Supporting Information

Nonspecific Heme-Binding Cyclase, AbmU, Catalyzes [4 + 2] Cycloaddition During Neoabyssomicin Biosynthesis

Qinglian Li,*[,†] Wenjuan Ding,[†,‡] Jiajia Tu,[†] Changbiao Chi,[§] Hongbo Huang,[†] Xiaoqi Ji,[†,‡] Ziwei Yao,[†,‡] Ming Ma,[§] and Jianhua Ju*[,†,‡]

[†]CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Rd., Guangzhou 510301, China

[‡]College of Oceanology, University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China

[§]State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, 38 Xueyuan Road, Haidian District, Beijing 100191 , China.

*liql@scsio.ac.cn; *jju@scsio.ac.cn.

Supporting Figures

Figure S1. Selection of known abyssomicin congeners.

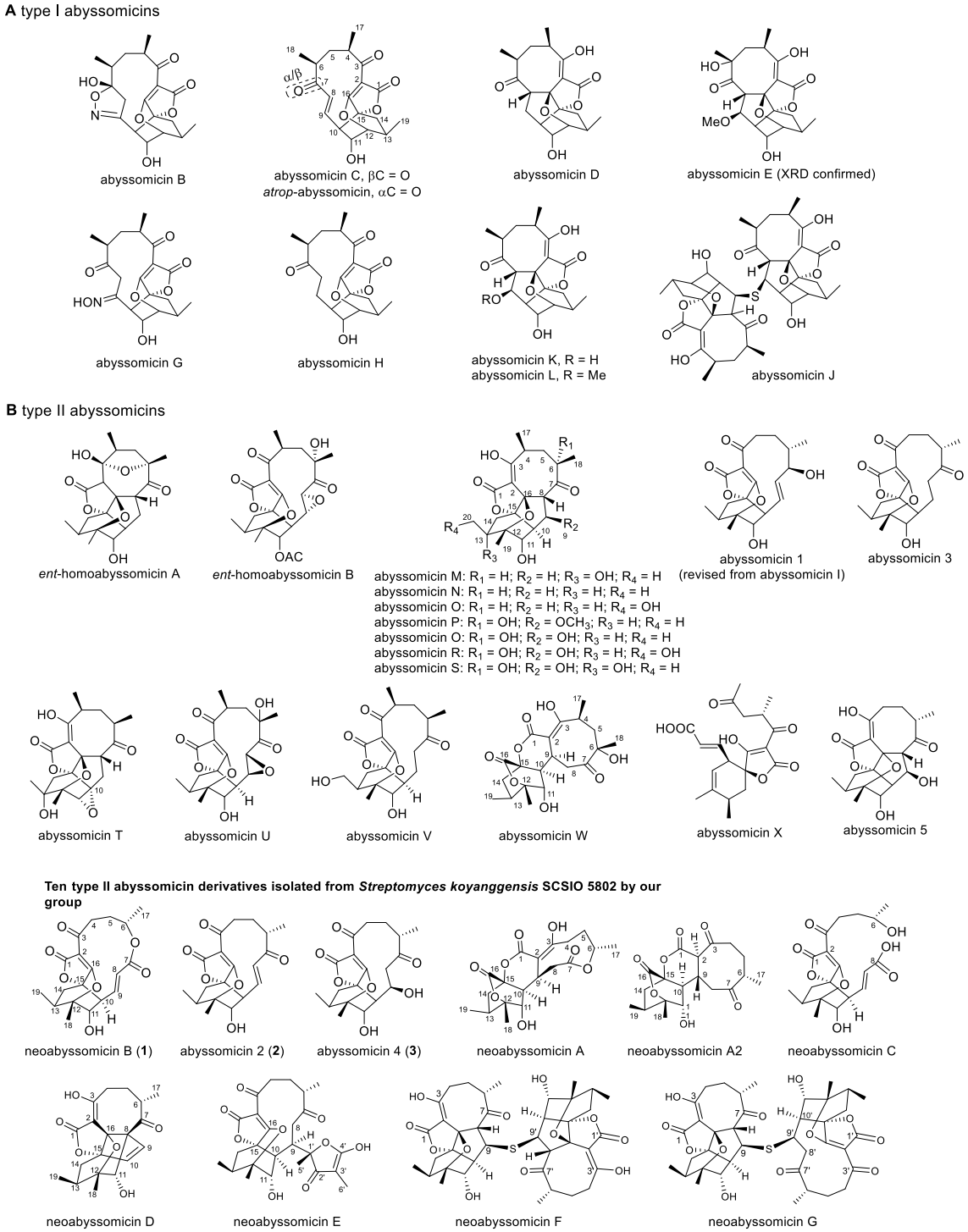


Figure S2. Structure-based sequence alignments of AbmU and AbyU.

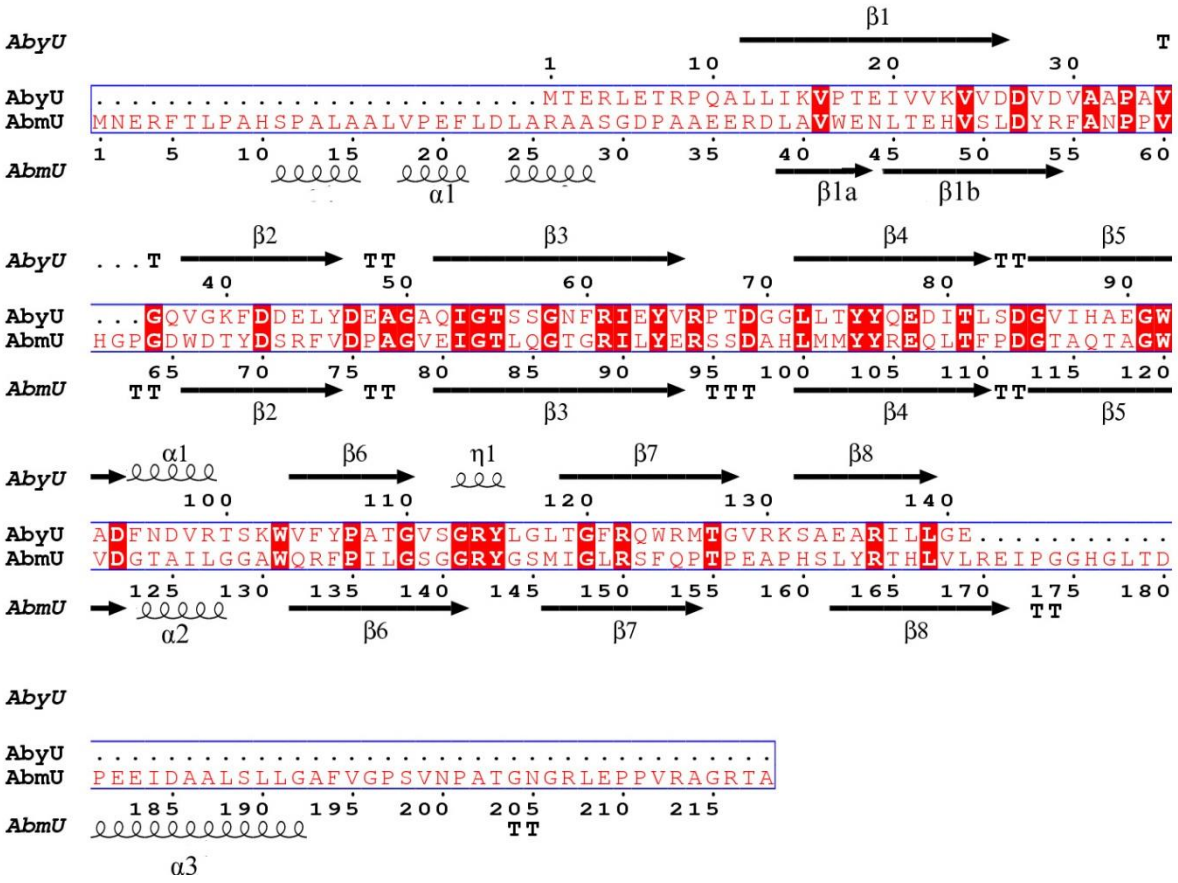


Figure S3. In-frame deletion of *abmU* in WT *S. koyangensis* SCSIO 5802 via PCR-targeting. (**A**) Schematic representation for in-frame deletion of *abmU*. (**B**) PCR analyses of the WT strain and the *abmU* double-cross mutant were carried out using the primers listed in **Table S2**. Marker: DNA molecular ladder; W: using the genomic DNA of *S. koyangensis* SCSIO 5802 as template; M: using the genomic DNA of Δ*abmU* mutant as template.

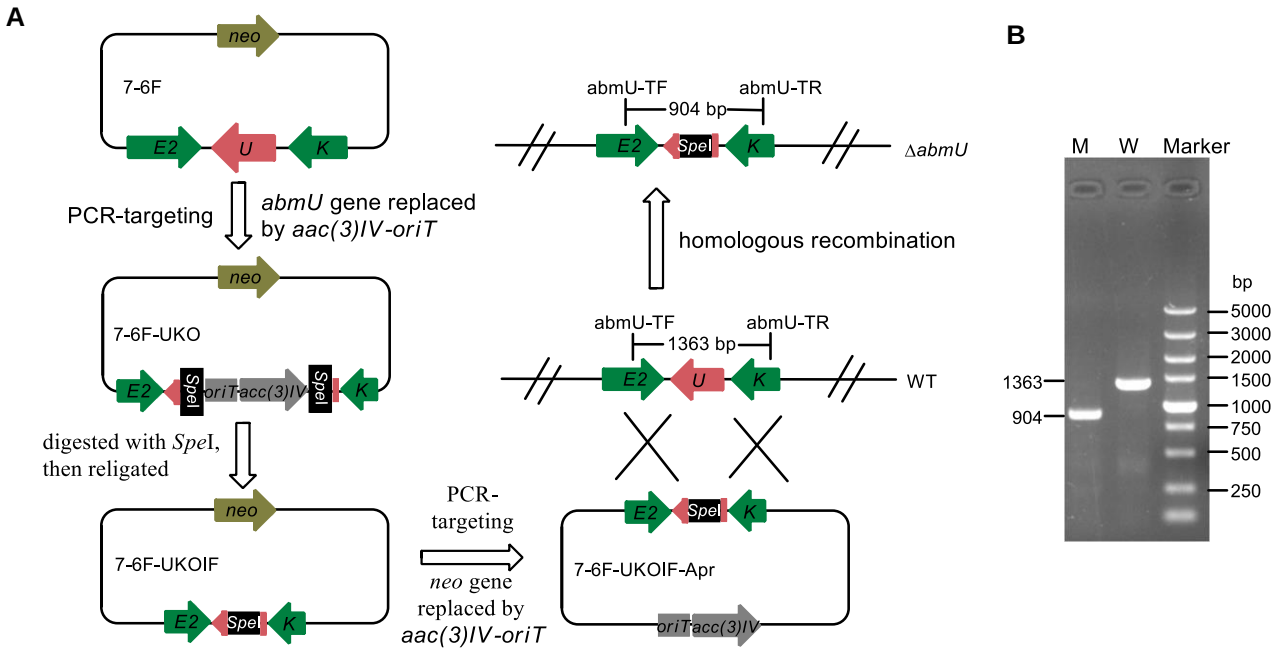


Figure S4. (–) HR-ESI-MS spectrum of compound **5**.

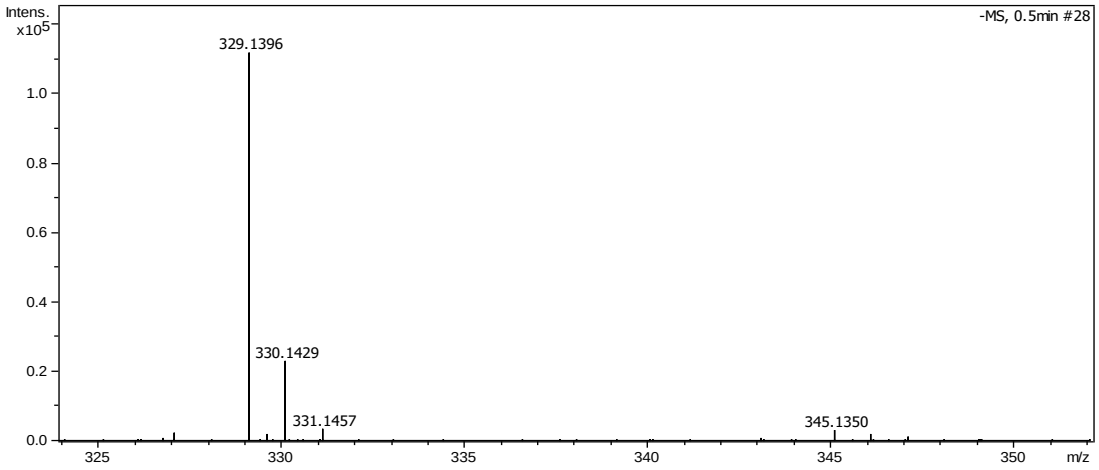


Figure S5. Structure of **5** and the key COSY, HMBC and NOESY correlations of **5**.

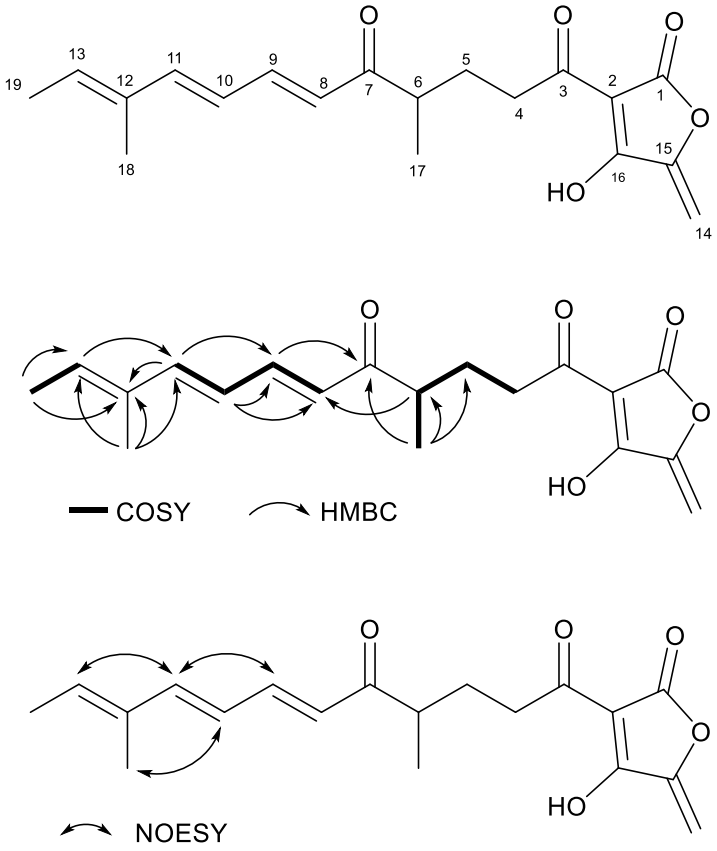
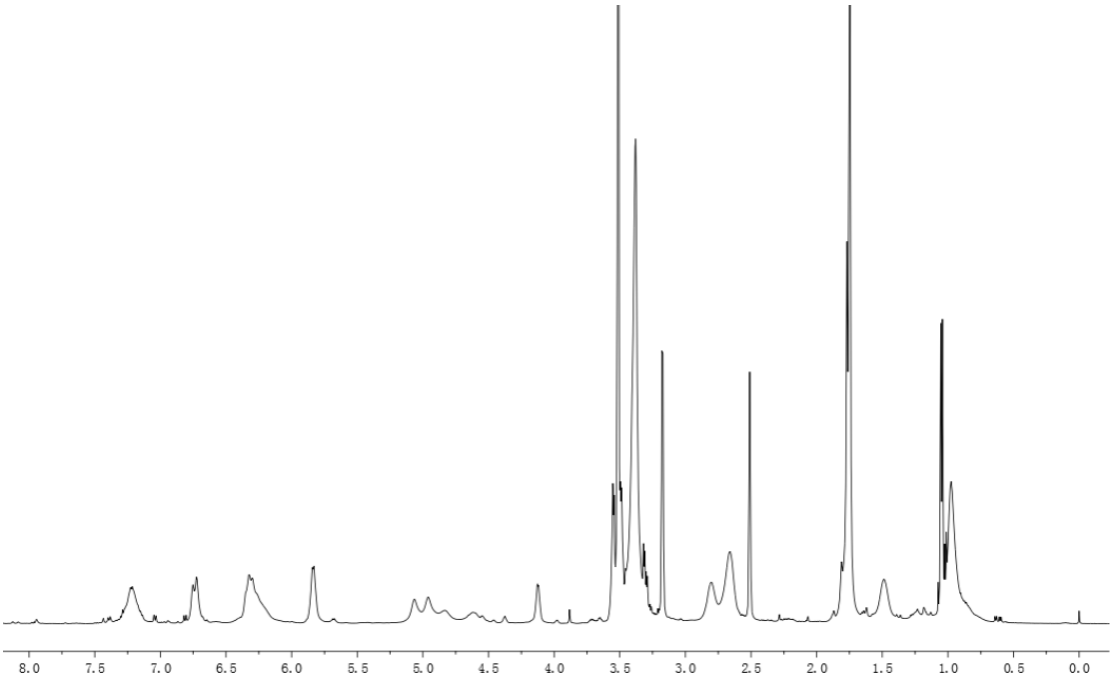
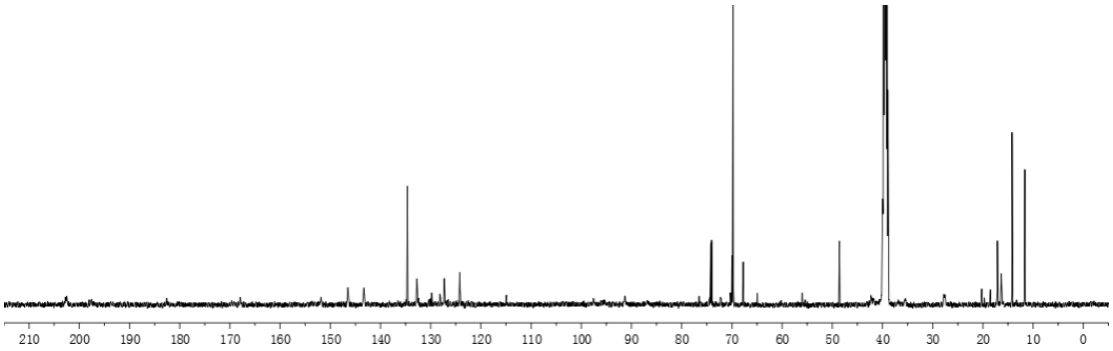


Figure S6. NMR spectra of compound **5**. (**A**) ^{1}H NMR spectrum. (**B**) ^{13}C NMR spectrum. (**C**) Dept 135 spectrum. (**D**) ^{1}H-^{1}H COSY spectrum. (**E**) HSQC spectrum. (**F**) HMBC spectrum. (**G**) NOESY spectrum.

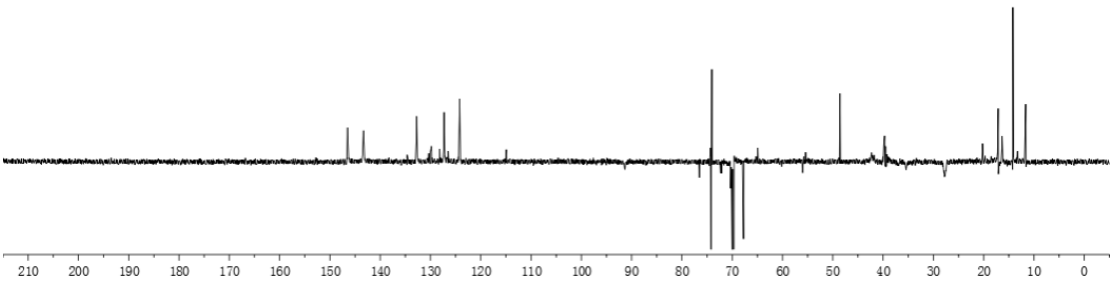
A



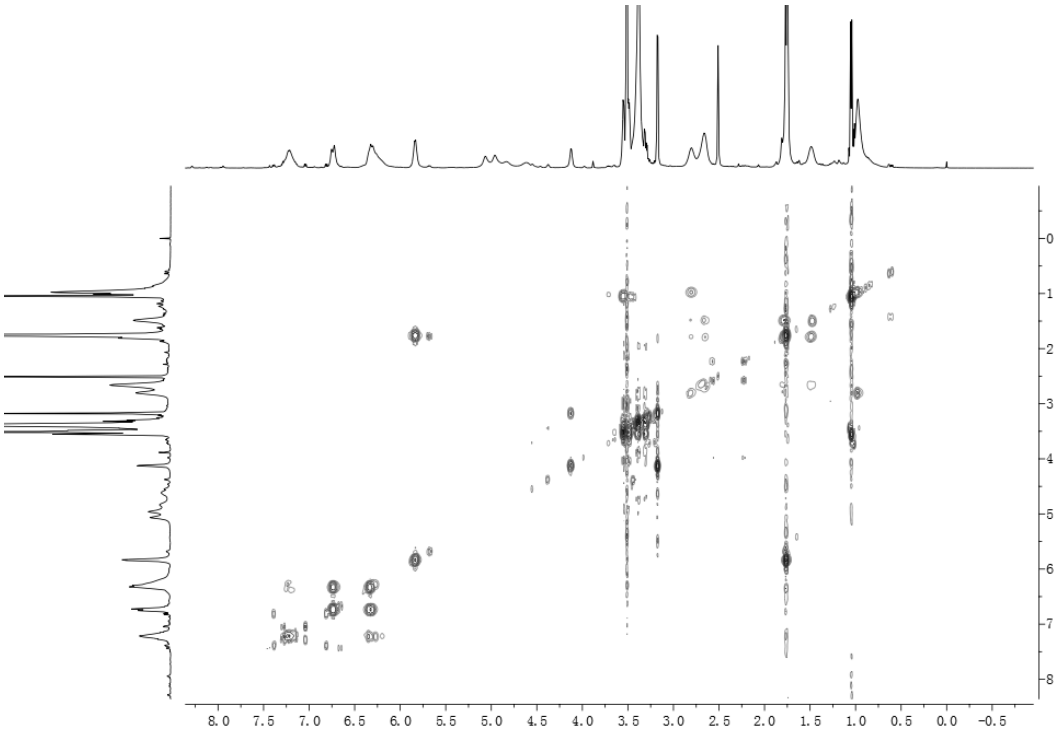
B



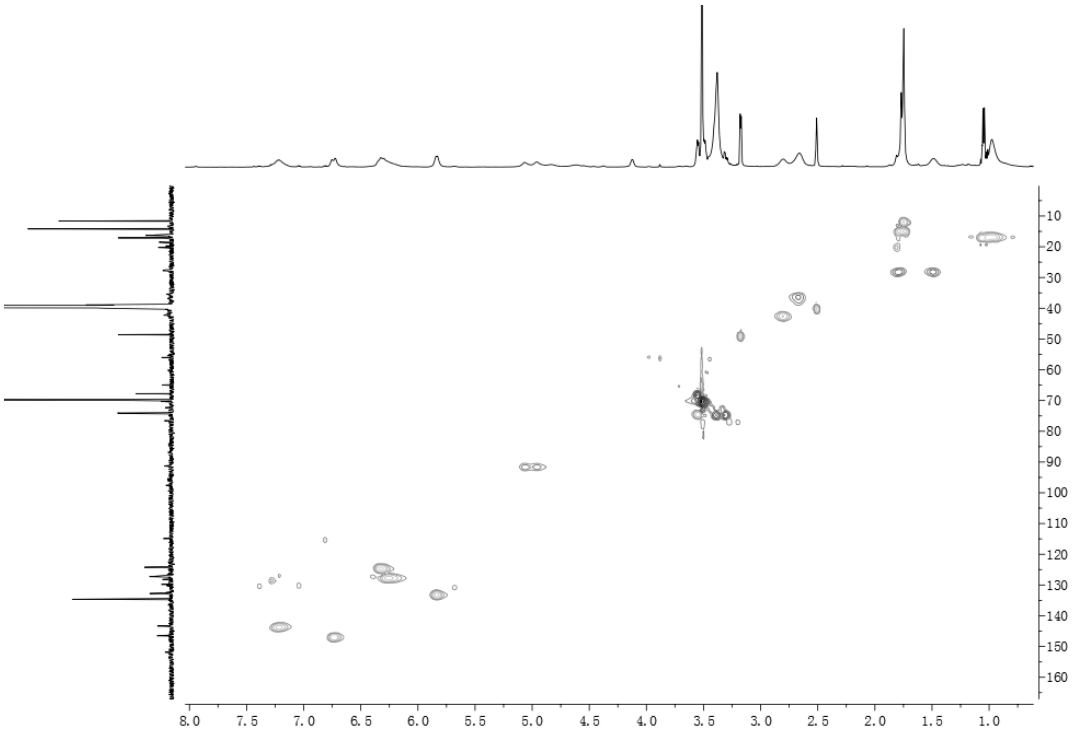
C



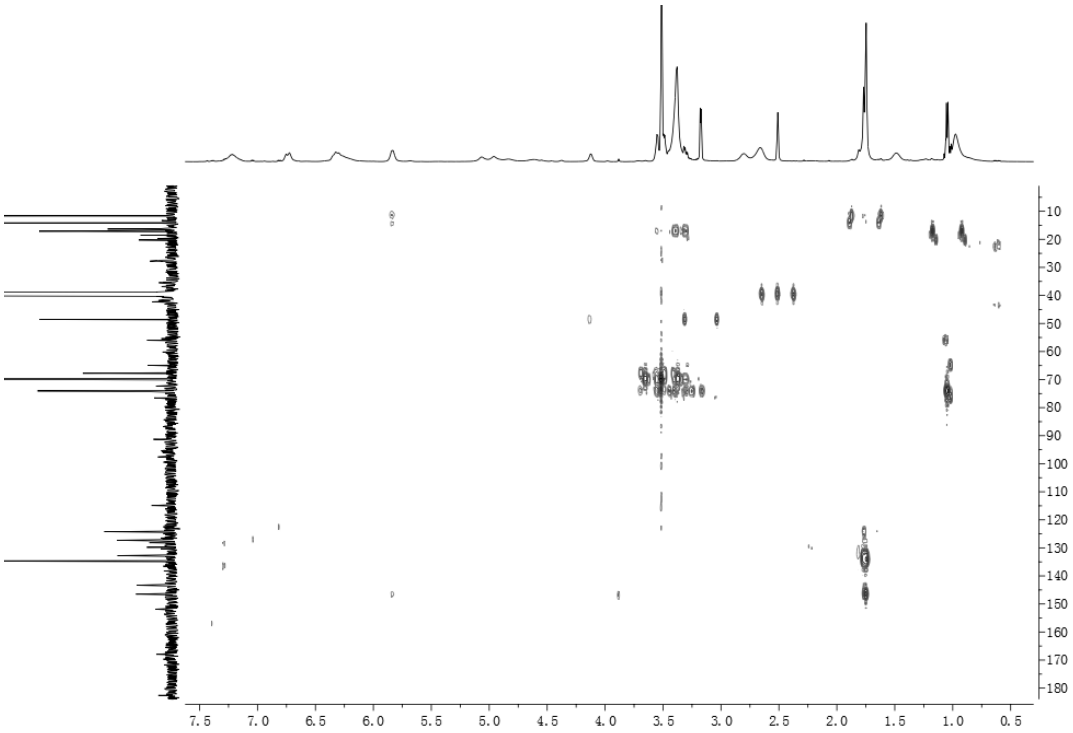
D


8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
0 1 2 3 4 5 6 7 8

E


8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160

F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120
130
140
150
160
170
180
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6.5
6.0
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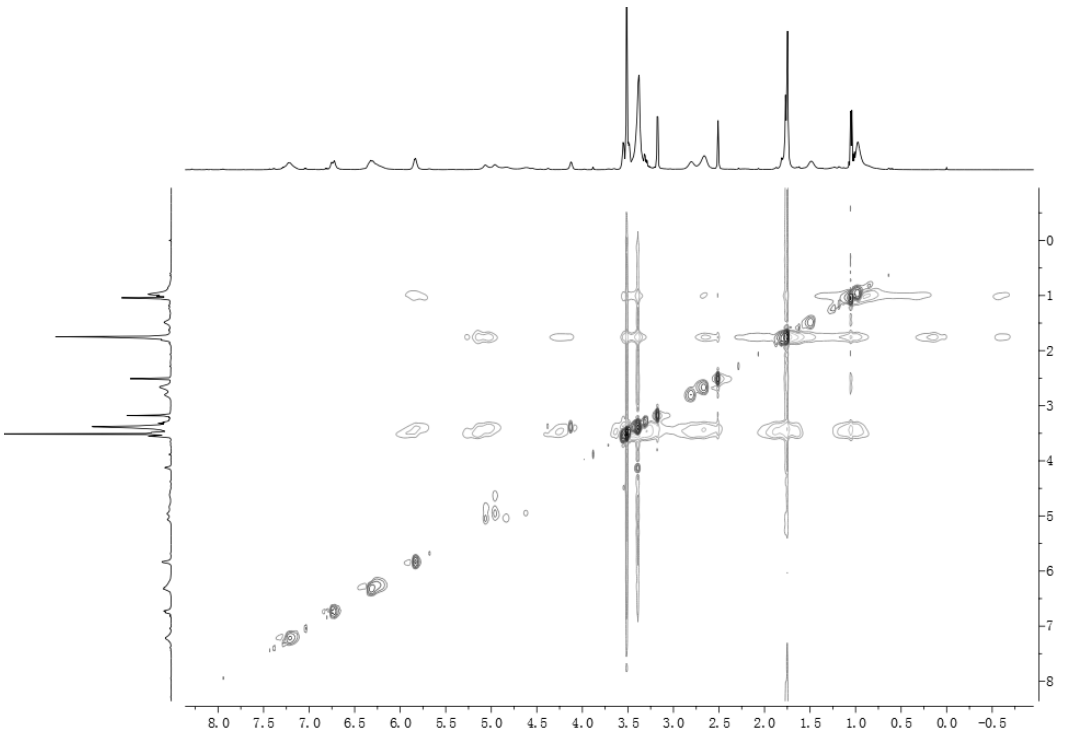
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5

G


0
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2
3
4
5
6
7
8
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5

Figure S7. Evaluation of protein overexpressions. SDS-PAGE analyses of wild-type (WT) AbmU and its 17 mutant variants purified from LB medium supplemented with the heme precursor 5-aminolevulinic acid (5-ALA).

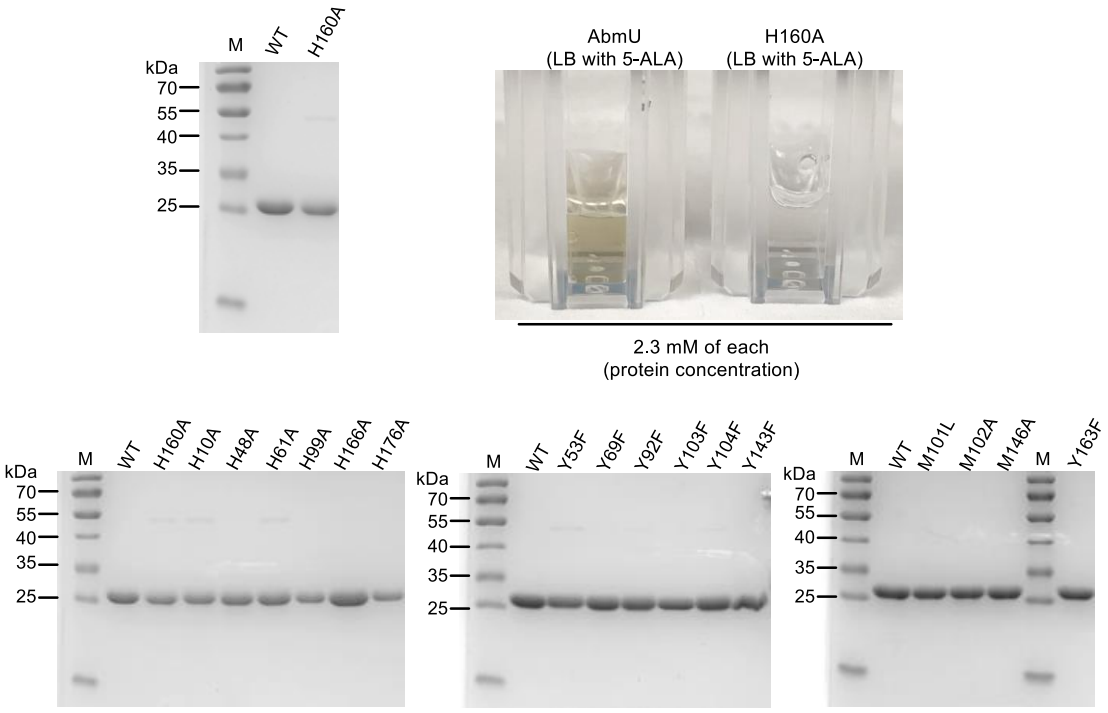


Figure S8. Mass spectrum of AbmU-catalyzed reaction product. Positive ion mode ESI-MS of compound recovered from AbmU-catalyzed [4 + 2] cycloaddition reaction (see **Figure 3C**, trace vi). The product mass is consistent with the proposed structure of abyssomicin 6 (**4**) with *m/z* of $[M + H]^+$ = 331.12 (calculated 331.15).

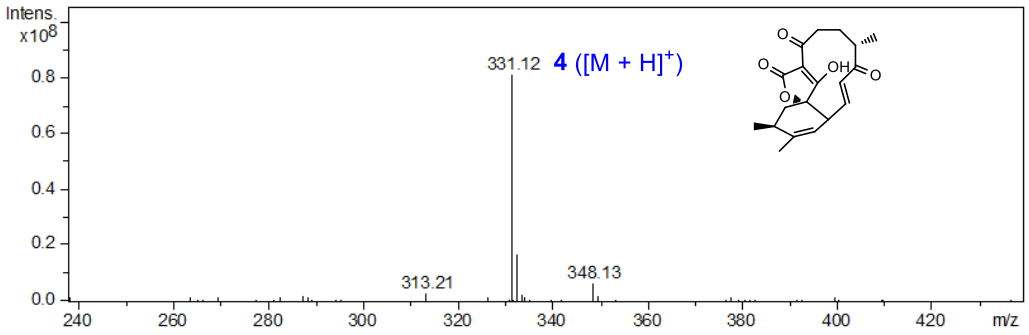


Figure S9. Kinetic investigations of AbmU activity under oxidizing conditions. All data points represent the mean value ± SD.

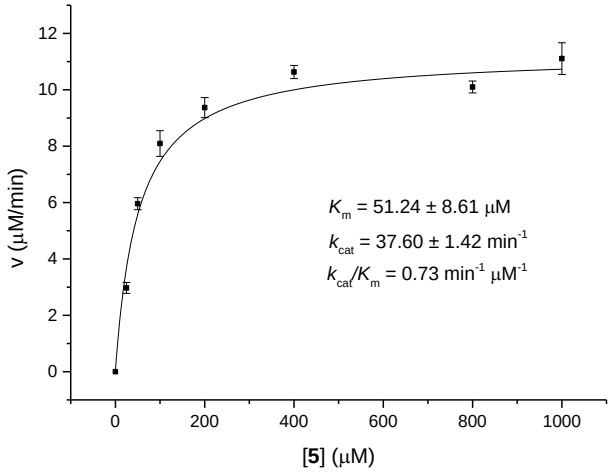


Figure S10. UV-Vis spectra of AbmU. (**A**) Pyridine hemochrome assay of AbmU showing the characteristic spectrum of heme *b*. (**B**) UV-vis spectra of AbmU purified from LB medium supplemented with 5-ALA (termed as-purified AbmU) and oxidized AbmU. The concentration of each protein is 100 μM. To generate the oxidized AbmU, 500 μM potassium ferricyanide ($K_3[Fe(CN)_6]$) was added to the as-purified AbmU. Since $K_3[Fe(CN)_6]$ absorbs strongly in the UV-vis region, the spectrum of oxidized AbmU shown here is actually the difference spectrum of potassium ferricyanide ($K_3[Fe(CN)_6]$)-oxidized AbmU minus $K_3[Fe(CN)_6]$ (500 μM).

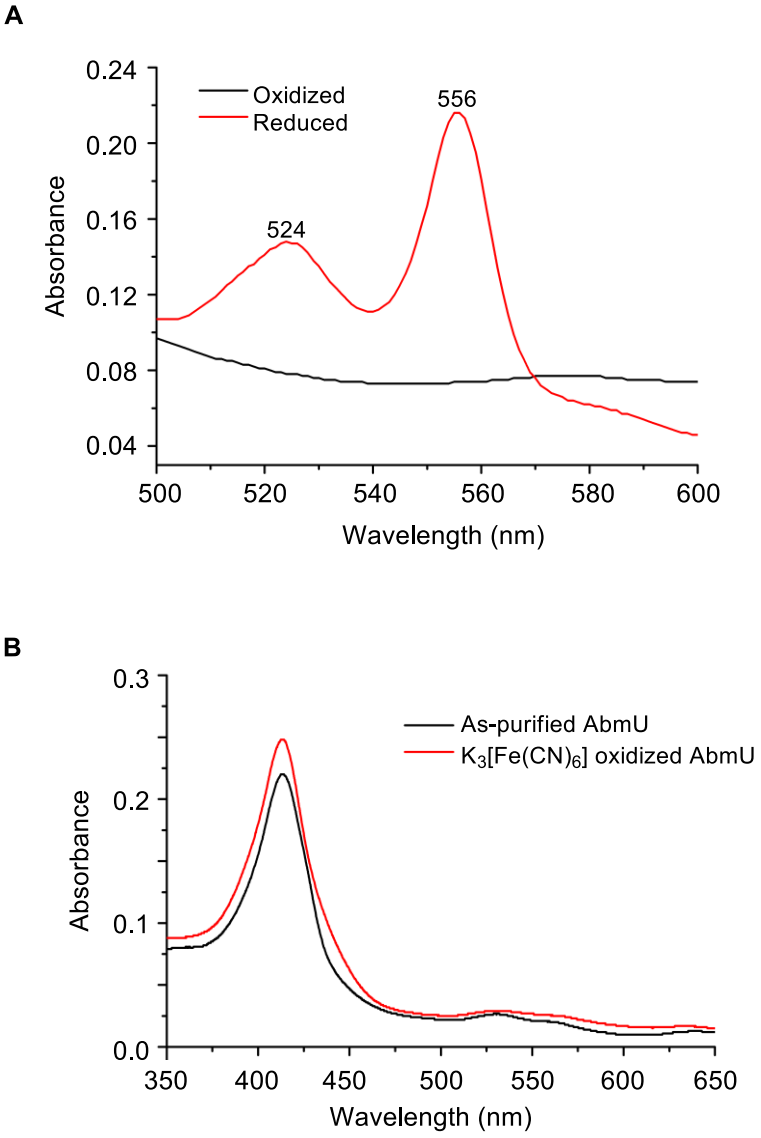


Figure S11. The effect of heme content on the activity of AbmU. (**A**) Comparison of the color and the UV-vis spectrum of AbmU purified from minimal LB medium (~0.1 % heme content) relative to that purified from LB medium supplemented with heme precursor 5-ALA (~ 1.5 % heme content). (**B**) HPLC analysis of the reaction mixtures of linear substrate **5** (200 μM) with AbmU (1 μM). i: AbmU purified from LB medium in the presence of 2 μM hemin; ii: AbmU purified from minimal LB medium; iii: AbmU purified LB medium supplemented with 5–ALA. The activity assays were performed in three independent replicates (each had at least two parallel samples); the set of traces shown is representative of the full dataset. There were no obvious differences in catalytic efficiencies for the AbmU purified from minimal LB medium and that purified LB medium supplemented with 5–ALA (traces ii and iii); however, additional supply of hemin (2 μM) in the reaction mixture inhibited the activity of AbmU purified from LB medium (traces i and ii), demonstrating that the non-specific binding of heme moiety at His160 residue facing the barrel precluded the substrate binding and thus catalysis.

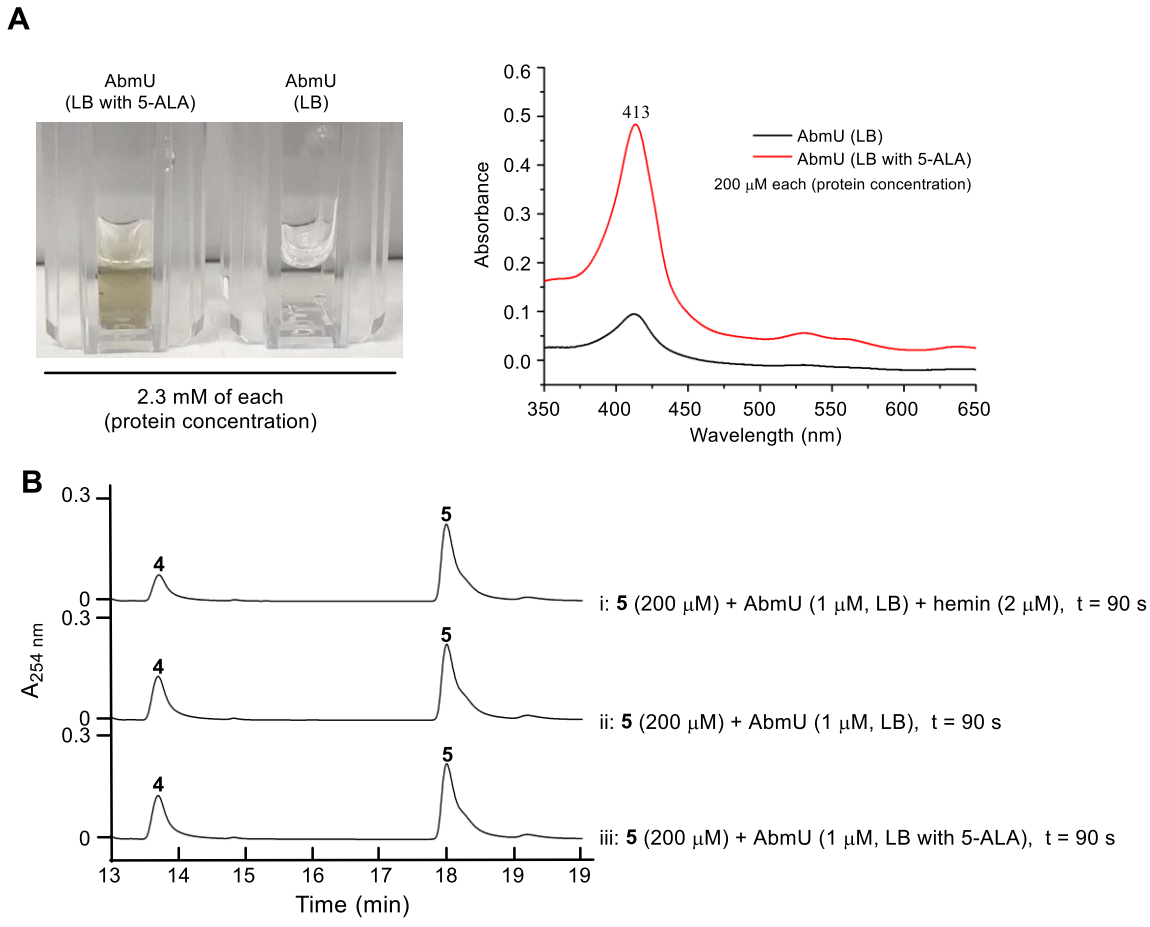
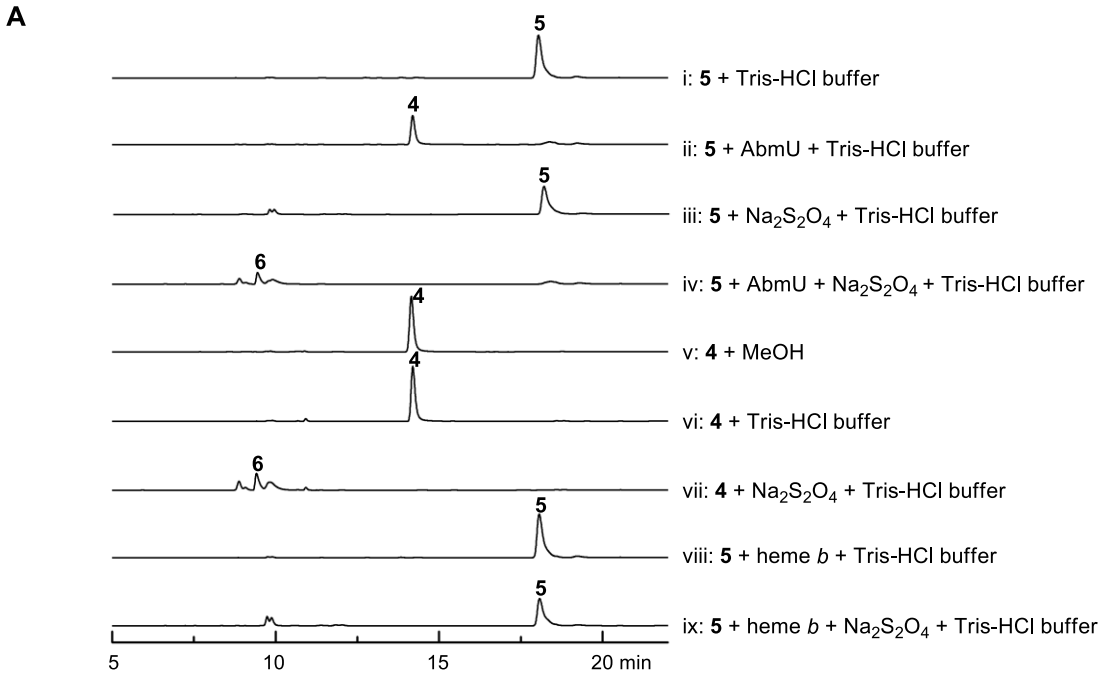
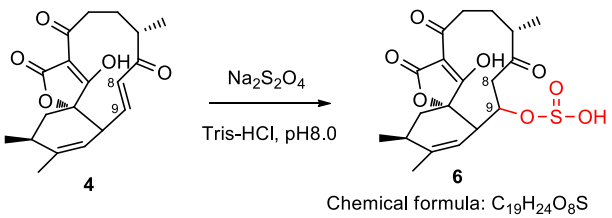
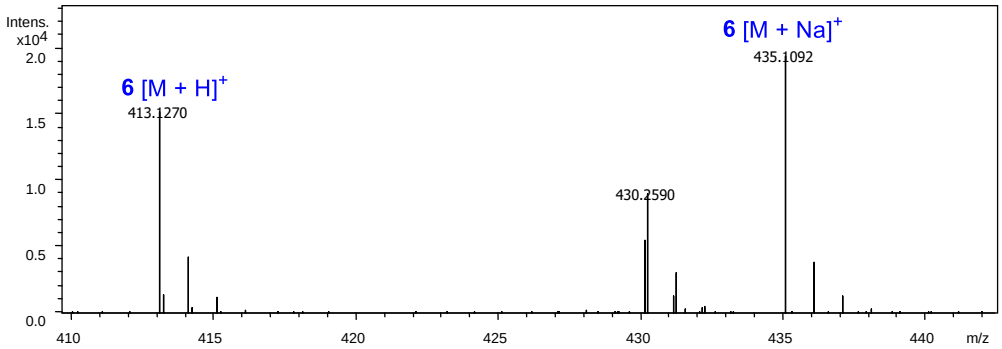


Figure S12. AbmU employs either heme iron oxidation state (+2 or +3) during the course of [4 + 2] cycloaddition. (**A**) HPLC analyses of AbmU-catalyzed reactions under oxidized or reduced conditions. As-purified AbmU (trace ii), sodium dithionite (1 mM)-reduced AbmU (trace iv), heme *b* (trace viii) and sodium dithionite (1 mM)-reduced heme *b* (trace ix) were each incubated with 200 μM **5** in Tris-HCl buffer (50 mM, pH 8.0) at 30 °C for 10 min. In the above reactions, the concentration of AbmU or heme *b* was set in 1 μM. In addition, authentic standard **4** (200 μM) was similarly subjected to the same reducing reaction condition (devoid of AbmU) comprising 1 mM sodium dithionite in Tris-HCl buffer (50 mM, pH 8.0), and used as a control (trace vii). (**B**) HPLC-HRESIMS analysis of the AbmU reaction product under reducing conditions. The formula of the product **6** was determined to be $C_{19}H_{24}O_8S$ (*m/z* 413.1270 $[M + H]^+$, calcd 413.1265) on the basis of LC-HRESIMS. Compound **6** is proposed to be spontaneously derived from AbmU-catalyzed [4 + 2] cycloaddition adduct **4** *via* a Michael-type addition of bisulfite to the C8-C9 olefin of **4**. (**C**) Summary of ^{1}H NMR (700 MHz) data for compound **6** in CD_3OD. (**D**) The ^{1}H NMR (700 MHz) spectrum of compound **6** in CD_3OD. (**E**) The ^{1}H-^{1}H COSY spectrum of compound **6** in CD_3OD.

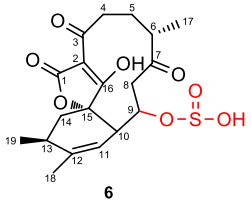


B



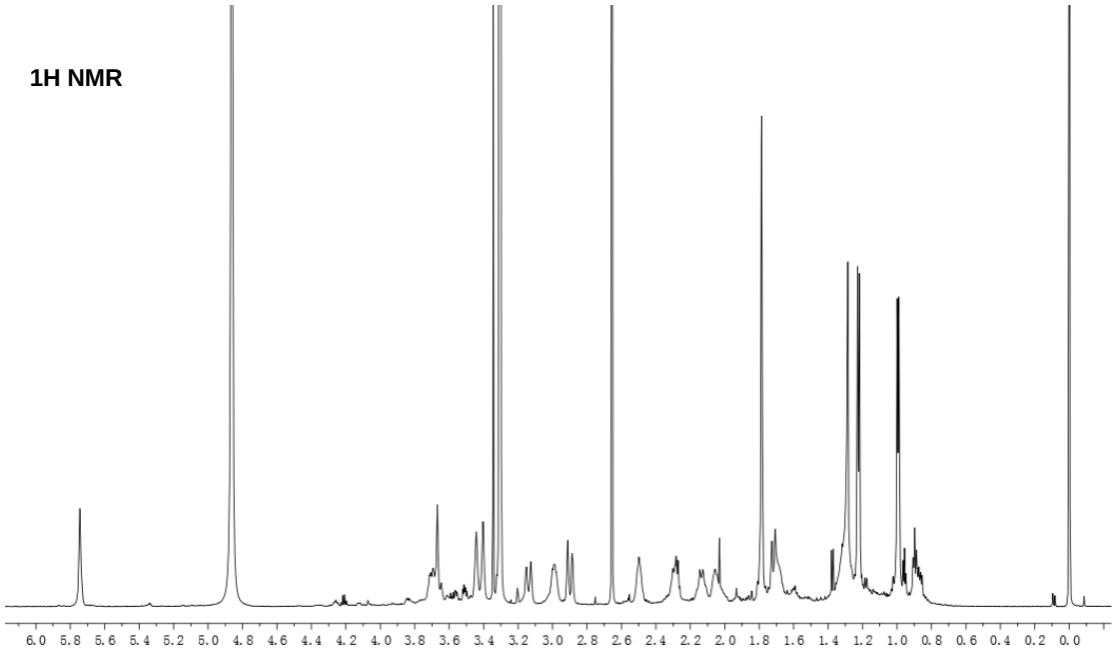


C

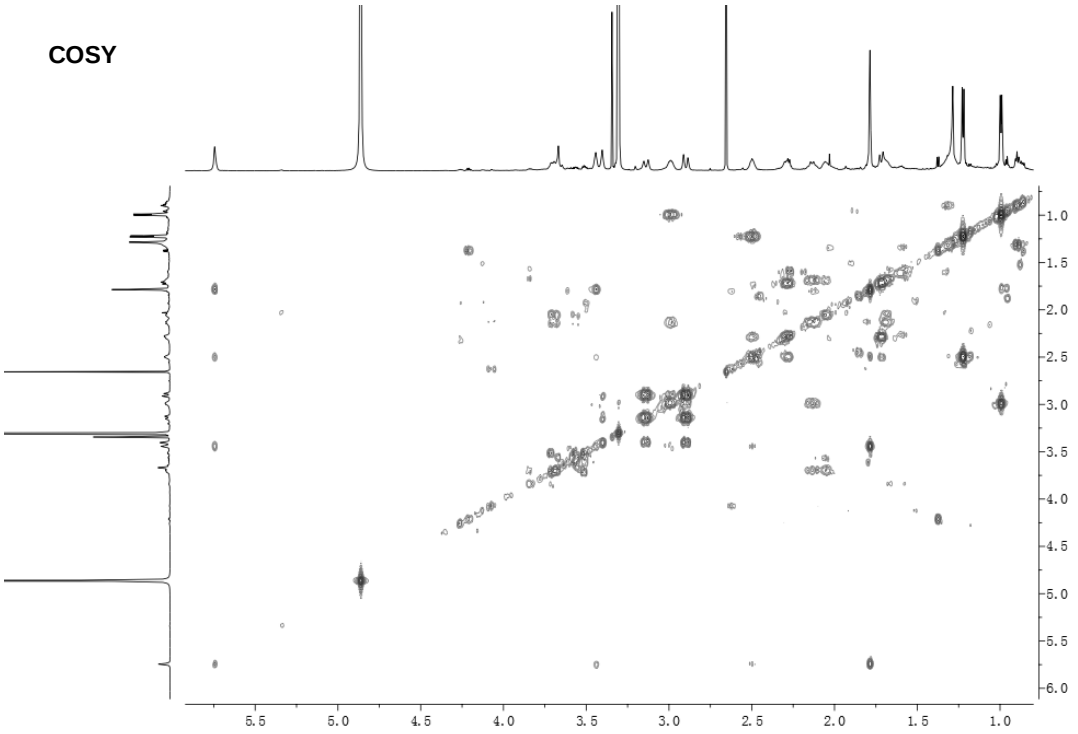


position	δ_H, mult. (J in Hz)
4	3.00, m; 2.30, m
5	1.73, m; 1.70, m
6	2.91, m
7	
8	3.40, m; 3.15, m
9	3.71, m
10	3.44, m
11	5.74, s
12	
13	2.51, m
14	2.16, m; 2.07, m
17	1.00, d (6.9)
18	1.79, s
19	1.23, d (7.3)

D


1H NMR
6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0

E


COSY
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4.5
5.0
5.5
6.0
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0

Figure S13. AbmU-catalyzed reactions under aerobic and anaerobic conditions. The as-purified AbmU (1 μM), sodium dithionite (1 mM)-reduced AbmU (1 μM) were each incubated with **5** (200 μM) in Tris-HCl buffer (50 mM, pH 8.0) at 30 °C for 10 min, and then subjected to HPLC analyses.

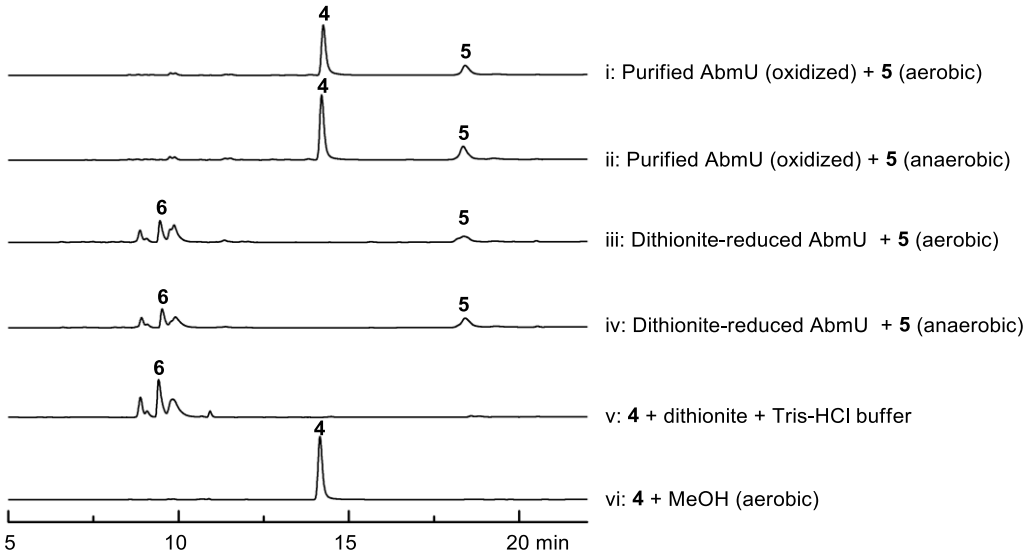


Figure S14. Mutagenesis and reaction analysis of putative AbmU heme ligands. A total of 16 amino residues were each mutated to nonpolar residues (alanine, phenylalanine or leucine). Each (1 μM) of the mutant proteins was incubated with substrate **5** (200 μM) in Tris-HCl buffer (50 mM, pH 8.0) at 30 °C for 10 min, and then subjected to HPLC analyses.

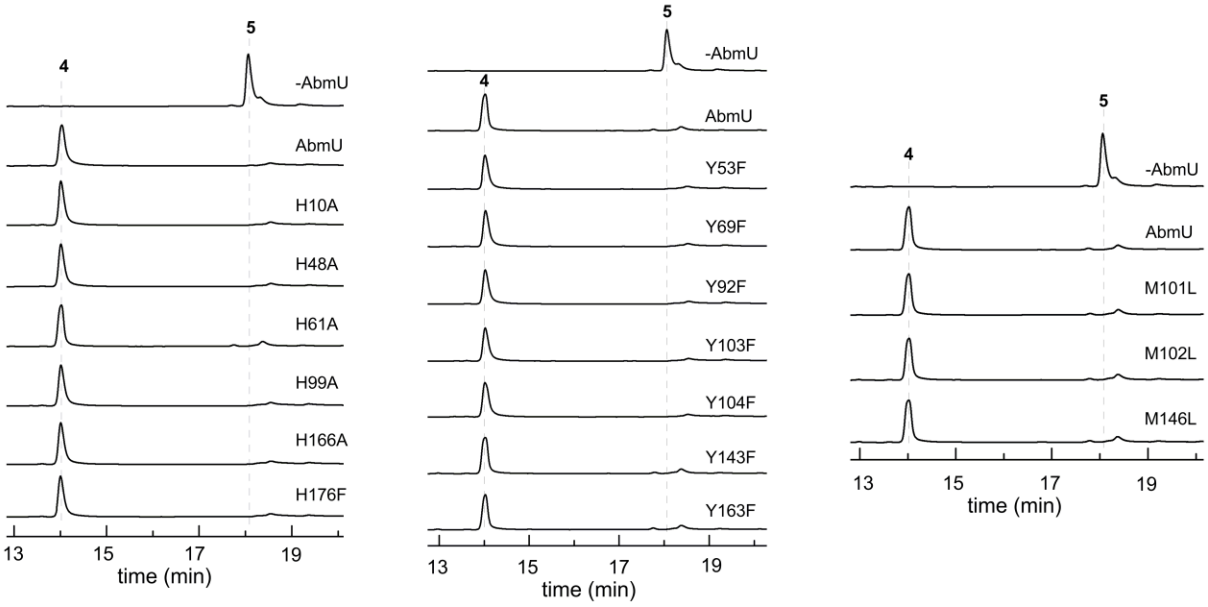
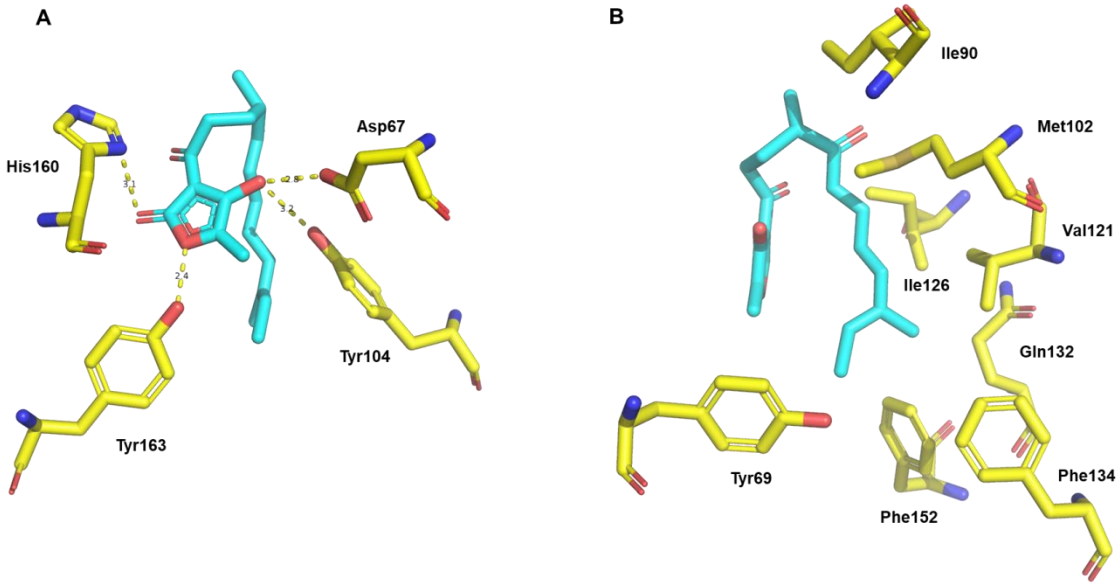


Figure S15. The modeling of substrate **5** of AbmU in the active site. (A) The enlarged details for the H-bond interactions between the tetronate moiety of **5** (cyan) with key residues of AbmU. H-bonds were labeled with yellow dashed lines. The four residues of AbmU with H-bonds to the tetronate moiety were labeled as yellow sticks. (B) The enlarged details for the hydrophobic interactions between the terminal $\Delta^{8,9}$, $\Delta^{10,11}$, $\Delta^{12,13}$-triene moiety of **5** (cyan) and residues (yellow sticks) of AbmU.



Supporting Tables

Table S1. Summary of ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for compound **5** in DMSO-d_6.

compound **5**			
position	δ_C	type	δ_H, mult. (J in Hz)
1	167.9	C	
2	97.6	C	
3	197.5	C	
4	35.5	CH_2	2.66,m
5	27.8	CH_2	1.49, m; 1.81, m
6	42.3	CH	2.80, m
7	202.5	C	
8	127.3	CH	6.28, overlapped
9	143.3	CH	7.22, dd (13.3, 6.0)
10	124.2	CH	6.33, overlapped
11	146.4	CH	6.74, d (14.1)
12	134.6	C	
13	132.8	CH	5.84, q (5.4)
14	91.0	CH_2	4.96, s; 5.06,s
15	151.8	C	
16	182.6	C	
17	17.1	CH_3	1.06, dd (6.2, 3.0)
18	11.6	CH_3	1.75, s
19	14.2	CH_3	1.77, d (5.4)

Table S2. X-ray diffraction data collection and refinement statistics.

	SeMet-AbmU
Data collection	
Wavelength	0.97893
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	66.61, 65.35, 101.40
α, *β*, *γ* (°)	90.00, 90.19, 90.00
Resolution (Å)	50.00-2.51 (2.58-2.51)[a]
No. of unique reflections	29528
R_{merge}[b]	0.081 (0.498)
I/σ_I	13.9 (3.0)
Completeness (%)	98.2 (98.9)
Redundancy	6.8 (7.0)
Refinement	
R_{work}/R_{free}	0.19/ 0.23
No. atoms	6427
Protein	6245
Water	182
B-factors	
Protein	51.4
Water	41.6
Ramachandran	
Favored (%)	98.1
Allowed (%)	1.6
Outlier (%)	0.0
RMSD	
Bond lengths (Å)	0.012
Bond angles (°)	1.426
Protein Data Bank entry	6LE0

[a] Numbers in parentheses are values for the highest-resolution bin. [b] $R_{merge} = \sum_{hkl}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl*, and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations of that reflection *hkl*.

Table S3. Bacteria and plasmids used in this study.

Strains/Plasmids	Description	Source/[References
Streptomycetes		
Streptomyces koyangensis SCSIO 5802	WT producer of neoabyssomicins/abyssomicins (**1**–**3**)	This work
Δ*abmU*	*abmU* in-frame deletion mutant; *S. koyangensis* SCSIO 5802 with a 459 bp fragment of *abmU* deleted	This work
Δ*abmU::abmU*	Δ*abmU* complemetation strain, expression of *abmU* under the control of a strong constitutive promoter *ermE**p	This work
E. coli		
DH5α	Host strain for general clone	
BW25113/pIJ790	K-12 derivative: *araBAD, rhaBAD*; host strain for Red/ET-mediated recombination	[1]
ET12567/pUZ8002	*dam*, *dcm*, *hsdM*, *hsdS*, *hsdR*, cat^R, tet^R; donor strain for conjugation between *E. coli* and *Streptomyces*	[2]
Plasmids		
pIJ773	*aac(3)IV*, *oriT;* used for amplifying the *aac(3)IV-oriT* gene cassette for gene inactivation	[3]
pL646	Derived from pSET152, containing *ermE**P promoter and the ribosome-binding site of the *tuf1* gene	[4]
cosmid 7-6F	A cosmid which contains partial *abm* biosynthetic cluster, used for in-frame deletion of *abmU*	[5]
7-6F-UKO	A 459 bp fragment of *abmU* in cosmid 7-6F was replaced by the *aac(IV) + oriT* cassette	This work
7-6F-UKOIF	The *aac(IV) + oriT* cassette in 7-6F-UKO was removed	This work
7-6F-UKOIF-Apr	The kanamycin resistance gene neo was replaced by the *aac(IV) + oriT* cassette	This work

Table S4. Primers used in this study.

Name	Sequence (5'-3')	Purpose
abmU-DelF	GACCGAGGGGCCCACGAAGGCGCCGAGCAGCGACAGTGCACTAGTATTCCGGGGATCCGTCGACC (*Spe*I site underlined)	For disrupting *abmU*
abmU-DelR	TTCCTCGACCTGGCGCGAGCCGCGAGCGGCGATCCGGCCACTAGTTGTAGGCTGGAGCTGCTTC (*Spe*I site underlined)	
abmU-TF	CCCGCCAGTTGCAGTTCTCG	For verifying the disruption of *abmU*
abmU-TR	CGGTTCGTTCCGCGTCATG	
abmU-expF	AAATTTCCCATATGATGAACGAACGCTTCACC	For cloning and expression of *abmU*
abmU-expR	ATATGGATCCTCAGGCGGTGCGCCCGGC	
AbmU-H10A-F	TTCACCCTGCCCGCCgcCAGCCCCGCCCTCG	For construction of AbmU/ H10A mutant
AbmU-H10A-R	gcGGCGGGCAGGGTGAAGCGTTCGTTC	
AbmU-H48A-F	GAGAACCTCACGGAAgcCGTCTCGCTGGAC	For construction of AbmU/ H48A mutant
AbmU-H48A-R	gcTTCCGTGAGGTTCTCCCAGACCGCGAGG	
AbmU-H61A-F	GCCAACCCGCCCGTGgcCGGTCCCGGCGAC	For construction of AbmU/ H61A mutant
AbmU-H61A-R	gcCACGGGCGGGTTGGCGAACCGGTAGTC	
AbmU-H99A-F	CGTTCGTCGGACGCGgcCCTGATGATGTAC	For construction of AbmU/ H99A mutant
AbmU-H99A-R	gcCGCGTCCGACGAACGCTCGTACAGGATG	
AbmU-H160A-F	ACCCCCGAGGCGCCGgcCAGCCTCTACCG	For construction of AbmU/ H160A mutant
AbmU-H160A-R	gcCGGCGCCTCGGGGGTGGGCTGGAAGG	
AbmU-H166A-F	AGCCTCTACCGCACCgcCCTGGTGCTCCGG	For construction of AbmU/ H166A mutant
AbmU-H166A-R	gcGGTGCGGTAGAGGCTGTGCGGCGCCTC	
AbmU-H176F-F	GAGATCCCCGGCGGGttCGGGCTGACCGAC	For construction of AbmU/ H176F mutant
AbmU-H176F-R	aaCCCGCCGGGGATCTCCCGGAGCACCAG	
AbmU-M101L-F	TCGGACGCGCACCTGcTGATGTACTACCGC	For construction of AbmU/ M101L mutant
AbmU-M101L-R	gCAGGTGCGCGTCCGACGAACGCTCGTAC	
AbmU-M102A-F	GACGCGCACCTGATGgcGTACTACCGCGAG	For construction of AbmU/ M102A mutant
AbmU-M102A-R	gcCATCAGGTGCGCGTCCGACGAACGCTCG	
AbmU-M146A-F	GGCCGGTACGGCTCCgcGATCGGGCTGCGC	For construction of AbmU/ M146A mutant
AbmU-M146A-R	gcGGAGCCGTACCGGCCGCCCGACCCCAGG	
AbmU-Y53F-F	ACGTCTCGCTGGACTtCCGGTTCGCCAAC	For construction of AbmU/ Y53F mutant

AbmU-Y53F-R	aAGTCCAGCGAGACGTGTTCCGTGAGG	
AbmU-Y69F-F	GCGACTGGGACACGTtCGACAGCCGCTTC	For construction of AbmU/ Y69F mutant
AbmU-Y69F-R	aACGTGTCCCAGTCGCCGGGACCGTGC	
AbmU-Y92F-F	CCGGACGCATCCTGTtCGAGCGTTCGTCG	For construction of AbmU/ Y92F mutant
AbmU-Y92F-R	aACAGGATGCGTCCGGTGCCCTGGAGGG	
AbmU-Y103F-F	CGCACCTGATGATGTtCTACCGCGAGCAG	For construction of AbmU/ Y103F mutant
AbmU-Y103F-R	aACATCATCAGGTGCGCGTCCGACGAAC	
AbmU-Y104F-F	ACCTGATGATGTACTtCCGCGAGCAGCTG	For construction of AbmU/ Y104F mutant
AbmU-Y104F-R	aAGTACATCATCAGGTGCGCGTCCGACG	
AbmU-Y143F-F	GGTCGGGCGGCCGGTtCGGCTCCATGATC	For construction of AbmU/ Y143F mutant
AbmU-Y143F-R	aACCGGCCGCCCGACCCCAGGATGGGG	
AbmU-Y163F-F	CGCCGCACAGCCTCTtCCGCACCCACC	For construction of AbmU/ Y163F mutant
AbmU-Y163F-R	aAGAGGCTGTGCGGCGCCTCGGGGGTGG	

Supporting References

1. Datsenko, K. A.; Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia Coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.U.S.A.* **2000**, *97*, 6640-6645.
2. MacNeil, D. J.; Gewain, K. M.; Ruby, C. L.; Dezeny, G.; Gibbons, P. H.; MacNeil, T. Analysis of *Streptomyces Avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **1992**, *111*, 61-68.
3. Gust, B.; Kieser, T.; Chater, K. F. *Redirect Technology: PCR-Targeting System in Streptomyces coelicolor A3 (2);* John Innes Centre: Norwich, 2002.
4. Hong, B.; Phornphisutthimas, S.; Tilley, E.; Baumberg, S.; McDowall, K. J. Streptomycin production by *Streptomyces griseus* can be modulated by a mechanism not associated with change in the *adpA* component of the A-factor cascade. *Biotechnol. Lett.* **2007**, *29*, 57-64.
5. Tu, J.; Li, S.; Chen, J.; Song, Y.; Fu, S.; Ju, J.; Li, Q. Characterization and heterologous expression of the neoabyssomicin/abyssomicin biosynthetic gene cluster from *Streptomyces koyangensis* SCSIO 5802. *Microb. Cell Fact.* **2018**, *17*, No. 28.