Supporting information

Contents

General Procedures

All DNA isolation and manipulation procedures in *Escherichia coli* and *Streptomyces* were performed according to standard procedures. Primers were synthesized at Eurofins Co., Ltd. (Germany). Restriction enzymes and DNA ligase were purchased from NEB Biotechnology Co. Ltd. (England). Plasmid, gel extraction and cycle-pure kits were acquired from Promega Corporation (USA).

Genome sequencing and bioinformatic Analysis

The complete genome of *Streptomyces asterosporus* DSM 41452 was sequenced using the Illumina HiSeq2000 technology, assembled and annotated by Shanghai Majorbio Biopharm Technology Co, Ltd.

(Shanghai, China). Prediction of the gene clusters was performed using antiSMASH (http://antismash.secondarymetabolites.org/). A large DNA fragment without any gaps was found to contain the putative *SAS* gene cluster. The orfs were determined by application of the FramePlot 4.0 beta program (http://nocardia.nih.go.jp/fp4/). Protein sequences were compared with BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Domain organization and substrate specificities for NRPSs were predicted by PKS/NRPS analysis software (http://nrps.igs.umaryland.edu/).

Construction of mutant strain *Streptomyces asterosporus* DSM 41452 :: pKC1132-InAnn3

An internal fragment of gene *ann3* was amplified by PCR using the genomic DNA of *Streptomyces asterosporus* DSM 41452 as template with primers InAnn3-F and InAnn3-R. The PCR product was ligated into the EcoRV-digested pBluescript KS (-) to yield pBSK-InAnn3. After restriction enzyme digestion by HindIII and XbaI, the corresponding fragment was cloned into plasmid pKC1132 to yield plasmid pKC1132-InAnn3. Then this plasmid was introduced into *Streptomyces asterosporus* DSM 41452 by intergeneric conjugation. An apramycin-resistant (50ug/ml) mutant was isolated. Apramycin-sensitive colonies were tested for target gene disruption by colony PCR using primers Vann3-F and Apra-R (Table S1).

Construction of mutant strain *Streptomyces asterosporus* DSM 41452 :: pUC19Δ3100spec

The plasmid pUC19Δ3100spec (See the Table S2) was introduced into *Streptomyces asterosporus* DSM 41452 by intergeneric conjugation. A spectinomycin-resistant (100 ug/ml) mutant was isolated. Spectinomycin-sensitive colonies were tested for target gene disruption by colony PCR using primers V3100-F and Vaad-R (Table S1).

Generation of gene *orf(-1)* disruption mutant in *Streptomyces asterosporus* DSM 41452

An internal fragment of gene *orf(-1)* was amplified by PCR using the genomic DNA of *Streptomyces asterosporus* DSM 41452 as template with primer pair Orf(-1)-F and Orf(-1)-R. The PCR product was ligated into the EcoRV-digested pBluescript SK (-) to yield pBSK-orf(-1). After restriction enzyme digestion by HindIII and XbaI, the corresponding fragment was cloned into plasmid pKC1132 to yield plasmid pKC1132-orf(-1). pKC1132-orf(-1) was introduced into *Streptomyces asterosporus* DSM 41452 by intergeneric conjugation. An apramycin-resistant (50ug/ml) mutant was isolated. Apramycin-sensitive colonies were tested for target gene disruption by colony PCR using primers VluxR1-F and Apra-R (Table S1).

Generation of gene *sas1* disruption mutant in *Streptomyces asterosporus* DSM 41452

An internal fragment of gene *sas1* was amplified by PCR using the genomic DNA of *Streptomyces asterosporus* DSM 41452 as template with primers LuxR2-F and LuxR2-R. The PCR product was ligated into the EcoRV-digested pBluescript SK (-) to yield pBSK-SAS1. After restriction enzyme digestion by HindIII and XbaI the corresponding fragment was cloned into the plasmid pKC1132 to yield plasmid pKC1132-SAS1. Then pKC1132-SAS1 was introduced into *Streptomyces asterosporus* DSM 41452 by intergeneric conjugation. The correct exconjugants carrying plasmid were screened for resistance against apramycin (50 ug/ml). Apramycin-sensitive colonies were tested for target gene disruption by colony PCR using primers VluxR2-F and Apra-R (Table S1).

Generation of gene *sas13* disruption mutant in *Streptomyces asterosporus* DSM 41452

An internal fragment of gene *sas13* was amplified by PCR using the genomic DNA of *Streptomyces asterosporus* DSM 41452 as template with primers SAS13F and SAS13R. The PCR product was ligated into the EcoRV-digested pBluescript SK (-) to yield pBSK-SAS13. The insertion fragment was excised by HindIII/XbaI restriction enzyme and subcloned into vector pKC1132 to generate plasmid pKC1132-SAS13. After conjugation of this plasmid into *Streptomyces asterosporus* DSM 41452, an apramycin-resistant mutant named *Streptomyces asteroporous* **::** pKC1132-SAS13 was obtained. The correct exconjugants carrying plasmid were screened for resistance against apramycin (50 ug/ml). Apramycin-sensitive colonies were tested for target gene disruption by colony PCR using primers Vsas13-F and Apra-R (Table S1).

Generation of gene *sas16* disruption mutant in *Streptomyces asterosporus* DSM 41452

An internal fragment of gene *sas16* was amplified by PCR using the genomic DNA of *Streptomyces asterosporus* DSM 41452 as template with primers P450S-F and P450S-R. The PCR product was ligated into the EcoRV-digested pBluescript SK(-) to yield pBSK-SAS16. The insertion fragment was excised by HindIII/XbaI fragment and subcloned into vector pKC1132 to generate plasmid pKC1132-SAS16. After conjugation of this plasmid into *Streptomyces asterosporus* DSM 41452, an apramycin-resistant mutant named *Streptomyces asteroporous* DSM 41452 **::** pKC1132-SAS16 was obtained. The correct exconjugants carrying plasmid were screened for resistance against apramycin(50ug/ml). Apramycin-sensitive colonies were tested for target gene disruption by colony PCR using primers Vsas16-F and Apra-R (Table S1).

Generation of gene ΔNmet mutant in *Streptomyces asterosporus* DSM 41452

A 4800bp DNA fragment including the domain encoding methyltransferase and its flanking region in gene *sas17* was amplified from the genome of *Streptomyces asterosporus DSM 41452* by PCR using primers Nmet4800bpF and Nmet4800bpR (Table S1). The PCR product was ligated into the EcoRV-digested pBluescript SK(-) to yield pBSK-Nmet. The loxP-site-flanked apramycin resistance cassette from plasmid pLERECJ was amplified by PCR using primes Nmet-ApraF and Nmet-ApraR. The resulting amplicon was used to replace the methyltransferase encoding sequence of pBSK-Nmet by recombination in E. coli BW25113 cell containing λ RED plasmid pIJ790, yielding pBSK-Nmet::aac(3)IV. The latter was amplified by PCR using primers Nmet4800bpF and Nmet4800bpR. The resulting fragment was cloned into the EcoRV-digested pKGLP2-GusA to generate pKGLP2-GusA-Nmet::aac(3)IV.

The wildtype *Streptomyces asterosporus* DSM 41452 was conjugated with *E. coli* ET 12567 (pUZ8002) harboring plasmid pKGLP2-GusA-Nmet::aac(3)IV, then the correct exconjugants carrying plasmid pKCLP2-gusA Nmet::aaa(3)IV were selected by antibiotic resistance screening against apramycin(50 ug/ml). In order to generate the mutant strain with doube-crossover, initial conjugants were incubated at 28°C for 4 days, and then screened for the apramycin resistance and hygromycin sensitivity. Replacement of Nmet domain encoding gene with *aac(3)IV* in *Streptomyces asterosporus DSM 41452* Δ Nmet:: aac(3)IV was confirmed by PCR using primers Nmet4800bpF and Nmet4800bpR (Table S1).

The *Cre* recombinase expression plasmid pUWLCre was then introduced into *Streptomyces asterosporus DSM 41452* ΔNmet::aac(3)IV to eliminate aac(3)IV gene from its genome. The resulting conjugants resistant to hygromycin were incubated on MS solid medium plates and selected for the apramycin sensitivity. Then the hygromycin resistance colony was cultured into liquid TSB medium at 37°C, then it was repeatedly passaged three times. Hygromycin-sensitive colonies were tested for the loss of plasmid pUWLCre. The correct excision of *aac(3)IV* gene from the genome of *Streptomyces asterosporus DSM 41452* ΔNmet genome was confirmed by PCR using primers Nmet4800bpF and Nmet4800bpR (Table S1).

Generation of gene Δ*sas16* mutant in *Streptomyces asterosporus* DSM 41452

A 4472bp DNA fragment including gene *sas16* and its flanking regions was amplified from the genome of *Streptomyces asterosporus DSM 41452* by PCR using primers SAS16F and SAS16R. The PCR product was ligated into the EcoRV-digested pBluescript SK (-) to yield pBSK-SAS16. The loxP-site-flanked apramycin resistance cassette from plasmid pLERECJ was amplified with the pair of primer SAS16-ApraF and SAS16-ApraR. The resulting amplicon was used to replace the coding sequence of *SAS16* in pBSK-SAS16 by gene recombination in *E. coli* BW25113 cell containing λ RED plasmid pIJ790, yielding pBKS-SAS16::*aac(3)IV*.

The latter was amplified using primers SAS16F and SAS16R, the resulting fragment was cloned into the EcoRV-digested pKGLP2-GusA to afford pKGLP2-GusA-SAS16::aac(3)IV.

Streptomyces asterosporus DSM 41452 was conjugated with *E. coli* ET 12567 (pUZ8002) harboring plasmid pKGLP2-GusA-SAS16::aac(3)IV, the correct exconjugants carrying plasmid pKGLP2-GusA-SAS16::*aac(3)IV* were screened for resistance against apramycin(50 ug/ml). To generate the mutant strain containing a doube-crossover, initial conjugants were incubated at 28°C for 4 days and then screened for apramycin resistance and hygromycin sensitivity. Replacement of *SAS16* with *aac(3)IV* in *Streptomyces asterosporus DSM 41452* Δsas16:: aac(3)IV was confirmed by PCR using primers SAS16F and SAS16R (Table S1).

The *Cre* recombinase expression plasmid pUWLCre was then introduced into *Streptomyces asterosporus DSM 41452* Δsas16::aac(3)IV to eliminate *aac(3)IV* gene from its genome. The resulting exconjugants resistant to hygromycin were incubated on MS solid medium plates and selected for the apramycin sensitivity. Then the hygromycin resistance colony was cultured into the TSB medium at 37°C, and was repeatedly passaged three times. Then hygromycin-sensitive colonies were tested for the loss of plasmid pUWLCre. The correct in-frame excision of *aac(3)IV* gene from the *Streptomyces asterosporus DSM 41452* Δsas16 genome was confirmed by PCR using primers SAS16F and SAS16R (Table S1).

Complementation of *Streptomyces asterosporus* DSM 41452 Δ*sas16* strain with SAS16

To construct plasmid pTESa-SAS16, a 1272bp fragment carrying entire *sas16* with its upstream 48bp region was amplified from *Streptomyces asterosporus* DSM 41452 genomic DNA using primers KpnI-RBSp450-F and EcoRI-p450-R. The 1272bp fragment was firstly cloned into EcoRV-digested pBluescript SK(-) to yield pBSK-SAS16. Then this pBSK-SAS16 was digested with KpnI and EcoRI, and then DNA fragment was ligated to pTESa with a constitutive promoter *ermEp*, yielding plasmid pTESa-SAS16. *Streptomyces asterosporus DSM 41452Δsas16* exconjugants carrying the plasmid pTESa-SAS16 were screened from MS plate with apramycin resistance (50 ug/ml).

Strain information, Fermentation, Extraction

The wildtype strain *Streptomyces asterosporus* DSM41452 was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and cell Cultures, Germany. The initial cultures were maintained on the Tryptic soy broth (TSB) solid medium (Tryptone 17g, Bacto Soytone 3g, Glucose 2.5g, Sodium Chloride 5g, K_2HPO_4 2.5g, Tap water 1000mL, pH 7.3). A small loop of spores growing on a TSB solid plate was inoculated into a 250 mL Erlenmeyer flask containing 75 mL liquid productive SG medium (Soy peptone 10.0 g, Glucose 20.0 g, L-Valine 2.34 g, $CaCO_3$ 2.0 g, $CoCl_2$-solution 1mg/mL 1 mL, Tap water 1000 mL, pH7.2) and cultured at 28 ℃ for 3 days on a rotary shaker at 180 $r \cdot min^{-1}$. Then, 10 mL of the preculture (at a volume ratio of 1:100) was inoculated into a 500 mL Erlenmeyer flask (40 flasks) containing 150 mL of the SG medium, then incubated for 4 days, 180rpm, 28℃. The fermentation broth (6L) was filtered

through high speed centrifugation (8000 rpm, 10min, 22℃), yielding the supernatant and cell pellet. Then the supernatant was extracted by double volume ethyl acetate using a separating funnel, then this organic solvent was evaporated under reduced pressure. The mycelium also was extracted by acetone, which was evaporate under reduced pressure. The crude extract from the supernatant and the cell pellet were combined.

Isolation of Compound WS9326A, B, D, E, F, G, and Annimycin B

The crude extract (~5g) was dissolved in 15 mL MeOH and fractionated by reversed-phase (RP) C18 liquid chromatography (Oasis® HLB 20 / 35cc), the starting elution solvent is 5% methanol, then the column was eluted using a stepwise gradient MeOH (30%, 40%, 50%, 60%, 70%, 80, 90% and 100%). Fractions afforded from SPE column were analyzed by LC-MS. Among those fractions, fractions (Nr.22-27) were subsequently subjected to a further purification by a semi-preparative HPLC (Agilent Technologies), equipped with a Waters ZORBAX SB-C18 column (9.4 x 150 mm, 5 µm) and a Zorbax 80SB-C8 guard column (9.4 x 15 mm, 7 µm), the fraction was eluted by an isocratic method (60% CH_3CN-40% H_2O, each solvent contains 0.5% acetic acid; flow rate 1 mL/min), to yield compounds WS9326A (10mg), WS9326B (4.9mg), WS9326D (10.9mg), WS9326E (2.4mg), WS9326F (9.4mg), and WS9326G (4.8mg). For purification of compound Annimycin B, the crude extract (~2g) of the cell culture of *Streptomyces asterosporus* DSM 41152 :: pUC19Δ3100spec was obtained using the same fermentation and extraction methods mentioned above. The crude extract firstly was dissolved in methanol, then it was subjected onto a silica gel column (40g separating silica gel) eluting with CH_2Cl_2-MeOH (100: 0-0: 100). The fractions (Fr.1-Fr.48) were collected and analyzed by LC-MS, the fractions(Fr.1-Fr.6) were combined and subjected to a silica gel column (10g separating gel) eluting with cyclohexane-ethyl acetate (100:0-0:100). The fractions were analyzed by LC-MS, and the subfractions containing target mass were further separated by semi-preparative HPLC, the fractions were eluted by an isocratic method (75% CH_3CN-25% H_2O, each solvent contains 0.5% acetic acid; flow rate 1.5 mL/min), to yield compound Annimycin B(7.3mg).

Sample analysis by HPLC-MS

The organic phase was evaporated, resuspended in MeOH(1mL), and filtered through syringe filters (LLG, PVDF, 0.45um) prior to LC-MS analysis. HPLC-MS analysis was performed on an Agilent 1100 series LC/MS system with electrospray ionization (ESI) and detection in the positive and negative modes. The LC system was equipped with a X Bridge C18 column (3.5 µm particle size; 4.6 x 100 mm) and a Zorbax XDB-C18 guard column (5-µm particle size; 12.5 x 4.6 mm; Agilent), maintained at room temperature. Detection wavelengths of the diode array detector were 254/360 nm, 480/800 nm, 360/580 nm, and 430/600 nm. The mobile system consisted of solvent A (acetic acid [0.5%, vol/vol] in acetonitrile) and solvent B (acetic acid [0.5%, vol/vol]). A 10 µL aliquot of the MeOH-soluble extract was injected for analysis each sample, a gradient elution method was used (A: CH_3CN with 0.1% HAc; B: H_2O with 0.1% HAc; 5% A over 4 min, 5–95% A from 4 to 20 min, 95% A from 20 to 22 min, 95-5% A from 22 to 23 min, and 5% A from 23 to 30 min; 0.5mL/min). MSD settings during the LC gradient were as follows: Acquisition—mass range m/z 150–

1000, MS scan rate $1s^{-1}$, MS/MS scan rate $2s^{-1}$, fixed collision energy 20 eV; ion source drying gas temperature 350 °C, drying gas flow 10 L/min; Nebulizer pressure 35 psig; ion source mode API-ES; capillary voltage 3000; The MS detector was autotuned using Agilent tuning solution in positive and positive mode before measurement. LC (DAD) and MS data were analyzed with ChemStation software (Agilent).

NMR methods and General instrumentation for structural characterization

Nuclear magnetic resonance (NMR) was employed to elucidate the structures of Compound WS9326A, B, D, E, F, G, Annimycin B. The 1D NMR spectra [^{1}H NMR(400 MHz) and ^{13}C NMR (100 MHz)] and 2D NMR spectra [^{1}H/^{1}H-COSY, HSQC and HMBC] of these compounds were detected on a Varian VNMR-S 600-MHz spectrometer in 150 µl DMSO-d_6 at T = 35°C or 25°C. Residual solvent signals were used as an internal standard (DMSO-d_6: δ_H = 2.5ppm, δ_C = 39.5 ppm). For WS9326F, WS9326G and Annimycin B, their high-resolution electron spray ionization mass spectra (HR-ESI-MS) were measured on a LTQ Orbitrap XL (Thermo Scientific).

Structure information of compound 1-7

WS9326A (1): White amorphous powder; UV(MeOH) λmax 231 nm, 288 nm; ESI-MS *m/z* $[M-H]^-$ 1035, $[M+Cl]^-$1071, (calcd. for $C_{54}H_{68}N_8O_{13}$, 1036.49); ^{1}H-NMR (400 MHz, DMSO-d_6) and ^{13}C-NMR (100 MHz, DMSO-d_6) data are shown in Table S7.

WS9326B (2): White amorphous powder; UV(MeOH) λmax 212 nm, 290 nm; ESI-MS *m/z* $[M-H]^-$1037, $[M+Cl]^-$1073, HRESI-MS *m/z* $[M+H]^+$ 1039.0229 (calcd. for $C_{54}H_{70}N_8O_{13}$, 1038.51);

WS9326D (3): White amorphous powder; UV(MeOH) λmax 209 nm, 289 nm; ESI-MS *m/z* $[M-H]^-$ 852.4(calcd. for $C_{47}H_{59}N_5O_{10}$, 853.43);

WS9326E (4): White amorphous powder; UV(MeOH) λmax 222 nm, 291 nm; ESI-MS *m/z* $[M-H]^-$ 838.4(calcd. for $C_{46}H_{57}N_5O_{10}$, 839.41);

WS9326F (5): White amorphous powder; UV(MeOH) λmax 210 nm, 290 nm; ESI-MS *m/z* $[M-H]^-$ 966.4, HRESI-MS *m/z* $[M+H]^+$ 968.4750 (calcd. for $C_{51}H_{65}N_7O_{12}$, 967.47); ^{1}H-NMR (400 MHz, DMSO-d_6) and ^{13}C-NMR (100 MHz, DMSO-d_6) data are shown in Table S7.

WS9326G (6): White amorphous powder; UV(MeOH) λmax 225 nm, 293 nm; ESI-MS *m/z* [M-H]- 952.4, HRESI-MS *m/z* $[M+H]^+$ 954.4644(calcd. for $C_{50}H_{63}N_7O_{12}$, 953.45);

Annimycin B (7): yellow amorphous powder; UV(MeOH) λmax 326 nm; ESI-MS *m/z* [M-H]-260, $[M+H]^+$ 262, HRESI-MS *m/z* [M+H]+262.1446 (calcd. for $C_{15}H_{19}NO_3$, 261.14); ^{1}H-NMR (400 MHz, DMSO-d_6) and ^{13}C-NMR (100 MHz, DMSO-d_6) data are shown in Table S8.

Determination of the absolute configuration of WS9326A by Marfey's method

The Marfey's method was carried out following the previous protocol[1], and the Marfey's reagent was purchased from Thermo Scientific (Number 48895). The amino acid standards involved in the assembly of WS9326A were obtained commercially from Carl Roth or Sigma-Aldrich. Each of them (1 mg) were dissolved in 100 ul H_2O, respectively. Then the amino acids were derivatized by adding 1 M $NaHCO_3$ (40 ul) and 1% FDAA (in acetone, 200 ul). The reaction mixture was heated at 40°C for 1 hour, then the mixture was neutralized with 2 M HCl after cooling at room temperature. The derivatives were then dried and redissolved in CH_3OH, then analyzed by HPLC-MS (ESI+ mode, Zorbax Eclipse XDB-C18 column,100 x 4.6 mm), a gradient elution method was used (A: CH_3CN with 0.1% HAc; B: H_2O with 0.1% HAc; 10%-40%-90% CH_3CN in 50 min, 0.5 ml/min at 25°C, detection at wavelengths of 270 nm, and 340 nm, *m/z* range from 100-2000). WS9326A (0.5 mg) was hydrolyzed with 2 M HCl (2.0 mL) at 45°C for 2 hours. The solution was evaporated to dryness, and derivatized with Marfey's reagent. The derivatives of WS9326A acid hydrolysates were analyzed by LC-MS. The retention times of amino acid standards derivitized with Marfey's reagent are as follows: 21.71 min (L-threonine), 19.48 min (L-asparagine), 41.92 min (L-leucine), 21.87 min (L-allo-threonine), 20.31 min (L-serine), 44.55 min (D-phenylalanine).

Antiparasite assay method and materials

Asexual, blood-stage parasites were cultured in vitro using standard conditions[2]. Briefly, parasites were maintained in 2% human O+ erythrocytes (Interstate Blood Bank, Memphis, TN) in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 0.5% Albumax (Life Technologies), 24 mmol/L sodium bicarbonate, and 10 µg/mL gentamycin. Tissue culture flasks were incubated at 37 °C under a gas mixture of 5% CO2, 5% O2, and 90% N2. Cultures were screened for mycoplasma using the Universal Mycoplasma Detection Kit (ATCC, Manassas, VA).

In vitro drug responses were measured using 72-hr SYBR Green staining assays as described previously with minor modifications[3]. Parasites were diluted to 0.5% final parasitemia with 2% final hematocrit. The diluted parasite culture (100 µL) was added to duplicate test wells in a 96-well plate containing 100 µL of the drug tested. IC50 values were determined by nonlinear regression analysis using Prism 5.0 software (GraphPad Software, San Diego, CA). Drug assays were performed on three independent occasions.

Table S1. Primers fragments used in this study

Name	**Sequence**

Primer for vector construction of pKC1132-InAnn3	
InAnn3-F	CCGCTGAACGTCATGTCGACCGC
InAnn3-R	CGTTCGGACCGCGCACCACGA
Primer for vector construction of pKC1132-orf(-1)	
Orf(-1)-F	GTCGGTGACGCCGTCGCCC
Orf(-1)-R	GAGATGTGCAGCTTCTCCGCGATG
Primer for vector construction of pKC1132-SAS1	
LuxR2-F	CGCCATGACGCGTCCACCGTG
LuxR2-R	GGTGCGGACGCTGCATCCCAGAC
Primer for vector construction of pKC1132-SAS16	
P450S-F	ATATaagcttGCGCCGCAGGGAACTGCTCGAC
P450S-R	ATATggatccTGGTGGACGCCGTAGCCGAAC
Primer for vector construction of pKC1132-SAS13	
SAS13F	ATATaagcttCCGTGACGCGGCTTCGTGCT
SAS13R	ATATtctagaGCACCTCGCCGAAGAAGCGG
PCR verification for gene *ann3* disruption	
Vann3-F	GTACTTCGGGGTGCTGGCGGCC
Apra-R	AGCTCAGCCAATCGACTGGCGAG
PCR verification for gene *orf3100* disruption	
V3100-F	TGGTCTCCGCGGGCAGGAGCCAGAA
Vaad-R	GTCGATCGTGGCTGGCTCGAAGATAC
PCR verification for gene *orf(-1)* disruption	
VluxR1-F	ACGGAGATCCGGGTGAGCCTGCG
Apra-R	AGCTCAGCCAATCGACTGGCGAG
PCR verification for gene *sas1* disruption	
VluxR2-F	AATCCGCCGACGGAGGAGGACACC
Apra-R	AGCTCAGCCAATCGACTGGCGAG
PCR verification for gene *sas16* disruption	
Vsas16-F	AGTCATGGGTCTGTCCACTCCAGTA
Apra-R	AGCTCAGCCAATCGACTGGCGAG
PCR verification for gene *sas13* disruption	
Vsas13-F	CGCACTCCGGGGACGTGGTGACC
Apra-R	AGCTCAGCCAATCGACTGGCGAG
Primers for in-frame deletion of *sas16* by λ Red-mediated recombination	
SAS16F	ATATaagctcCCGACTACACCGGCATCCTC 3'
SAS16R	ATATgaattcGCTCGTCGTCCACCGTGTC 3'
SAS16-ApraF	CGCAAGAAATGACCTCAGCTCAGATATAGGGGTAACGTCATGGATATCTCTAGATACCG
SAS16-ApraR	GATGCAGCGGTCGCGTTCGGCGTGAACCTTCATGGCGCCTAAACAAAAGCTGGAGCTC
Primers for in-frame deletion of N-methyltransferase domain in *sas17* gene by λ Red-mediated recombination	
Nmet4800bpF	GACCTGGTCGGCTTCCTCGT
Nmet4800bpR	GTCGGCGCGGTAGGTGAAG
Nmet-ApraF	GAGAACTTCGCCGGCTGGCACAGCAGTTACGACGGCTCGGTGGATATCTCTAGATACCG
Nmet-ApraR	CAGGCCAGGACGGGCACCGAGGCGAGGGAGACGGCTTCCGCAACAAAAGCTGGAGCTC
Primer for amplifying a 1224bp fragment carrying the entire *sas16* with its upstream 48bp region from the *Streptomyces asterosporus* DSM 41452 genome	

KpnI-RBSp450-F	GGTACCGGATCGGCCCGCAAGAAATGA
EcoRI-p450-R	GAATTCCTACCAGCCGATCGTCAGCTTGTG

Table S2. Plasmid information

Name	Description	Reference
pIJ790	λ-Red plasmid, temperature sensitive replicon, Cm^r	[4]
pBluescript SK(-)	Cloning vector, *lacZ*(α-complementation), Amp^r	Stratagene
pKC1132	Conjugative vector, Non-replicative in Streptomyces, $Apra^r$	[5]
pKC1132-orf(-1)	Vector for gene disruption of orf(-1), based on pKC1132, $Apra^r$	This study
pKC1132-SAS1	Vector for gene disruption of *sas1*, based on pKC1132, $Apra^r$	This study
pKC1132-SAS16	Vector for gene disruption of *sas16*, based on pKC1132, $Apra^r$	This study
pKC1132-SAS13	Vector for gene disruption of *sas13*, based on pKC1132, $Apra^r$	This study
pUC19	Cloning and sequencing vector for *E. coli*, Amp^r	[6]
pUC19Δ3100spec	Vector for gene disruption of *ann5*, based on pUC19, *aadA* from pLERE-Spec-oriT, $Spec^r$	[7]
pUZ8002	Helper plasmid for conjugating plasmid containing the *oriT* sequence, $Kana^r$	[8]
pBSK-Nmet	Plasmid containing N-MTase encoding gene for subcloning, Amp^r	This study
pBSK-SAS16	Plasmid containing gene *sas16* for subcloning, Amp^r	This study
pKGLP2-GusA	pKCLP2 derivative with *gusA* gene, $Hygr^r$	[9]
pKGLP2-GusA-Nmet::*aac3(IV)*	Vector for double crossover of NMTase encoding gene in *Streptomyces*, $Apra^r$, $Hygr^r$	This study
pKGLP2-GusA-SAS16::*aac3(IV)*	Vector for double crossover of gene *sas16* in *Streptomyces*, $Apra^r$, $Hygr^r$	This study
pTESa-SAS16	an integrative plasmids pTESa base on ϕ31-based integrase, $Apra^r$	This study
pTESa	pSET152 derivatives; *attP* flanked by loxP site, *ermEp* promoter flanked by *tfd* terminator sequences, $Apra^r$	[9]
pLERECJ	Carrying aac(3)IV flanked by loxP-sites, Amp^r, $Apra^r$	[10]
pUWLCre	pUWLoriT derivative carrying *cre* under *ermEp*, $Hygr^r$, Tsr^r	[11]
pKC1132-InAnn3	Vector for gene disruption of *ann3*, based on pKC1132, $Apra^r$	This study

Table S3. Strain constructed and used in this study

Strain	Relevant characteristics	Reference
***E. coli* DH5α**	General cloning host	Invitrogen
***E. coli* ET12567(pUZ8002)**	Methylation-deficient *E. coli* strain for conjugation with the helper plasmid	Invitrogen
***E. coli* BW25113**	Host for DNA recombination	[10]
Streptomyces asterosporus DSM 41452	Wild type strain of WS9326A producer	DSMZ
***Streptomyces asterosporus* DSM 41452::pUC19Δ3100spec**	*Streptomyces asterosporus DSM 41452* strain containing plasmid pUC19Δ3100spec, $Spec^r$	This study

***Streptomyces asterosporus DSM 41452*::pKC1132-orf(-1)**	Gene *orf(-1)* inactivation in the WT strain, Apra^r	This study
***Streptomyces asterosporus DSM 41452*::pKC1132-SAS1**	Gene *sas1* inactivation in the WT strain, Apra^r	This study
***Streptomyces asterosporus DSM 41452*::pKC1132-SAS16**	Gene *sas16* inactivation in the WT strain, Apra^r	This study
***Streptomyces asterosporus DSM 41452*::pKC1132-SAS13**	Gene *sas13* inactivation in the WT strain, Apra^r	This study
***Streptomyces asterosporus DSM 41452*::pKC1132-InAnn3**	Gene *ann3* inactivation in the WT strain, Apra^r	This study
***Streptomyces asterosporus DSM 41452* Δsas16**	Gene *sas16* knockout in the WT strain, Apra^r	This study
Streptomyce asterosporus DSM 41452Δsas16::pTESa-SAS16	SAS16 overexpression in the mutant *Streptomyces asterosporus DSM 41452* Δsas16, Apra^r	This study
***Streptomyces asterosporus DSM 41452* Δ Nmet**	Gene encoding NMTase knockout in the WT strain, Apra^r	This study

Table S4. Predicted highly conserved core motifs of A domain binding pockets in NRPSs within the *SAS* cluster

Domian	**Residues in the Binding Pocket**								**Amino acid residues**	
	235	236	239	278	299	301	322	330	Predicted	in WS9326As
SAS17-A1	D	F	W	N	I	G	M	V	Thr	Thr/Ser
SAS17-A2	D	A	S	T	T	A	A	V	Tyr	Tyr
SAS18-A1	D	A	Y	T	W	G	A	V	Leu	Leu
SAS18-A2	D	A	W	T	V	A	A	V	Phe	Phe
SAS19-A1	D	V	W	H	L	G	X	X	Ser	Ser
SAS19-A2	D	V	W	H	L	S	L	V	Ser	Ser
SAS22-A	D	F	W	S	V	G	M	V	Thr	Thr
SAS23-A	D	L	T	K	V	G	E	V	Asn	Asn

Table S5. List of putative biosynthesis genes involved in the biosynthesis of the side chain of the WS9326As and their homologues in the biosynthesis gene cluster of Skyllamycin[13].

No.	**SAS**	**Homologues in Skyllamycin gene cluster**	**Function**	**Identity/Coverage(%)**
1	SAS21	---	Short chain dehydrogenase/reductase	---
2	SAS24	---	Oxidoreductase	---

3	SAS27	Sky5	Isomerase	62/99
4	SAS28	Sky4	Phytoene dehydrogenase/oxidoreductase	56/96
5	SAS29	Sky16	Acyl-carrier protein	71/96
6	SAS30	Sky17	3-oxoacyl-ACP synthase	77/96
7	SAS31	Sky18	3-oxoacyl-ACP synthase	62/100
8	SAS32	Sky19	3-oxoacyl-ACP synthase	54/99
9	SAS33	Sky22	3-oxoacyl-ACP synthase	53/98
10	SAS34	Sky23	Acyl-carrier protein	55/86
11	SAS35	Sky24	3-oxoacyl-ACP dehydratase	53/85
12	SAS36	Sky25	3-oxoacyl-ACP dehydratase	46/99
13	SAS37	Sky26	3-oxoacyl-ACP reductase	68/100
14	SAS7	---	3-oxoacyl-ACP synthase	---
15	SAS8	---	3-oxoacyl-ACP synthase	---
16	SAS9	---	Acyl-carrier protein	---
17	SAS25	Sky20	Hydrolase	---
18	SAS26	Sky21	Thioesterase	---
19	---	Sky11	Carboxyltransferase	---
20	---	Sky13	ACP-acyltransferase	---

Table S6: Proposed Functions of Open Reading Frames of WS9326As Biosynthesis Gene Cluster in *Streptomyces asterosporus* DSM41452.

Protein	Size(a.a.)	Homologous Protein and origin	Identity [%]	predicted function
Orf(+1)	220	SDD75964.1 [Streptomyces emeiensis]	87%	DedA family membrane protein
SAS1	407	Cal1 [Streptomyces calvus]	99%	Sensor histidine kinase
SAS2	211	Cal2 [Streptomyces calvus]	100%	LuxR transcriptional regulator
SAS3	79	Cal3 [Streptomyces calvus]	99%	LysR family transcriptional regulator
SAS4	72	Cal4 [Streptomyces calvus]	99%	MbtH domain protein
SAS5	248	Cal5 [Streptomyces calvus]	99%	Thioesteraase
SAS6	293	Cal6 [Streptomyces calvus]	99%	Hypothetical protein
SAS7	334	Cal7 [Streptomyces calvus]	99%	3-oxoacyl-ACP synthase II
SAS8	413	Cal8 [Streptomyces calvus]	99%	3-oxoacyl-ACP synthase II
SAS9	83	Cal9 [Streptomyces calvus]	99%	polyketide-8 synthase ACP
SAS10	123	Cal10 [Streptomyces calvus]	100%	hypothetical protein
SAS11	136	Cal11 [Streptomyces calvus]	100%	hypothetical protein
SAS12	140	Cal12 [Streptomyces calvus]	99%	translation initiation factor IF-2
SAS13	314	Cal13 [Streptomyces calvus]	99%	3-hydroxyacyl-ACP dehydratase
SAS14	257	Cal14 [Streptomyces calvus]	98%	hypothetical protein
SAS15	65	Cal15 [Streptomyces calvus]	100%	ferredoxin

SAS16	407	Cal16 [Streptomyces calvus]	100%	cytochrome P450
SAS17	2567	Cal17 [Streptomyces calvus]	99%	NRPS(C-A-PCP-C-A-Nmet-PCP)
SAS18	2588	Cal18 [Streptomyces calvus]	99%	NRPS(C-A-PCP-C-A-PCP-E)
SAS19	3389	Cal19 [Streptomyces calvus]	99%	NRPS(C-PCP-C-A-PCP-C-A-PCP-TE)
SAS20	233	Cal20 [Streptomyces calvus]	100%	Thioesterase
SAS21	274	Cal21 [Streptomyces calvus]	100%	Oxidoreductase
SAS22	974	Cal22 [Streptomyces calvus]	99%	NRPS(A-PCP)
SAS23	601	Cal23 [Streptomyces calvus]	99%	NRPS(A-PCP)
SAS24	400	Cal24 [Streptomyces calvus]	99%	ferredoxin reductase or FAD-dependent oxidoreductase
SAS25	264	Cal25 [Streptomyces calvus]	99%	alpha/beta hydrolase
SAS26	333	Cal26 [Streptomyces calvus]	99%	Acyl-CoA thioesterase
SAS27	241	Cal27 [Streptomyces calvus]	99%	isomerase
SAS28	574	Cal28 [Streptomyces calvus]	99%	Dehydrogenase or reductase
SAS29	86	Cal29 [Streptomyces calvus]	99%	Acyl carrier protein
SAS30	416	Cal30 [Streptomyces calvus]	99%	3-oxoacyl-ACP synthase
SAS31	379	Cal31 [Streptomyces calvus]	99%	3-oxoacyl-ACP synthase
SAS32	314	Cal32 [Streptomyces calvus]	99%	3-oxoacyl-ACP synthase
SAS33	371	Cal33 [Streptomyces calvus]	99%	3-oxoacyl-ACP synthase
SAS34	87	Cal34 [Streptomyces calvus]	100%	polyketide-8 synthase ACP
SAS35	133	Cal35 [Streptomyces calvus]	99%	3-oxoacyl-ACP dehydratase
SAS36	159	Cal36 [Streptomyces calvus]	99%	3-oxoacyl-ACP dehydratase
SAS37	248	Cal37 [Streptomyces calvus]	100%	beta-ketoacyl-ACP reductase
SAS38	178	Cal38 [Streptomyces calvus]	100%	tRNA synthetase
SAS39	318	Cal39 [Streptomyces calvus]	99%	ABC transporter
SAS40	280	Cal40 [Streptomyces calvus]	100%	multidrug ABC transporter permease
Orf(-1)	319	WP_040907349.1 [Streptomyces griseoflavus]	91%	transcriptional regulator
Orf(-2)	358	KES07412.1 [Streptomyces toyocaensis]	81%	polyprenyl diphosphate synthase
Orf(-3)	717	WP_004931928.1 [Streptomyces griseoflavus]	93%	putative drug exporter

Table S7. Summary of NMR Data for WS9326A and WS9326F in DMSO-d_6

position	**WS9326A**		**WS9326F**		
	δ_H	**δ_C**	**δ_H**	**δ_C**	**HMBC**

Acyl	1		165.7		165.6	
	2	6.69,1H,d(15.5)	123.0	6.88, 1H, d(15.1)	128.5	
	3	7.42,1H,d(15.5)	127.7	7.53, 1H, d(15.1)	137.4	165.6
	4		133.5			
	5	7.20,1H,m	126.5	-		
	6	7.26,1H	127.2	-		
	7	7.27,1H	128.8	-		
	8	7.20,1H	130.1	-		
	9		139.1			
	10	6.50,1H,d(11.4)	127.4	6.53, 1H, d(11.4)	127.1	30.4
	11	5.83,1H,dt(11.4,7.4)	134.7	5.79, 1H, dt(11.4, 7.4)	134.5	
	12	1.98,2H,m	30.5	1.98, 2H, m	30.4	127.1, 134.5, 22.1
	13	1.36,2H,m	22.7	1.34, 2H, m	22.1	134.5, 30.4, 14.2
	14	0.80,3H,t(7.2)	14.2	0.79, 3H, t(7.3)	14.2	30.4, 22.1
[1]Thr	NH	8.73,1H,d(9.4)		-		
	α	5.33,1H,t(9.8)	53.6	4.29, 1H	52.4	
	β	5.02,1H,dq(9.8,6.2)	73.9	3.69, 1H	66.9	
	γ	1.15,3H,d(6.0)	17.3	1.05, 3H, brs	20.3	52.4, 66.9
	C=O		169.4		170.8	
[2] Δ MeTyr	NMe	2.98,3H,s	34.9	2.87, 3H,s	34.8	170.8
	α		128.4		130.1	
	β	6.13,1H,s	132.1	6.63, 1H, s	132.3	165.2, 132.2
	1		123.4		124.0	
	2,6	7.39,2H,d(8.6)	132.3	7.27, 2H, d(8.0)	132.2	130.1, 132.3, 158.5
	3,5	6.58,2H,d(8.6)	115.4	6.65, 2H, d(8.0)	115.4	124.0, 158.5
	4		158.5		158.5	
	C=O		166.1		165.2	
[3]Leu	NH	9.25,1H.br.s		-		
	α	4.07,1H,m	54.1	4.49	49.6	
	β	1.26,2H,m	39.7	1.23, 2H, m	40.1	
	γ	0.85,1H,m	24.0	0.81, 1H, m	24.0	
	σ	0.76,3H	22.6	0.70, 3H	22.6	49.6, 40.1

		0.63,3H	23.3	0.65,3H	23.3	
	C=O		172.5		171.8	
[4]Phe	NH	9.17,1H,d(8.0)		-		
	α	4.33,1H,m	56.5	4.62,1H, m	54.6	
	β	3.28,1H,m	36.8	3.11, 1H, m	38.4	138.1
		2.72,1H,m		2.65, 1H, m		
	1		139.1		138.1	
	2,6	7.33,2H,d	129.4	7.35, 2H, m	129.4	138.1, 127.2
	3,5	7.28,2H,d	128.4	7.27, 2H, m	128.4	
	4		127.2		127.2	
	C=O		170.6		-	
[5]Thr	NH	7.59,1H,d(9.8)				
	α	4.34,1H,m	56.8	4.23, 1H, m	58.7	170.4
	β	4.28,1H,m	68.6	4.02, 1H, m	67.5	
	γ	0.63,3H	22.7	0.92, 3H	22.7	
	OH	5.18,1H,d(3.0)		-		
	C=O		170.4		170.4	
[6]Asn	NH	8.34,1H,d(7.3)		-		
	α	4.44,1H,m	51.3	3.17, 1H, m	48.8	
	β	2.45,2H	37.2	2.45, 2H	37.2	48.8, 171.2
	γ (C=O)		171.6		171.2	
	γ (NH2)	6.93,1H, 7.30,1H		-		
	C=O		172.0		-	
[7]Ser	NH	8.49,1H,d(9.5)				
	α	4.34,1H	56.9			
	β	3.15,1H	61.5			
		3.24,1H				
	OH	4.79,1H,br				
	C=O		169.3			

Note: Signal assignments based on the 1D and 2D NMR data.

Table S8. Summary of ^{1}H NMR(400MHz) and ^{13}C NMR(100MHz) Data for Annimycin B in $CDCl_3$

Position	δ_C	δ_H	COSY	HMBC
1	165.4			
2	118.7	5.97, d, 14.9Hz	H3	C1, C4
3	145.3	7.38, dd, 14.9, 11.3 Hz	H2, H4	C1, C5
4	123.3	6.24, dd, 15.3, 11.3 Hz	H5	
5	147.5	6.62, d, 15.3 Hz		
6	133.3			
6a	12.2	1.79, s		C5, C7
7	141.4	5.76, t, 7.4	H8	C5
8	22.1	2.21, m	H7, H9	
9	13.8	1.04, t, 7.3	H8	C7
NH		7.49,s		C5′, C1′
1′	115.2			
2′	174.5			
2′-OH		13.7, s		C1′, C2′, C3′
3′	25.7	2.62, m	H4′	
4′	32.3	2.53, m	H3′	C2′, C5′
5′	197.7			

Figure S1.

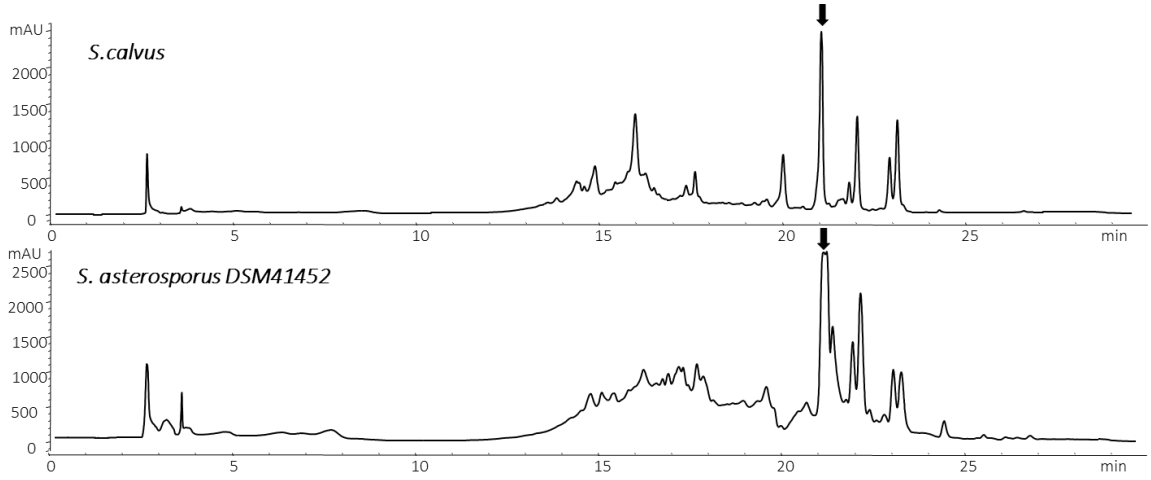

Figure S1. Comparative HPLC Profiles analysis of extracts from the cultures of *Streptomyces calvus* ATCC 13382 and *Streptomyces asterosporus* DSM 41452. The peak of WS9326A is marked with an arrow.

Figure S2.

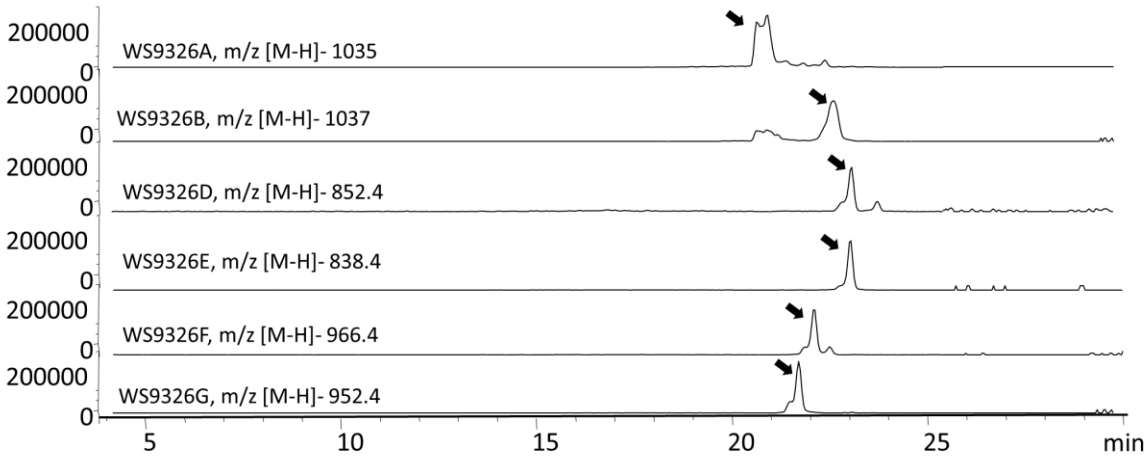

Figure S2. HPLC-MS analysis of the cultures of the *Streptomyces asterosporus* DSM 41452. (A) Chromatogram monitored at 254 nm. (B) Corresponding extracted ion chromatograms for WS9326A, B, D, E, F, and G. The peaks of interest are marked with an arrow.

Figure S3.

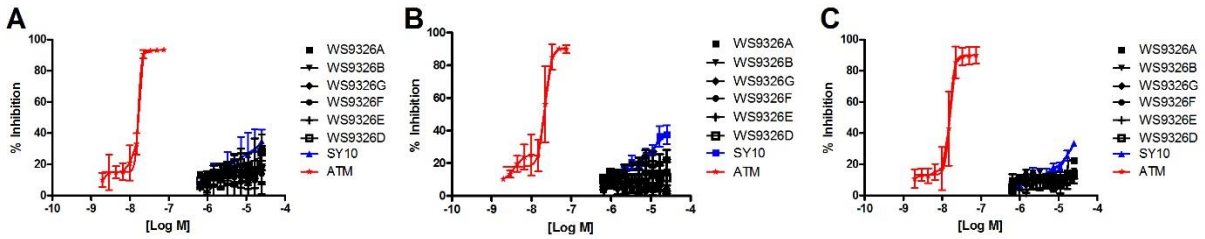

Figure S3. Drug response phenotypes for Plasmodium falciparum Dd2 (A), HB3 (B) and 3D7 (C) strains. Annimycin B was the only compound that demonstrate significant antimalarial growth inhibition, with ~30% growth inhibition, against all three strains at the highest concentration tested (25 uM). Artemether (ATM) is shown as a positive antimalarial control. The results shown are the average of three independent experiments conducted in duplicate per concentration, shown as the mean and standard error.

Figure S4.

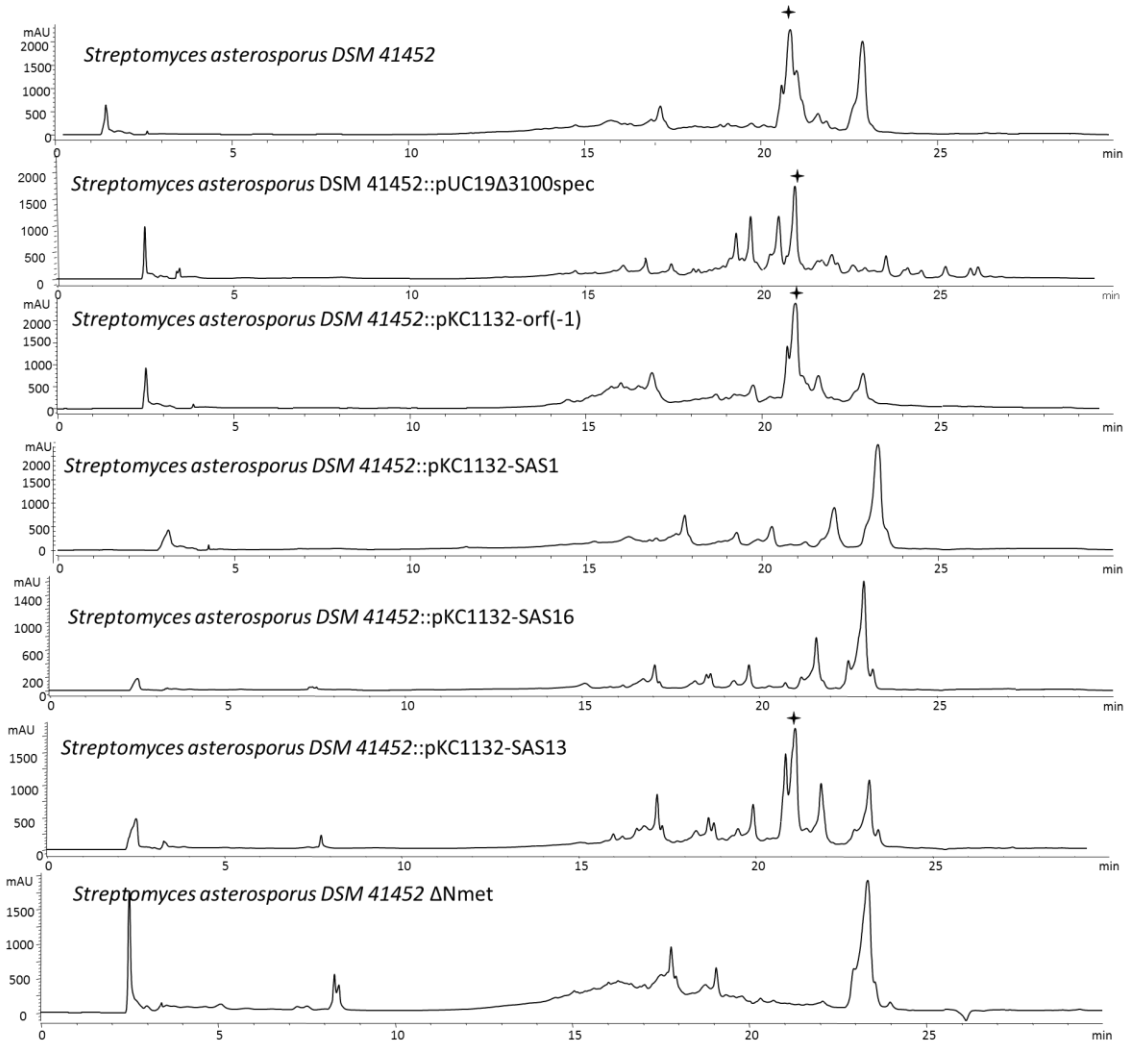

Figure S4. HPLC chromatograms of *Streptomyces asterosporus* DSM 41452 and its mutant strains (monitored at 254nm wavelength). The peak corresponding to WS9326A is marked with a star.

Figure S5.

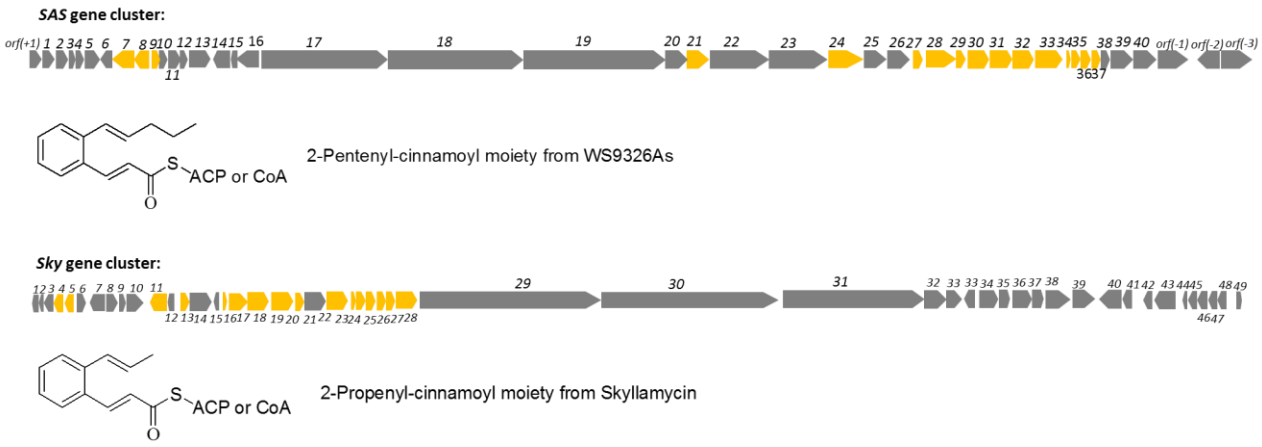

Figure S5. Comparison of the gene clusters encoding the cinnamoyl side chain biosynthesis in the SAS and SKY gene clusters (highlighted in yellow).

Figure S6.

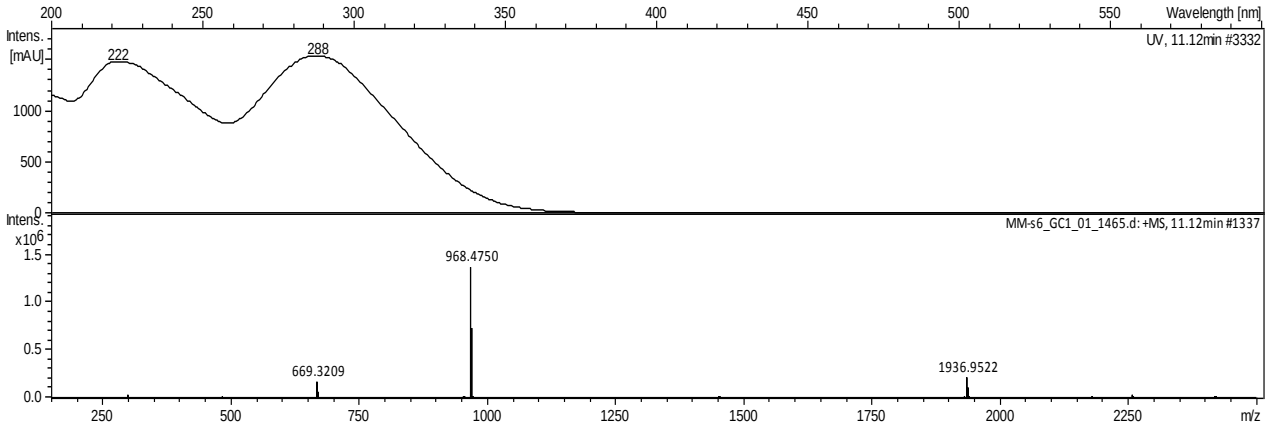

Figure S6. UV-Visible spectrum and HR-ESI-MS spectrum of WS9326F

Figure S7.

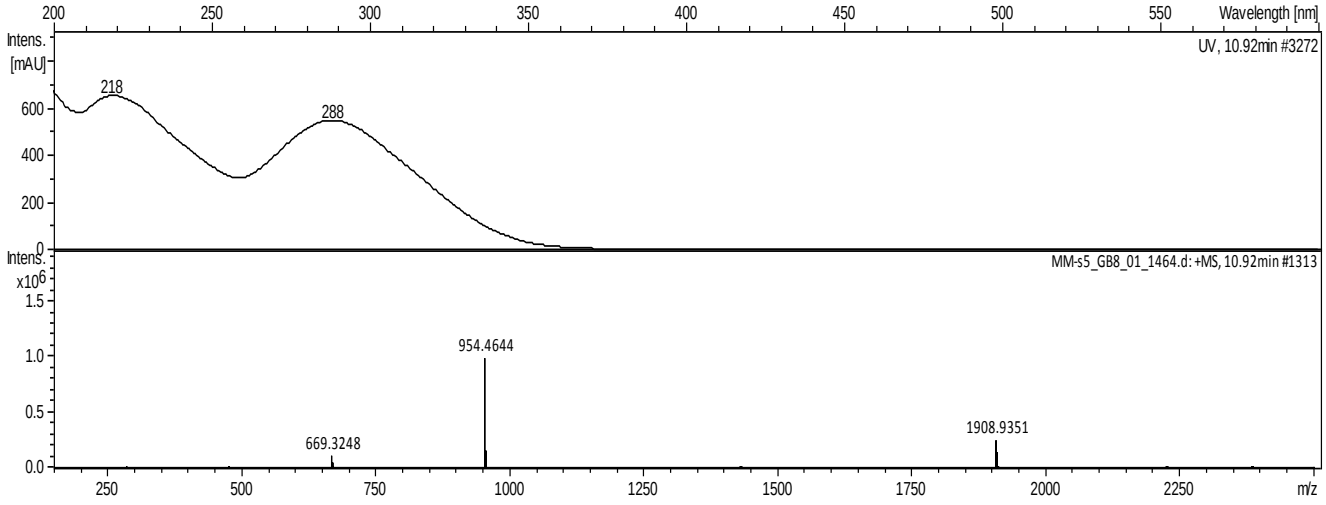

Figure S7. UV-Visible spectrum and HR-ESI-MS spectrum of WS9326G

Figure S8.

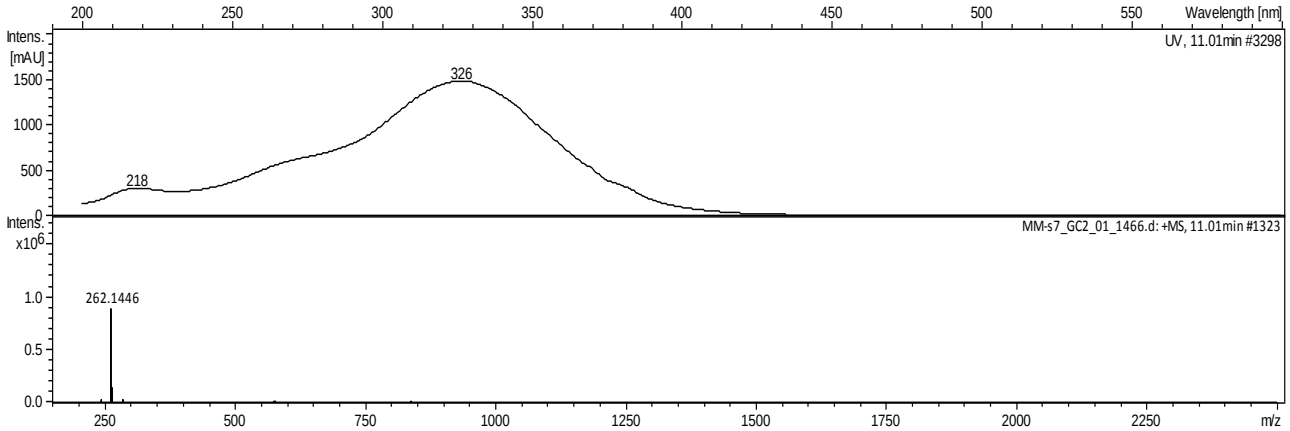

Figure S8.UV-Vis spectrum and HR-ESI-MS spectrum of Annimycin B

Figure S9.

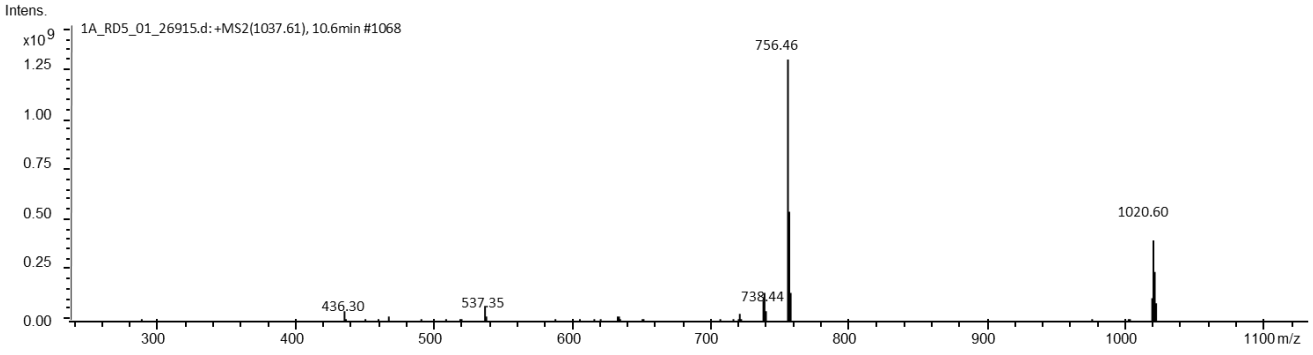

Figure S9. ESI-MS/MS spectrum of WS9326A

Figure S10.

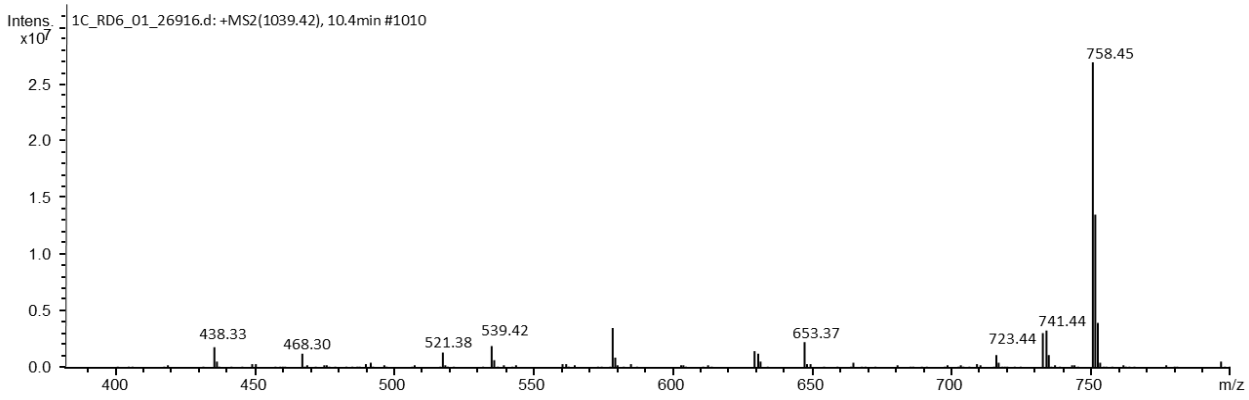

Figure S10. ESI-MS/MS spectrum of WS9326B

Figure S11.

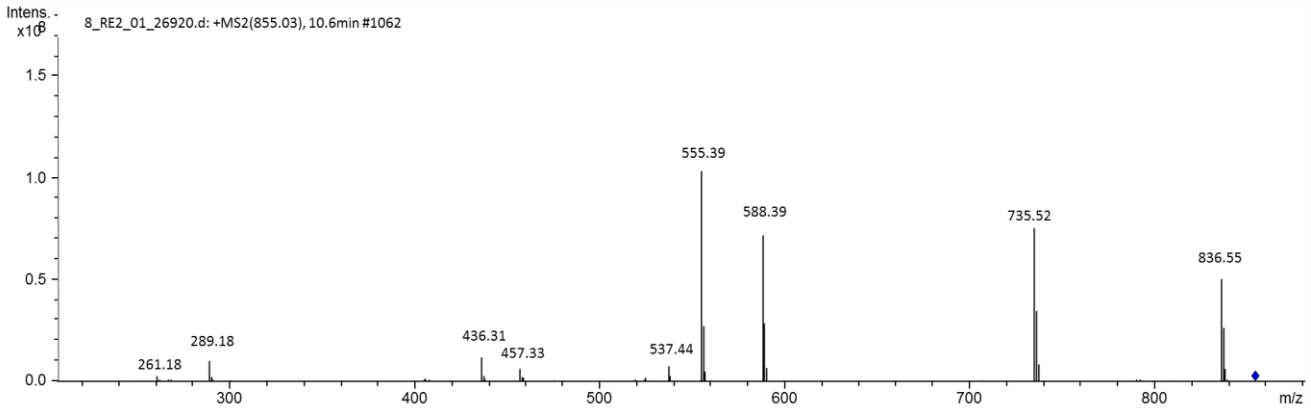

Figure S11. ESI-MS/MS spectrum of WS9326D

Figure S12.

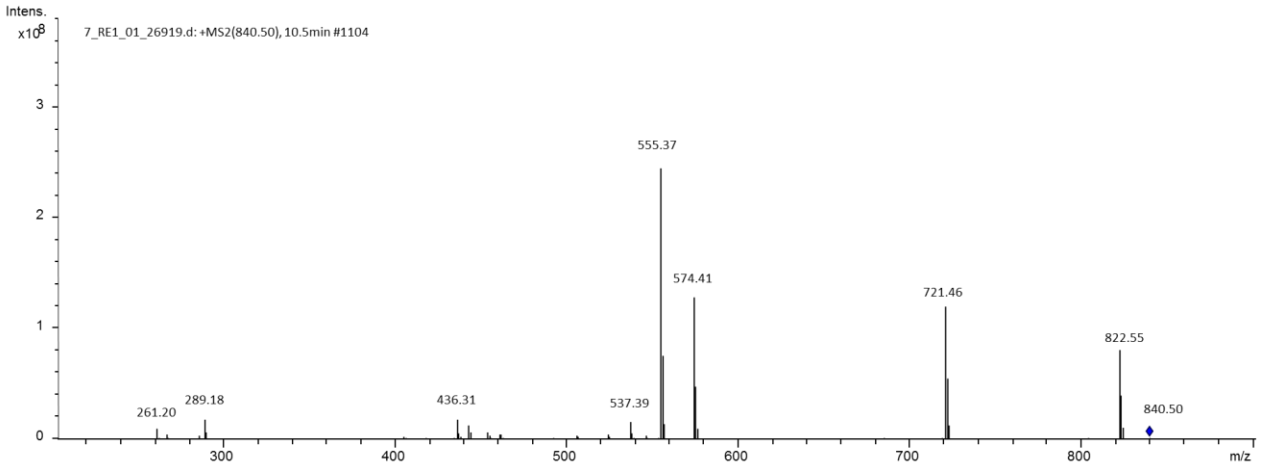

Figure S12. ESI-MS/MS spectrum of WS9326E

Figure S13.

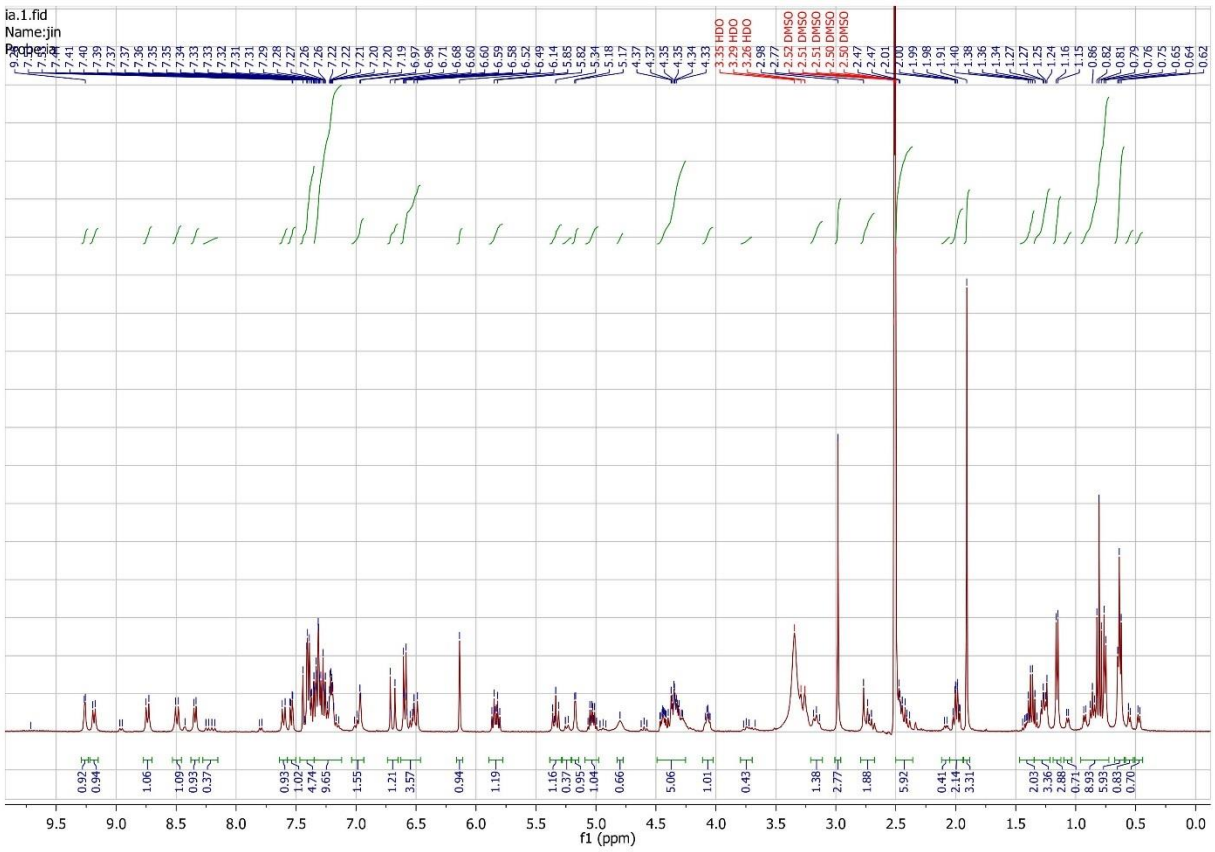

Figure S13. H NMR spectrum of WS9326A

Figure S14.

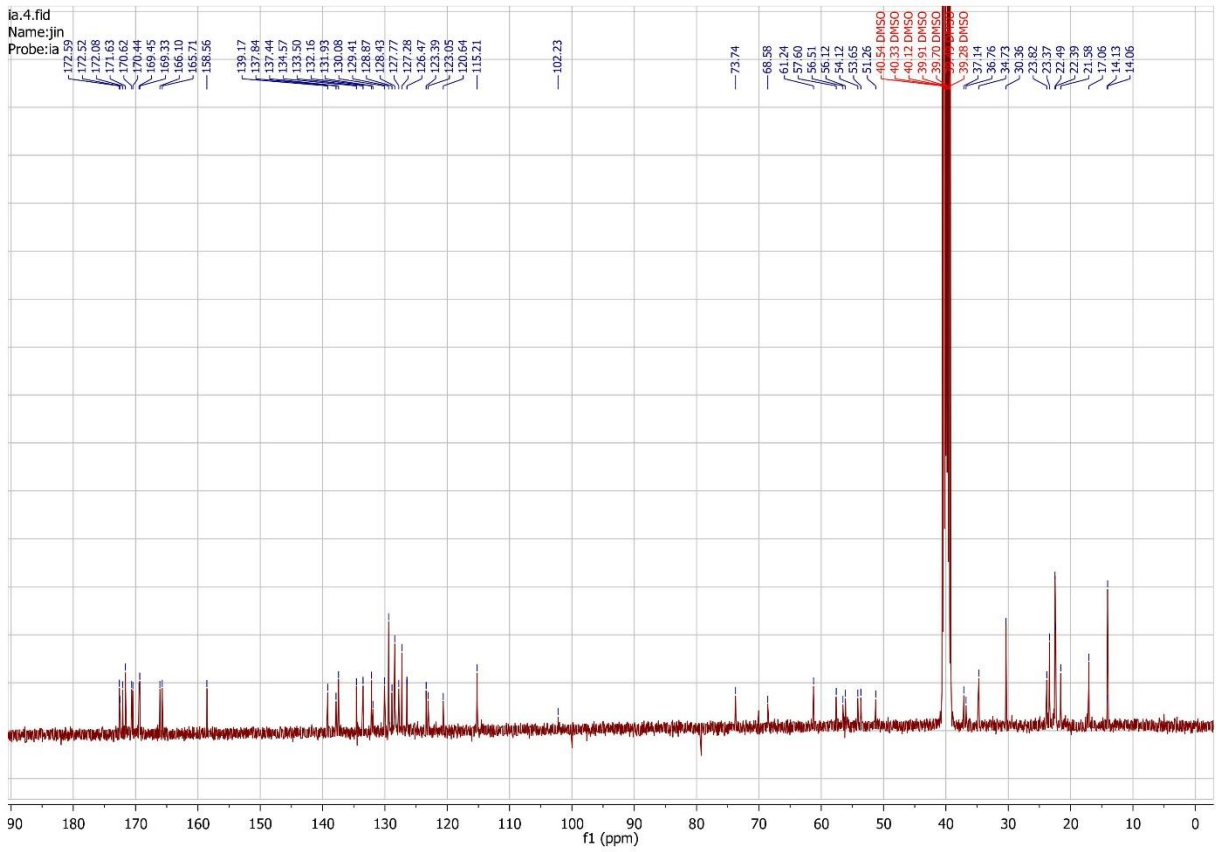

Figure S14. C NMR spectrum of WS9326A

Figure S15.

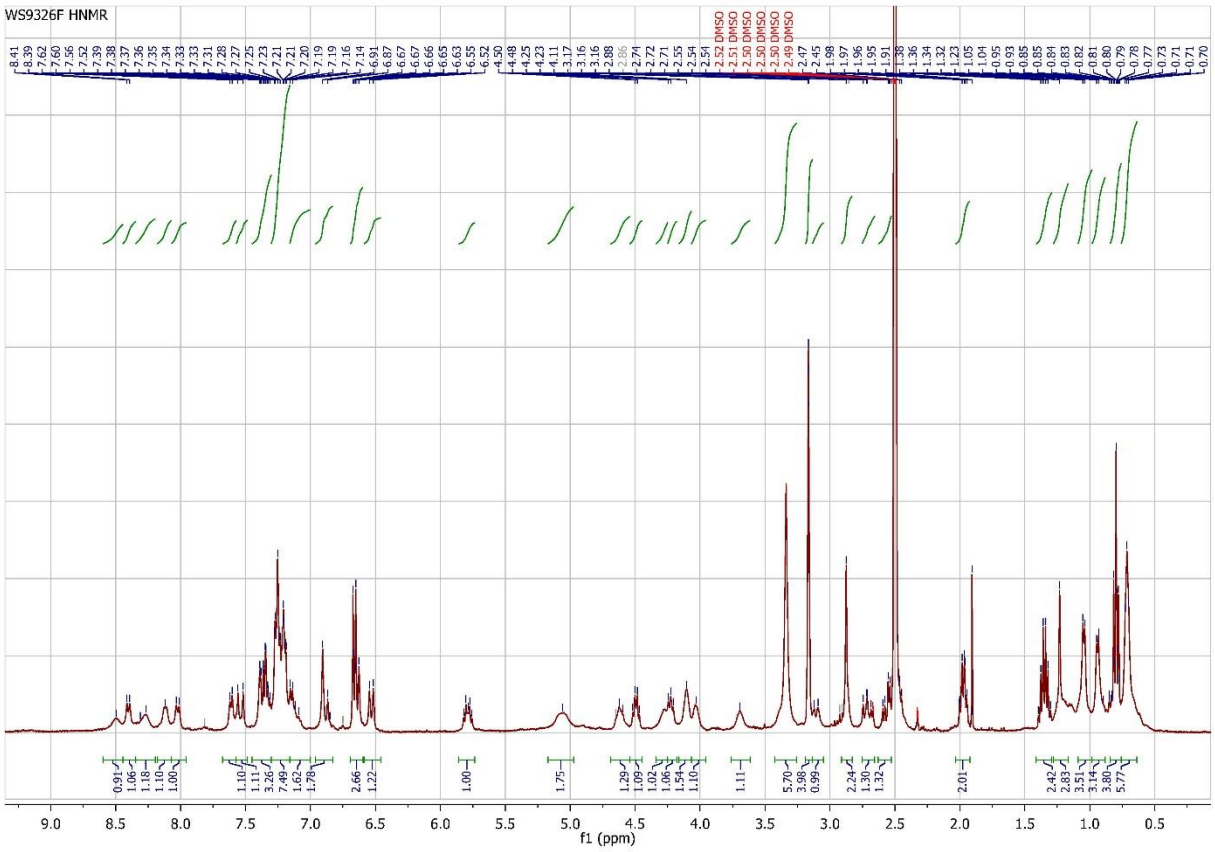

Figure S15. H NMR spectrum of WS9326F

Figure S16.

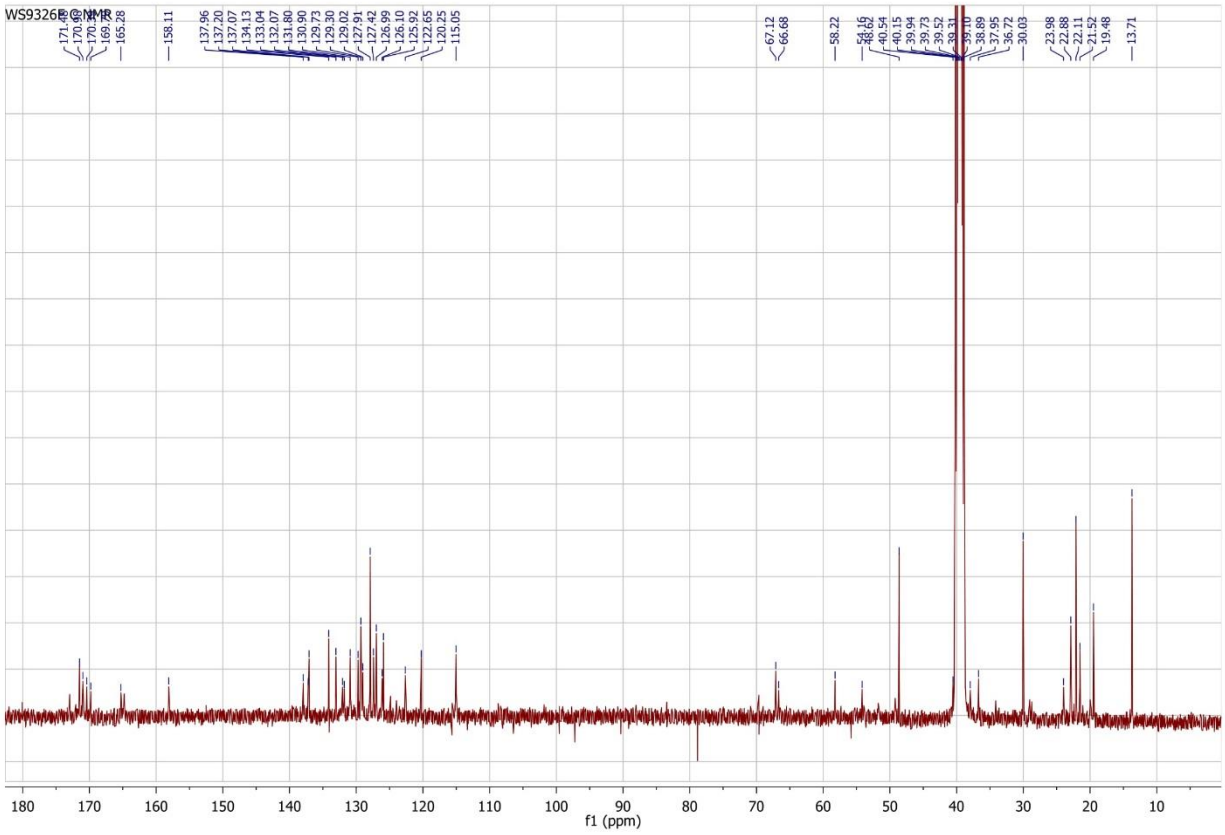

Figure S16. C NMR spectrum of WS9326F

Figure S17.

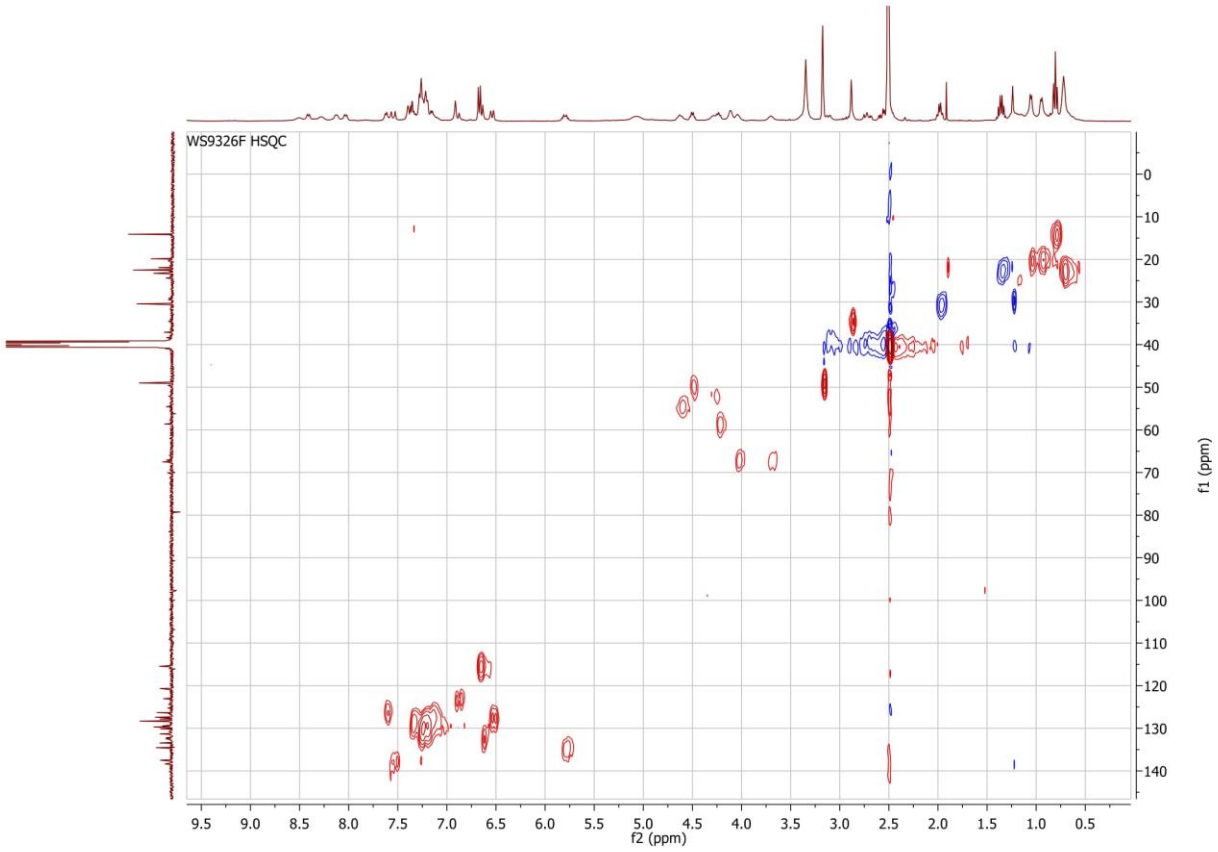

Figure S17. HSQC spectrum of WS9326F

Figure S18.

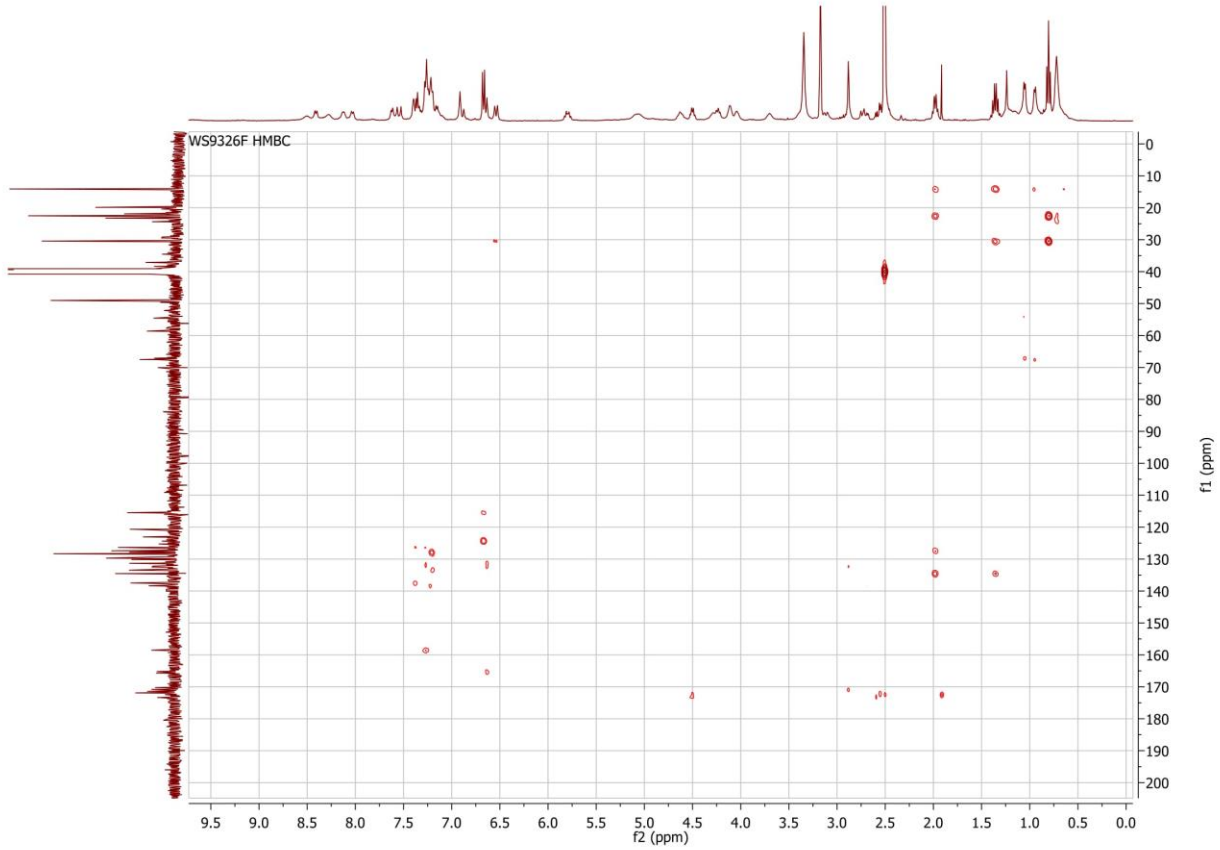

Figure S8. HMBC spectrum of WS9326F

Figure S19.

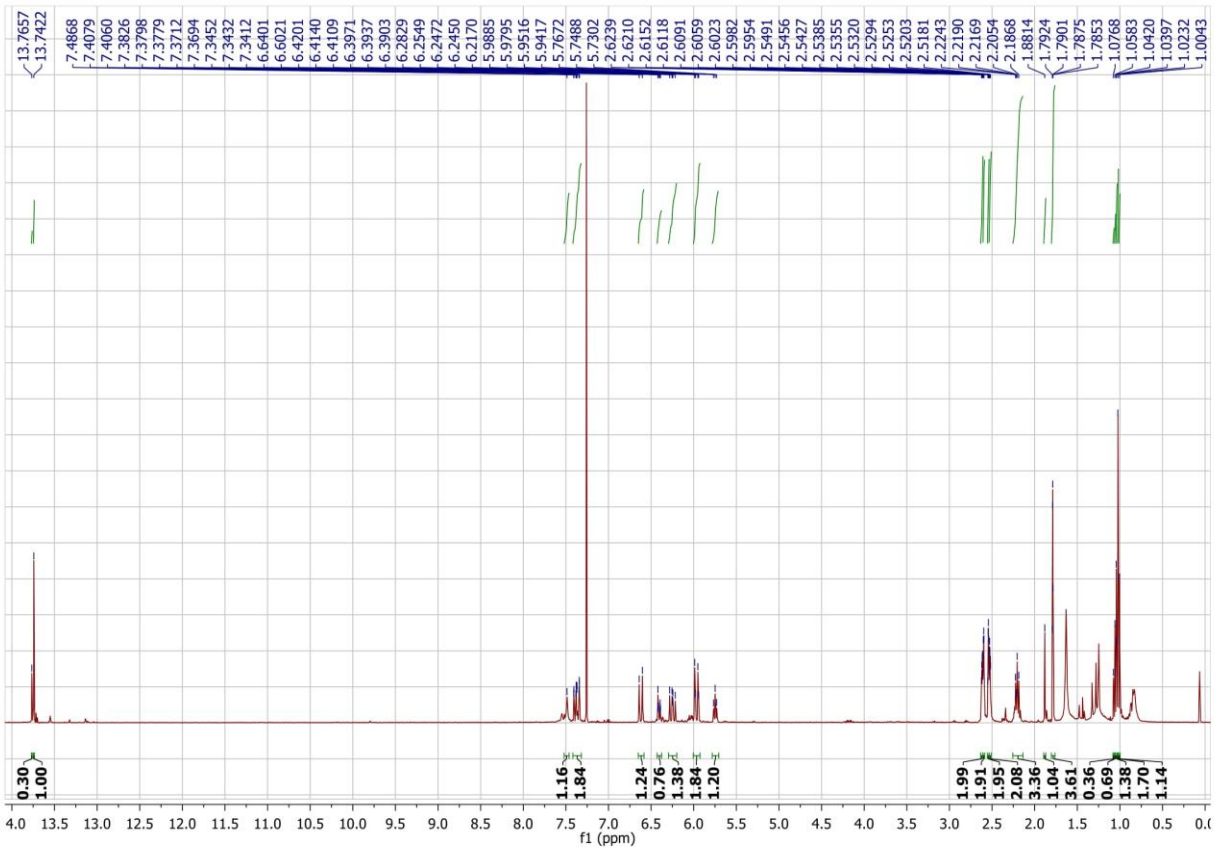

Figure S19. H NMR spectrum of Annimycin B

Figure S20.

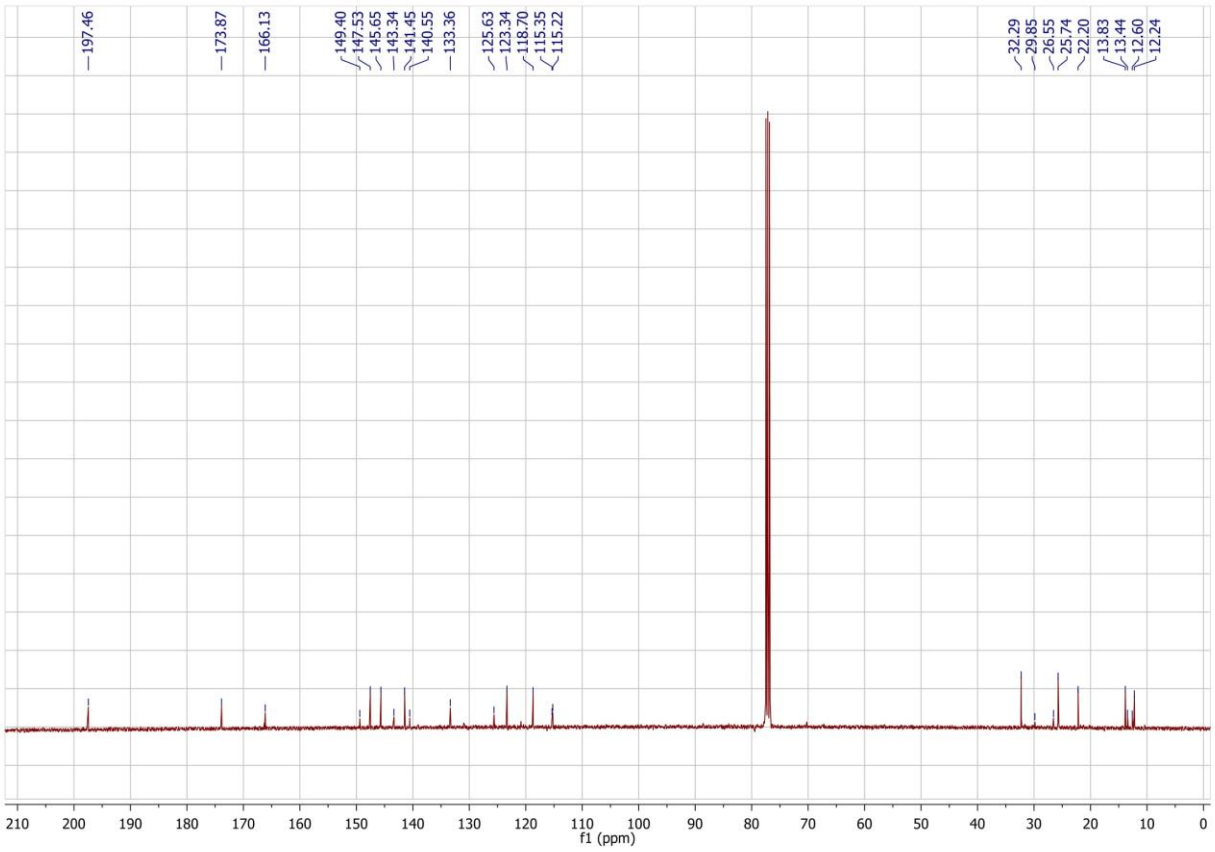

Figure S20. C NMR spectrum of Annimycin B

Figure S21.

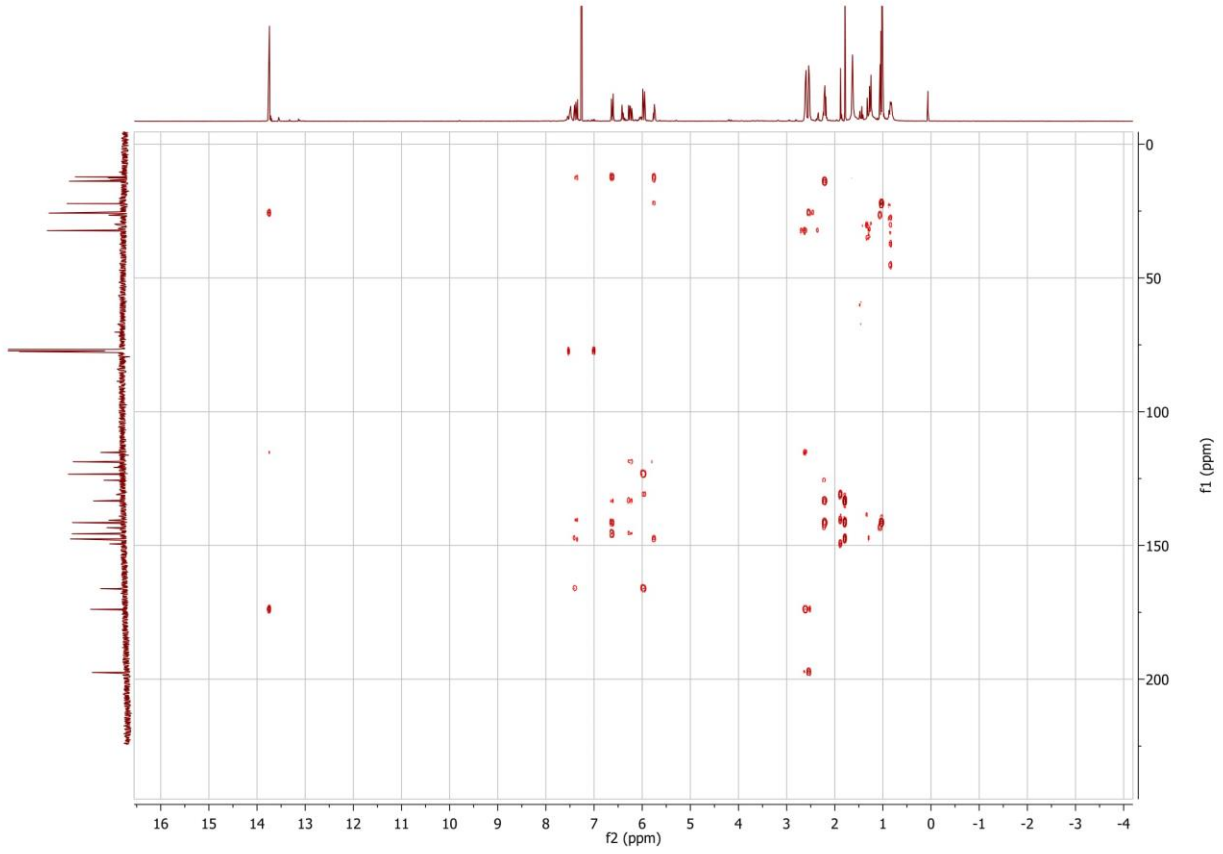

Figure 21. HMBC spectrum of Annimycin B

Figure S22.

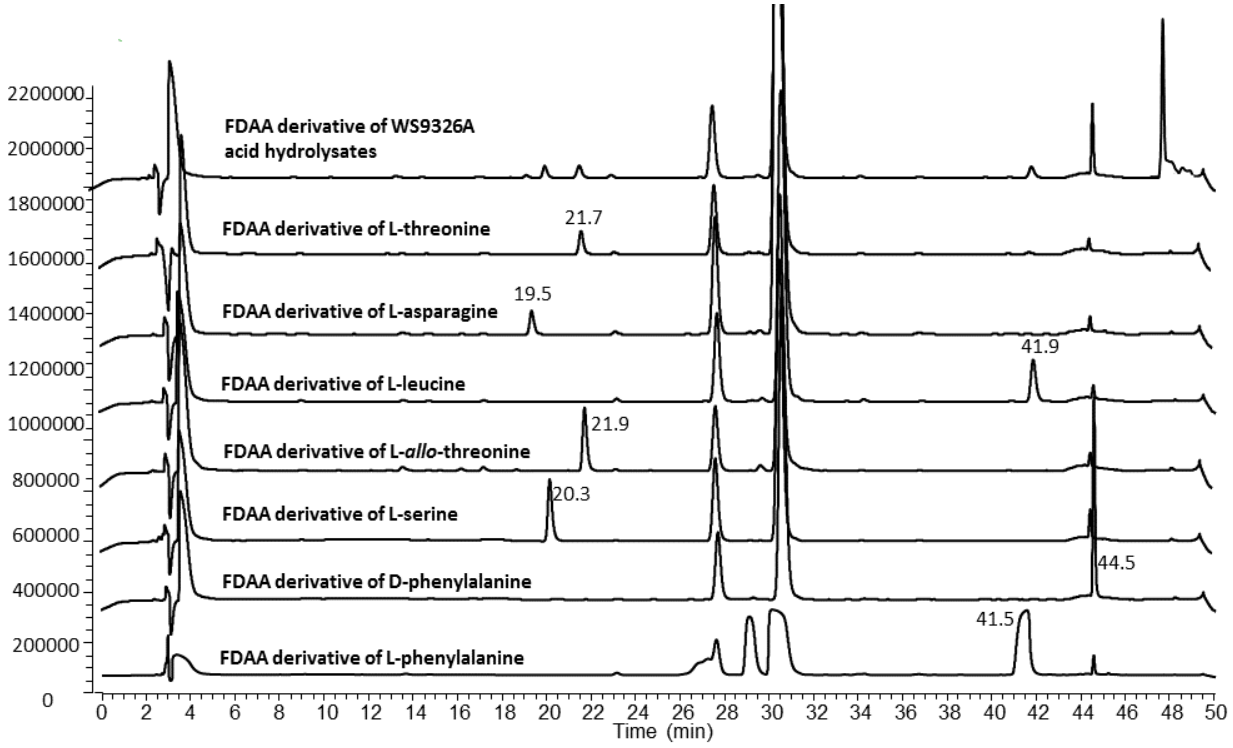

Figure S22. HPLC chromatogram of FDAA derivative of WS9326A and the corresponding standard amino acids (number representing the retention time). The eluent for each chromatogram was monitored at 256 nm. Retention times for FDAA derivatives are shown next to the respective peaks.

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