Identification and Biosynthetic Characterization of Natural Aromatic Azoxy Products from *Streptomyces chattanoogensis* L10

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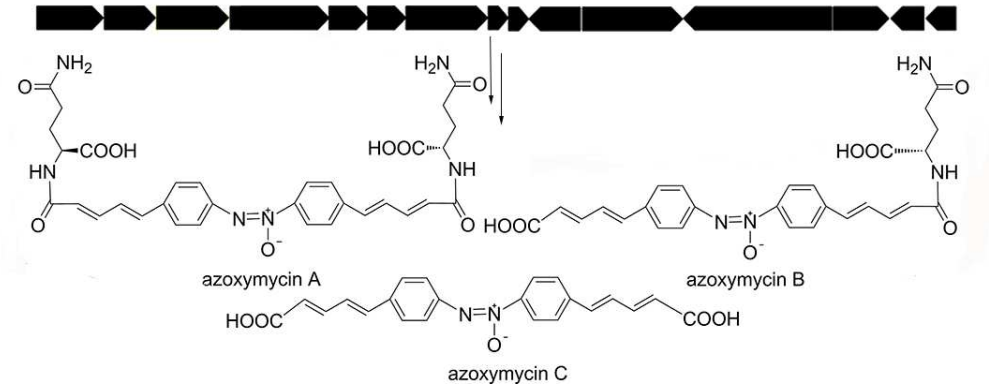
azoxymycin A
azoxymycin B
azoxymycin C

Experimental section

1. General analytical procedures

HPLC analysis was carried out on agilent 1260 infinity system with a DAD detector, column used was agilent ZORBAX SB-C18 (5 μm，4.6*150 mm), mobile phase A was 0.1% TFA in water, mobile phase B was 0.1% TFA in methanol, flow rate was 1mL min^{-1}, during the analysis procedure mobile phase B was raised from 10% to 100% in 30 min. Analysis of azoxymycins A, B and C was carried out at 400 nm, while compounds A and B were analyzed at 280 nm.

HPLC analysis for photo and heat induced isomerizations (figures S37-S42) was also carried out on agilent 1260 infinity system with a DAD detector, column used was agilent poroshell 120 EC-C18 (2.7 μm，3.0*50mm), mobile phase A was 0.1% TFA in water, mobile phase B was 0.1% TFA in acetonitrile, flow rate was 0.5 mL min^{-1}, azoxymycins 1 and 2 were analyzed under 80% mobile phase B for 10 minutes at 280 nm, while the azoxymycin 3 was analyzed under 100% mobile phase B for 10 minutes at 280 nm.

ESI-MS was conducted on a Thermo Finnigan LCQ Deca XP MAX system.

HR-TOF-MS was conducted on a AB TripleTOF 5600plus System (AB SCIEX, Framingham,USA).

NMR spectrums were tested with Bruker Advance 600 spectrometer. DMSO-D6 was used as the solvent of NMR test.

Rotation was tested on Jasco P-1000 polarimeter at 589nm.

2. Azoxymycins producing condition

Seed culture medium (YEME culture medium): yeast extract 3g L^{-1}, malt extract 3g L^{-1}, trypton 5g L^{-1}, glucose 10g L^{-1}.

Fermentation culture medium(optimized SMM culture medium): 10g L^{-1} glucose, 7g $L^{-1}NH_4NO_3$, 1g $L^{-1}NaH_2PO_4 \cdot 2H_2O$, 2.3g $L^{-1}Na_2HPO_4 \cdot 12H_2O$, 0.1mL L^{-1} trace element solution.

Streptomyces chattanoogensis L10 was inoculated into sterilized seed medium and incubated at 30℃, 200 rpm for 24 h, after that 0.1% of the seed culture was incubated to the optimized medium, and further incubation was carried out at 30℃ 200rpm for 96 h.

All the cultured medium used above should be sterilized at 115℃ for 20min, and added with 5 to 20 glass beads, the medium should be less than 20% of the flask's maximum volume.

3. Purification and physical description of azoxymycins.

Fermentation broth of the *S.chattanoogensis* L10 was mixed with equal volume of methanol, and sustained for 12 h, after that the mixture was centrifuged at 12000rpm for 10min, the supernate was collected and the residue was washed with water until no yellow color observed, the washing solution was centrifuged and combined with the supernate, and vacuumed dried at 40℃.The dried residue was redissolved in DMSO and referred to HPLC separation.

Finally, 8.9 mg azoxymycin A, 10.3 mg azoxymycin B, and 2.7 mg azoxymycin C were isolated

from 2 L culture.

Isotope labeled azoxymycins were purified with the same procedure. And 0.2 mg 13C labeled azoxymycin A, trace amount of ^{13}C labeled azoxymycin B and C were obtained from 100 mL ^{13}C labeled SMM culture. 0.4 mg ^{15}N labeled azoxymycin A, 0.2 mg ^{15}N labeled azoxymycin B, and trace amount of ^{15}N labeled azoxymycin C were isolated from 200 mL ^{15}N labeled SMM culture.

The azoxymycins A, B and C were yellow solid powder.

Azoxymycin A and C have a melting point above 350℃（decomposed while temperature above 350）, and azoxymycin B have a melting point at 226℃。

4. Primer list and description

GYY1: TCAACGTCGGCAAGTTCAAC

GYY2: GTGGTTGCCCATGAACTTGAG

GYY3: CCCCCTCACCTACCACTCA

GYY4: CCCAGGTGTCCCATGATCT

GYY5: cgcgccggtacccgtacccgaagtacaaggagaatcacgATTCCGGGGATCCGTCGACC

GYY6: ttcccgggggtctgggtgtctgaaggttgggtctgggggTGTAGGCTGGAGCTGCTTC

GYY7: gcggttcacccccagggccgccgcgaggacgtccagcaATTCCGGGGATCCGTCGACC

GYY8: cgaccggtgtcttggcgagcttctcggtttcctgcgcggTGTAGGCTGGAGCTGCTTC

GYY9: ccggcggtcattctcagctttcggaattgtggagcaccgATTCCGGGGATCCGTCGACC

GYY10: cgagcgggcccgaggccgccgggtcccgtctggaagtgcTGTAGGCTGGAGCTGCTTC

GYY11: GATCGTCAATCCTGCGTTCCA

GYY12: ATGTCGTCGAGCATCTCGTGA

GYY13: TGGCCGTGGTGCTCGTCAG

GYY14: CGGACGCCGATTCGAGGATCA

GYY15: TGGGTCGACGACACGCTGTTG

GYY16: CTTGTCAGACGCGGCAGTTGG

GYY17: ATTCCGGGGATCCGTCGACC

GYY18: TGTAGGCTGGAGCTGCTTC

GYY19: GCGAGCTTCTCGGTTTCCTG

GYY20: CCACACGATGGAGGAGCGCTA

GYY21: CCGCTCCCGAAGGATTCGTGC

GYY22: CGGCGGTCATTCTCAGCTTTC

GYY1/GYY2 were a pair of primers for screening the upstream of azoxymycins biosynthetic gene cluster, PCR product length was 500bp.

GYY3/GYY4 were a pair of primers for screening the downstream of azoxymycins biosynthetic gene cluster, PCR product length was about 500bp.

GYY5/GYY6 were a pair of primers for replacing *azoC* gene with *aac(3)IV* gene.

GYY7/GYY8 were a pair of primers for replacing *azoFG* gene with *aac(3)IV* gene.

GYY9/GYY10 were a pair of primers for replacing *azoJ* gene with *aac(3)IV* gene.

GYY11/GYY12 were a pair of primers located inside *azoC* gene. Wild type strain's PCR product length was about 550bp, mutant strain should have no PCR product.

GYY13/GYY14 were a pair of primers located inside *azoFG* gene. Wild type strain's PCR product length was about 720bp, mutant strain should have no PCR product.

GYY15/GYY16 were a pair of primers located inside *azoJ* gene. Wild type strain's PCR product length was about 710bp, mutant strain should have no PCR product.

GYY17/GYY18 were a pair of primers for *aac(3)IV* gene test. Mutant strain's PCR product length was about 1200bp, wild type strain should have no PCR product.

GYY19/GYY20 were a pair of primers located outside *azoFG* gene. Wild type strain's PCR product length was about 1850bp, Mutant strain's PCR product length should be about 1250bp.

GYY21/GYY22 were a pair of primers located outside *azoJ* gene. Wild type strain's PCR product length was about 1550bp, Mutant strain's PCR product length should be about 1300bp.

5. Construction of ΔazoC, ΔazoFG and ΔazoJ mutant.

First, a cosmid 36E1, which harbored the azoxymycins biosynthesis gene cluster, was screened out with PCR amplification using two primer pairs GYY1/GYY2 and GYY3/GYY4, Second, three disruption cassette *aac(3)IV* genes were individually PCR amplified by using primer pairs GYY5/GYY6, GYY7/GYY8 and GYY9/GYY10 from pHY773, with the resulting product carrying 39 ends with homology to the corresponding region of the *azoC* gene, *azoFG* gene and *azoJ* gene, respectively. Three PCR products were then individually introduced into *E. coli* BW25113 carrying pIJ790/36E1, and the transformed cells carrying mutagenized 36E1 were selected on LB agar containing apramycin. Three mutagenized 36E1 cosmids, in which the *azoC* gene, *azoFG* gene and *azoJ* gene were replaced with *aac(3)IV*, were designated AZO-C, AZO-EF, AZO-J and confirmed by PCR analysis using primer pairs GYY11/GYY12, GYY13/GYY14 and GYY15/GYY16, respectively. Third, AZO-C, AZO-EF, AZO-J were individually transformed into E. coli ET12567/pUZ8002 respectively. Finally, after conjugal transfer of AZO-C, AZO-EF, AZO-J individually from E. coli ET12567/pUZ8002 into S. chattanoogensis L10, the single-crossover exconjugants were obtained after selection for apramycin. The single-crossover exconjugants were then inoculated onto YMG plates for two rounds of nonselective growth. The double-crossover exconjugants were obtained after selection for apramycin-resistant and thiostrepton-sensitive colonies. The resulting mutants, in which *azoC* gene, *azoFG* gene and *azoJ*

gene were in-frame deleted, were designated Δ*azoC*, Δ*azoFG* and Δ*azoJ*, the Δ*azoFG* and Δ*azoJ* confirmed by PCR analysis using primer pairs GYY19/GYY20 and GYY21/GYY22 respectively, and Δ*azoC* was confirmed by PCR analysis using primer pairs GYY11/GYY12 and GYY17/GYY18.

Table S1: NMR data of azoxymycins A and B (in DMSO, δ value in ppm)

No.	Azoxymycin A		Azoxymycin B	
	δH	δC	δH	δC
1	–	143.15, C	–	147.20, C
1′	–	146.75, C	–	143.33, C
2, 6	2H, 8.17, d, *J*=8.3 Hz	125.69, CH	2H, 8.28, d, *J* = 8.7 Hz	122.68, CH
2′, 6′	2H, 8.26, d, *J* = 8.4 Hz	122.41, CH	2H, 8.19 ,d, *J* = 8.6 Hz	125.87, CH
3, 5	2H, 7.75, d, *J*=8.5 Hz	127.38, CH	2H, 7.81, d, *J* = 8.8 Hz	127.86, CH
3′,5′	2H, 7.81, d, *J* = 8.5 Hz	127.5, CH	2H, 7.77, d, *J* = 8.8 Hz	127.60, CH
4	–	137.78	–	139.79, C
4′	–	139.97	–	137.97, C
7	7.04, d, *J* = 14.5 Hz,	136.91, CH	7.06, d, *J* = 14.6 Hz	137.25, CH
7′	7.10, d, *J* = 14.2 Hz	136.13, CH	7.20, d, *J* = 15.6 Hz	137.89, CH
8	7.23, dd, *J* = 20.6, 13.2 Hz	128.72, CH	7.31, dd, *J* = 10.3, 4.9 Hz	129.54, CH
8′	7.23, dd, *J* = 20.6, 13.2 Hz	129.78, CH	7.24, m	128.86, CH
9	7.23, dd, *J* = 20.6, 13.2 Hz	139.09, CH	7.26, m	139.51, CH
9′	7.23, dd, *J* = 20.6, 13.2 Hz	138.82, CH	7.39, dd, *J* = 14.9 Hz	143.76, CH
10	6.32, d, *J* = 13.8 Hz	126.25, CH	6.31, d, *J* = 14.2 Hz	126.06, CH
10′	6.35, d, *J* = 13.8 Hz	126.86, CH	6.12, d, *J* = 15.1 Hz	123.82, CH
11	–	164.73, C	–	165.09, C
11′	–	164.63, C	–	167.45, C
12,12′	2H, 8.32, s	–	8.43, d, *J* = 7.7 Hz	–
13,13′	2H, 4.22, dd, *J* = 13.0,7.7Hz	52.17, CH	4.27,d, *J* = 4.5 Hz,	51.81, CH
14,14′	–	173.53, C	–	167.45, C
15,15′	2H, 2.0,dt, *J* = 13.1, 7.6Hz, 2H,1.8,dt, *J* = 14.8, 7.8 Hz	27.31, CH2	2.01, dd, *J* = 15.0, 6.9 Hz, 1.8, dd, *J* = 15.6, 8.9 Hz	26.96, CH2

16,16′	4H, 2.13, m	31.45, CH2	2H, 2.15, m	31.42, CH2
17,17′	–	173.53, C	–	173.55, C
18,18′	2H, 7.34, s, 2H, 6.75, s	–	6.78, s, 7.31, s	–

Table S2. *In silico* annotation of the ORFs in the putative azoxymycin biosynthesis gene cluster

Protein (GenBank Accession)	Predicted function	Homolog in *asu* PKS(identity)	Homolog in *S. lydicus* （identity）	Homolog in *S. auratus* （identity）
AzoA（AKQ24641）	esterase		WP_052686964(99%)	EJJ08104(84%)
AzoB(AKQ24640)	ketoreductase	AsuC7 (67%)	WP_046924478(99%)	EJJ08105(94%)
AzoC(AKQ24642)	*p*-aminobenzoate N-oxidase		WP_046924477(99%)	EJJ08106(91%)
AzoD(AKQ24643)	EmrB/QacA subfamily drug resistance transporter		WP_052686963(99%)	EJJ08107(87%)
AzoE(AKQ24644)	ABC substrate binding protein		WP_046924475(99%)	EJJ08108(70%)
AzoF(AKQ24645)	beta-ketosynthase, beta subunit (CLF)	AsuC14 (41%)	WP_052686962(99%)	EJJ08109(78%)
AzoG(AKQ24646)	beta-ketoacyl synthase, alpha subunit	AsuC13 (34%)	WP_046924473(99%)	EJJ08110(89%)
AzoH(AKQ24647)	3,4-AHBA carrier protein	AsuC12(34%)	WP_046924472(99%)	EJJ08111(91%)
AzoI(AKQ24648)	KS I/II associated ACP	AsuC11 (34%)	WP_046924471(99%)	EJJ08112(88%)
AzoO(AKQ24654)	4′-phosphopantetheinyl transferase		WP_052686961(99%)	EJJ08113(69%)
AzoJ(AKQ24649)	3,4-AHBA carboxyl group adenylation	AsuA2 (41%)	WP_046924470(99%)	EJJ08114(86%)
AzoK(AKQ24650)	*p*-aminobenzoate synthase		WP_046924469(99%)	EJJ08115(90%)
AzoL(AKQ24651)	4-amino-4-deoxychorismate lyase		WP_046924468(99%)	EJJ08116(82%)
AzoM(AKQ24652)	acyl dehydratase	AsuC8(33%)	WP_046924467(99%)	EJJ08117(88%)
AzoN(AKQ24653)	acyl dehydratase	AsuC9 (51%)	WP_046924466(99%)	EJJ08118(80%)

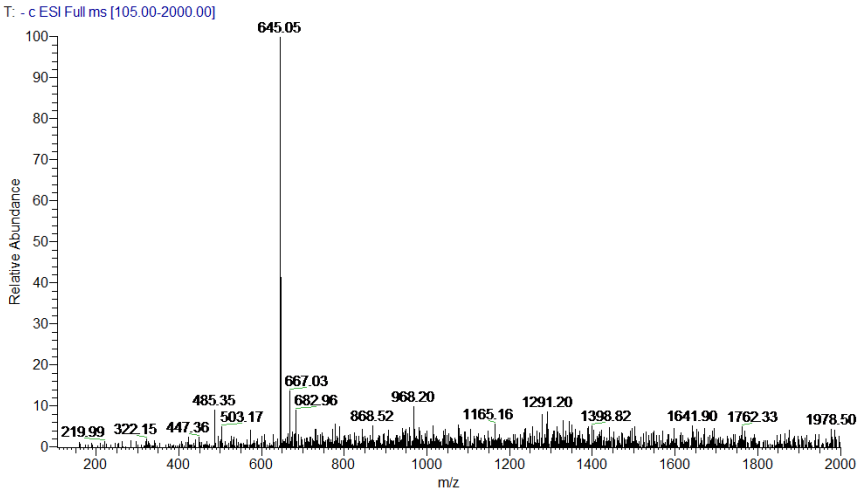

Figure S1. MS of azoxymycin A.

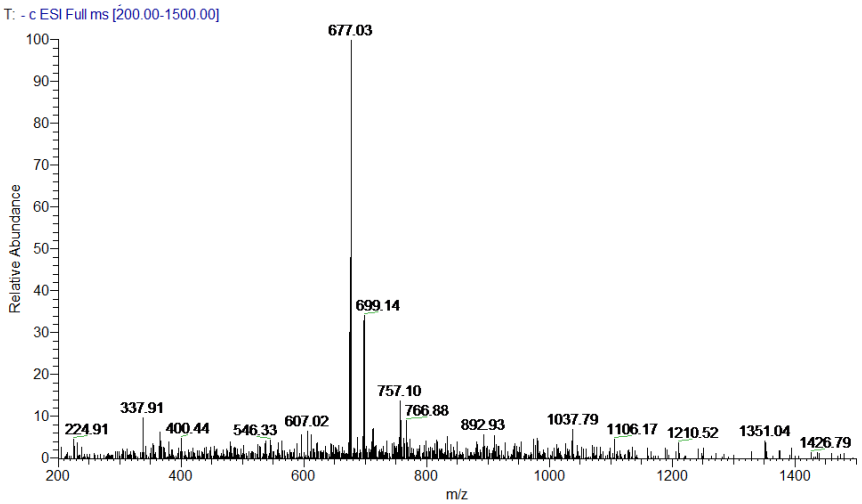

Figure S2. MS of ^{13}C-labled azoxymycin A.

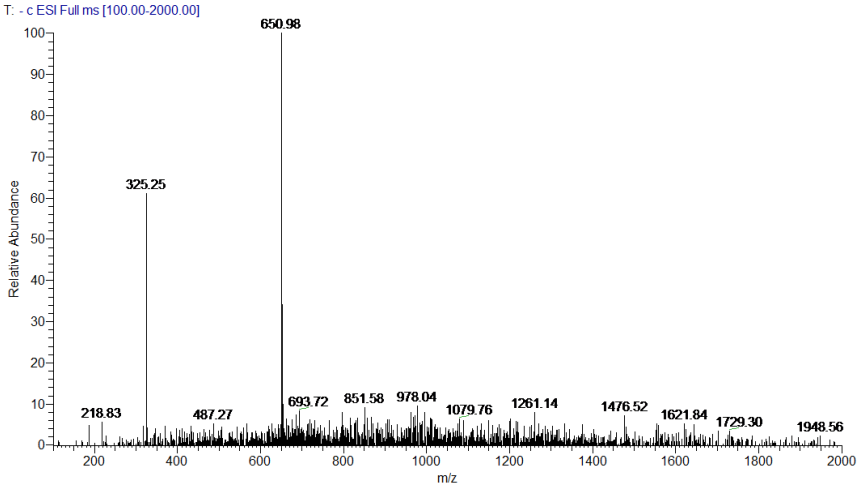

Figure S3. MS of ^{15}N-labled azoxymycin A.

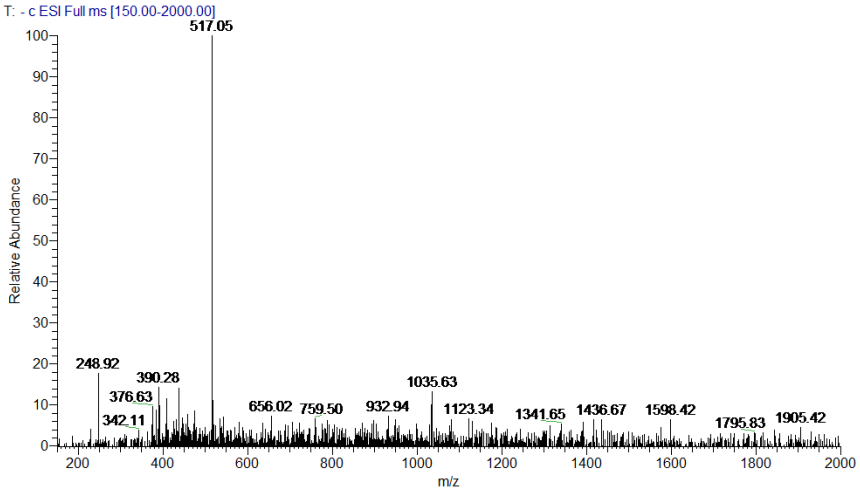

Figure S4. MS of azoxymycin B.

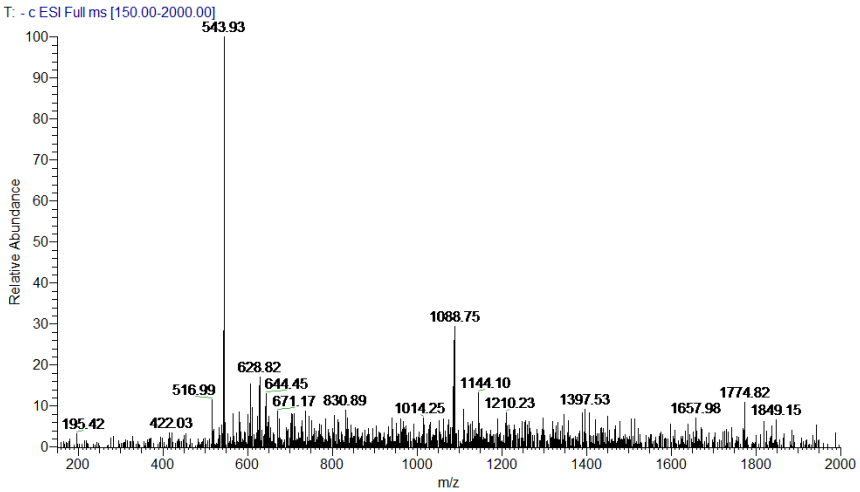

Figure S5. MS of ^{13}C-labled azoxymycin B.

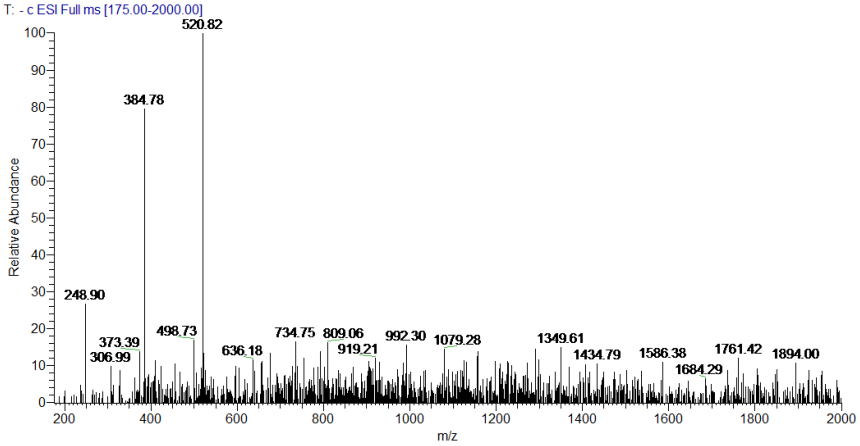

Figure S6. MS of ^{15}N-labled azoxymycin B.

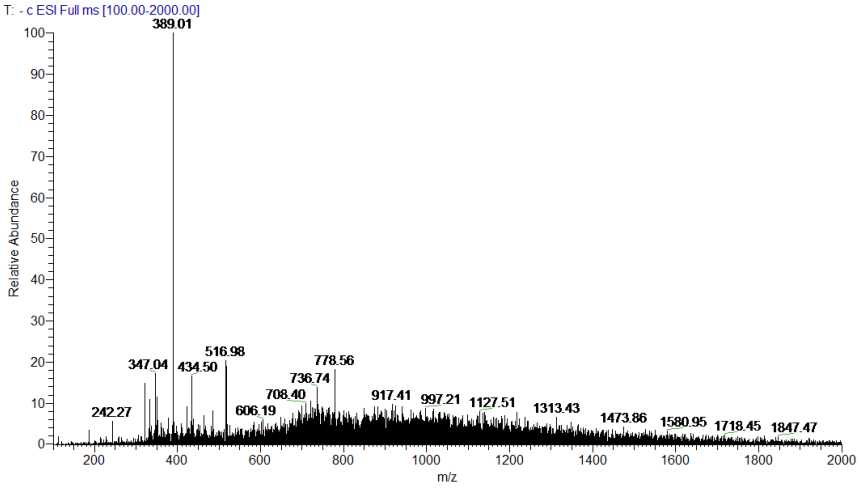

Figure S7. MS of azoxymycin C.

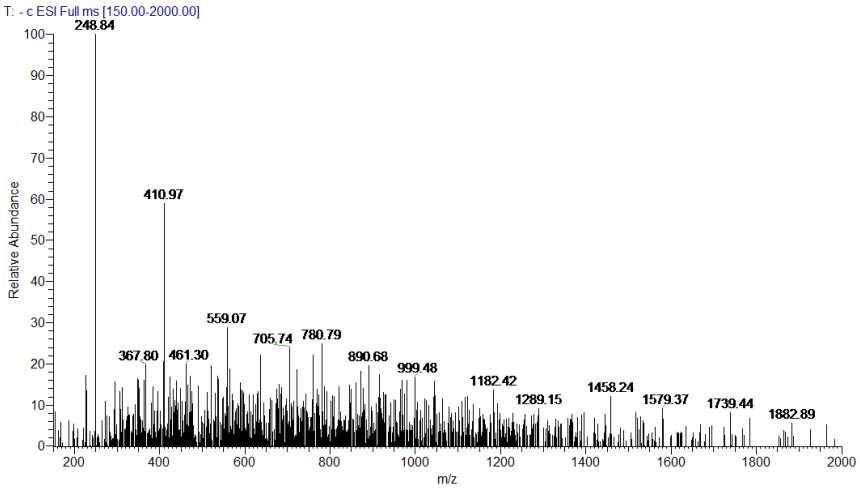

Figure S8.MS of ^{13}C-labled azoxymycin C

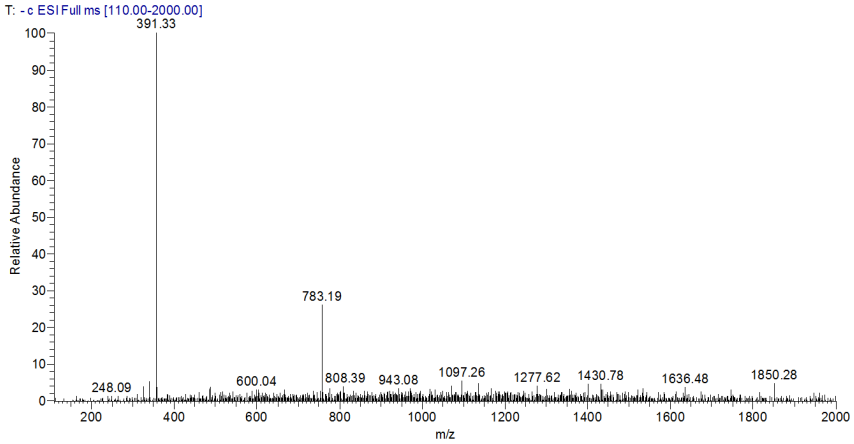

Figure S9.MS of ^{15}N-labled azoxymycin C

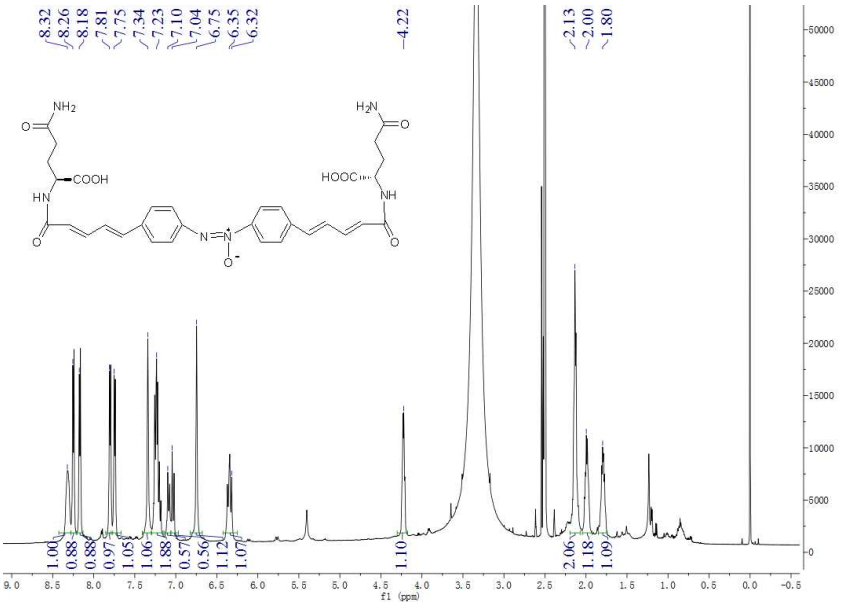

Figure S10. ^{1}H NMR spectrum of azoxymycin A.

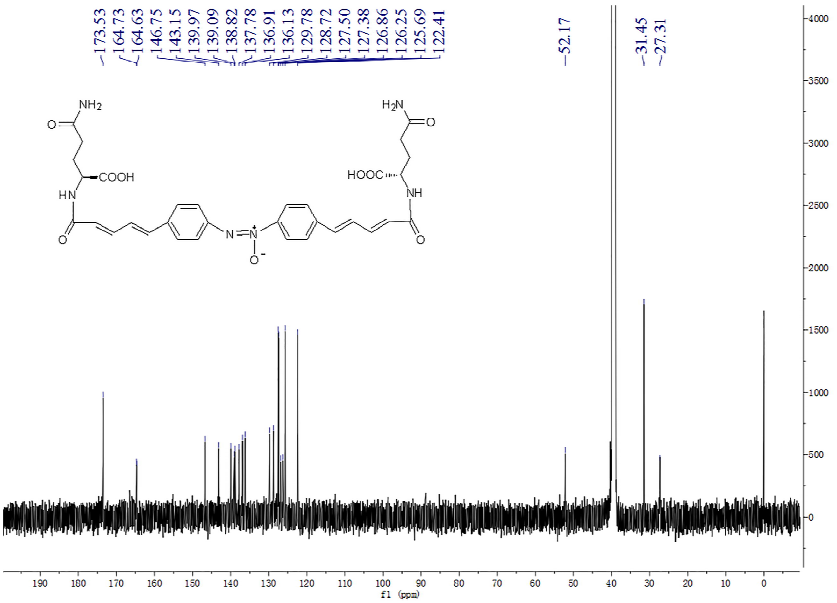

Figure S11. ^{13}C NMR spectrum of azoxymycin A.

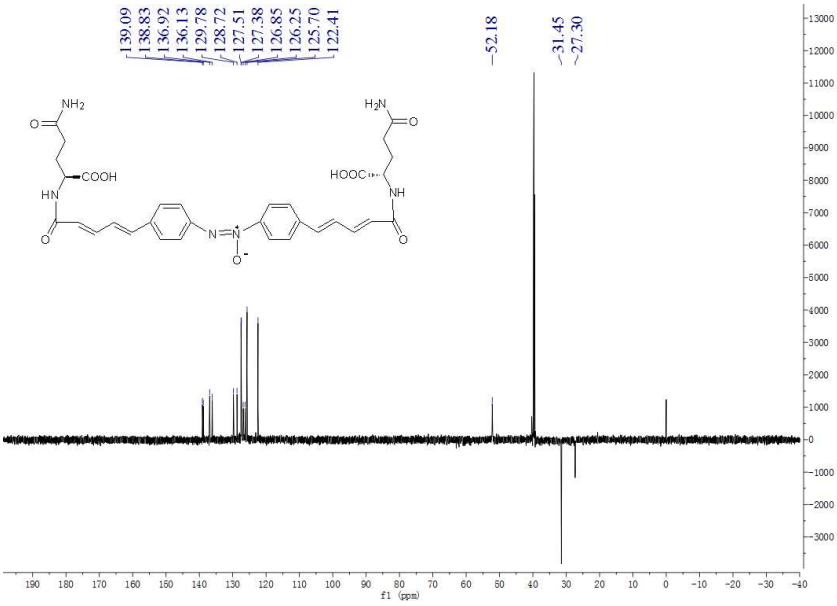

Figure S12. DEPT 135 spectrum of azoxymycin A.

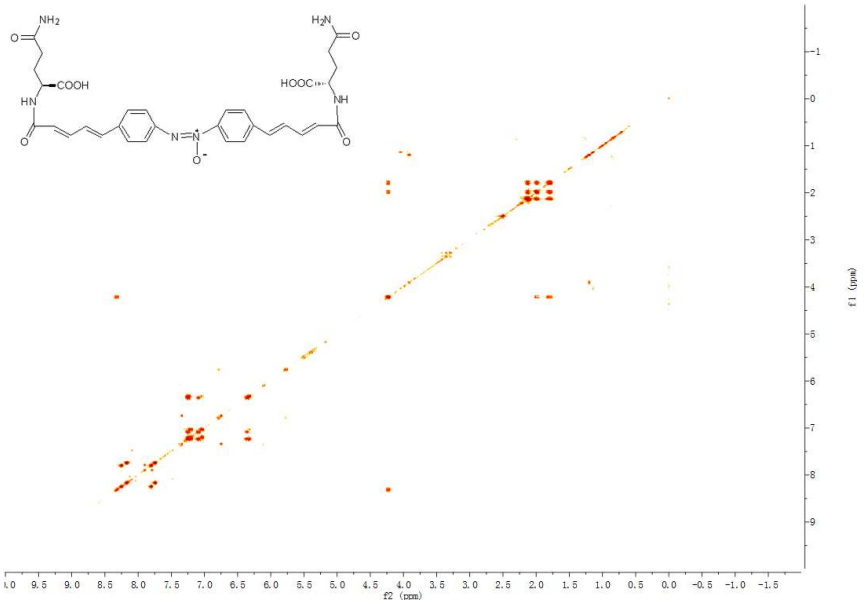

Figure S13. COSY spectrum of azoxymycin A.

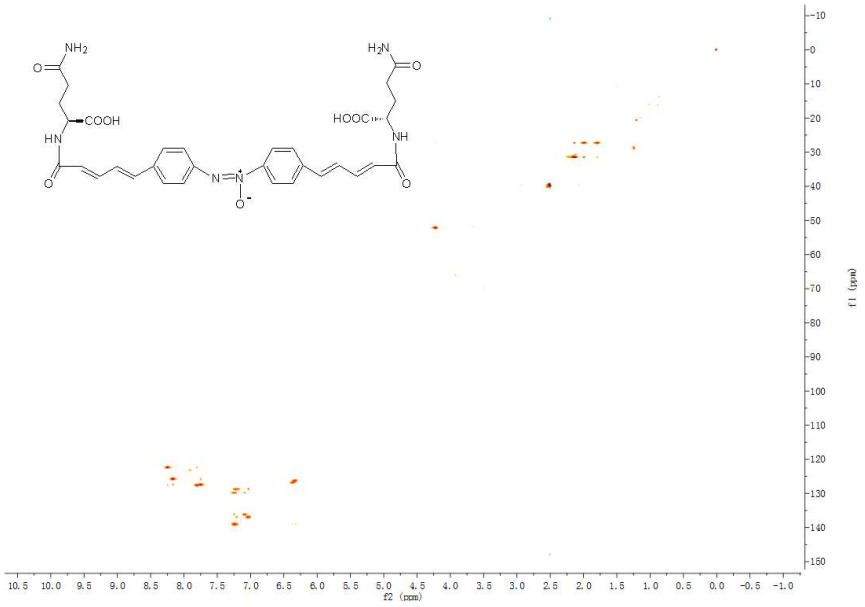

Figure S14. HSQC spectrum of azoxymycin A.

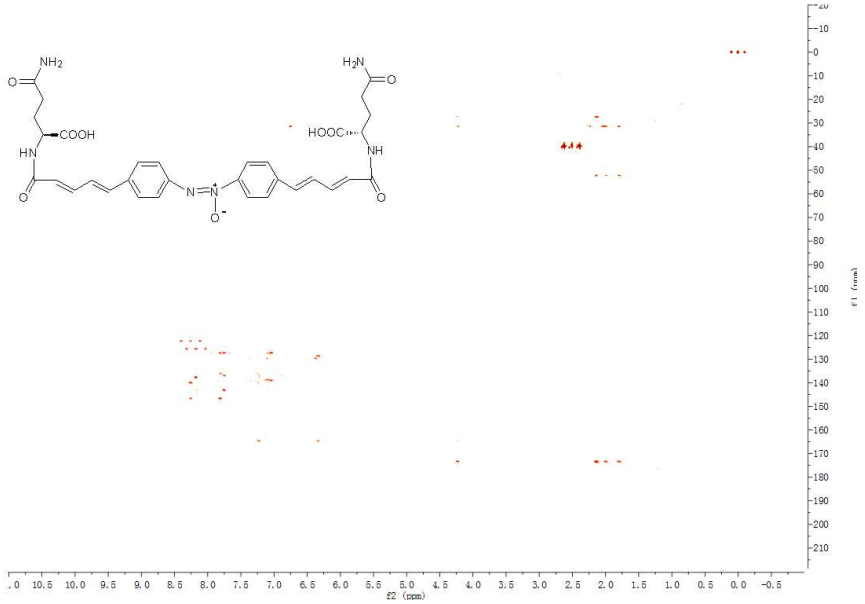

Figure S15. HMBC spectrum of azoxymycin A.

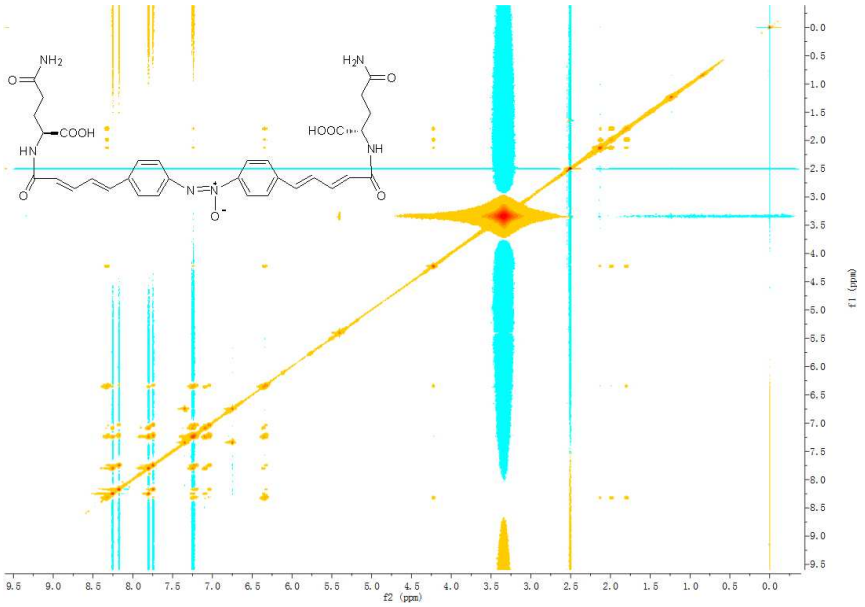

Figure S16. NOESY spectrum of azoxymycin A.

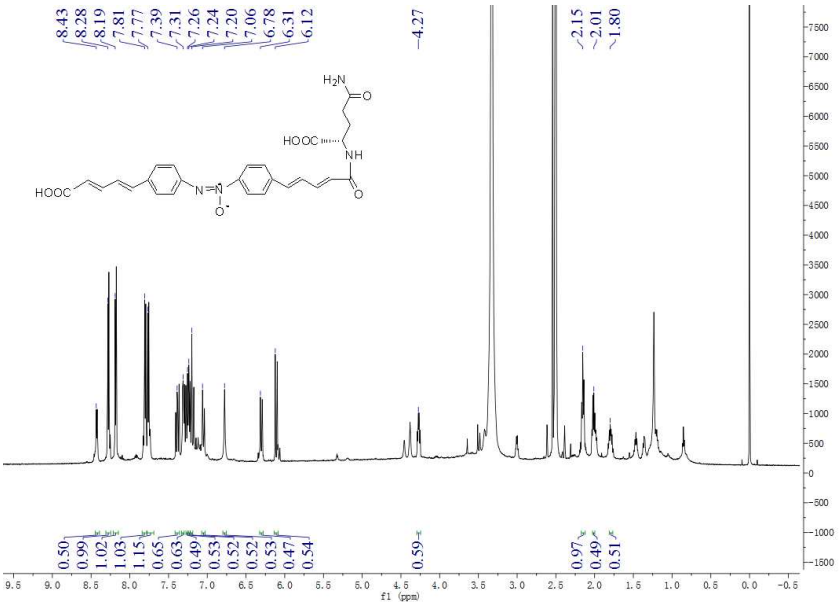

Figure S17. ^{1}H NMR spectrum of azoxymycin B.

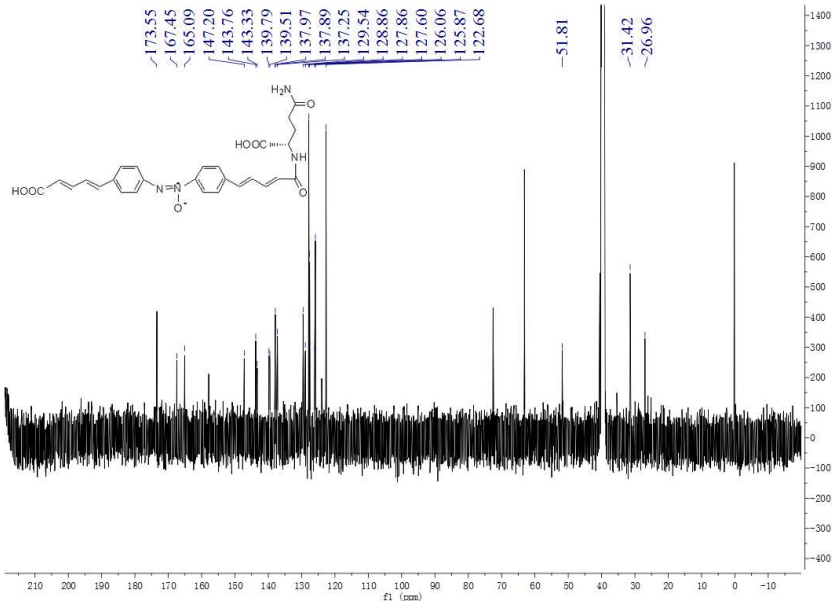

Figure S18. ^{13}C NMR spectrum of azoxymycin B.

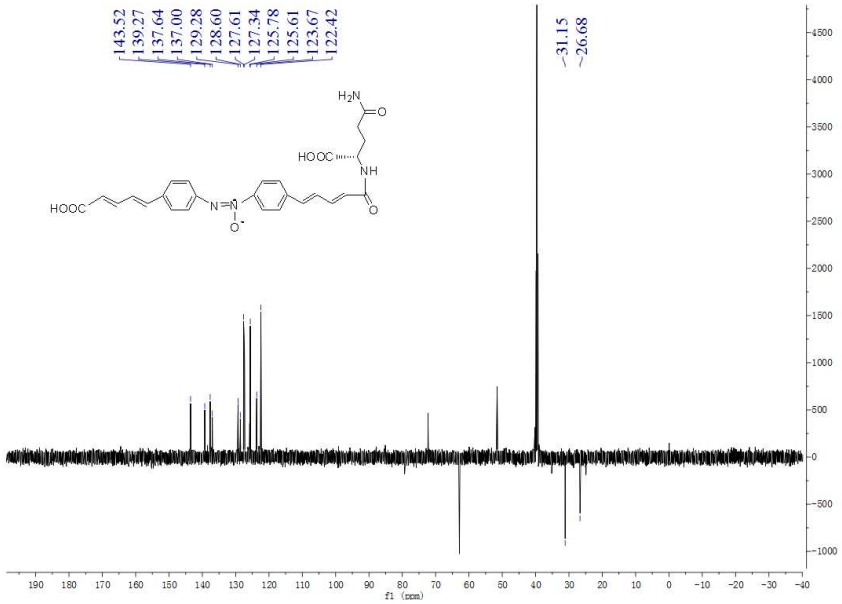

Figure S19. DEPT 135 spectrum of azoxymycin B.

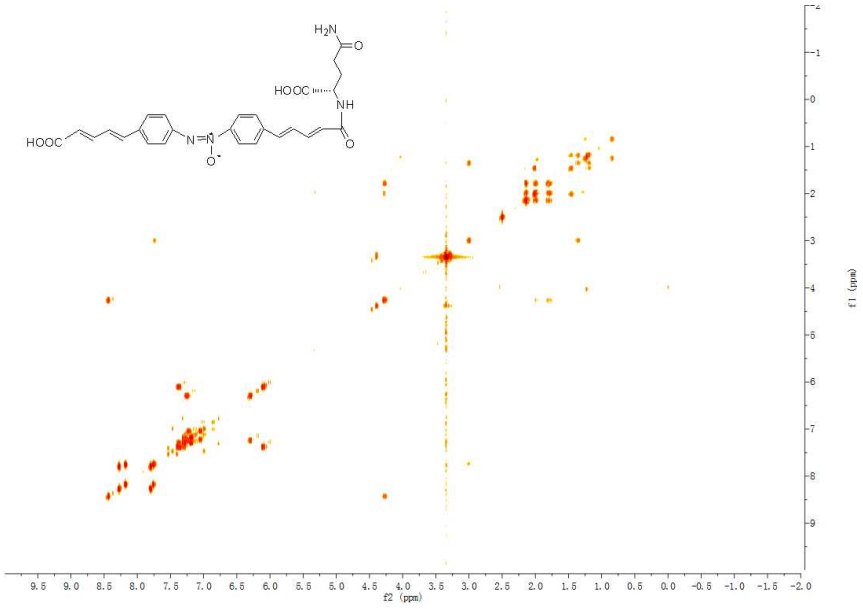

Figure S20. COSY spectrum of azoxymycin B.

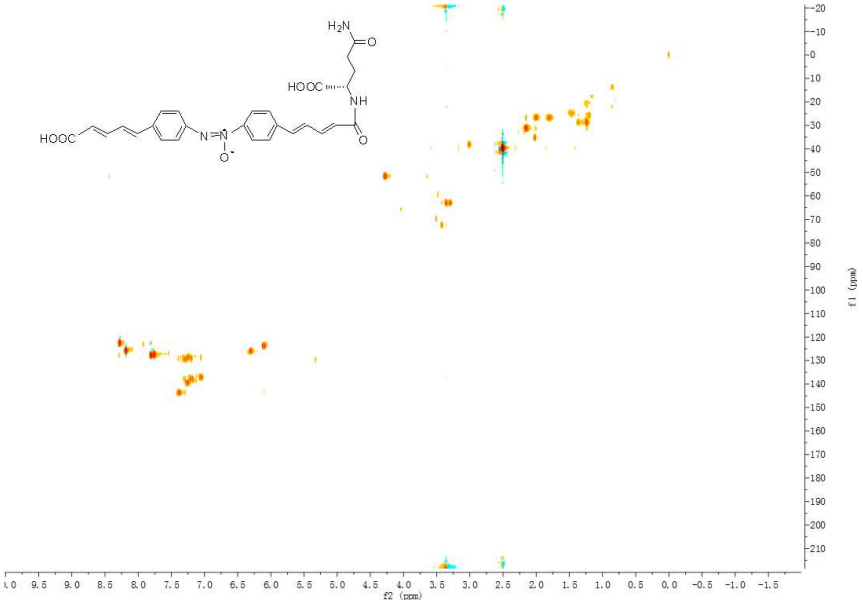

Figure S21. HSQC spectrum of azoxymycin B.

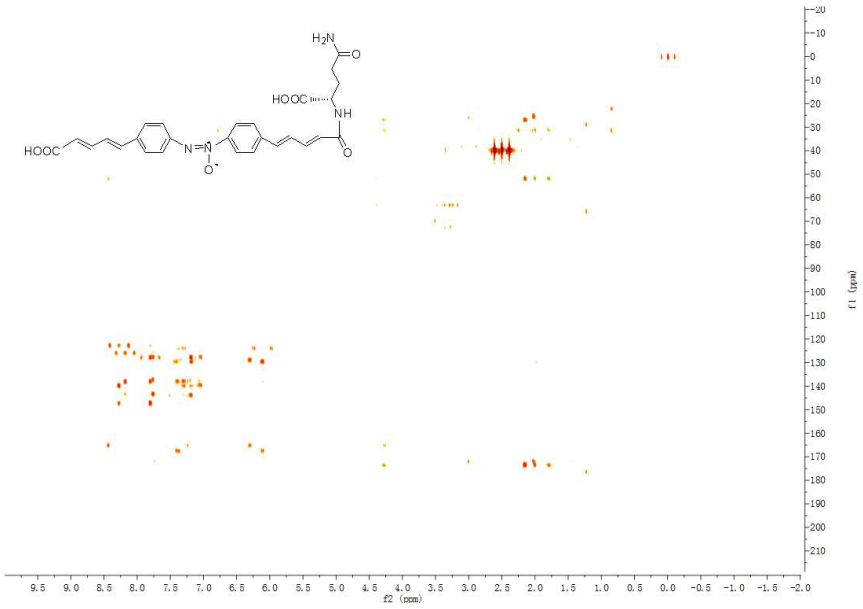

Figure S22. HMBC spectrum of azoxymycin B.

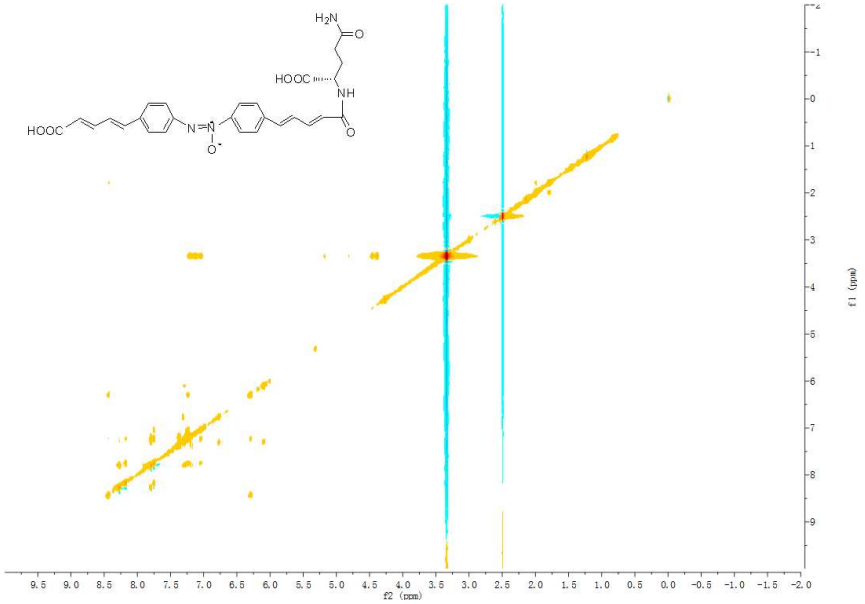

Figure S23. NOESY spectrum of azoxymycin B.

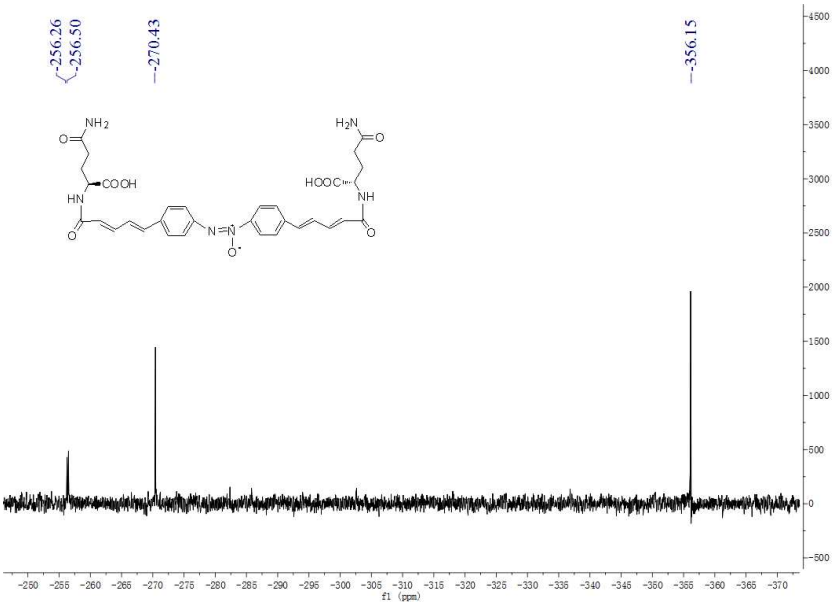

Figure S24. ^{15}N spectrum of ^{15}N-labeled azoxymycin A.

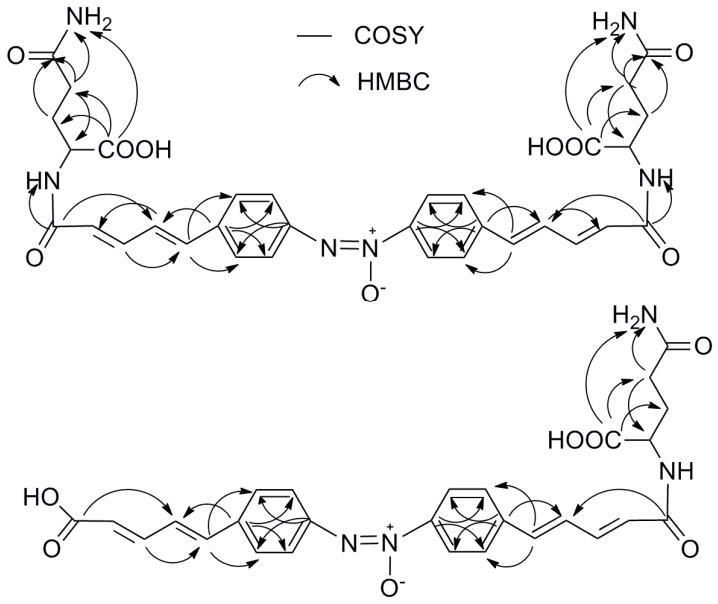

Figure S25. COSY and HMBC correlation of azoxymycins A and B.

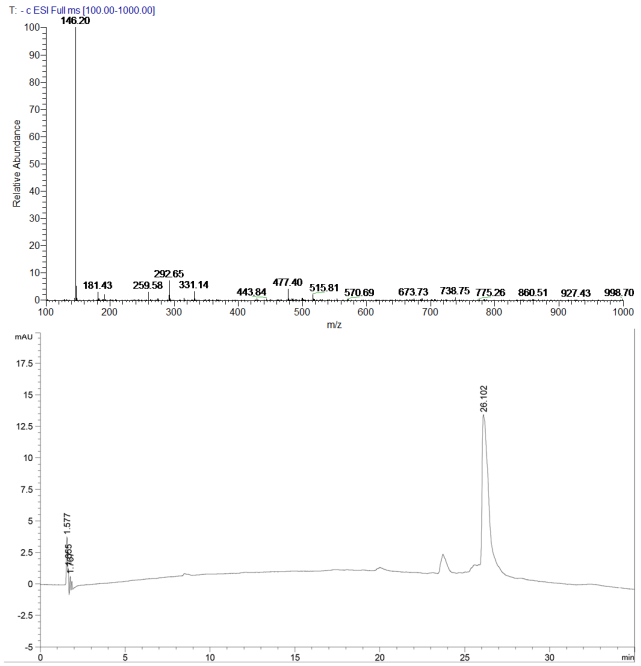

Figure S26. MS and HPLC spectrum of glutamate and azoxymycin C hydrolysed from azoxymycins A and B.

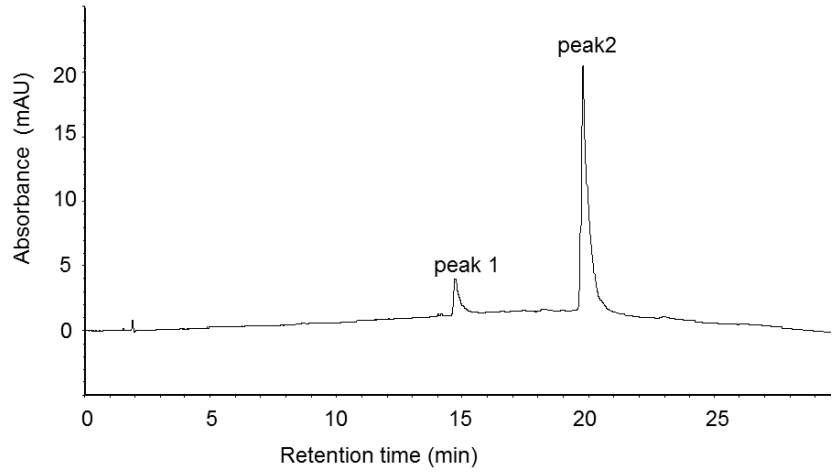

Figure S27. HPLC split of azoxymycin A(400nm)

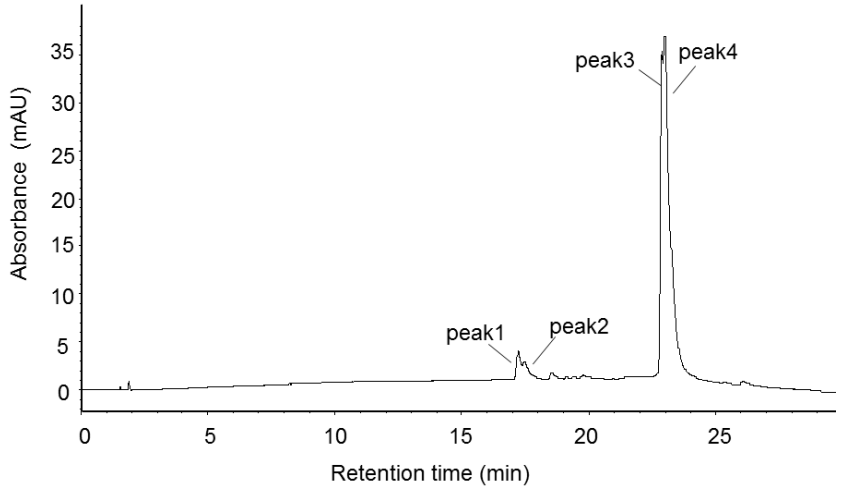

Figure S28. HPLC split of azoxymycin B(400nm)

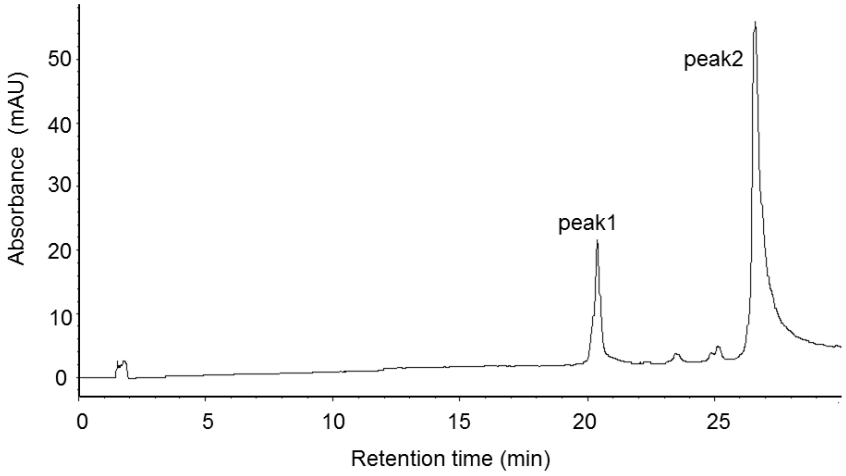

Figure S29. HPLC split of azoxymycin C(400nm)

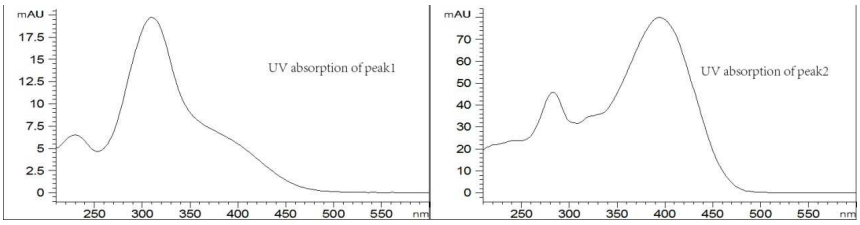

Figure S30. UV/Vis spectrums of azoxymycin A.

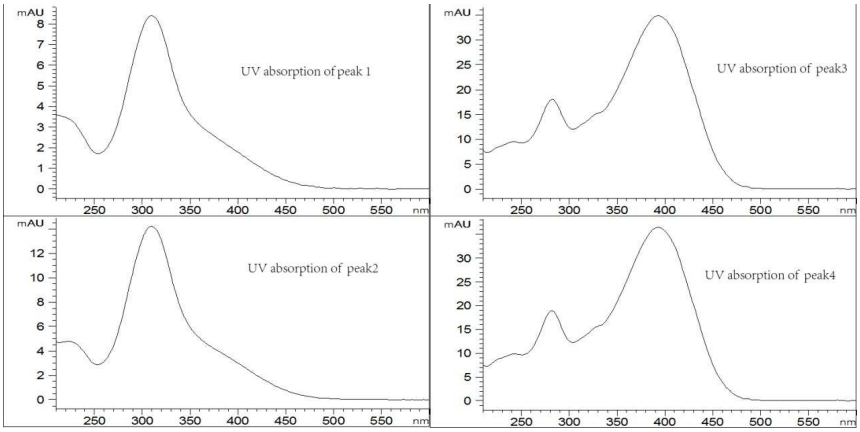

Figure S31. UV/Vis spectrums of azoxymycin B.

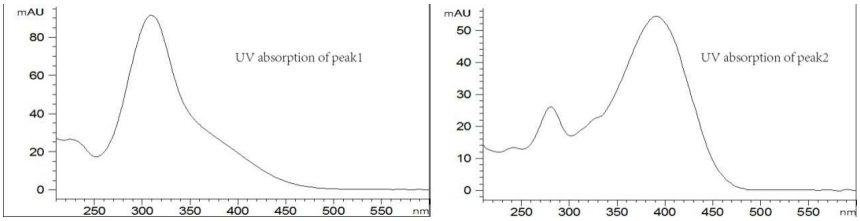

Figure S32. UV/Vis spectrums of azoxymycin C.

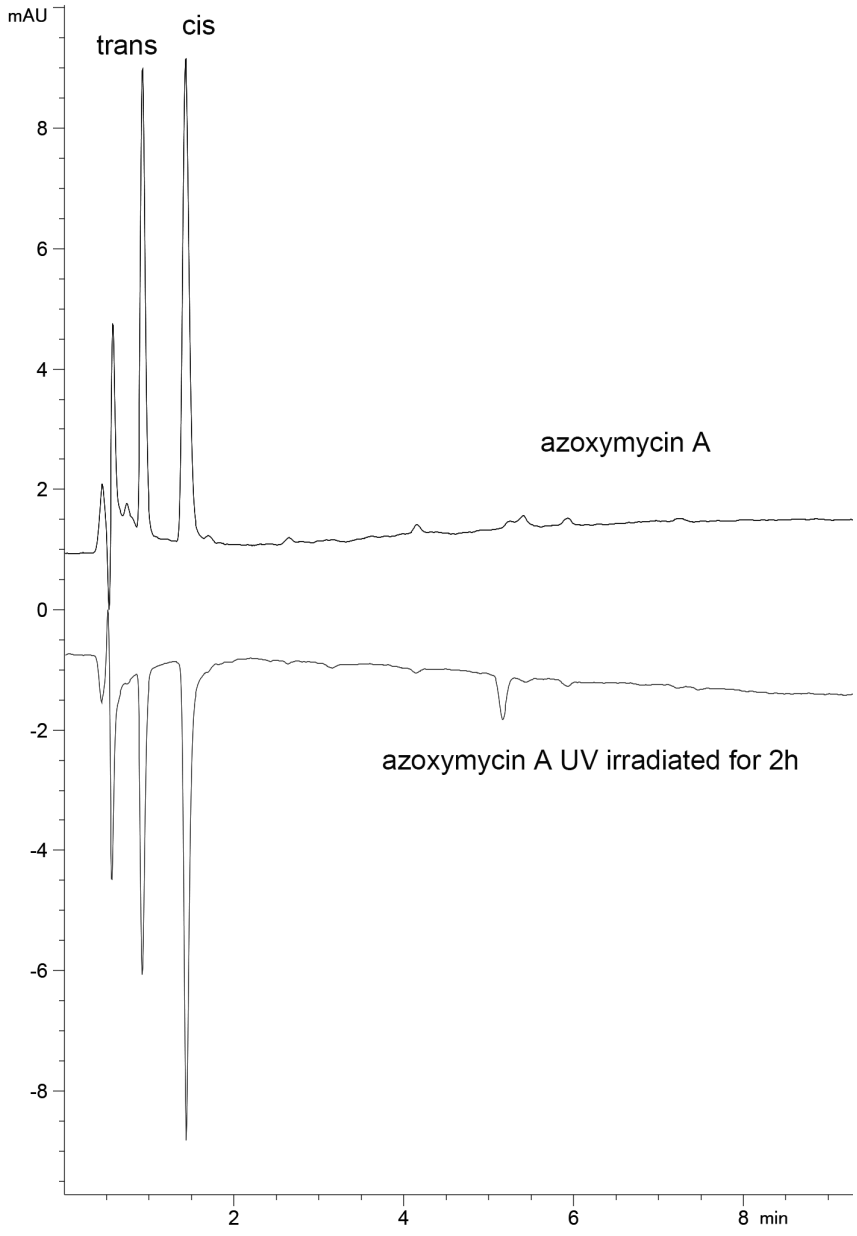

Figure S33. UV induced *trans-cis* isomerization of azoxymycin A.

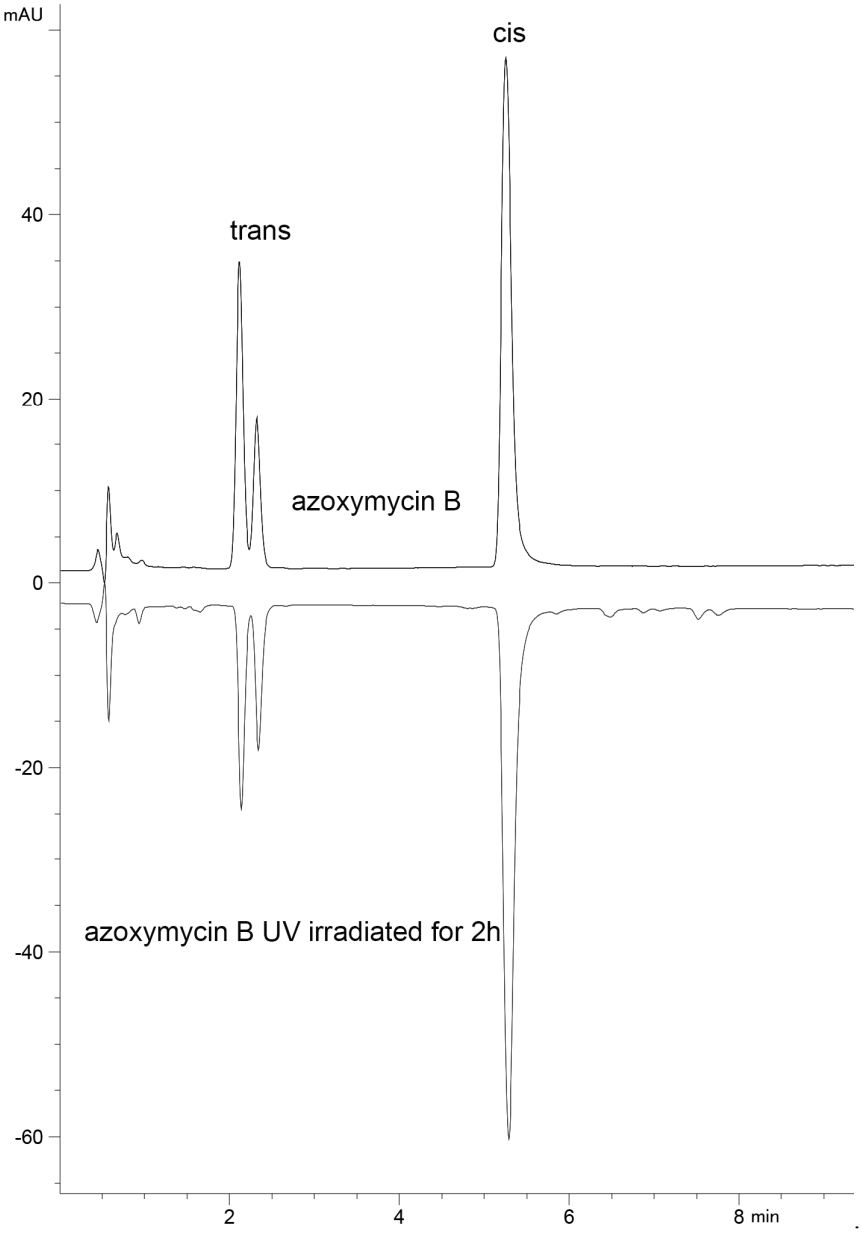

Figure S34. UV induced *trans-cis* isomerization of azoxymycin B.

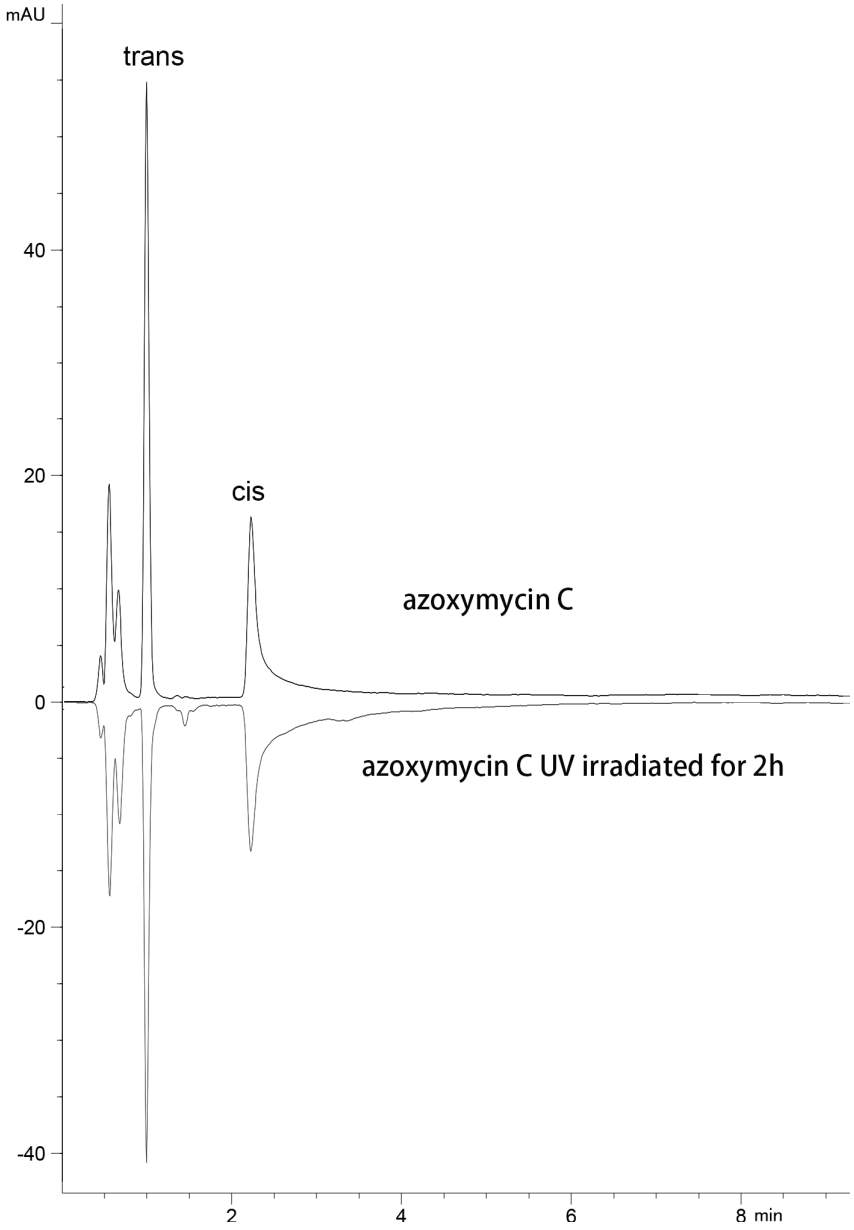

Figure S35. UV induced *trans-cis* isomerization of azoxymycin C.

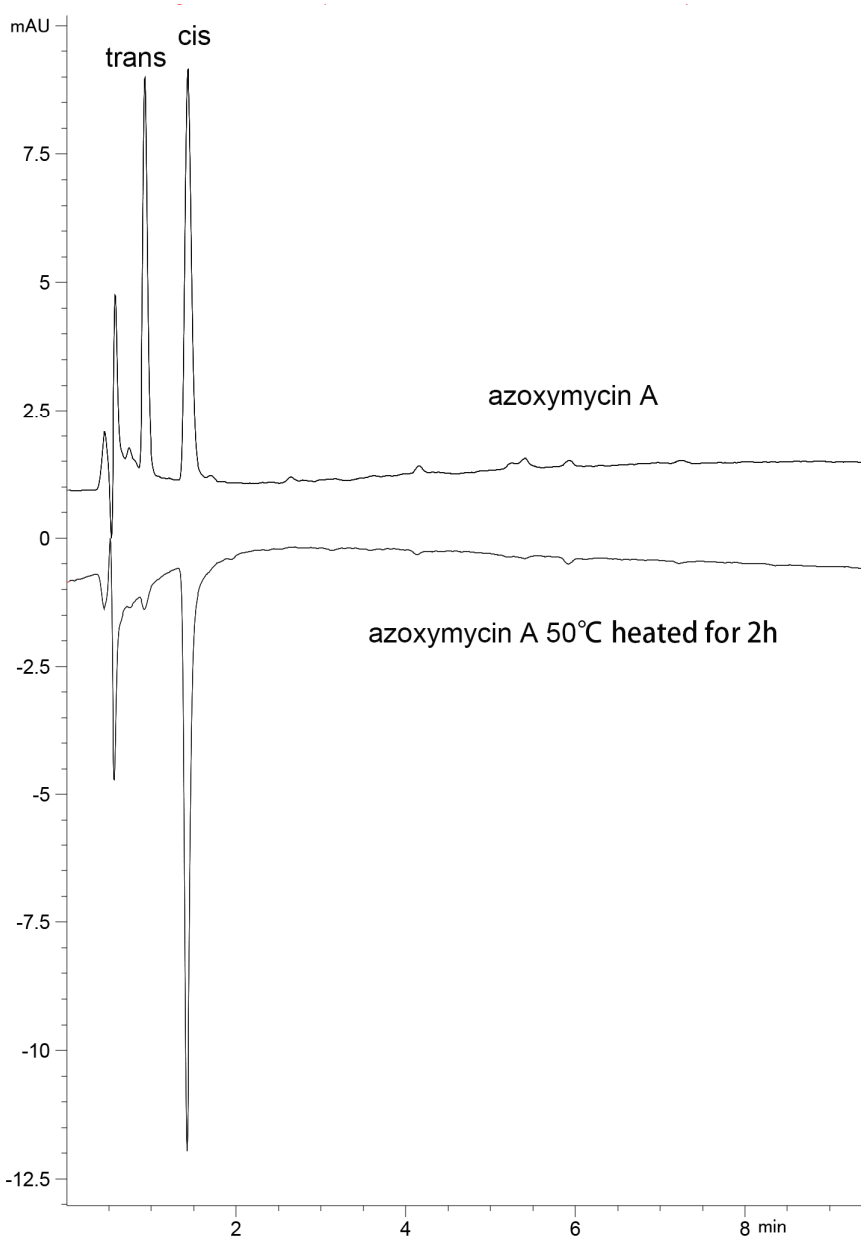

Figure S36. Heat induced *trans-cis* isomerization of azoxymycin A.

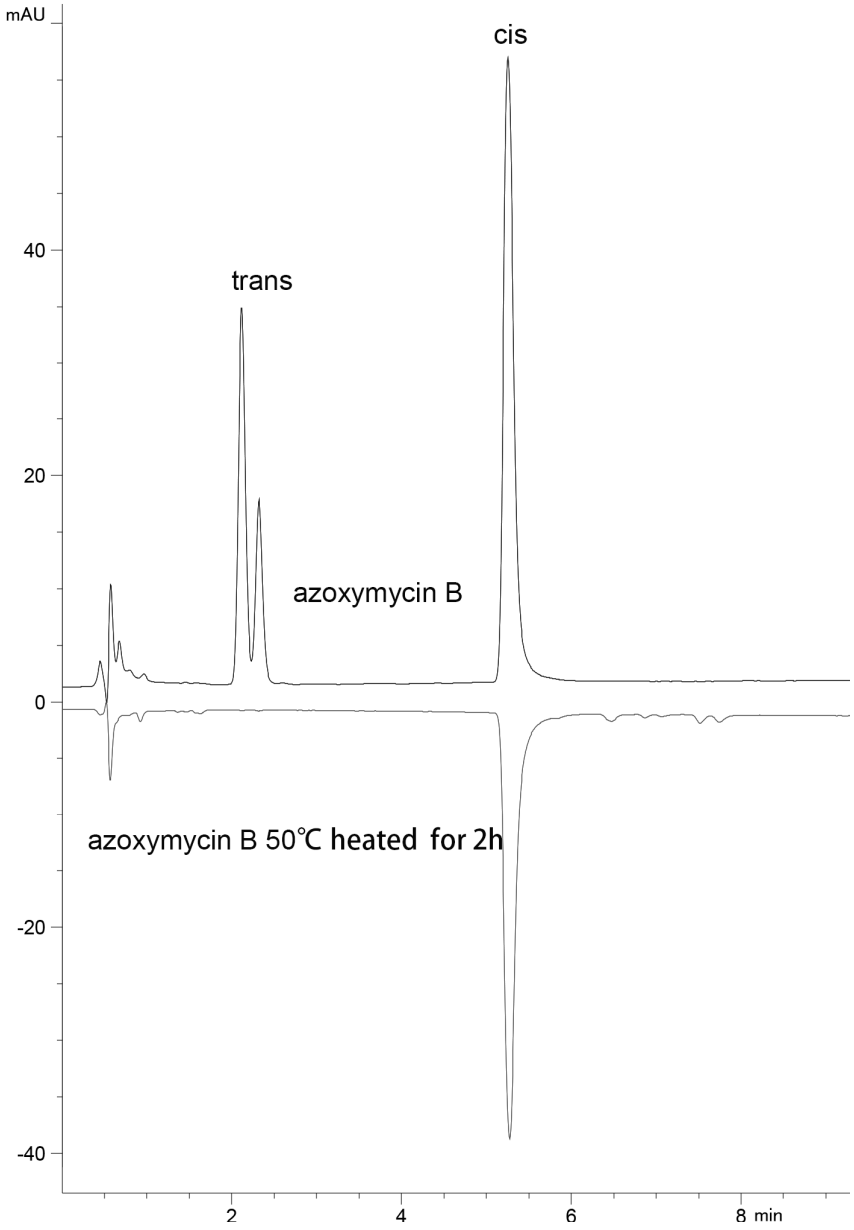

Figure S37. Heat induced *trans-cis* isomerization of azoxymycin B.

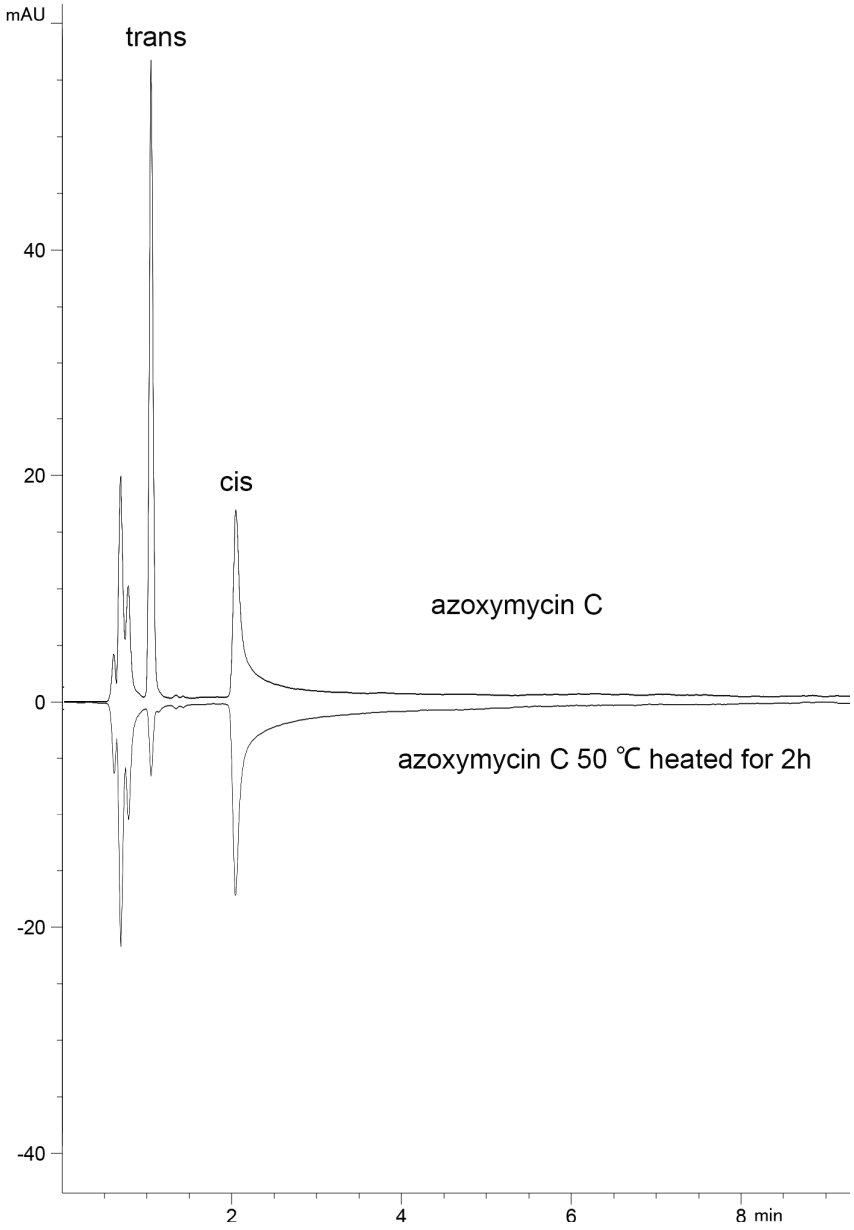

Figure S38. Heat induced *trans-cis* isomerization of azoxymycin C.

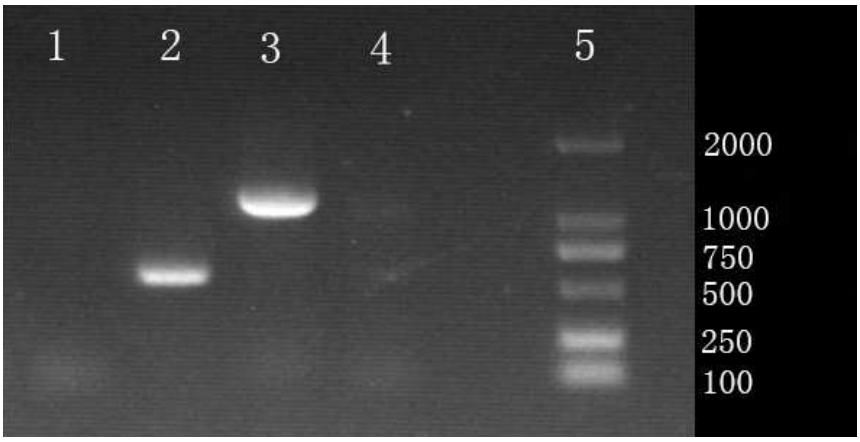

Figure S39. PCR test of the *ΔazoC*∷*aac(3)IV*. 1 was the PCR result of mutant strain with GYY11/GYY12 primers, 2 was that of wild type strain with GYY11/GYY12 primers, 3 was that of mutant strain with GYY17/GYY18, 4 was that of wild type strain with GYY17/GYY18.

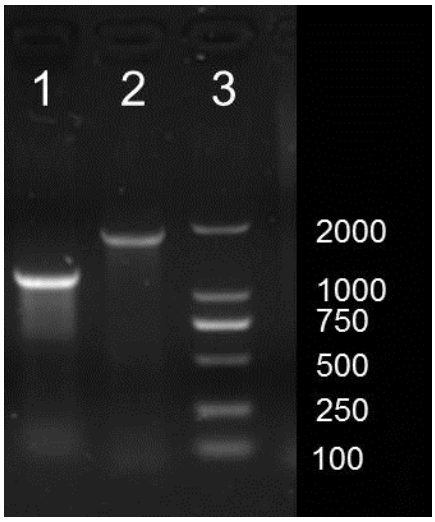

Figure S40. PCR test of the *ΔazoFG*∷*aac(3)IV*. 1 was the PCR result of mutant strain with GYY19/GYY20 primers, 2 was that of wild type strain with GYY19/GYY20 primers.

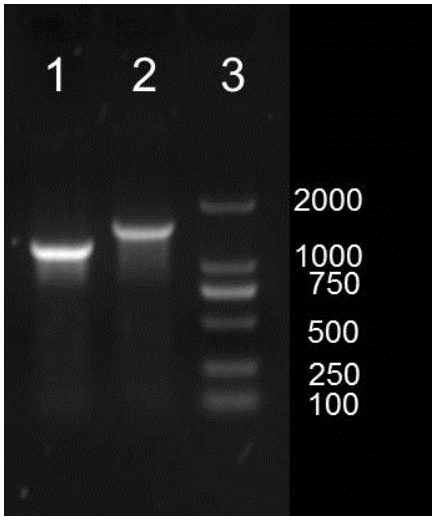

Figure S41. PCR test of the *ΔazoJ*::*aac(3)IV*. 1 was the PCR result of mutant strain with GYY21/GYY22 primers, 2 was that of wild type strain with GYY21/GYY22 primers.

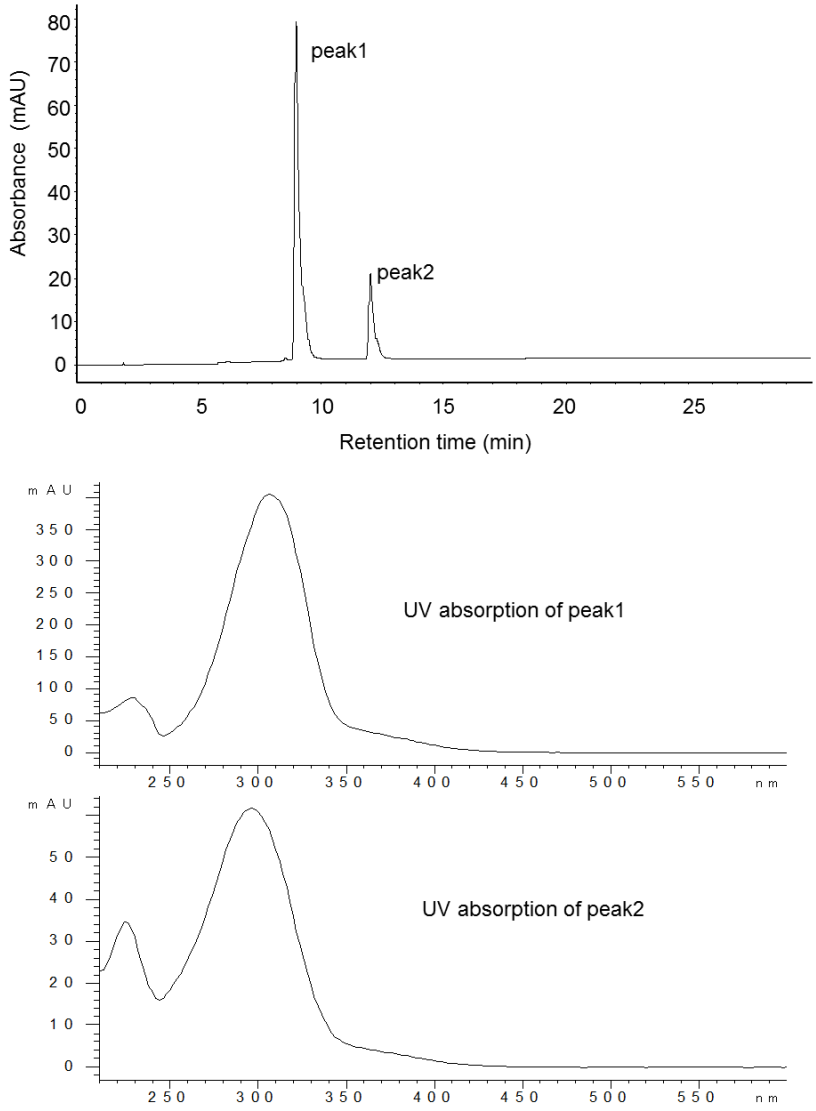

Figure S42. HPLC Split and UV/Vis absorption of compound A

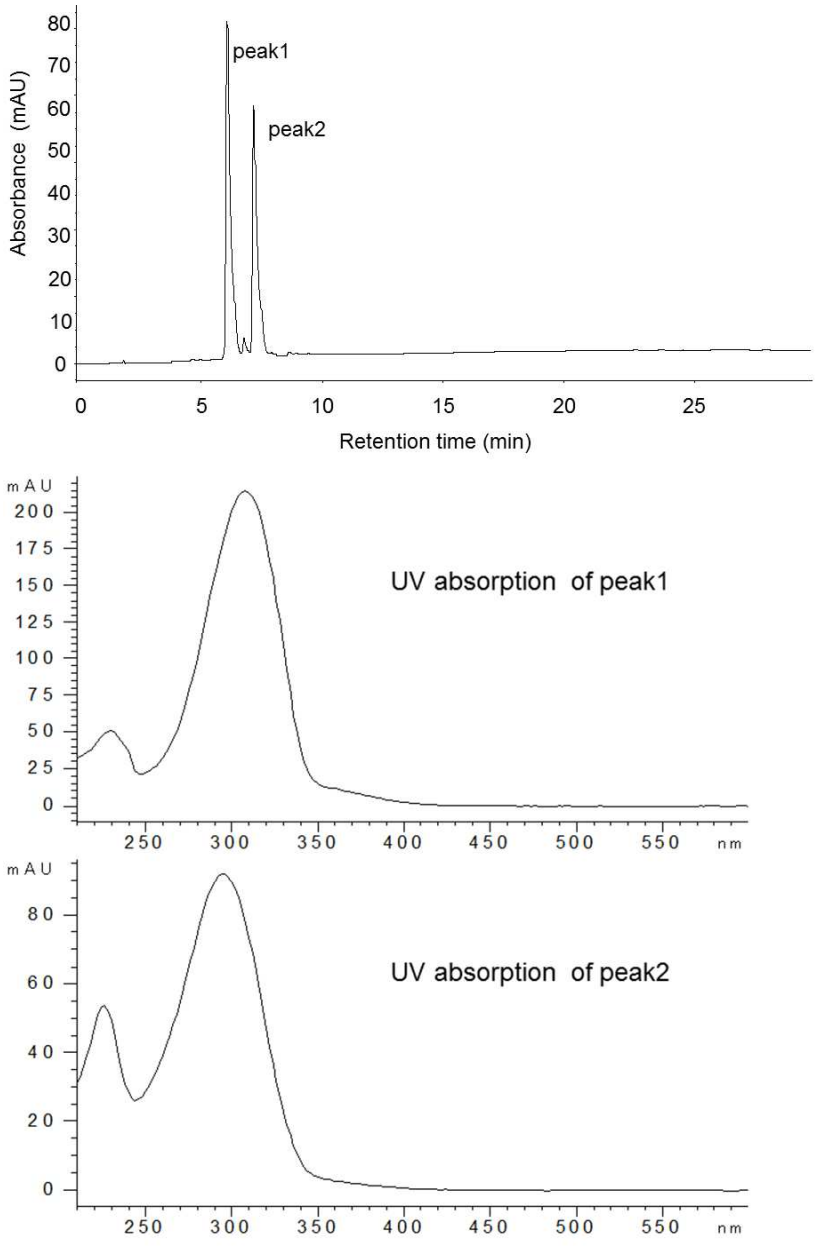

Figure S43. HPLC Split and UV absorption of compound B.

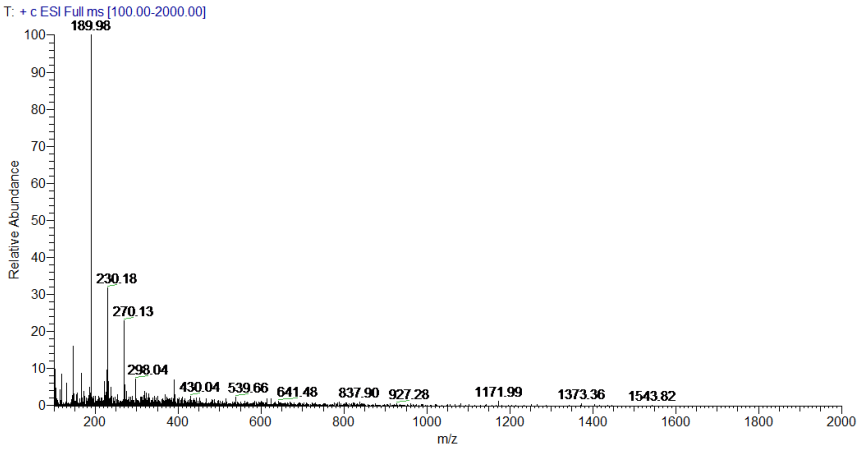

Figure S44. MS of compound A.

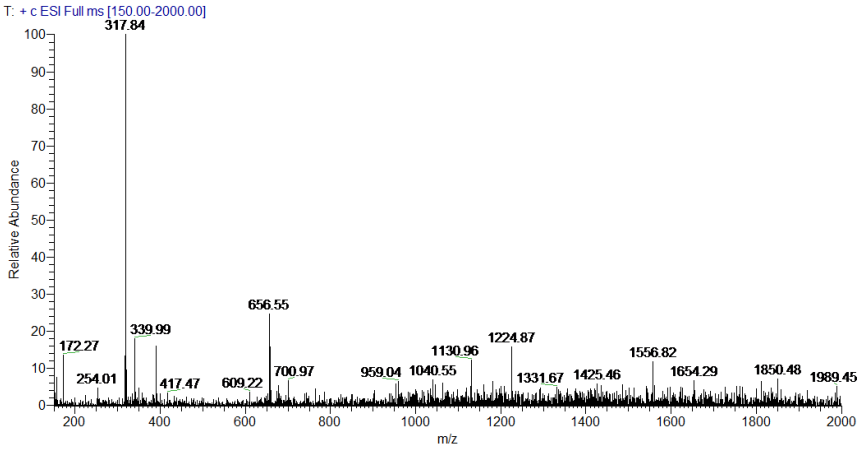

Figure S45. MS of compound B.