Supporting Information

The Enzymes Mediating Maturation of the Seryl-tRNA Synthetase Inhibitor SB-217452 during Biosynthesis of Albomycins

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Experimental Procedures

S1. General notes

Materials: All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were used without further purification unless otherwise specified. Standard genetic manipulations of *Escherichia coli* were performed as described by Sambrook and Russell.[1] DNA sequencing was performed at the core facility of the Institute of Cellular and Molecular Biology, the University of Texas at Austin. Oligonucleotide primers were prepared by Integrated DNA Technologies (Coralville, IA). Kits for DNA gel extraction and spin minipreps are products of Qiagen (Valencia, CA). PureLink Genomic DNA Mini Kit was acquired from Invitrogen (Carlsbad, CA). KOD DNA polymerase was purchased from Novagen (Madison, WI). Enzymes and molecular weight standards used in the cloning experiments were obtained from New England Biolabs (Ipswich, MA). Reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA). Sterile syringe filters were bought from Fisher Scientific. Amicon YM-10 ultrafiltration membranes are products of Millipore (Billerica, MA).

Bacterial Strains and Plasmids: *Streptomyces* sp. strain ATCC 700974 was obtained from American Type Culture Collection (Manassas, VA). *E. coli* DH5α from Bethesda Research Laboratories (Gaithersburg, MD) was used for routine cloning procedures.[1] The protein overexpression host *E. coli* BL21 star (DE3) was obtained from Invitrogen. The protein overexpression host *E. coli* Lemo21 (DE3) was purchased from New England Biolabs. Vectors pET28b(+) and pET30a(+) for protein overexpression were purchased from Novagen. Vector pYH7 was used for the gene inactivation experiments.[2] Methylation deficient *E. coli* ET12567/pUZ8002 was used for the intergeneric conjugation.[3] Plasmid pIB139 is a *Streptomyces*-*E. coli* shuttle vector that can site-specifically integrate into *Streptomyces* chromosomes.[4]

Instrumentation: DNA concentrations were measured using a NanoDrop ND-1000 UV−vis instrument from Thermo Fisher Scientific. Fast protein liquid chromatography (FPLC) was performed using BioLogic DuoFlow (Bio-Rad) equipped with Superdex 200 column (GE Healthcare, IL). LC-ESI-TOFMS analysis was performed using an Agilent Technologies HPLC system equipped with a pump (G1311C), an auto sampler (G1329B), and a ToF mass spectrometer (G6230B) with an electrospray ionization (ESI) source. LCMS separations were performed using Poroshell 120 EC-C18 column (2.7 μm, 4.6 × 100 mm) with Eclipse plus C18 guard column (1.8 μm, 2.1 × 5 mm) at a flow rate of 0.4 mL/min using 0.1% formic acid in H_2O (solvent A) and acetonitrile (solvent B) with the following gradient program: 0–10 min 0–30% B, 10–12 min, 30–100% B, 12–13 min, 100-0% B). The obtained LCMS data were analyzed using MassHunter software (Agilent Technologies). NMR spectra were recorded using a Varian DirectDrive 600 MHz and a Bruker Avance III HD 500 MHz NMR equipped with CryoProbeTM Prodigy NMR spectrometer at the Nuclear Magnetic Resonance Facility at the University of Texas at Austin. Anaerobic work was performed in an anaerobic glovebox (Coy, Grass Lake, MI) under an atmosphere of > 98% N_2 and ca. 2% H_2.

S2. Genetic manipulation in *Streptomyces*

S2.1 Gene deletion experiments

The inactivation of *abmC* in *S.* sp. ATCC 700974 was performed using an in-frame deletion method.[5,6] A 2 kb fragment was first amplified using primers, abmCLF and abmCLR. Another 2 kb fragment was also amplified using primers, abmCRF and abmCRR. The two fragments were then ligated into the *Eco*RV site of linearized pYH7 using Gibson Assembly® Master Mix (NEB) according to the manufacturer's protocol. To transfer the resulting plasmid into *S.* sp. ATCC 700974, conjugation using *E. coli* ET12567/pUZ8002 was carried out following the standard procedure.[6] The colonies that were apramycin resistant were identified as

conjugants. These mutants were cultured in the absence of apramycin, and the resulting apramycin-sensitive colonies were analyzed by polymerase chain reaction (PCR). Sequencing of the PCR product confirmed the in-frame deletion of *abmC*. The inactivation of *abmF* in *S.* sp. ATCC 700974 was also performed based on the same procedure described for the inactivation of *abmC*. The fermentation culture of each deletion strain was analyzed as previously described.[5,7] The obtained results are shown in Figures 1 and S1.

abmCLF: 5′- GCCAGGTTCGGCGATCCGGAGTGGGTTCAGCGGTCGTGCC -3′
abmCLR: 5′- CACGCCTGTGATGGGACTCGCCTGAGCGGACGGGA -3′
abmCRF: 5′- CGAGTCCCATCACAGGCGTGTTGACAGTCAGGTTC -3′
abmCRR: 5′- ACGCCGGCTCGCGATGACGGAAGCCCTCCACCATGTGCTC -3′

abmFLF: 5′- GCCAGGTTCGGCGATCGACCATGACGGCCTGACTGGACGG -3′
abmFLR: 5′- TGAGATGGGCGTGACGGAAGGAGCGGACCATTGAT -3′
abmFRF: 5′- CTTCCGTCACGCCCATCTCAGCCCACCACCAGCGA -3′
abmFRR: 5′- ACGCCGGCTCGCGATGCTGAACCTGGTGCCGAGCGAGAAC -3′

S2.2 Gene complementation experiments

For complementation of *abmC*, a plasmid containing the *abmC* gene under the control of a constitutive promoter P*ermE** was prepared. The *abmC* gene was amplified by PCR using abmC-complementation-F and abmC-complementation-R as primers, and inserted into the P*ermE**-containing pIB139 which was first digested with *Nde*I and *Eco*RI. The gene insertion was achieved using seamless ligation cloning extract (SLiCE).[8] The prepared plasmids were then introduced into the Δ*abmC* mutant strain by conjugation based on the standard procedure.[5] The resulting recombinant strain, which is apramycin-resistant, was cultured and the produced metabolites were analyzed as previously described (Figure 1).

abmC-complementation-F: 5′- GTTGGTAGGATCCACATATGGAGAACCTGACTGTCAACACGCC -3′
abmC-complementation-R: 5′- TATGACATGATTACGAATTCTCAGGCGAGTCCCATGACCG -3′

For complementation of *abmF*, a complementation plasmid containing the *abmF* gene under the control of a native promoter in *S.* sp. ATCC700974 (P*abmJ*) was prepared. The *abmF* gene fragment was amplified by PCR using primers, abmF-complementation-F and abmF-complementation-R. A fragment containing P*abmJ* (424 bp sequence upstream from the start codon of *abmJ*) was also amplified using primers, PabmJ-abmF-F and PabmJ-abmF-R. The two fragments were inserted into pIB139 after digestion with *Nis*I and *Eco*RI by the same cloning method as described above. It should be noted that the digestion of pIB139 with *Nis*I and *Eco*RI removed the promoter P*ermE** from the vector. The prepared plasmid was then introduced into the Δ*abmF* mutant strain by conjugation. The resulting recombinant strain, which is apramycin-resistant, was cultured and the produced metabolites were analyzed as previously described (Figure 1).

abmF-complementation-F: 5′- ATGGGCCGGCCGGCCCCG -3′
abmF-complementation-R: 5′- TATGACATGATTACGAATTCTCAATGGTCCGCTCCTTCCGTCACGCG -3′
PabmJ-abmF-F: 5′- AGGTCGACTCTAGTATGCATTCCGCCTCGACCTTGGTCAGTTGC -3′
PabmJ-abmF-R: 5′- GCCGGGGCCGGCCGGCCCATCCGGCACCGGGCGTTGCATC -3′

S3. Gene cloning for protein expression

The *abmF* gene was PCR-amplified from the genomic DNA using primers carrying engineered *Kpn*I and *Hind*III restriction sites. The sequences of the primers are shown below. The PCR-amplified gene fragment was digested with *Kpn*I and *Hind*III and ligated into pET30a(+) vector, which had also been digested with the same

restriction enzymes. The resulting plasmid abmF/pET30a (+) was sequenced using the T7 or T7 terminal universal primers.

The *abmK* gene was PCR-amplified from the genomic DNA using primers abmK-F and abmK-R. The sequences of the primers are shown below. The PCR-amplified gene fragment was purified using Gel Extraction Kit from Qiagen and ligated into pET28b(+) vector after digestion with *Nde*I and *Xho*I according to the cloning method using SLiCE.[8] The resulting plasmid *abmK/*pET28b(+) was sequenced using the T7 or T7 terminal universal primers.

The *serRS1* gene was also PCR-amplified from the genomic DNA using primers carrying engineered *Nde*I and *Xho*I restriction sites. The sequences of the primers are shown below. The PCR-amplified gene fragments were purified using Gel Extraction Kit from Qiagen, digested with *Nde*I and *Xho*I, and ligated into pET28b(+) vector, which had also been digested with the same restriction enzymes. The resulting plasmid *serRS1*/pET28b(+) was sequenced using the T7 or T7 terminal universal primers.

The *abmJ* gene was PCR-amplified from the genomic DNA using primers carrying engineered *EcoR*I and *Hind*III restriction sites. The sequences of the primers are shown below. The PCR-amplified gene fragments were digested with *Eco*RI and *Hind*III, and ligated into pET30a(+) vector, which had also been digested with the same restriction enzymes.

For the construction of the AbmJ C12A/C16A/C19A expression plasmid, the *abmJ* 1-75 bp was amplified and mutated to the designed sequence using primers, abmJ-mut-LF and abmJ-mut-LR. The *abmJ* 16-1029 bp was also amplified and mutated to the designed sequence using primers, abmJ-mut-RF and abmJ-mut-RR. Each amplified fragment contains an overlap sequence with each other and a homologous sequence with pET30a(+). The plasmid abmJ-mut/pET30a(+) was constructed using Gibson assembly with the two amplified fragments and pET30a(+) vector digested with *Eco*RI and *Hind*III. The resulting plasmid was sequenced using the T7 or T7 terminal universal primers.

The genes encoding aminoacyl tRNA synthetases (TryRS, ThrRS, LysRS, and CysRS) were individually PCR-amplified from the genomic DNA of *E. coli* K12 using primers carrying engineered *Kpn*I and *Not*I restriction sites. The sequences of the primers are shown below. The PCR-amplified gene fragment was digested with *Kpn*I and *Not*I and ligated into pET30a(+) vector, which had also been digested with the same restriction enzymes. The resulting plasmids were sequenced using the T7 or T7 terminal universal primers.

abmF-F: 5′- CGGGGTACCATGGGCCGGCCGGCCCCG -3′
abmF-R: 5′- GCTACCCAAGCTTTCAATGGTCCGCTCCTTCCGTCACGCGGCGC -3′

abmK-F: 5′- TGCCGCGCGGCAGCCATATGCTTGATCTGGACCTGATTCGTAAGG -3′
abmK-R: 5′- TGGTGGTGGTGGTGCTCGAGTCAACGCTTGGGGCGGAC-3′

serRS1-F-5′- ATATCATATGATTGACCTTCGCCTGCTCCG -3′
serRS1-R-5′- ATATCTCGAGTCACTTGGCTACCGGTTCCAGGATC -3′

abmJ-F-5′- CCCGAATTCGTGAGCCGGATCTTCTTCTGG -3′
abmJ-R-5′- CACAAGCTTTCACCGGATCGCCTTGATGGC -3′

abmJ-mut-LF: 5′- GCTGATATCGGATCCGAATTCGTGAGCCGGATCTTCTTCTGG -3′
abmJ-mut-LR:
5′- GGCCTCCCGCCAGATGTCGGCCATCCGGGCCCGGGCGTTGGCTCCGCTGTTGATCCAGAA -3′

abmJ-mut-RF:
5′- TTCTGGATCAACAGCGGAGCCAACGCCCGGGCCCGGATGGCCGACATCTGGCGGGAGGCC -3′
abmJ-mut-RR: 5′- CTCGAGTGCGGCCGCAAGCTTTCACCGGATCGCCTTGATGGC -3′

TyrRS-F: 5′- CGGGGTACCATGGCAAGCAGTAACTTGATT -3′

TyrRS-R: 5′- ATAAGAATGCGGCCGCTTATTTCCAGCAAATCAGACAGTA -3′

ThrRS-F: 5′- CGGGGTACCATGCCTGTTATAACTCTTCCTG -3′
ThrRS-R: 5′- ATAAGAATGCGGCCGCTTATTCCTCCAATTGTTTAAGACTG -3′

LysRS-F-5′- CGGGGTACCATGTCTGAACAACACGCAC -3′
LysRS-R-5′- ATAAGAATGCGGCCGCTTATTTTACCGGACGCATCG -3′

CysRS-F-5′- CGGGGTACCATGCTAAAAATCTTCAATACTCTGACA -3′
CysRS-R-5′- ATAAGAATGCGGCCGCTTATTGCAACAGCGAAATATCC -3′

S4. Expression and purification of the gene products

S4.1 AbmF

The plasmid *abmF*/pET30a(+) was used to transform the *E. coli* Lemo21 (DE3) strain for protein expression. An overnight culture of *E. coli* Lemo21 (DE3)-*abmF*/pET30a(+) grown in the LB medium containing 50 μg/mL of kanamycin and 25 μg/mL of chloramphenicol was used to inoculate the same growth medium for protein expression. The culture was incubated at 37 °C with shaking (220 rpm) until the OD600 reached ~0.6. Protein expression was then induced by the addition of β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, and the cells were allowed to grow at 16 °C and 220 rpm for an additional 18 h. The cells were harvested by centrifugation at 4,000 rpm for 10 min and lysed by sonication in a buffer containing 20 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 10% (v/v) glycerol. The resulting lysate was centrifuged at 20,000 × *g* for 20 min, and the supernatant was subjected to Ni-nitrilotriacetic acid (NTA) resin. Bound protein was washed with the same buffer and eluted using increasing concentrations of imidazole (50 mM, 100 mM, 150 mM and 350 mM). The fractions containing the *N*-His_6 AbmF as determined by SDS-PAGE were collected. The obtained protein fraction was further purified by FPLC with a Superdex 200 (10/300 GL) gel filtration column using a buffer containing 50 mM HEPES (pH 7.5) and 300 mM NaCl at a flow rate of 0.3 mL/min. Fractions containing AbmF as determined by SDS-PAGE were collected. (Figure S2). TryRS, ThrRS, LysRS, and CysRS were also expressed and purified using the same procedure as described for AbmF (Figure S2). These proteins were used for enzyme assays without purification by a gel filtration column.

S4.2 AbmK

The plasmid *abmK*/pET28b(+) was used to transform the *E. coli* BL21 (DE3) strain for protein expression. An overnight culture of *E. coli* BL21 (DE3)-*abmK*/pET28b(+) grown in the LB medium (10 mL) containing 50 μg/mL of kanamycin was used to inoculate 1 L of the same growth medium. The culture was incubated at 37 °C with shaking (220 rpm) until the OD600 reached ~0.6. Protein expression was then induced by the addition of IPTG to a final concentration of 0.1 mM, and the cells were allowed to grow at 16 °C and 120 rpm for an additional 20 h. The cells were harvested by centrifugation at 4,000 × *g* for 10 min and stored at –80 °C until lysis. All purification steps were carried out at 4 °C. The thawed cells were resuspended in 50 mM HEPES buffer (pH 7.5) buffer containing 10% (v/v) glycerol, 10 mM imidazole, and NaCl (300 mM). After incubation with lysozyme (1 mg/mL) for 30 min, the cells were disrupted by sonication using 10 × 10 s pulses with a 30 s cooling pause between each pulse. The resulting lysate was centrifuged at 20,000 × *g* for 20 min, and the supernatant was subjected to Ni-NTA resin. Bound protein was eluted using 50 mM HEPES buffer (pH 7.5) buffer containing 10% (v/v) glycerol, 250 mM imidazole, and NaCl (300 mM). The collected protein solution was dialyzed against 3 × 1 L of 50 mM HEPES buffer (pH 7.5) containing 300 mM NaCl and 10% glycerol. The protein solution was then flash-frozen in liquid nitrogen and stored at –80 °C until use. (Figure S2).

S4.3 SerRS1

The *N*-His_6-SerRS1 was expressed using *E. coli* BL21 (DE3)-*serRS1*/pET28b(+) and purified as described for *N*-His_6-AbmK. (Figure S2).

S4.4 AbmJ

The plasmid *abmJ*/pET30a(+) was used to transform the *E. coli* BL21 (DE3) strain carrying pRKSUF017 plasmid.[9] The resulting *E. coli* strain was grown in 6 L LB medium containing 30 μg/mL of kanamycin and 5 μg/mL of tetracyclin at 37 °C with 200 rpm agitation until OD600 reached 0.5. Protein expression was then induced by the addition of 0.1 mM IPTG, 0.2 mM $FeSO_4(NH_4)_2SO_4$, and 0.2 mM L-cysteine, and the cells were allowed to grow at 18 °C and 120 rpm for an additional 20 h. The cells were harvested by centrifugation at 4,000 × *g* for 10 min, resuspended with 50 mM HEPES (pH 7.5) buffer containing 150 mM NaCl, 10 mM imidazole, 20% (v/v) glycerol, and lysed by ultra-sonication on ice. After centrifugation at 13,000 × *g* for 60 min, 2 mL of Ni-NTA resin was added to the supernatant followed by incubation at 4 °C for 2 h. The resulting mixture was loaded onto a filter column and the protein-bound Ni-NTA resin was washed with 50 mM HEPES buffer (pH 7.5) containing 150 mM NaCl, 20 mM imidazole, and 20% (v/v) glycerol. Protein was eluted with 50 mM HEPES buffer (pH 7.5) containing 150 mM NaCl, 250 mM imidazole, and 20% (v/v) glycerol. The *N*-His_6-AbmJ was collected, concentrated, and exchanged into 50 mM HEPES buffer (pH 7.5) containing 150 mM NaCl and 20% (v/v) glycerol (Figure S2).

S5. *In vitro* assays

S5.1 Typical AbmF/AbmK assay conditions and procedures

Compound **11** (0.2 mM) was incubated with AbmF (3.8 μM), AbmK (3.3 μM), L-serine (2.5 mM), ATP (2.5 mM), and total tRNA (5 μg/μL) in the buffer containing HEPES (pH 7.5, 50 mM), KCl (100 mM), $MgCl_2$ (10 mM). After 5 h, the mixture was filtered through an YM-10 membrane using an Amicon ultrafiltration unit to remove protein. The filtrate was analyzed by LCMS (Figure 2A).

S5.2 Chemical reconstitution of AbmJ

In the anaerobic glovebox, varied concentrations of AbmJ (26.2 μM, 13.1 μM, 10.5 μM, 5.2 μM) in a reconstitution buffer containing 25 mM HEPES (pH 7.5, 1.7 mL) and 15% glycerol were stirred on ice to remove molecular oxygen from the mixture. After 3 h, the AbmJ solution was added 0.1 mL of 0.1 M DTT and stirred on ice for 15 min. The resulting solution was added 10 μL of 5 mM $Fe(NH_4)_2(SO_4)_2$ 10 times in 10 min and then 5 μL of 5 mM Na_2S 20 times in 20 min. The mixture was stirred on ice for additional 3 h and then desalted using an Econo-Pac 10DG desalting column (Bio-Rad). The reconstituted AbmJ was eluted with the same buffer containing 25 mM HEPES (pH 7.5) and 15% glycerol. The Fe content in the reconstituted AbmJ was determined based on the known procedure[10] and the obtained results are summarized in Table S1.

S5.3 Typical AbmJ assay conditions and procedures

In vitro AbmJ assays were performed in the anaerobic glovebox. Compound **17** (0.25 mM) was incubated with reconstituted AbmJ (5 μM), SAM (0.5 mM), and sodium dithionite (2 mM) in HEPES buffer (pH 7.5, 25 mM). After 24 h, the mixture was filtered through an YM-10 membrane using an Amicon ultrafiltration unit to remove protein. The filtrate was analyzed by LCMS (Figures 2B and S9).

Based on the peak areas of **7** and 5'-dA, the ratio between the two products generated during the AbmJ reaction of **17** was determined to be 1 : 1.05 (average of two measurements). The ratio of the ionization efficiencies of **7** and 5'-dA (1 : 11) in the positive ionization mode under the LCMS conditions employed was used for the quantification.

S6. Preparation of tested compounds

S6.1 Preparation of 4

Compound **4** was prepared as previously reported.[5]

S6.2 Preparation of 11

Compound **11** was generated via digestion of 3′-*epi*-albomycin δ_2 (**18**) produced in the Δ*abmJ* strain using the same method described for **4**.[5] Compound **11** was purified by HPLC using an analytical C18 column (Agilent, C18, 5 μm, 4.6 × 250 mm). The HPLC column was eluted using H_2O as mobile phase A and acetonitrile as mobile phase B at a flow rate of 1 mL/min with the following gradient program: 0–2 min 0% B, 2–12 min 0–50% B, 12–13 min 50–80% B, 13–16 min 80% B, 16–17 min 80–0% B, 17–25 min 0% B. Elution of **11** was monitored by setting the UV-detector to 306 nm. Fractions containing compound **11** were collected and lyophilized. The residue was further purified by HPLC using an analytical Dionex CarboPac PA1 (4 × 250 mm, Thermo). The HPLC column was eluted using H_2O as mobile phase A and 2 M NaCl as a mobile phase B at a flow rate of 1 mL/min with the following gradient program: 0–2 min 0% B, 2–10 min 0–10% B, 10–11 min 10–50% B, 11–13 min 50% B, 13–14 min 50–0% B, 14–20 min 0% B. Fractions containing compound **11** were collected and desalted using the same C18 column to give **11** after lyophilization. ^{1}H NMR (D_2O, 600 MHz) δ 8.26 (1H, d, J = 8.3 Hz, H-6), 6.37 (1H, d, J = 8.3 Hz, H-5), 6.10 (1H, d, J = 4.7 Hz, H-1′), 4.57 (1H, dd, J = 3.8 Hz, J = 4.7 Hz, H-2′), 4.37 (1H, dd, J = 4.6 Hz, J = 4.6 Hz, H-5′), 4.36 (1H, dd, J = 3.8 Hz, J = 5.5 Hz, H-3′), 4.58 (1H, d, J = 4.6 Hz, H-6′), 3.91 (1H, J = 4.6 Hz, J = 5.5 Hz, H-4′), 3.49 (3H, s, NMe). ^{13}C NMR (D_2O, 151 MHz) δ 167.4, 155.4, 152.1, 137.4, 96.9, 77.2, 74.6, 70.1, 65.5, 58.3, 52.9, 29.8. ESI-HRMS calcd for $C_{13}H_{20}N_5O_7S^+$ $[M + H]^+$ 390.1078, found 390.1073.

S6.3 Preparation of 7

A 400 mL of TSB medium was inoculated with the spores of the wild type *S.* sp. ATCC700974 and incubated at 28 °C. After 24 h, 50 mL of the TSB seed culture was used to inoculate 6 × 1 L of the albomycin-producing medium.[6] The culture was incubated at 28 °C for 96 h and centrifuged at 4,000 × *g*. The cell-free broth was applied to a column (5 cm × 15 cm) packed with Amberlite XAD4 pre-equilibrated with water. The column was washed with water and eluted with 50% aqueous methanol. Fractions containing **7** as determined by ESIMS analysis were concentrated under reduced pressure. The obtained residue was then loaded to a Biogel P2 (Bio-Rad) size exclusion column (2.5 cm × 75 cm) using 25 mM NaCl as the running solvent. Fractions containing **7** were collected and concentrated under reduced pressure. The resulting residue was dissolved in 5 mL water and loaded on a reverse phase Cosmosil 140C18-OPN (Nacalai USA, Inc. (San Diego, CA)) column (2.5 cm × 10 cm) pre-equilibrated with water. The column was washed and eluted using 0–30% methanol in water. Evaporation of the elution fractions containing **7** gave a brown residue. The residue was purified by HPLC using a semipreparative C18 column (ZORBAX, ODS, 5 μm, 9.4 mm × 250 mm). The HPLC column was eluted using 0.1% TFA in H_2O as mobile phase A and 0.1% TFA in acetonitrile as mobile phase B at a flow rate of 4 mL/min with the following gradient program: 0–1 min 0% B, 1–10 min 0–4% B, 10–11 min 4–100% B, 11–15 min 90% B, 15–16 min 90–0% B, 16–21 min 0% B. Elution of **7** was monitored by setting the UV-detector to 280 nm. Elution fractions were collected and lyophilized to give **7**. ^{1}H NMR spectrum of the obtained sample is in agreement with the literature data with only slight differences in chemical shifts and coupling constants.[11] The purity of **7** was determined to be approximately 85% based on ^{1}H NMR and HPLC-UV analysis. ^{1}H NMR (D_2O, 500 MHz) δ 8.56 (1H, d, J = 8.3 Hz, H-6), 6.88 (1H, d, J = 8.3 Hz, H-5), 5.87 (1H, d, J = 3.9 Hz, H-1′), 4.58 (1H, d, J = 5.3 Hz, H-6′), 4.41 (1H, dd, J = 4.1 Hz, J = 5.0 Hz, H-2′), 4.37 (1H, m, H-5′), 4.36 (1H, m, H-3′), 4.15 (1H, dd, J = 4.2 Hz, J = 5.1 Hz, H-2″), 3.92 (1H, dd, J = 4.2 Hz, J = 12.4 Hz, H-3″), 3.91 (1H, dd, J = 5.1 Hz, J = 12.4 Hz, H-3″), 3.91 (1H, m, H-4′), 3.44 (3H, s, NMe). ESI-HRMS calcd for $C_{16}H_{25}N_6O_9S^+$ $[M + H]^+$ 477.1398, found 477.1473.

S6.4 Preparation of 17

Compound **17** was generated in the Δ*abmJ* strain using the same method described for **7** and purified by HPLC using the same method described for **11**. ^{1}H NMR (D_2O, 500 MHz) δ 8.14 (1H, d, *J* = 8.3 Hz, H-6), 6.20 (1H, d, *J* = 8.3 Hz, H-5), 5.87 (1H, d, *J* = 4.9 Hz, H-1′), 4.41 (1H, dd, *J* = 4.0 Hz, *J* = 4.9 Hz, H-2′), 4.58 (1H, d, *J* = 7.1 Hz, H-6′), 4.37 (1H, dd, *J* = 2.9 Hz, *J* = 7.1 Hz, H-5′), 4.36 (1H, dd, *J* = 4.0 Hz, *J* = 5.1 Hz, H-3′), 3.92 (1H, d, *J* = 5.3 Hz, H-3″), 3.91 (1H, d, *J* = 5.3 Hz, *J* = 12.4 Hz, H-3″), 4.15 (1H, t, *J* = 5.2 Hz, H-2″), 3.91 (1H, dd, *J* = 2.9 Hz, *J* = 5.3 Hz, H-4′), 3.44 (3H, s, NMe). ^{13}C NMR (D_2O, 126 MHz) δ 175.5, 172.6, 167.5, 155.4, 152.2, 137.5, 97.0, 77.4, 74.4, 70.6, 65.2, 62.5, 58.2, 55.6, 52.9, 29.8. ESI-HRMS calcd for $C_{16}H_{25}N_6O_9S^+$ $[M + H]^+$ 477.1398, found 477.1379.

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Supplementary Table

Table S1. Iron quantification using the reconstituted AbmJ. Varied concentrations of AbmJ were used for the Fe-S cluster reconstitution experiment.

AbmJ (μM)	Determined Fe content per monomer[a]
26.2	5.1 ± 0.2
13.1	6.8 ± 0.1
10.5	10.3 ± 0.2
5.2	10.1 ± 0.1

[a] Average values are reported (n = 3).

Supplementary Figures

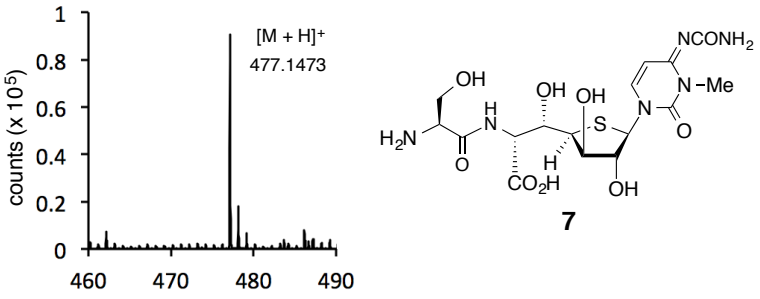

Figure S1. ESI-MS spectrum of **7** produced in the Δ*abmC* strain. See Figure 1 for the LC traces.

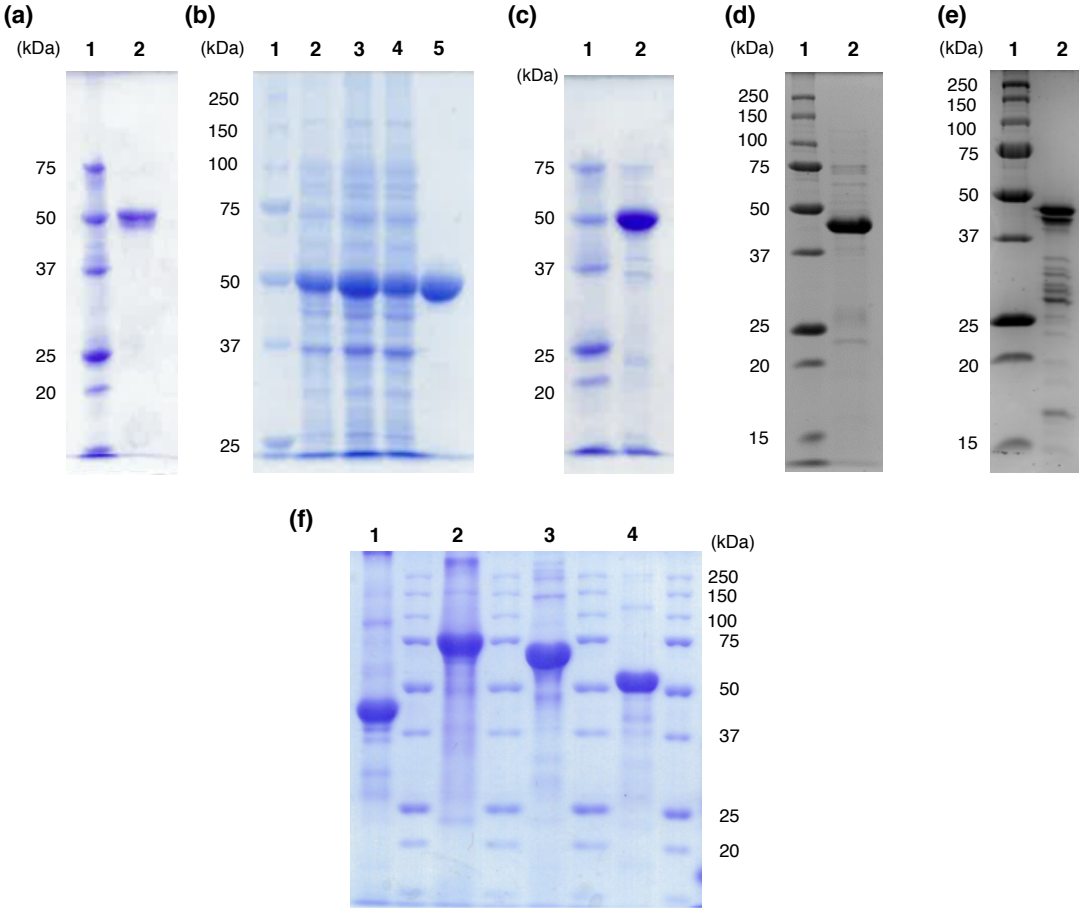

Figure S2. SDS-PAGE of purified proteins. (a) *N*-His$_6$-AbmF (53.3 kDa). Lane 1: Protein standards, Lane 2: Purified AbmF after gel filtration column chromatography. (b) *N*-His$_6$-AbmK (49.6 kDa). Lane 1: Protein standards, Lane 2: Whole cell lysate, Lane 3: Supernatant fraction after centrifugation of the lysate, Lane 4: Flow through fraction of Ni-NTA affinity chromatography, Lane 5: Elution fraction of Ni-NTA affinity chromatography. (c) *N*-His$_6$-SerR1 (49.1 kDa). Lane 1: Protein standards, Lane 2: Purified SerR1. (d) *N*-His$_6$-AbmJ (43.1 kDa). Lane 1: Protein standards, Lane 2: Purified AbmJ. (e) Lane 1: Protein standards, Lane 2: Partially purified *N*-His$_6$-AbmJ mutant (C12A/C16A/C19A, 43.0 kDa). (f) Lane 1: *N*-His$_6$-TryRS (47.5 kDa), Lane 2: *N*-His$_6$-ThrRS (74.0 kDa), Lane 3: *N*-His$_6$-LysRS (57.6 kDa), Lane 4: *N*-His$_6$-CysRS (52.2 kDa).

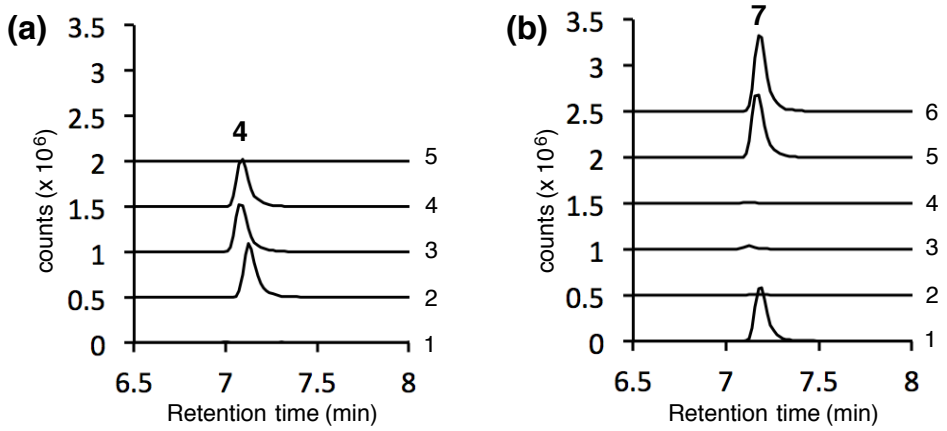

Figure S3. LCMS analysis of AbmF/AbmK reaction of **4**. Extracted ion chromatogram (EIC) traces corresponding to $[M - H]^-$ signals from (a) substrate **4** (*m*/*z* = 388.1) and (b) product **7** (*m*/*z* = 475.1) are overlaid. (1) Full reaction. (2) without AbmF. (3) without AbmK. (4) without ATP. (5) without tRNAs (6) standard sample of **7**. It should be noted that substrate **4** and product **7** co-eluted at around 7.2 min under the LC conditions. Nevertheless, the identity of the peaks could be verified by mass spectra.

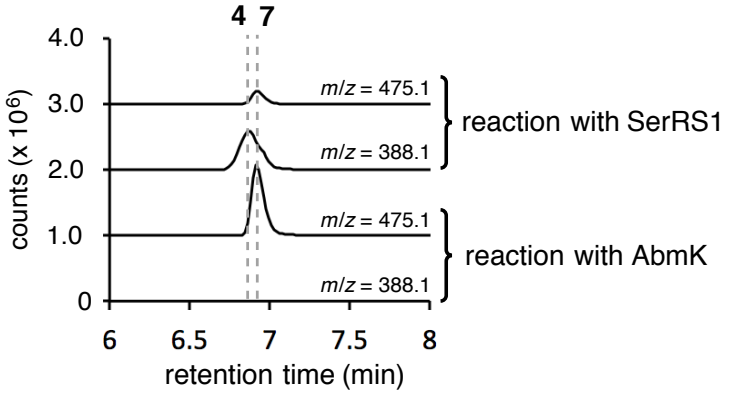

Figure S4. AbmF reaction of **4** using AbmK and SerRS1. EIC traces Two EIC traces corresponding to $[M - H]^-$ signals from substrate **4** (*m*/*z* = 388.1) and product **7** (*m*/*z* = 475.1) are shown.

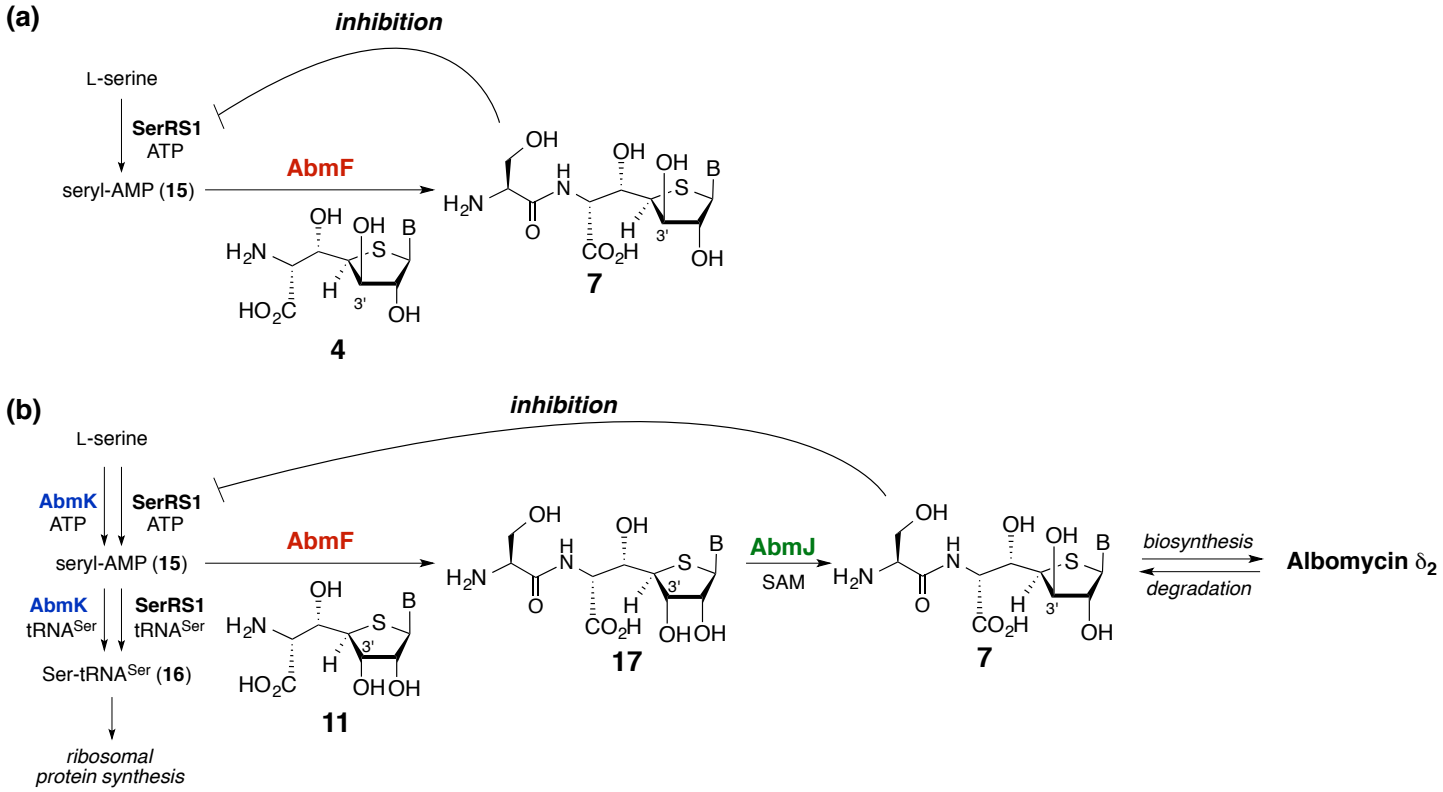

Figure S5. Proposed mechanisms of the inhibition of SerRS1 by SB-217452. (a) *In vitro* inhibition of SerRS1 by SB-217452 generated from the incubation of nucleoside **4** with AbmF and SerRS1. (b) A physiologically relevant model of the SerRS1 inhibition.

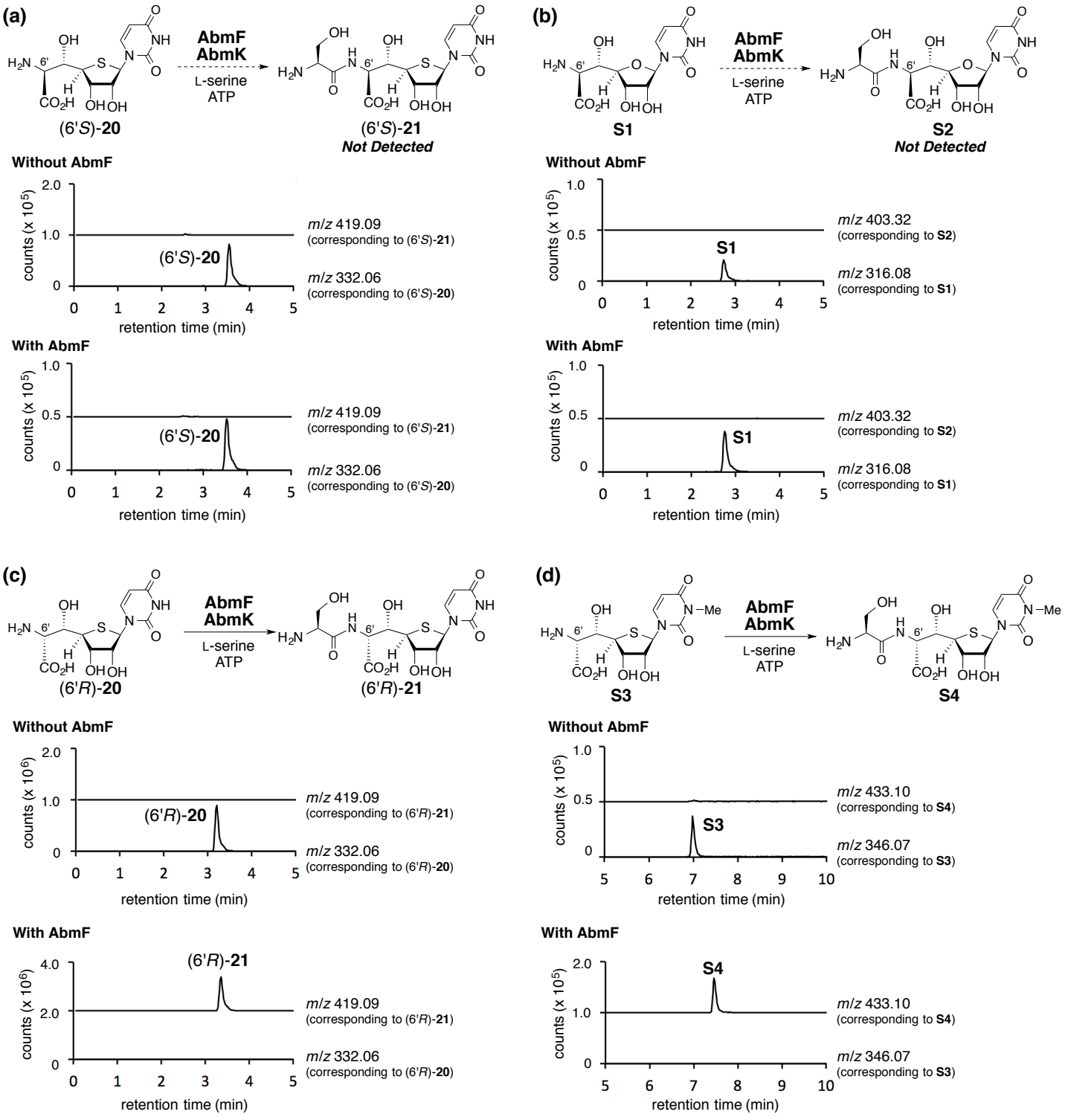

Figure S6. LCMS analysis of AbmF/AbmK reactions of substrate analogues. (a) (6′*S*)-**20**, (b) **S1**, (c) (6′*R*)-**20**, (d) **S3**. Note that substrate analogues **S1** and **S3** have the (6′*S*) and (6′*R*) stereoconfiguration, respectively, and that the former was not accepted by AbmF while the latter was fully converted to the corresponding serine-conjugated product **S4**. These results suggested that the stereochemistry of C6′ is critical for substrate recognition of AbmF.

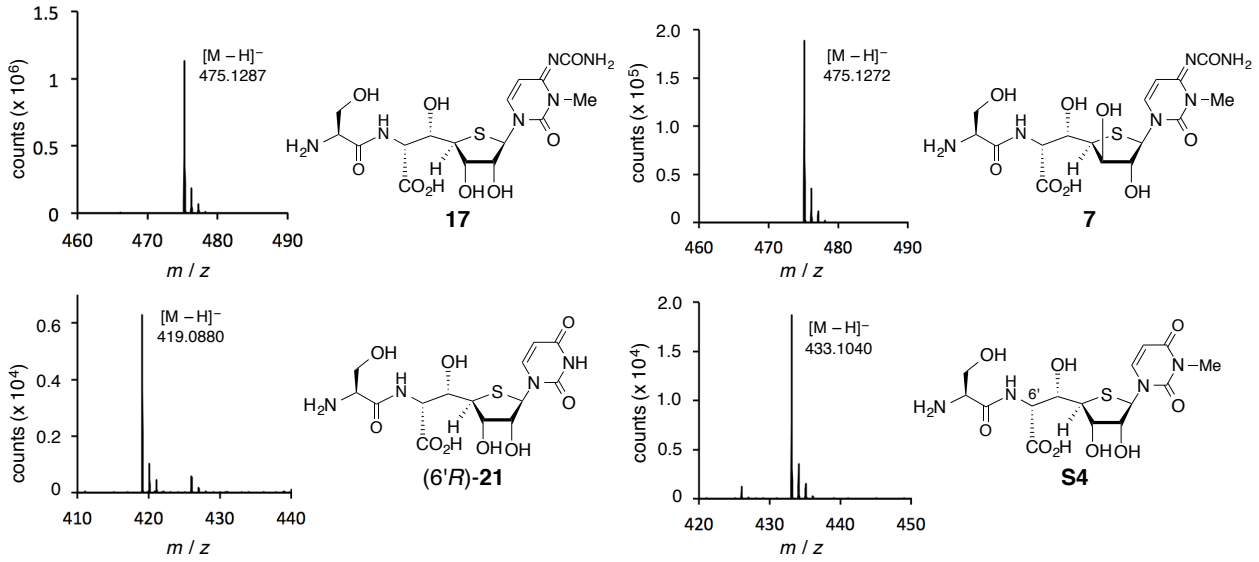

Figure S7. ESI-MS spectra of AbmF products. (a) **17** from **11**. (b) **7** from **4**. (c) (6′*R*)-**21** from (6′*R*)-**20**. (d) **S4** from **S3**. See Figure 2, S3, and S6 for the LC traces.

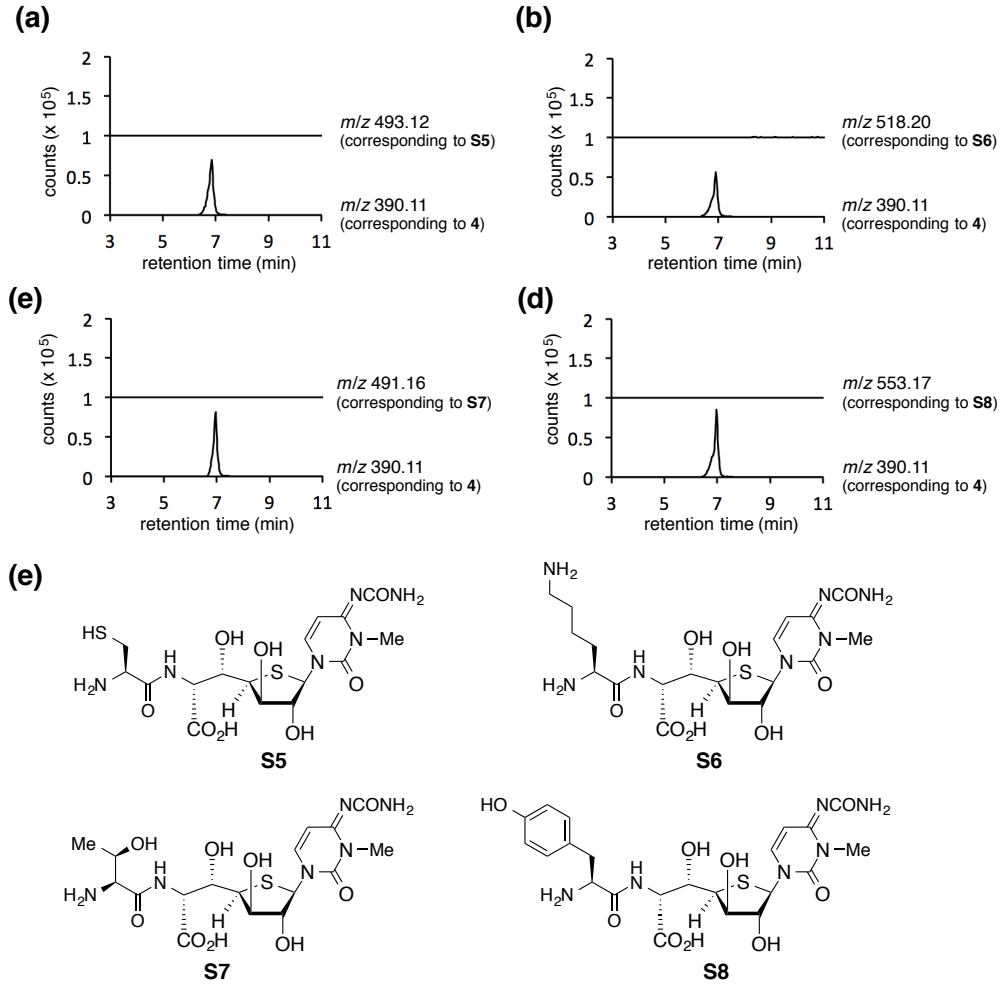

Figure S8. LCMS analysis of the AbmF reaction of **4** using different L-amino acids and aaRSs. EIC traces corresponding to $[M + H]^+$ species of **4** and the possible dipeptide product (**S5**, **S6**, **S7**, or **S8**) are shown. (a) With L-cysteine and cysteinyl-tRNA synthetase. (b) With L-lysine and lysyl-tRNA synthetase. (c) With L-threonine and threonyl-tRNA synthetase. (d) With L-tyrosine and tyrosyl-tRNA synthetase. (e) Structures of the possible dipeptide products **S5**–**S8**.

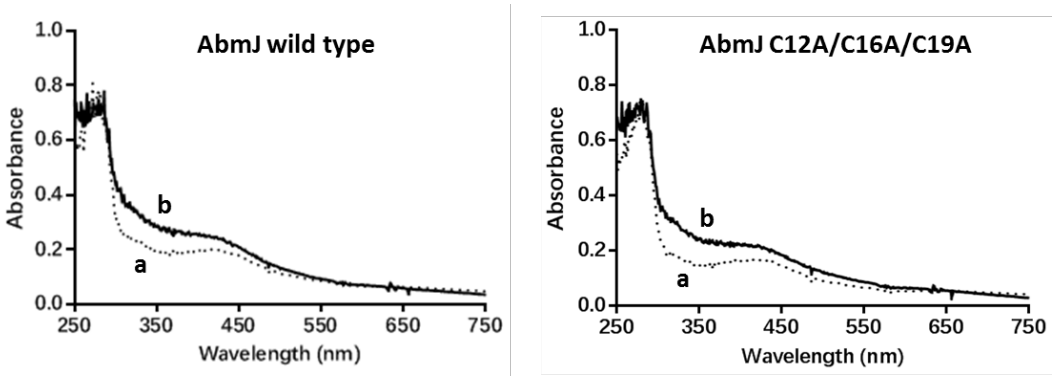

Figure S9. UV analysis of the wild type AbmJ and the C12A/C16A/C19A mutant (a) before and (b) after the Fe-S cluster reconstitution.

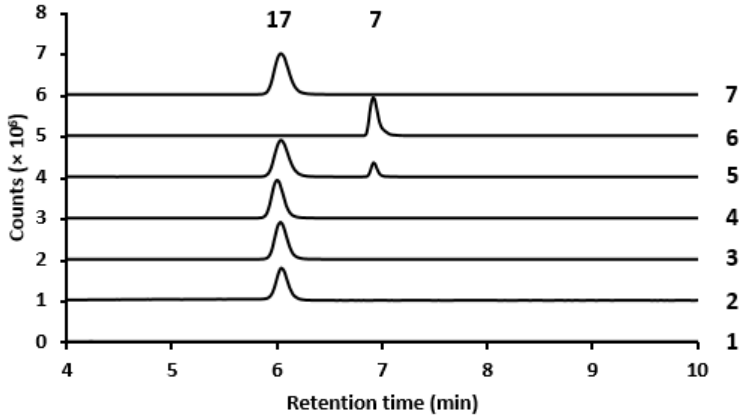

Figure S10. Incubation of AbmJ with **17** under different conditions. (1) Without substrate. (2) Without AbmJ. (2) Without SAM. (4) Without $Na_2S_2O_4$. (5) Full reaction. (6) Standard sample of **7**. (7) Incubation of the C12A/C16A/C19A mutant of AbmJ with **17** in the presence of SAM and $Na_2S_2O_4$.

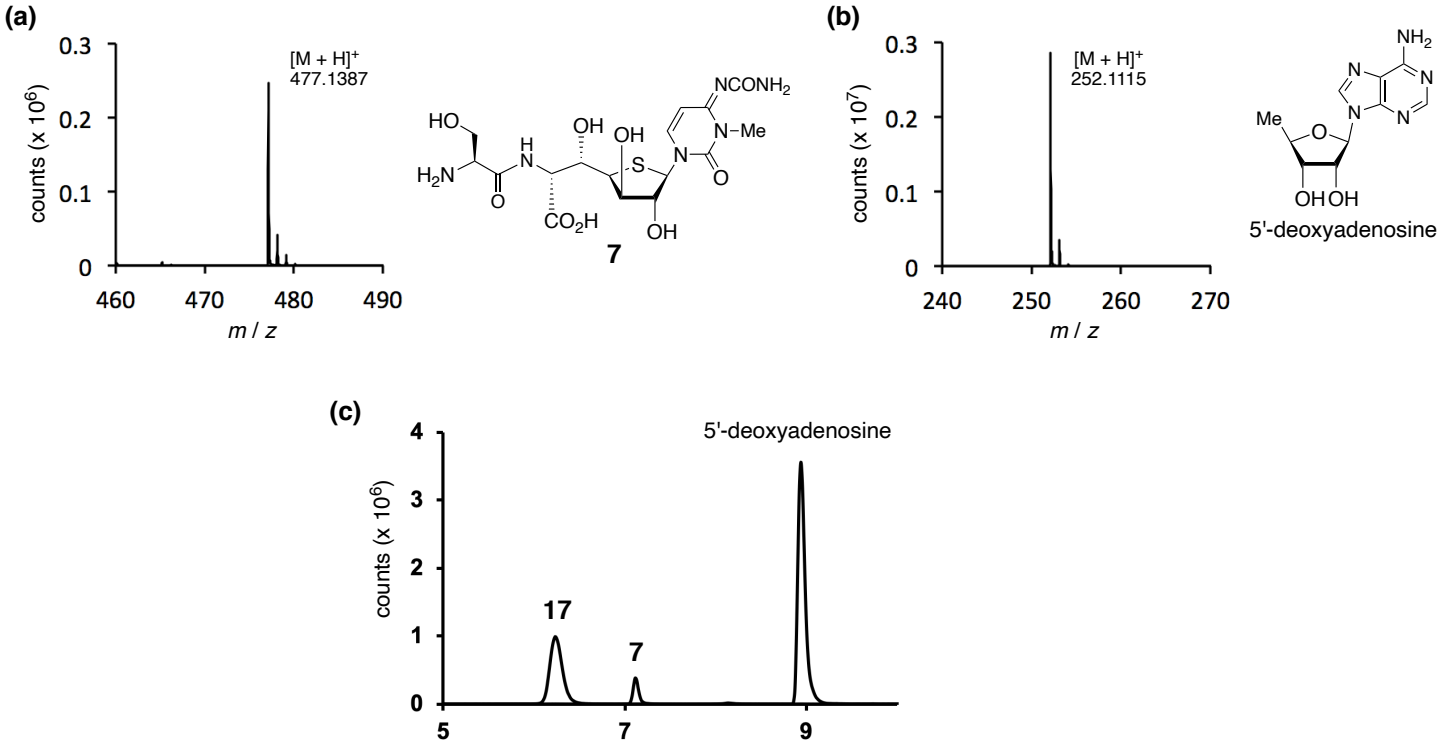

Figure S11. ESI-MS spectra of AbmF products (a) **7** and (b) 5′-dA observed in the AbmJ reaction of **17**. (c) Overlaid EIC traces corresponding to **17**, **7**, and 5'-deoxyadenosine. The ratio of the ionization efficiencies of **7** and 5'-dA in the positive ionization mode is 1 : 11 under the LCMS conditions employed.

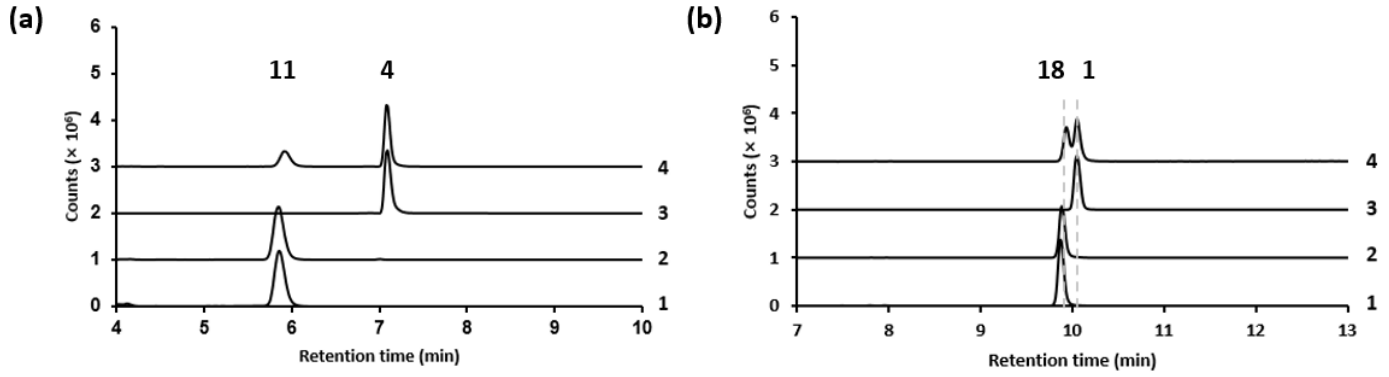

Figure S12. LCMS analysis of AbmJ reactions of (a) **11** and (b) **18**. EIC traces corresponding to $[M + H]^+$ signals from **11** and possible product **4** (*m*/*z* = 390.1) and **18** and possible product **1** (*m*/*z* = 1046.3) are shown. (1) Without AbmJ. (2) With AbmJ. (3) Standard sample of **4** in (a) or **1** (b). (4) Coinjection

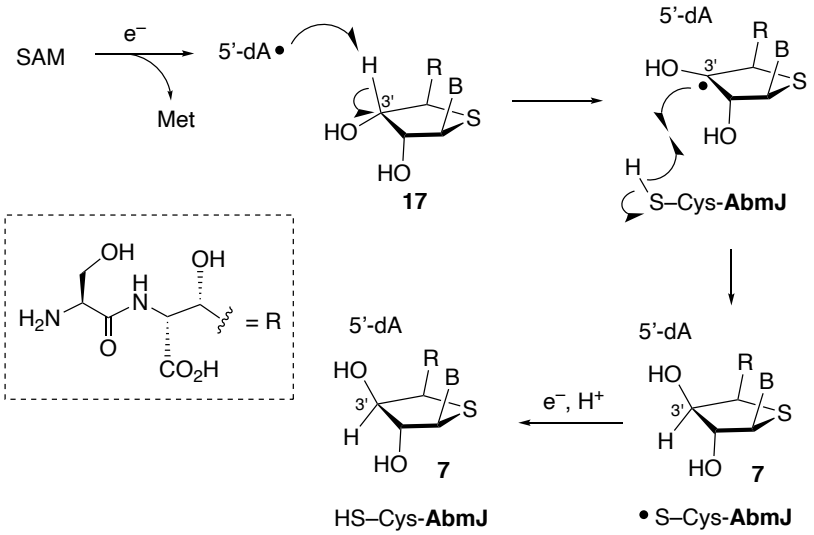

Figure S13. A possible mechanism of AbmJ-catalyzed C3' epimerization of **7**.

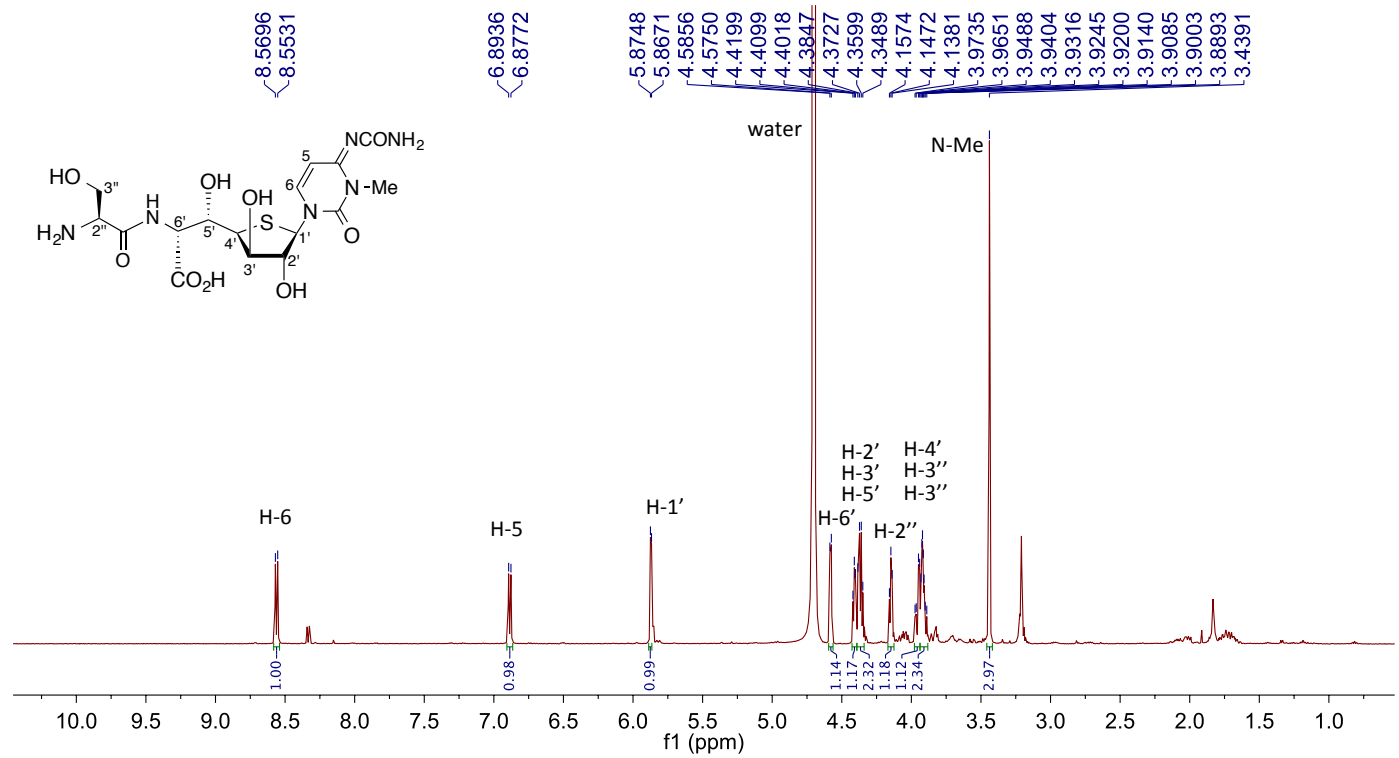

Figure S14. ^{1}H NMR spectrum of **7**.

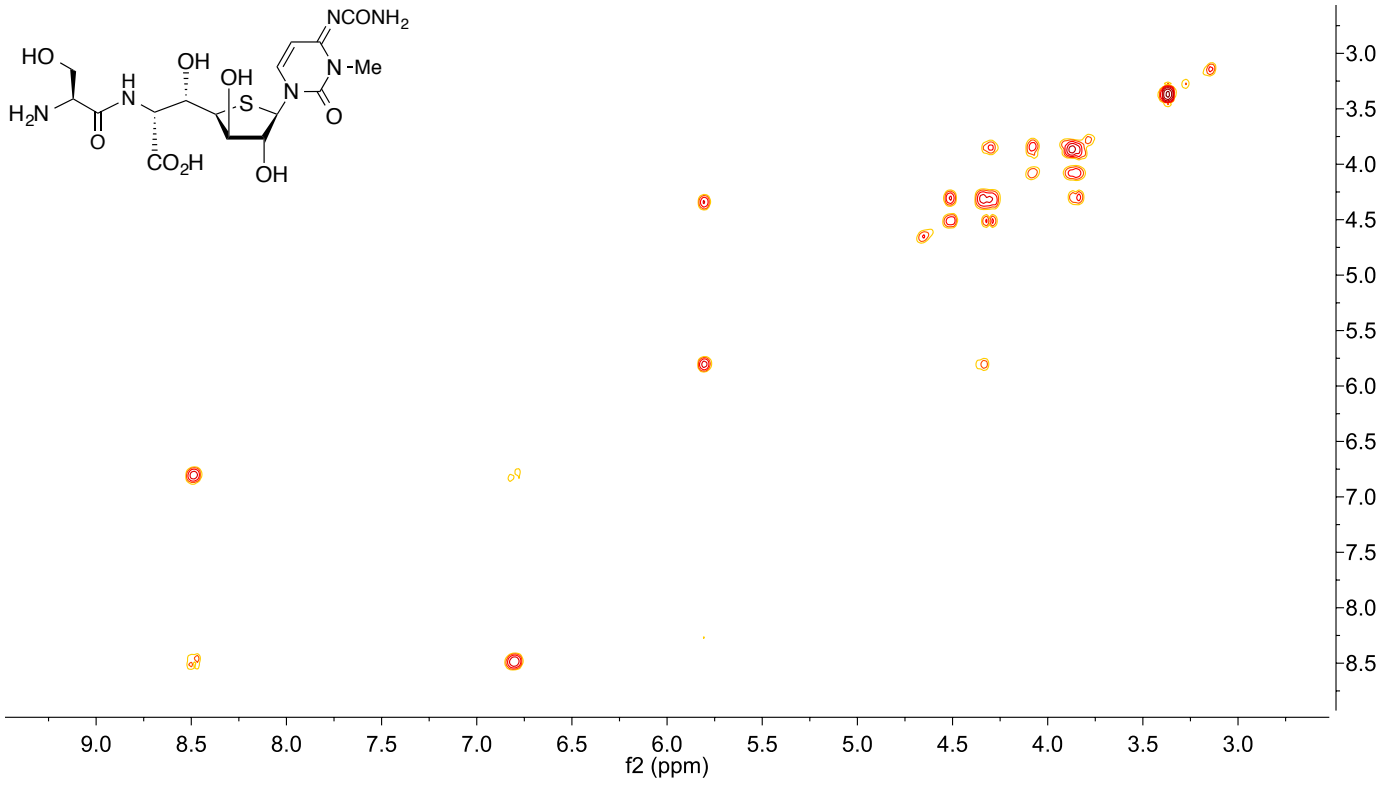

Figure S15. COSY spectrum of **7**.

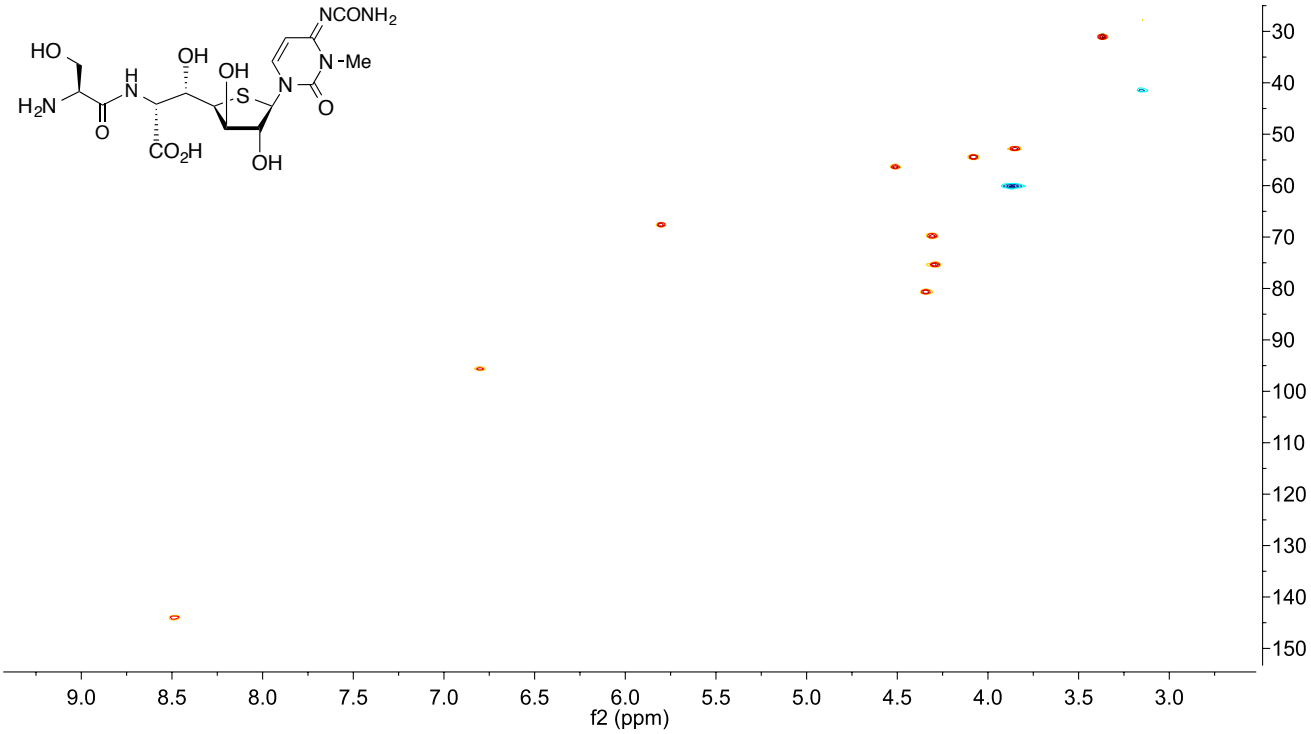

Figure S16. HSQC spectrum of **7**.

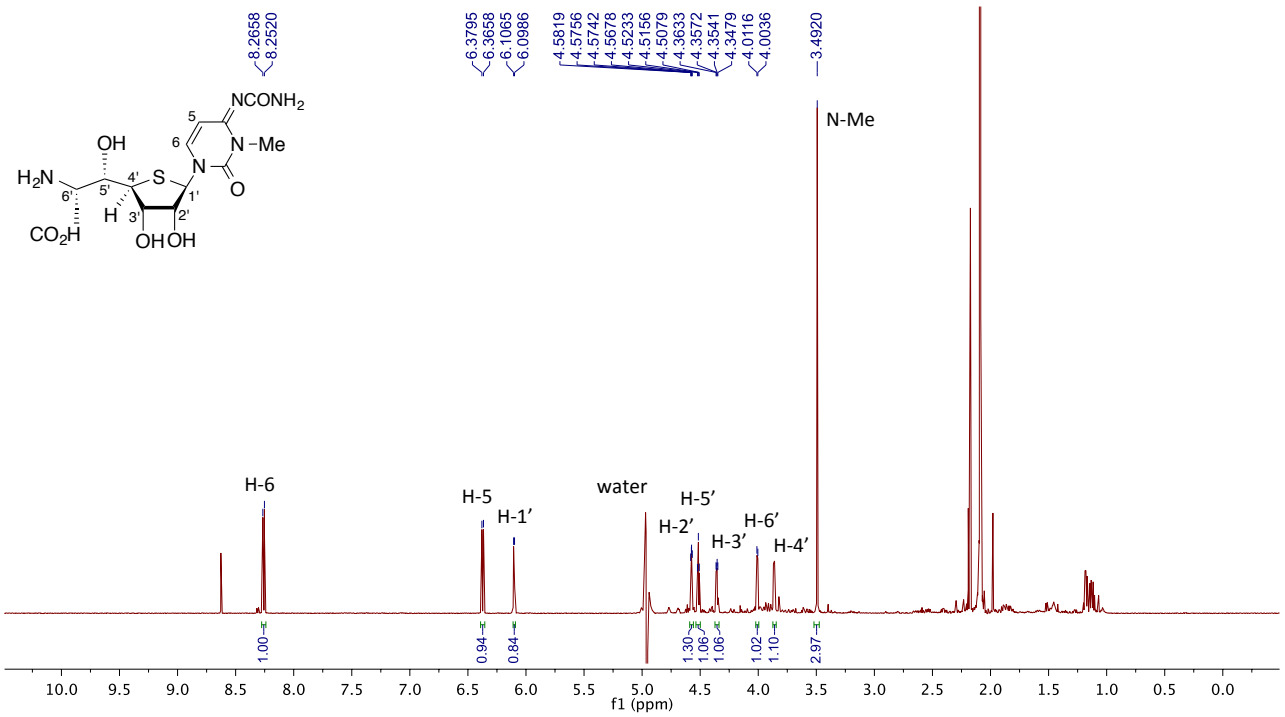

Figure S17. ^{1}H NMR spectrum of **11**.

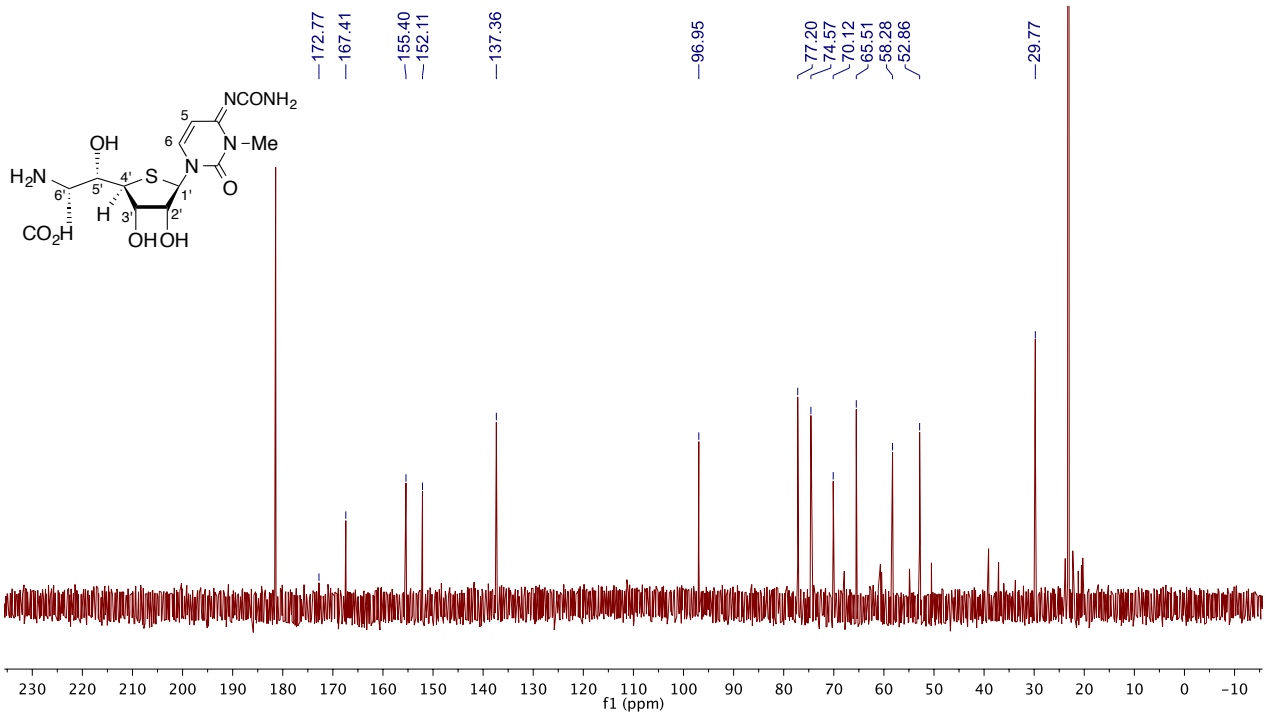

Figure S18. ^{13}C NMR spectrum of **11**.

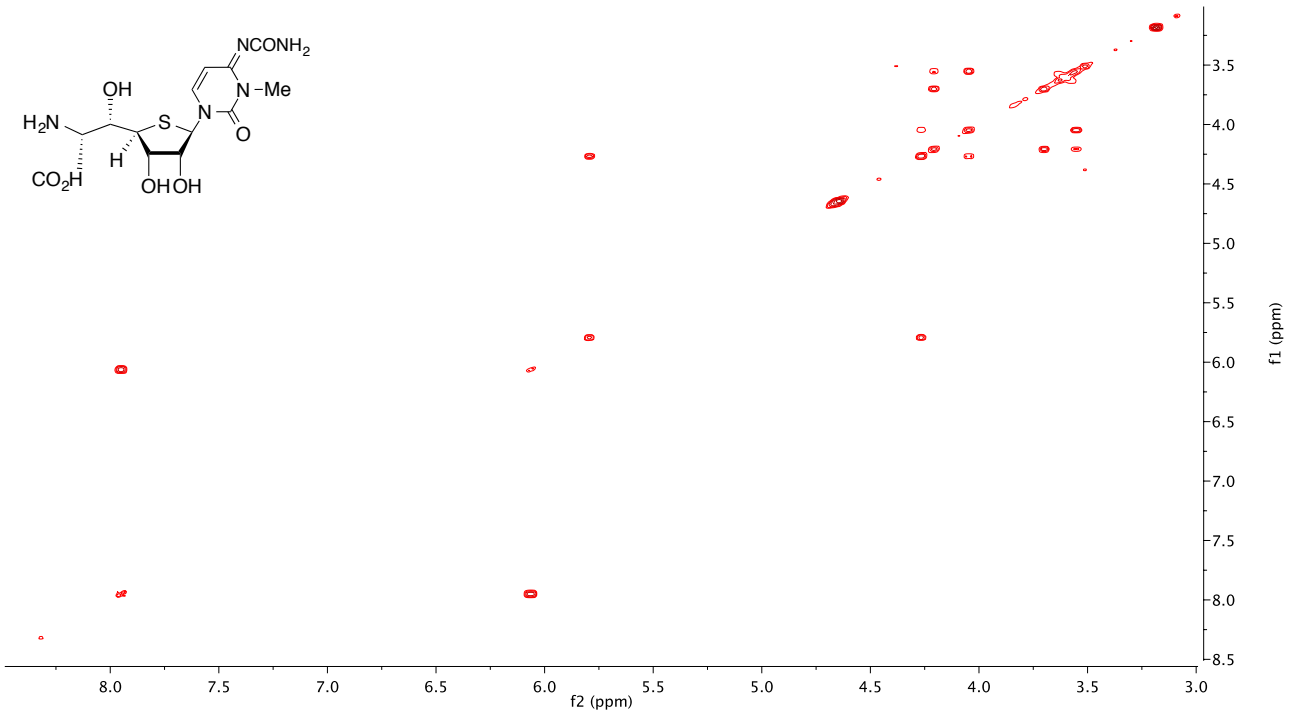

Figure S19. COSY of **11**.

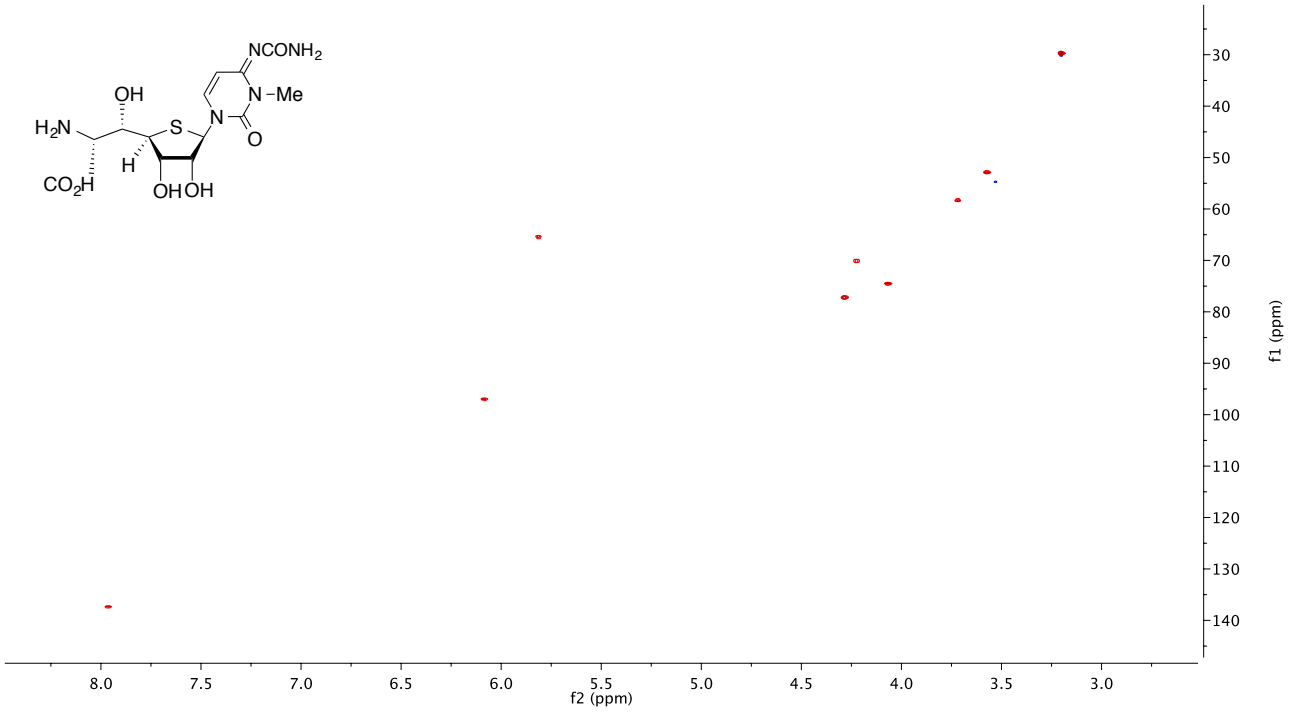

Figure S20. HSQC of **11**.

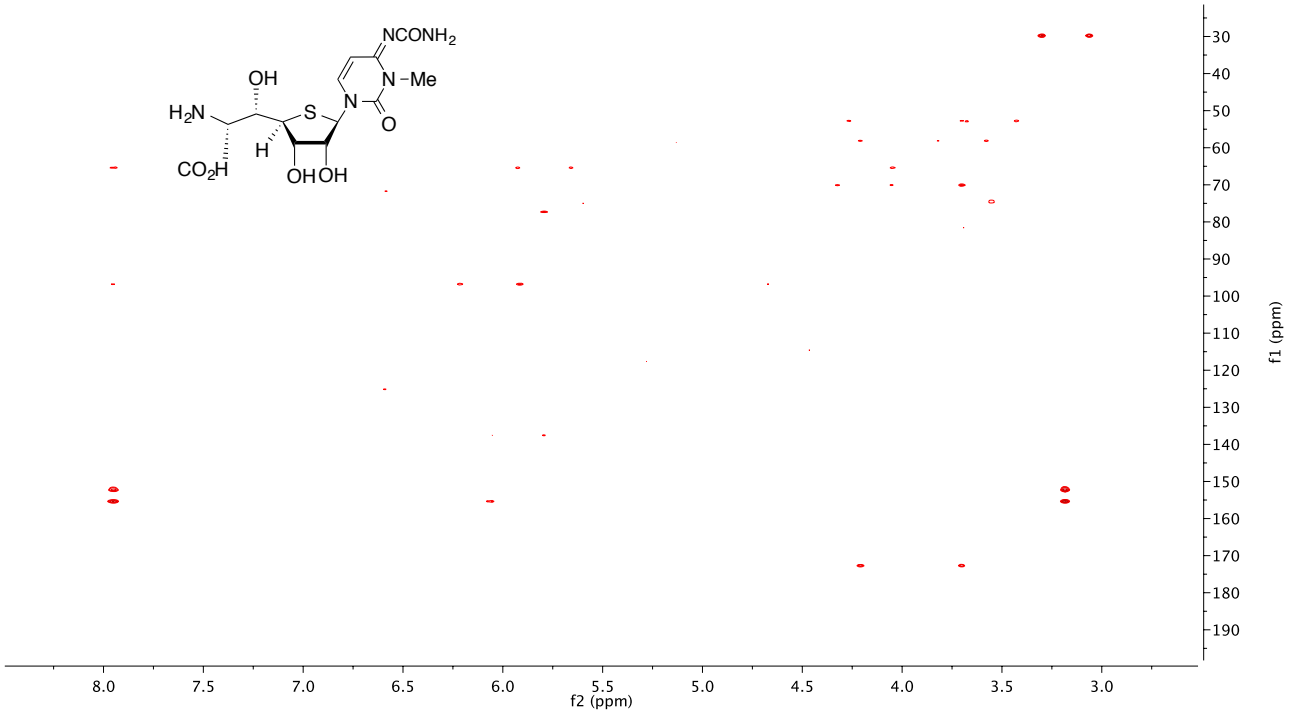

Figure S21. HMBC of **11**.

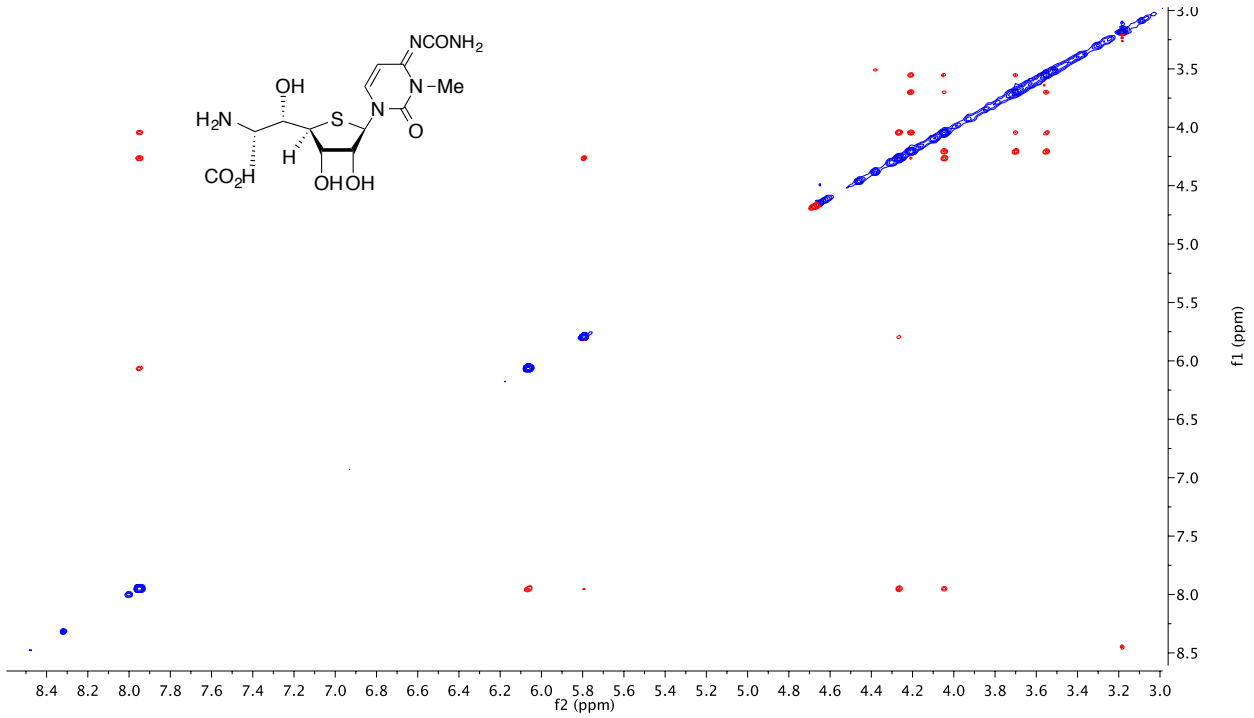

Figure S22. NOESY of **11**.

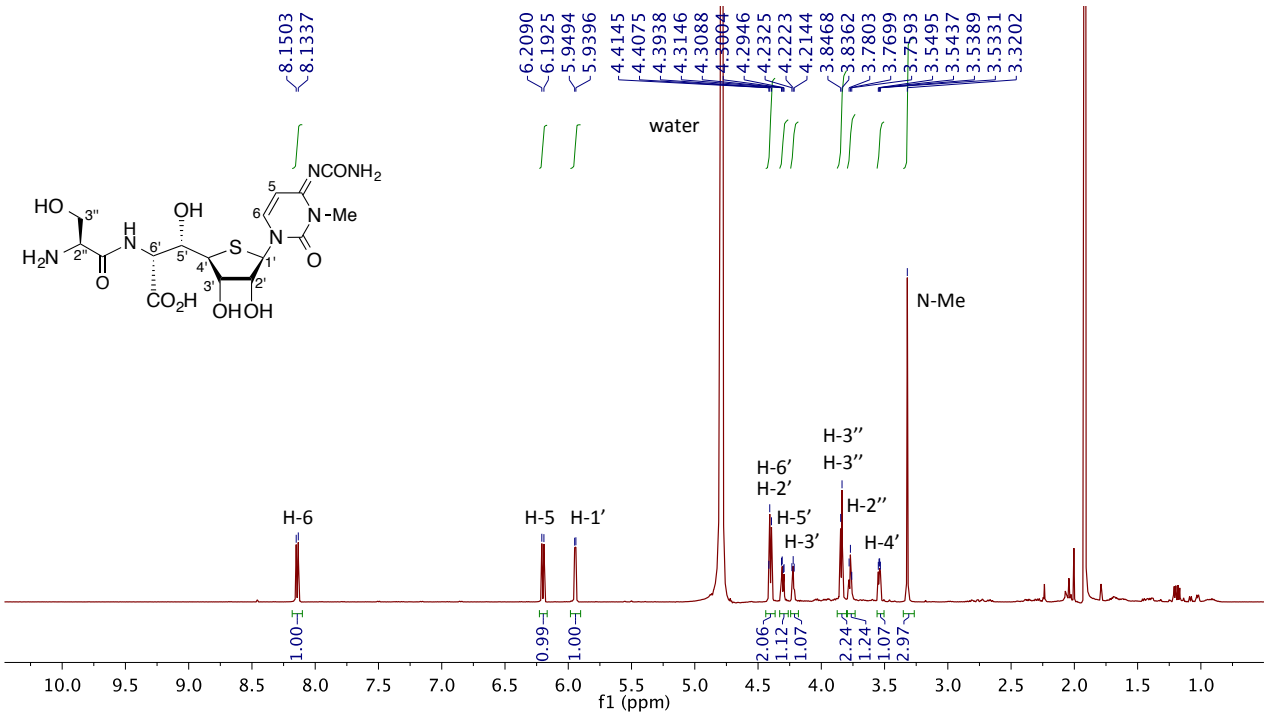

Figure S23. ^{1}H NMR spectrum of **17**.

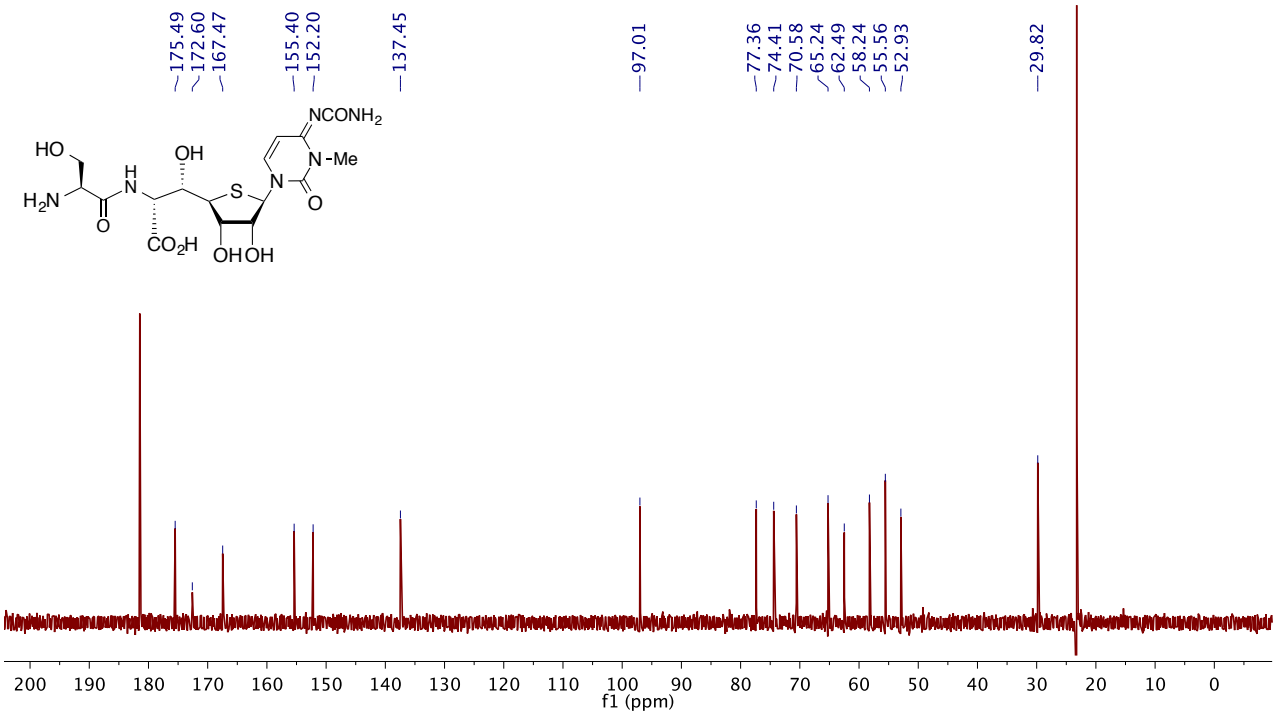

Figure S24. ^{13}C NMR spectrum of **17**.

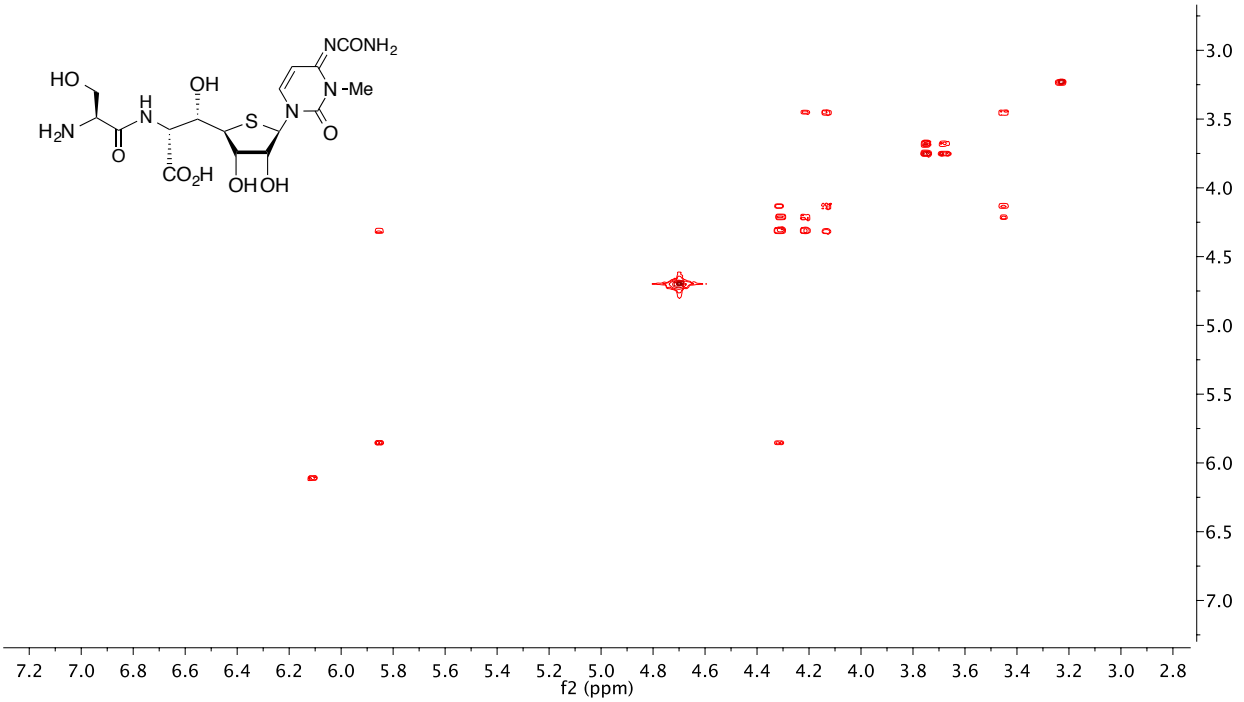

Figure S25. COSY of **17**.

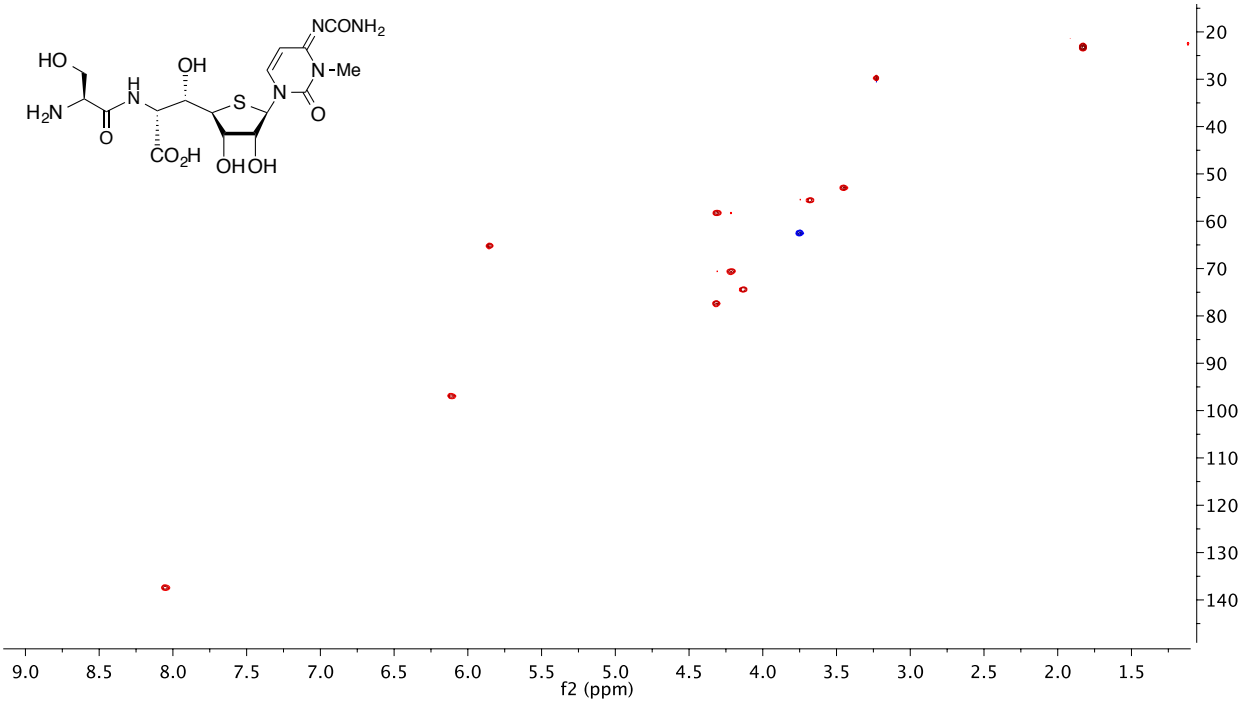

Figure S26. HSQC of **17**.

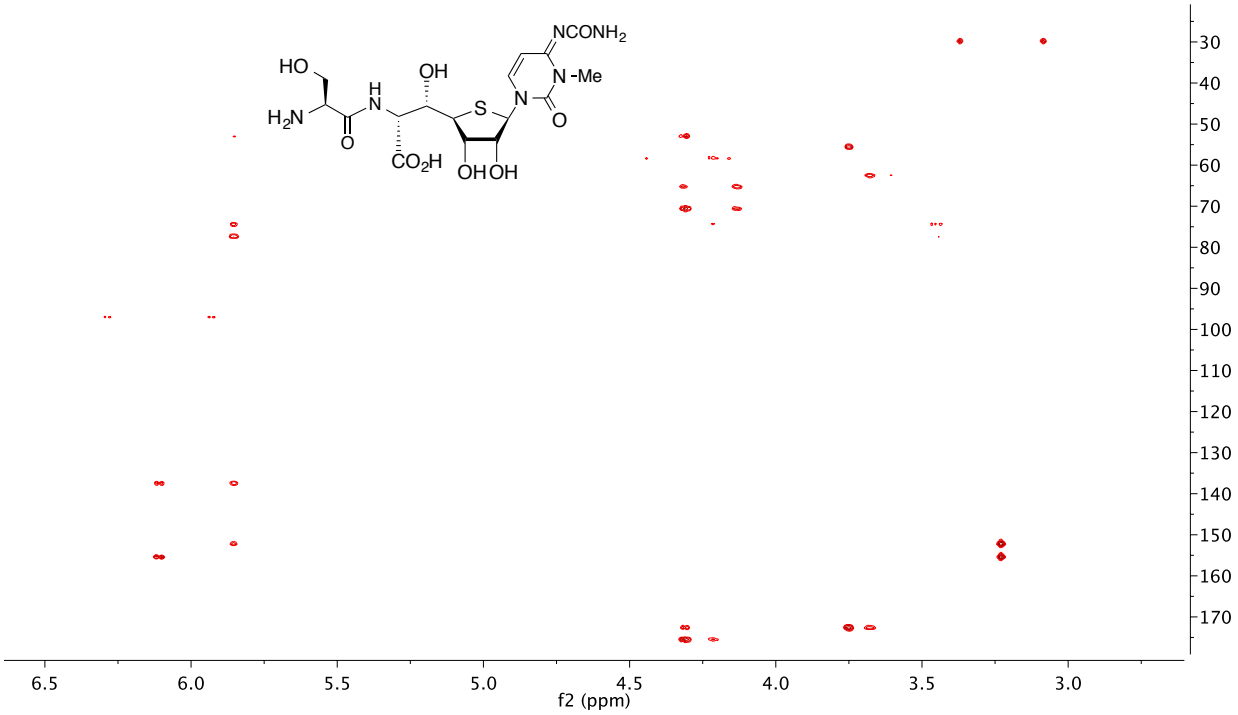

Figure S27. HMBC of **17**.