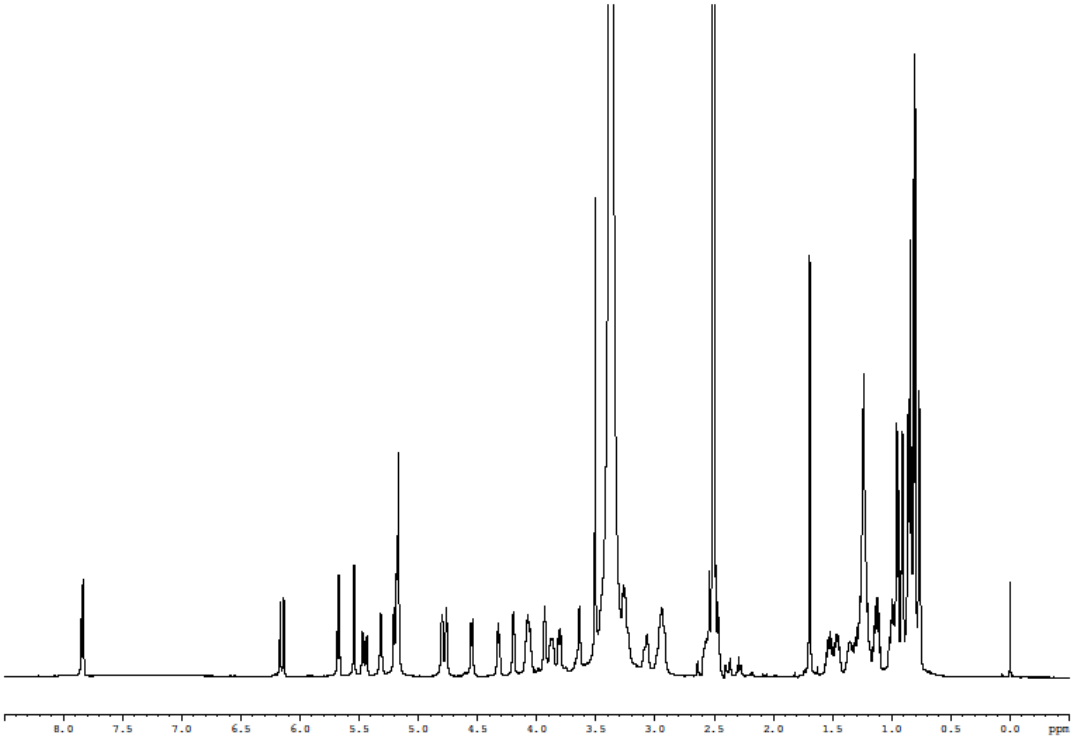


Figure S9. ^{1}H NMR spectrum of **11** in DMSO-*d6*.

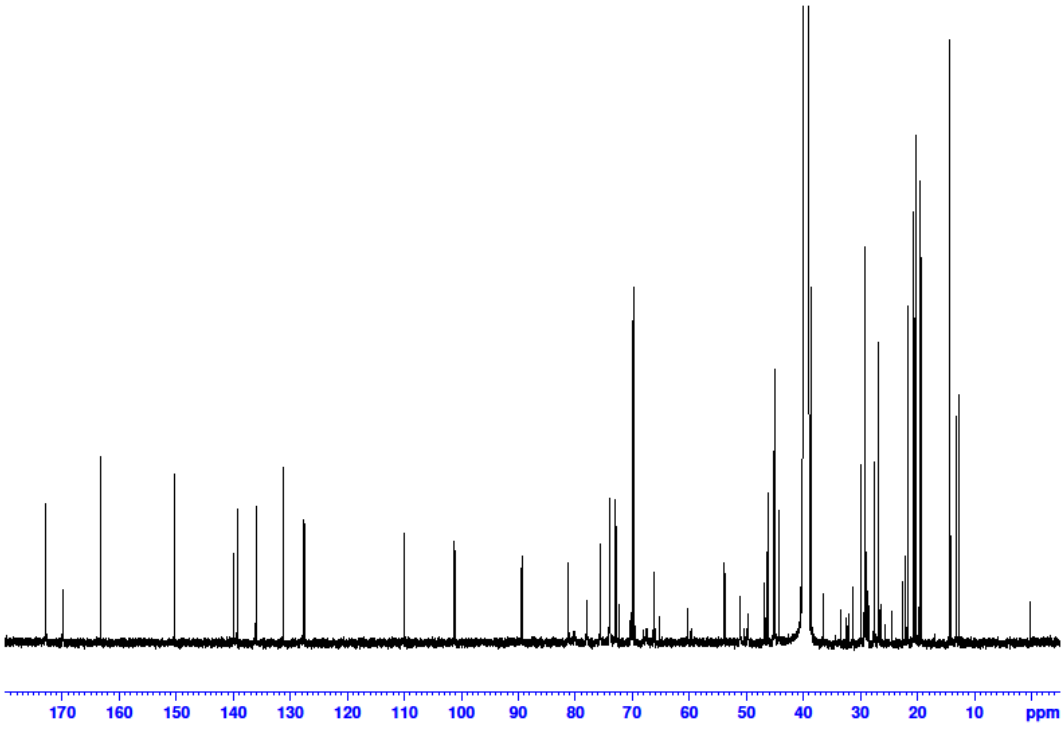


Figure S10. ^{13}C NMR spectrum of **11** in DMSO-*d6*.

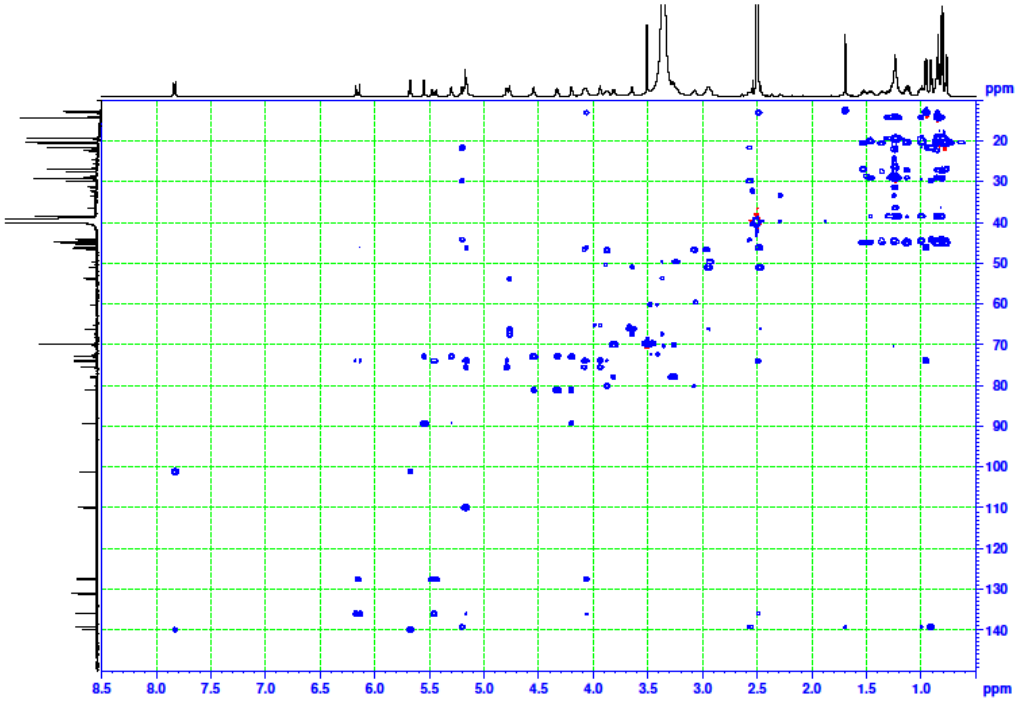


Figure S11. HSQC-TOCSY NMR spectrum of **11** in DMSO-*d6*.

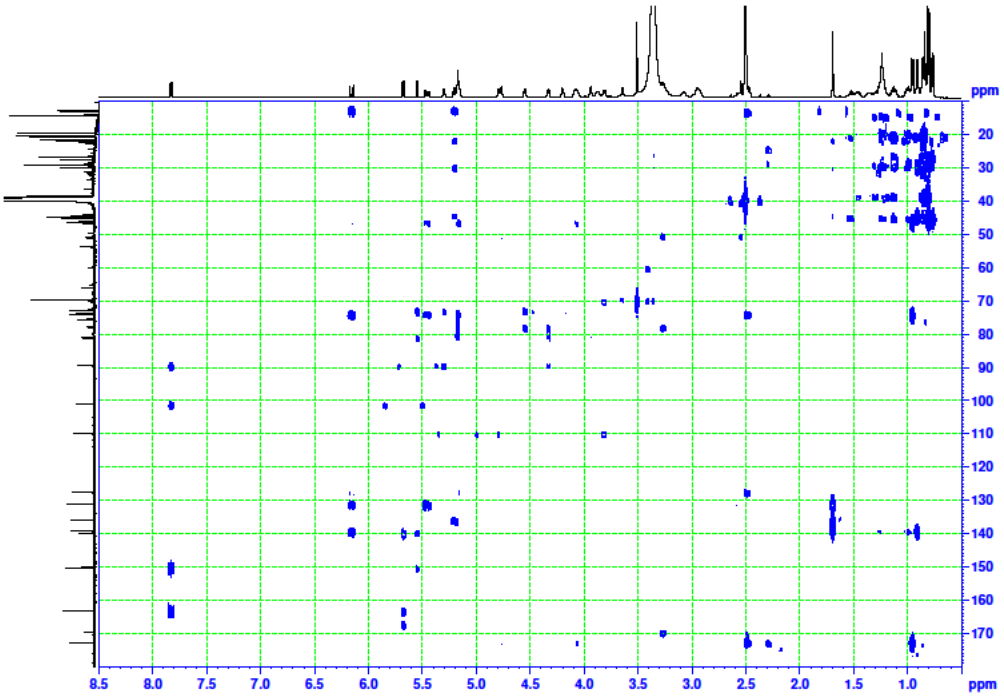


Figure S12. ^{1}H-^{13}C HMBC NMR spectrum of **11** in DMSO-*d6*.

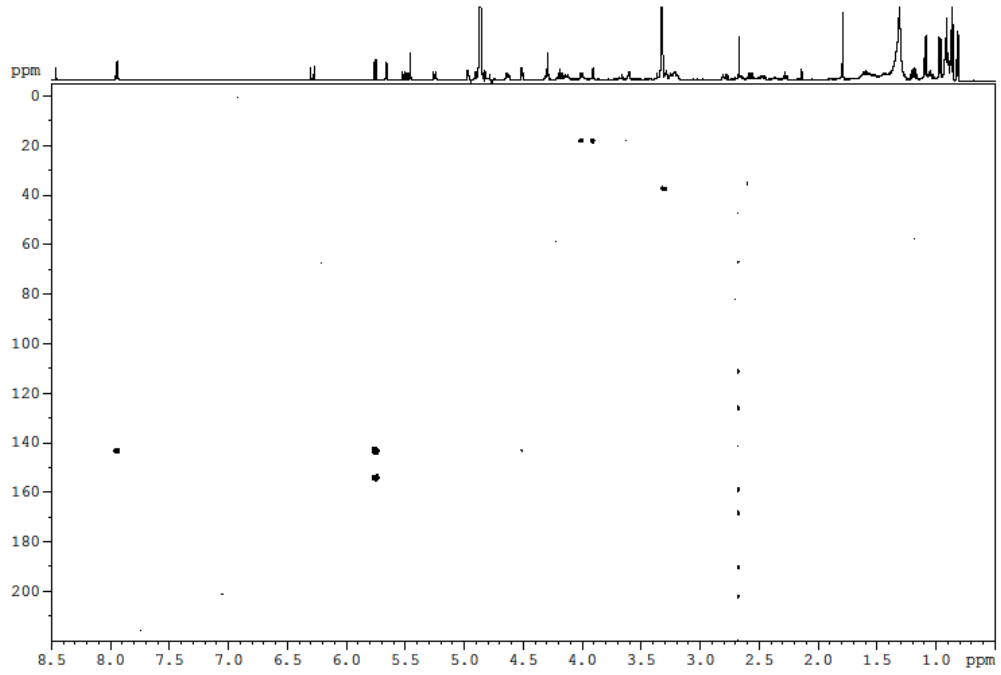


Figure S13. ^{1}H-^{15}N HMBC NMR spectrum of **11** in DMSO-*d6*.

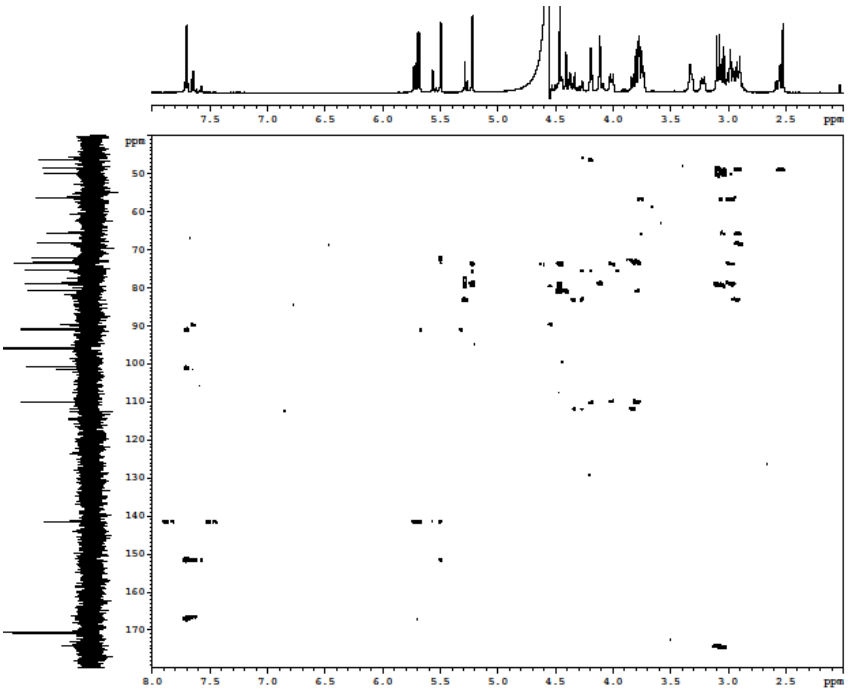


Figure S14. ^{1}H-^{13}C HMBC NMR spectrum of **15** in D_2O.

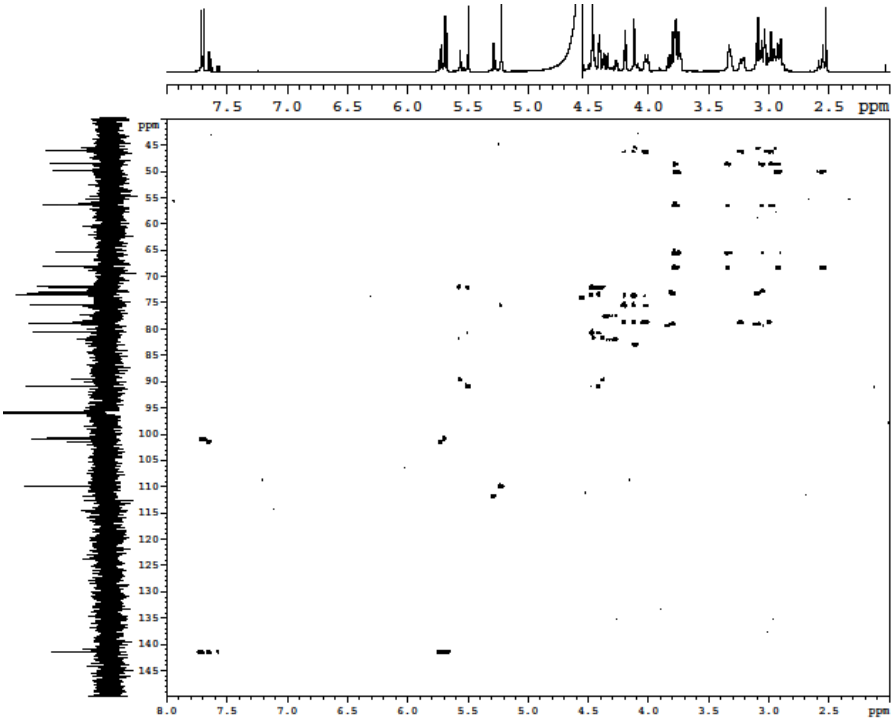


Figure S15. HSQC-TOCSY NMR spectrum of **15** in D_2O.

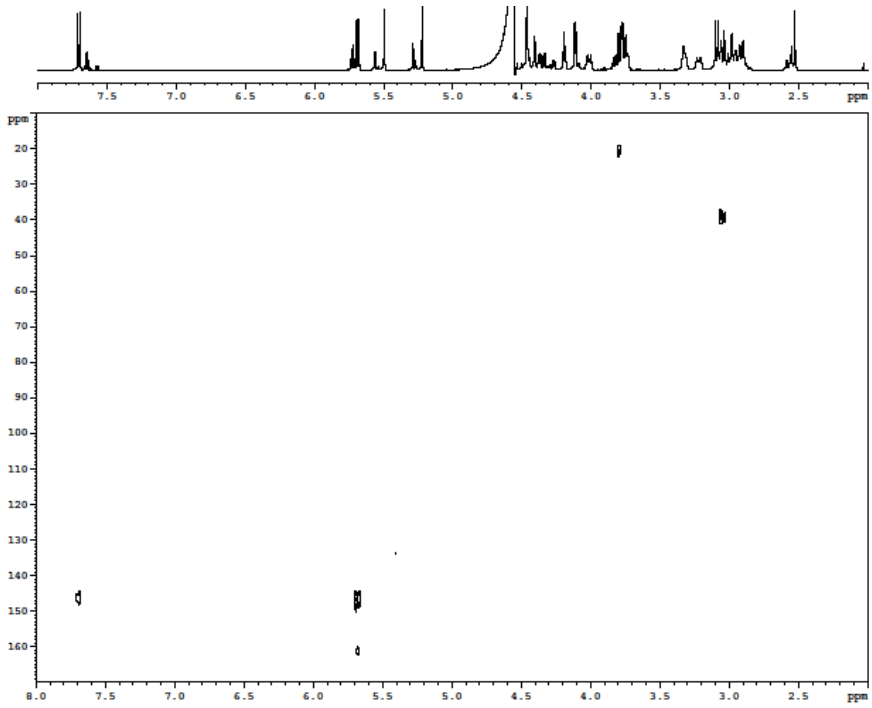


Figure S16. ^{1}H-^{15}N HMBC NMR spectrum of **15** in D_2O.

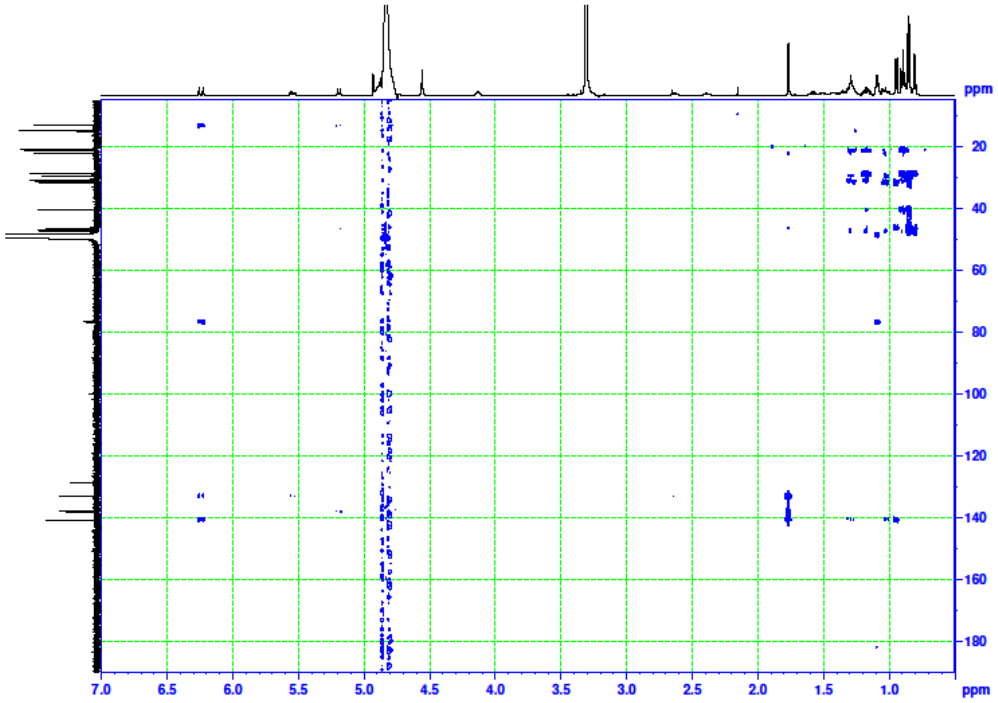


Figure S17. ^{1}H-^{13}C HMBC NMR spectrum of **16** in methanol-*d4*.

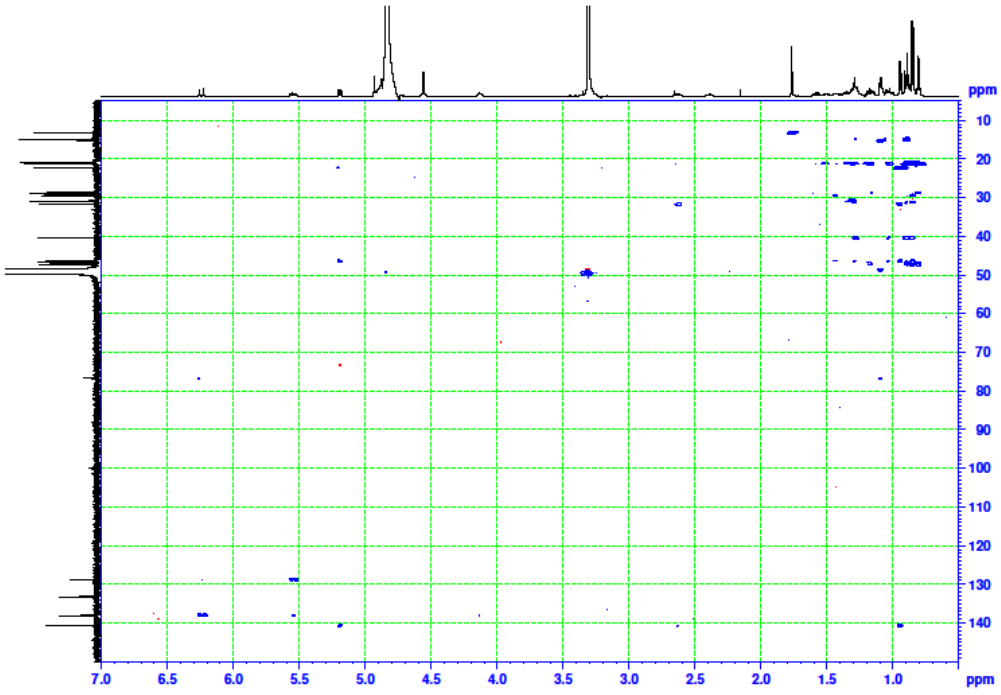


Figure S18. HSQC-TOCSY NMR spectrum of **16** in methanol-*d4*.

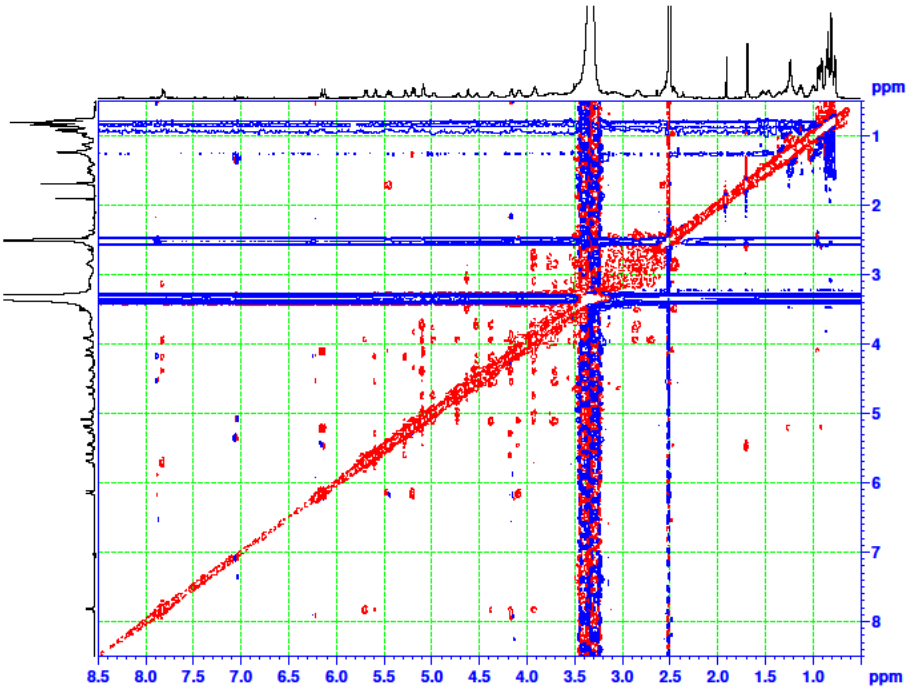


Figure S19. NOESY NMR spectrum of **11** in methanol-*d4*.

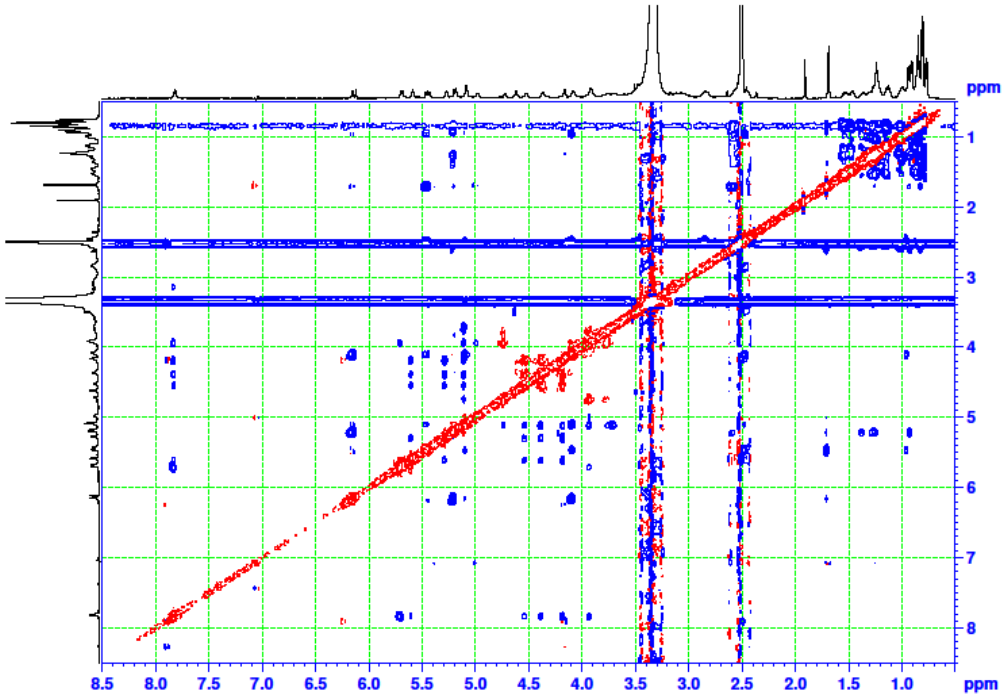
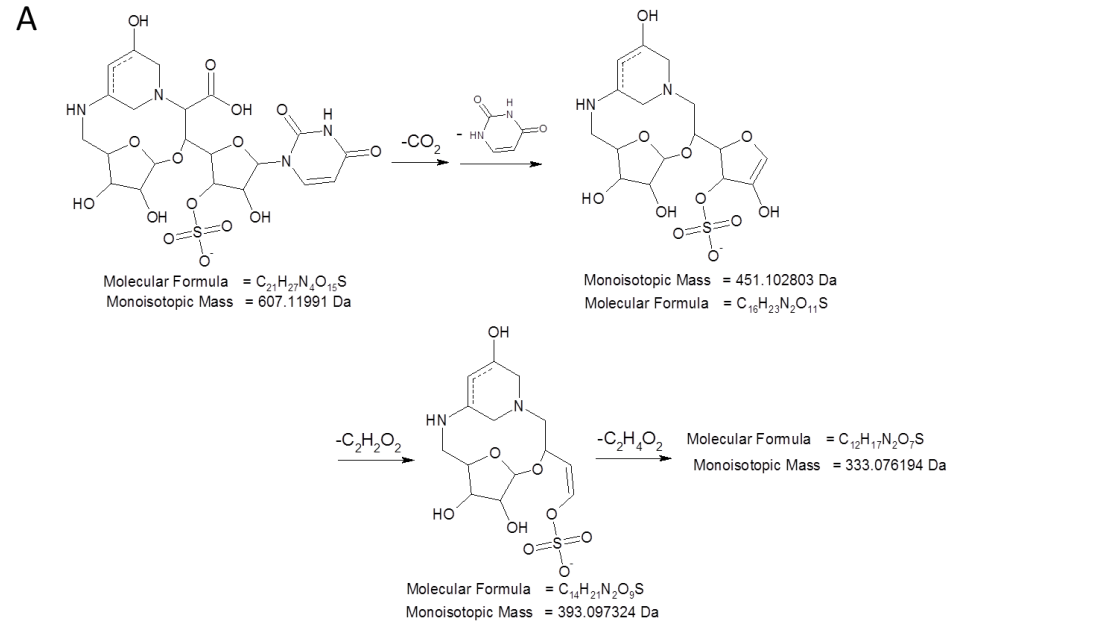


Figure S20. ROESY NMR spectrum of **11** in methanol-*d4*.



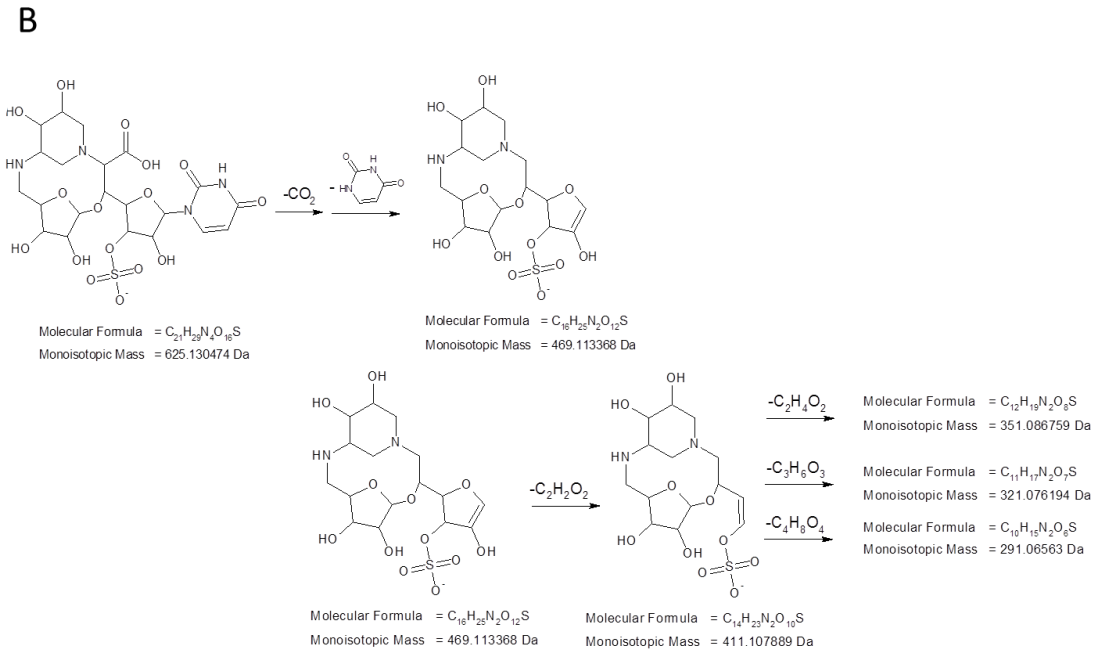


Figure S21. MS/MS/MS analysis of **11**. Fragment ions generated from parent ion of A) *m/z* 607 and B) *m/z* 625.

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[1] J. Sambrook J., D. W. Russell, *Molecular Cloning: A Laboratory Manual 3rd Ed.* (Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001).

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