Supplementary Information

NocU is a Cytochrome P450 Oxygenase Catalyzing *N*-Hydroxylation of the Indolic Moiety during the Maturation of the Thiopeptide Antibiotics Nocathiacins

Heng Guo,[a,‡] Xuebing Bai,[b,‡] Qian Yang,[a,‡] Yufeng Xue,[a] Dandan Chen,[a,c,*] Jiang Tao,[b,*] and Wen Liu[a,c,*]

[‡] These authors equally contributed to this work.

[a] State Key Laboratory of Bioorganic and Natural Products Chemistry, Center for Excellence on Molecular Synthesis, Shanghai Institute of Organic Chemistry, University of Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, China.

[b] Department of General Dentistry, Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine; National Clinical Research Center for Oral Diseases; Shanghai Key Laboratory of Stomatology & Shanghai Research Institute of Stomatology, 639 Zhizaoju Road, Shanghai 200011, China.

[c] Huzhou Center of Bio-Synthetic Innovation, 1366 Hongfeng Road, Huzhou 313000, China.

* To whom correspondence should be addressed:

Dandan Chen, Email: ddchen@sioc.ac.cn, Tel: 86-21-54925539;

Jiang Tao, E-mail: taojiang_doctor@hotmail.com, Tel: 86-21-53515202;

Wen Liu, Email: wliu@mail.sioc.ac.cn, Tel: 86-21-54925111, Fax: 86-21-64166128.

Table of Contents

Supplementary Material and Methods

Bacterial strains, plasmids and cultures.

The bacterial strains and plasmids used in this study are listed in Table S1. *Escherichia coli* strains were cultivated in Luria-Bertani (LB) medium supplemented with ampicillin or kanamycin. *Streptomyces actuosus* strains were grown on Mannitol Soya Flour (MS) agar plates for sporulation. For fermentation, 1×1 cm^2 MS agar consisting of *S. actuosus* spores were inoculated into 100 mL of seed medium (sucrose 2.0%, corn steep liquor 3.0%, peptone 0.5% and $CaCO_3$ 0.5%, pH 7.3-7.6) and incubated at 28℃ and 220 rpm for 24 hrs. 10 mL of seed culture was then transferred into 100 mL of fermentation medium (Pharmamedia cotton meal 1.0%, NaCl 0.3%, glucose 3.0% and $CaCO_3$ 0.3%, pH 7.3-7.6) and incubated at 28℃ for 4 days.

Construction of mutant and recombinant strains

All the primers used in this study are listed in Table S2. To heterologous express *nocU*, the genome of *Nocardia* sp. ATCC202099 was used as the template for PCR amplification [1]. A 1.1 kb *nocU*-containing fragment was obtained using the primers NocU-DC-1-SE (containing the *Bam*HI site) and NocU-DC-1-RE. Another 1.1 kb *nocU*-containing fragment was obtained using the primers NocU-DC-2-SE and NocU-DC-2-RE (containing the *Xba*I site). After sequencing to confirm fidelity, the DNA fragments were co-ligated into the *Bam*HI/*Xba*I site in a pSET152 derivative containing the constitutive promoter *PermE**, yielding the recombinant plasmid pSL6001, in which two copies of *nocU* were ligated and under the control of *PermE**. Introduction of pSL6001 into SL5001 was carried out via *E. coli*-Streptomyces intergeneric conjugation[1], generating the recombinant strain SL6001.

Structure examination and compound isolation

Each *S. actuosus* fermentation broth was subjected to structure examination. After removal of the supernatant by centrifugation, the mycelia cake was soaked with acetone of an equal volume, processed in an ultrasonic machine for 20 min, and then centrifuged for 2 min at 12,000 rpm to

collect the acetone sample. The acetone sample was subjected to HPLC analysis on an Agilent Zorbax column (SB-C18, 4.6×250 mm, 5 μm, Agilent Technologies Inc., USA) by gradient elution of solvent A (H_2O + 10 mM CH_3COONH_4) and solvent B (acetonitrile) with a flow rate of 1 mL/min over a 26 min period as follows: T = 0 min, 20% B; T = 2 min, 20% B; T = 22 min, 80% B; T = 23 min, 20% B; T = 26 min, 20% B. Absorbance was monitored at 330 nm. Related data were analyzed using Thermo Xcalibur software.

A total of 35 L of *S. actuosus* fermentation broth was subjected to compound isolation. The acetone sample was evaporated in a vacuum to remove acetone. The solvent was dissolved in H_2O, and then extracted four times with an equal volume of EtOAc together with the supernatant. Upon monitoring via HPLC analysis, the organic layer was sequentially subjected to silica chromatography (300-400 mesh, Qingdao Marine Chemical Inc., Qingdao, China) with a dichloromethane/methanol system (1:0, 50:1, 30:1, 15:1, 10:1, 7:1). The target fraction was eluted with dichloromethane/methanol 10:1, and was further purified by semi-preparative HPLC performed on an Agilent 1100 with a Zorbax SB-C18 column (9.4 mm×25 cm) via gradient elution of solvent A (H_2O + 10m M CH_3COONH_4) and solvent B (acetonitrile) at a flow rate of 3 mL/min over a 35 min period as follows: T = 0 min, 35% B; T = 8 min, 35% B; T = 13 min, 40% B; T = 18 min, 45% B; T = 20 min, 45% B; T=23 min, 50% B; T=25 min, 95% B; T=29 min, 95% B; T=35 min, 35% B.

Protein expression and purification

For over-expression of *nocU in E. coli*, the genome of *Nocardia* sp. ATCC20209 was used as the template for PCR amplification [1]. A 1.1 kb DNA fragment containing *nocU* obtained using the primers NocU-pET37b-1 (containing the *Nde*I site) and NocU-pET37b-2 (containing the *Xho*I site) was ligated into pET37b, yielding pSL6002. The fidelity of *nocU* in pSL6002 was confirmed by DNA sequencing.

The plasmid pSL6002 were introduced into *E. coli* BL21(DE3) for over-expression of NocU. The *E. coli* cells were grown at 37°C and 250 rpm until the cell density reached 0.5-0.6 at OD_{600}. Then, isopropyl-β-D thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After

incubation at 16°C and 250 rpm for 32 hrs, the *E. coli* cells were harvested and re-suspended in lysis buffer (50 mM phosphate, 10 mM imidazole and 300 mM NaCl, pH 7.5). After sonication, the soluble protein fraction was loaded onto a Ni-NTA resin (Qiagen, Valencia, CA) column for affinity purification. The purified protein fraction was then dialyzed in TSG buffer (50 mM Tris-HCl, 25 mM NaCl, 10% glycerol and 0.02% NaN_3, pH 7.5) at 4°C. Finally, the resulting NocU protein was concentrated for *in vitro* assays.

Characterization of *in vitro* enzymatic reactions

Spectra of purified NocU in storage buffer were recorded from 300 to 600 nm. The ferric heme in NocU was prepared in a reduced condition by adding saturated solution of dithionite ($Na_2S_2O_4$) and was exposed to CO for 1 min; the spectrum of the reduced enzyme was then recorded.

The reaction mixture (100 μL) contained substrate (50 μM), ferredoxin-$NADP^+$ reductase (0.1 U/mL), ferredoxin (50 μg/mL), NADPH (1.0 mM), glucose-6-phosphate (glucose-6-P, 10 mM), glucose-6-P dehydrogenase (1 U/mL), and 10 μM NocU. Three different types of ferredoxin-$NADP^+$ reductase and ferredoxin (from Cyanophyta, Spinach, or Micromonospora) were tested. The reactions were incubated at 30 °C for 3 hrs. To quench the reaction, an equal volume of methanol was added. NocU that was boiled at 100 °C for 10 min were used in the control reactions. After removal of the precipitate by centrifugation, the supernatant was subjected to HPLC and HPLC-ESI-MS analyses.

Bioassays for antibacterial activities against oral pathogens

Oral Gram-positive (*Streptococcus mutans* UA159, *Lactobacillus acidophilus* ATCC4356, *Actinomyces viscosus* ATCC19246, and *Enterococcus faecalis* ATCC29212) and Gram-negative (*Fusobacterium nucleatum* ATCC25286 and *Porphyromonas gingivalis* ATCC33277) bacteria were used to determine the antibacterial activities of NOS, NOS1260, NOS-U. Experiments were carried out according to the methods described previously [2, 3]. The MIC value was defined as the lowest concentration that inhibited visible bacterial growth. All tests were carried out 3 times.

Table S1. Strains and plasmids used in this study.

Strains/Plasmids	Description	Source / Reference
Strains		
Escherichia coli		
DH5α	Host for general cloning	Transgen
BL21(DE3)	Host for protein expression	Novagen
ET12567 (pUZ8002)	Donor strain for conjugation between *E.coli* and *Streptomyces actuosus*	[4]
Streptomyces actuosus		
ATCC25421	Wild-type nosiheptide (NOS)-producing strain	from H. G. Floss
SL5001	*nosABC* in-frame deletion mutant of ATCC 25421, NOS1260 producing strain	[3]
SL6001	SL5001 derivative for heterologous expression of *nocU*, NOS-U producing strain	This study
Oral pathogenic bacteria		
Streptococcus mutans	Oral caries pathogenic bacteria, Gram-positive	UA159
Lactobacillus acidophilus	Oral caries pathogenic bacteria, Gram-positive	ATCC4356
Actinomyces viscosus	Oral caries pathogenic bacteria, Gram-positive	ATCC19246
Enterococcus faecalis	Oral caries pathogenic bacteria, Gram-positive	ATCC29212
Fusobacterium nucleatum	Oral periodontopathic bacteria, Gram-negative	ATCC25286
Porphyromonas gingivalis	Oral periodontopathic bacteria, Gram-negative	ATCC33277
Plasmids		
pSET152	*E. coli-Streptomyces actuosus* shuttle vector for gene heterologous expression	[5]
pSL6001	pSET152 derivative containing a 2.2 kb fragment, construct for over-expressing *nocU*	This study
pET37b	*E. coli* protein expression vector	Novagen
pSL6002	pET37b derivative containing a 1.1kb fragment, construct for NocU expression	This study

Table S2. Primers used in this study. Small letters indicate the homologous sequences. Underlined letters indicate the recognition sites of restriction endonucleases.

Primer	Sequence
NocU-DC-1-SE	gaaatcgataagcttggatccGGAGATCGCCGCGCGCGC (*Bam*HI)
NocU-DC-1-RE	tggtggtcatGTCAGCCATCGCTCCTCGC
NocU-DC-2-SE	gatggctgacATGACCACCACGATCCCCG
NocU-DC-2-RE	gggctgcaggtcgactctagaACGGCCGCCCGGTCGGGG (*Xba*I)
NocU-pET37b-1	atacatatgATGACCACCACGATCCCCG (*Nde*I)
NocU-pET37b-2	gtgctcgagGCCATCGCTCCTCGCCGG (*Xho*I)

Table S3. ^{1}H and ^{13}C NMR data for NOS-U in Tetrahydrofuran-d8 (δ in ppm, *J* in Hz).

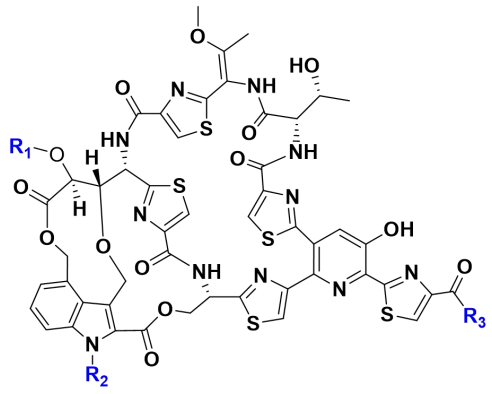
Substructure (No.)	δ_C	δ_H (mult., *J*, Hz)
Thz 1 (2)	164.3 s	
Thz 1 (4)	148.6 s	
Thz 1 (5)	122.8 d	8.09 (s, 1H)
Thz 1 (C=O)	159.0 s	
Thr (1)	169.3 s	
Thr (2)	53.8 d	5.40 (m, overlap, 1H)
Thr (3)	67.4 d	4.43 (m, 1H)
Thr (4)	17.9 q	1.11 (br s, 3H)
Thr (NH)		7.84 (d, J = 7.7 Hz, 1H)
But (2)	131.2 s	
But (3)	132.0 d	6.63 (m, overlap, 1H)
But (4)	12.2 q	1.84 (br s, 1H)
But (NH)		10.57 (s, 1H)
Thz 2 (2)	169.8 s	
Thz 2 (4)	153.2 s	
Thz 2 (5)	120.4 d	8.07 (s, 1H)
Thz 2 (C=O)	159.2 s	
Glu (1)	169.9 s	
Glu (2)	31.2 t	2.46 (m, 1H)
		2.38 (m, overlap, 1H)
Glu (3)	29.7 t	2.36 (m, overlap, 1H)
		2.21 (m, 1H)
Glu (4)	48.0 d	5.48 5.48 (t, J = 4.6 Hz, 1H)
Glu (NH)		8.21 (br s, overlap, 1H)
Thz 3 (2)	168.8 s	
Thz 3 (4)	149.4 s	
Thz 3 (5)	123.2 d	8.21 (br s, overlap, 1H)
Thz 3 (C=O)	163.2 s	
Cys (2)	48.7 d	5.98 (m, overlap, 1H)
Cys (3)	28.3 t	4.03 (m, 1H)
		3.86 (m, 1H)
Cys (NH)		7.91 (d, J = 9.8 Hz, 1H)
Thz 4 (2)	168.1 s	

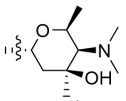
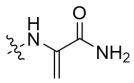
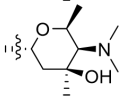
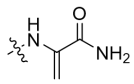
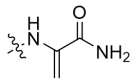
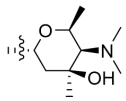
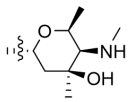
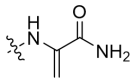
Thz 4 (4)	150.5 s	
Thz 4 (5)	126.2 d	8.52 (m, 1H)
Pyr (2)	151.4 s	
Pyr (3)	128.4 s	
Pyr (4)	139.3 d	8.33 (d, J = 8.0 Hz, 1H)
Pyr (5)	117.4 d	8.45 (d, J = 8.0 Hz, 1H)
Pyr (6)	149.3 s	
Thz 5 (2)	167.2 s	
Thz 5 (4)	150.2 s	
Thz 5 (5)	123.8 d	8.63 (s, 1H)
Thz 5 (C=O)	157.8 s	
Dha 2 (1)	161.0 s	
Dha 2 (2)	134.0 s	
Dha 2 (3)	100.0 t	6.82 (m, 1H)
		5.60 (m, overlap, 1H)
Dha 2 (NH)		10.14 (s, 1H)
Dha 1 (1)	163.9 s	
Dha 1 (2)	137.3 s	
Dha 1 (3)	106.3	6.66 (m, overlap, 1H)
		5.99 (m, overlap, 1H)
Dha 1 (NH)		8.98 (br s, 1H)
Ind (2)	130.0 s	
Ind (3)	118.5 s	
Ind (3a)	124.8 s	
Ind (4)	129.7 s	
Ind (5)	122.0 d	7.10 (d, J = 7.0 Hz, 1H)
Ind (6)	124.0 d	7.26 (m, 1H)
Ind (7)	113.1 d	7.70 (br s, 1H)
Ind (7a)	137.2 s	
Ind (3')	11.2 q	2.71 (s, 3H)
Ind (4')	64.7 t	5.58 (m, overlap, 1H)
		5.40 (m, overlap, 1H)
Ind (N-OH)		10.93 (s, 1H)
Ind (C=O)	180.1 s	

Table S4. MIC values of NOS-U, NOS, and NOS1260 against oral pathogens.

Oral pathogen	**MIC** (μg mL^{-1})		
	NOS1260	NOS-U	NOS
Streptococcus mutans UA159	0.052	1.041	0.009
Lactobacillus acidophilus ATCC 4356	0.026	4.160	0.032
Actinomyces viscosus ATCC 19246	0.039	0.104	0.001
Enterococcus faecalis ATCC 29212	0.312	10	0.104
Fusobacterium nucleatum ATCC 25286	10	10	5
Porphyromonas gingivalis ATCC 33277	0.208	0.521	0.020

Fig. S1. Chemical structures of NOC-series metabolites [6-10].



	R_1	R_2	R_3
Nocathiacin I (MJ347-81F4 A):		OH	
Nocathiacin II:		H	
Nocathiacin III:	H	OH	
Nocathiacin IV:		OH	NH_2
MJ347-81F4 B:		OH	

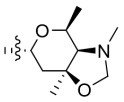
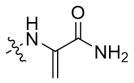
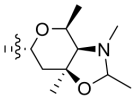
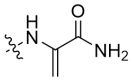
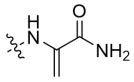
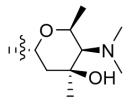
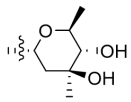
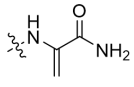
	R_1	R_2	R_3
Thiazomycin:		OH	
Thiazomycin A:		OH	
Thiazomycin B:	H	H	
Thiazomycin C:		H	NH_2
Thiazomycin D:		OH	

Fig. S2. Biosynthetic gene clusters of NOCs and NOS [1, 11]. Organization of the *noc* biosynthetic gene cluster in comparison with the *nos* biosynthetic gene cluster. The deduced functions of biosynthetic genes are labeled in pattern, and the sequence homologies are indicated by a dashed line for each pair. Different from *nosJ* and *nosK* that are two contiguous gene, *nocJ* and *nocK* are fused into one gene.

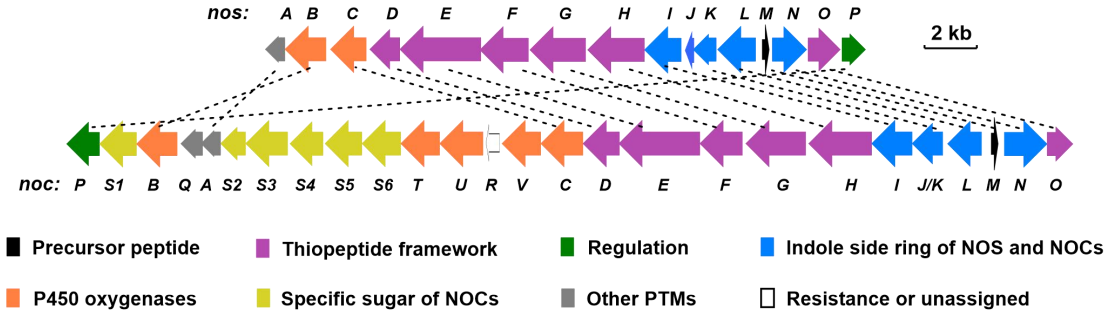
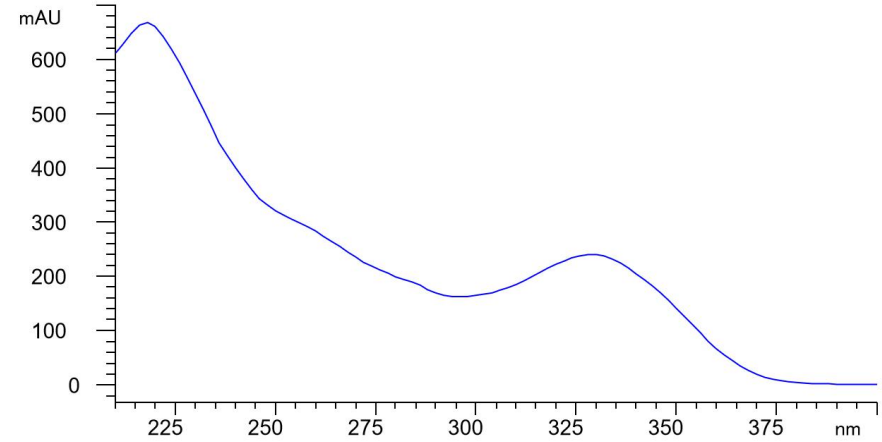


Fig. S3. UV spectra of NOS-U and NOS1260. A, UV spectrum of NOS-U; B, UV spectrum of NOS1260.

A



B

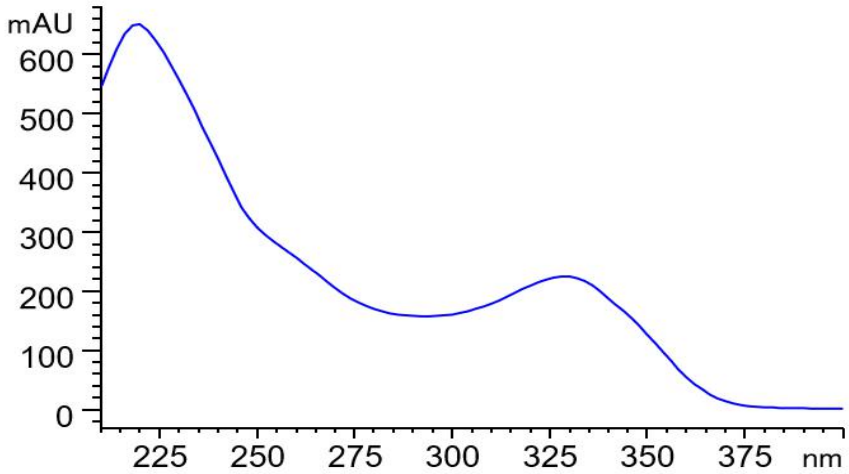


Fig. S4. HR-ESI-MS analysis of NOS-U.

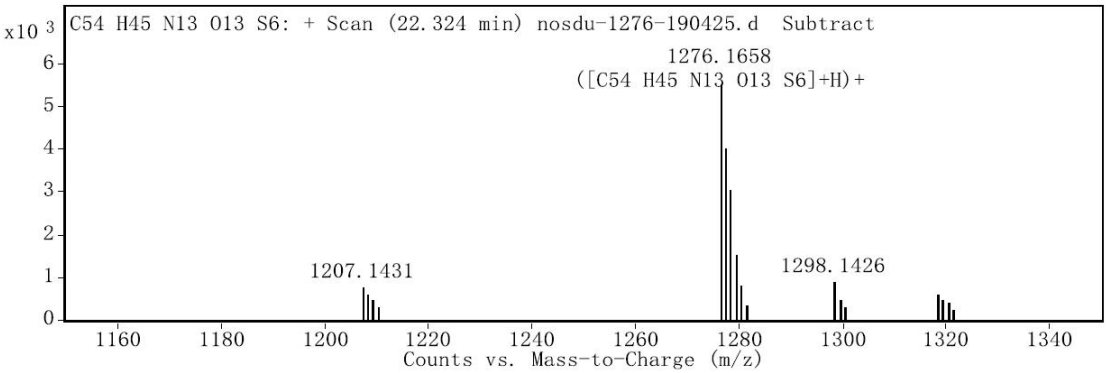


Fig. S5. Structure of NOS-U alongside the HR MS^2 data. The fragmental masses are in accordance with the characterized structural moieties. After fragmentation and protonation, the indolic moiety would undergo complex transformations, such as N-O bond cleavage.

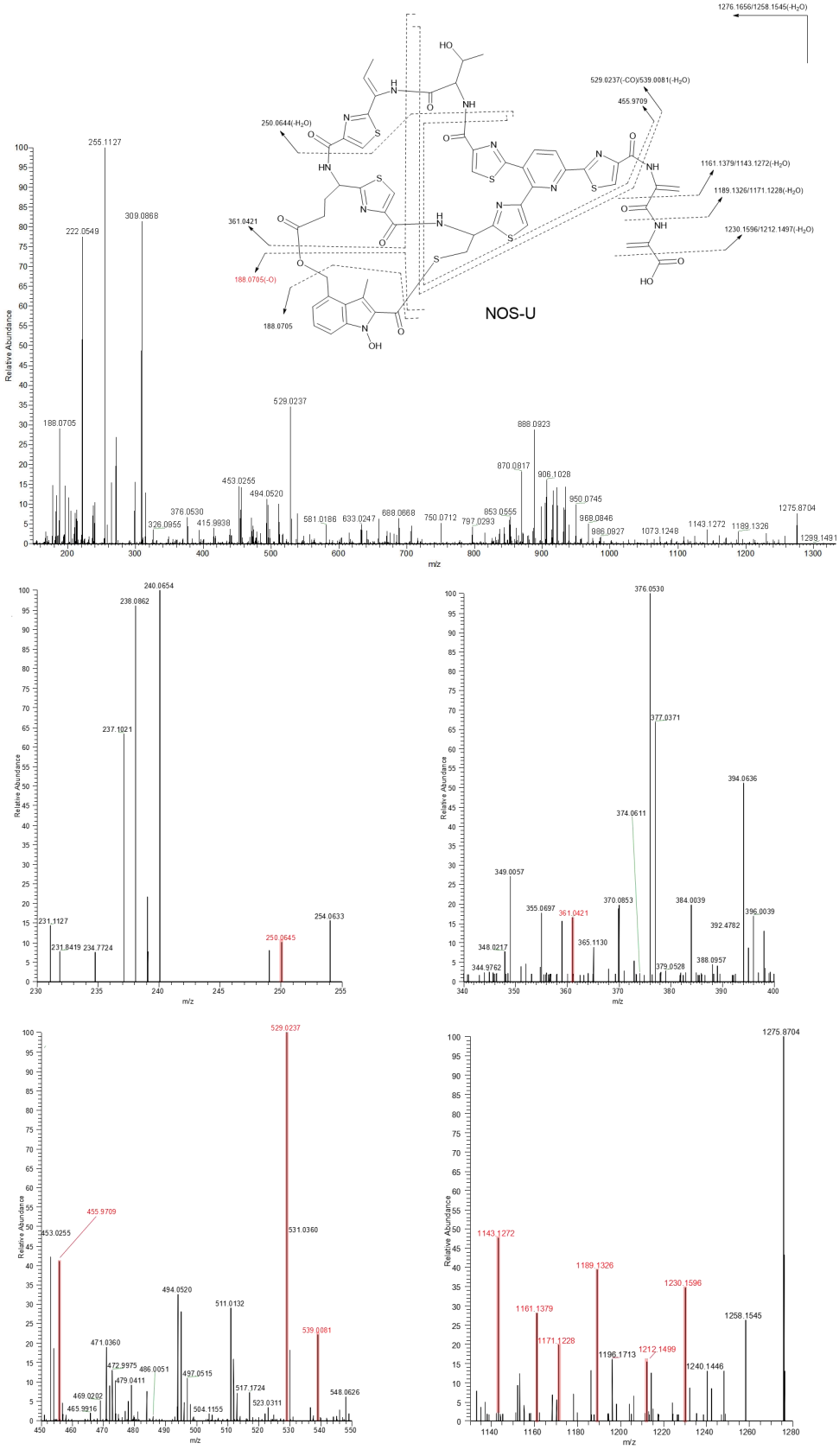


Fig. S6. Structure of NOS1260 alongside the HR MS^2 data. The fragmental masses are in accordance with the characterized structural moieties.

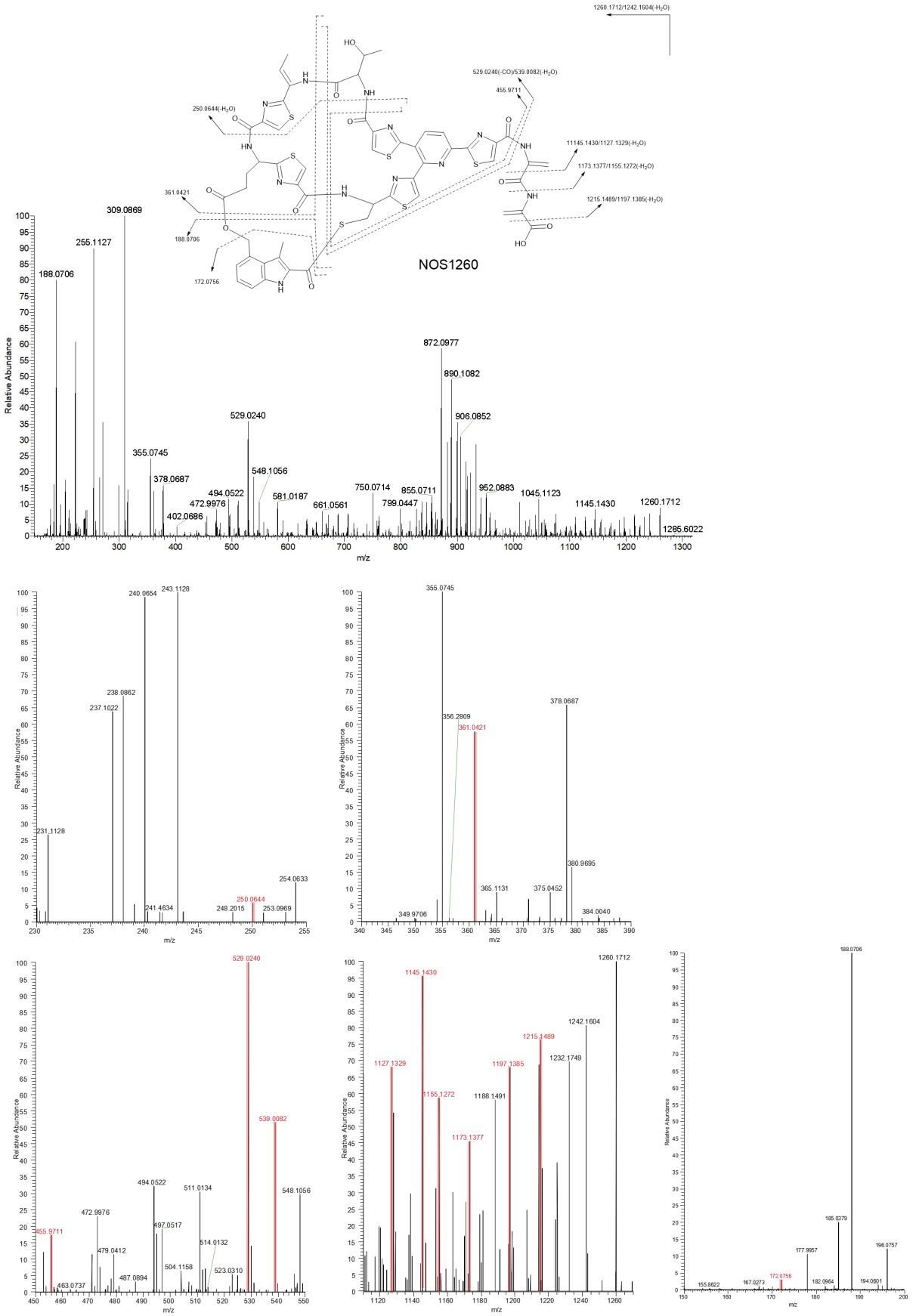


Fig. S7. Structure of NOS-U.

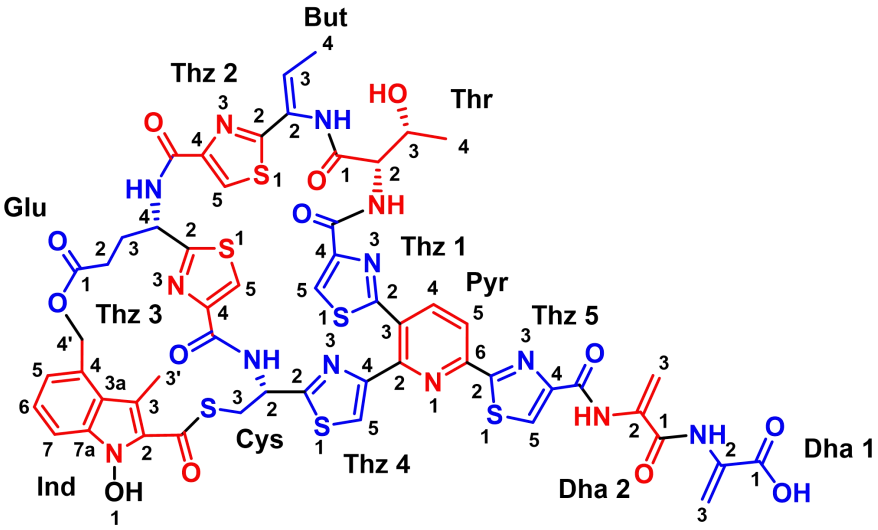
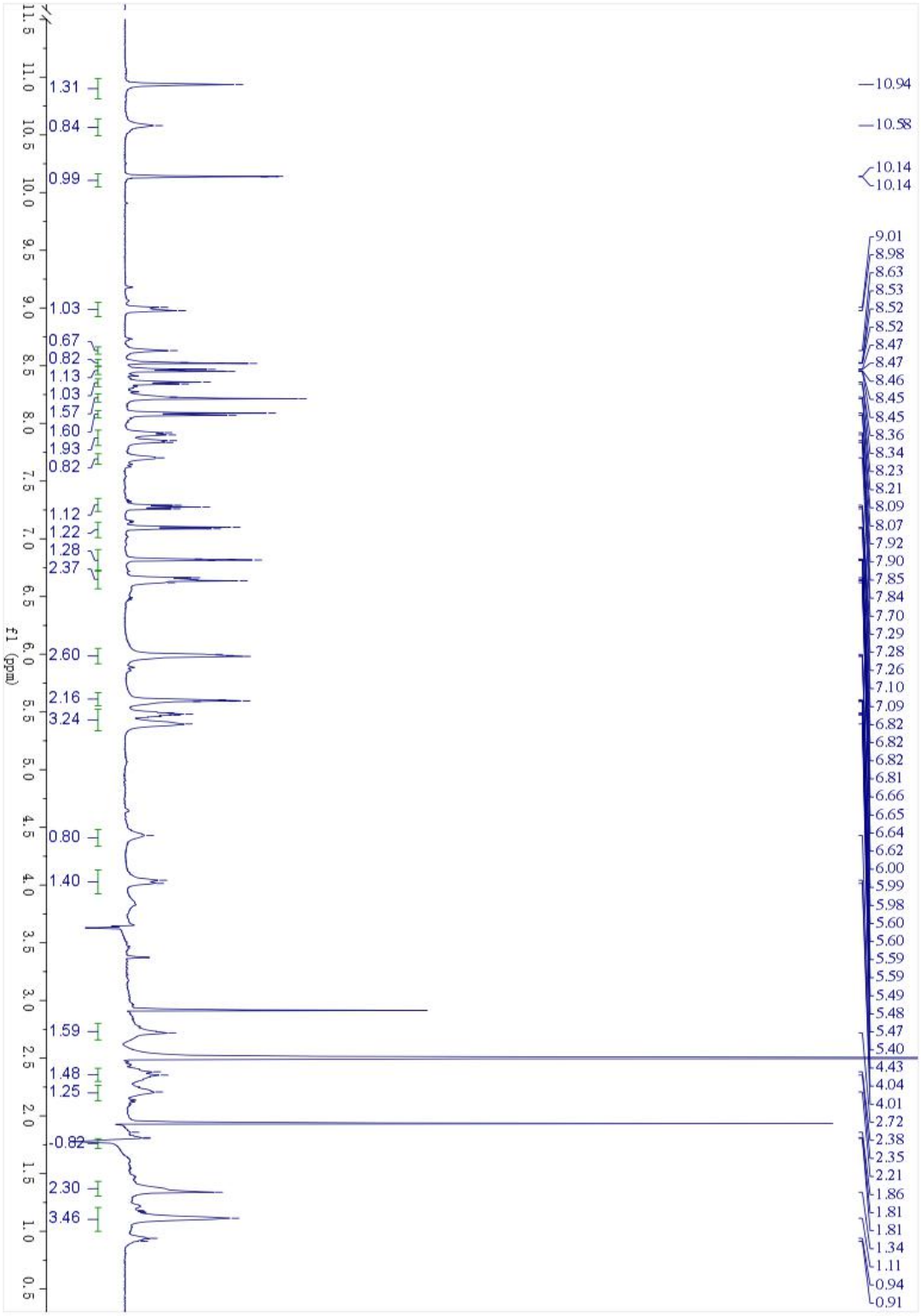
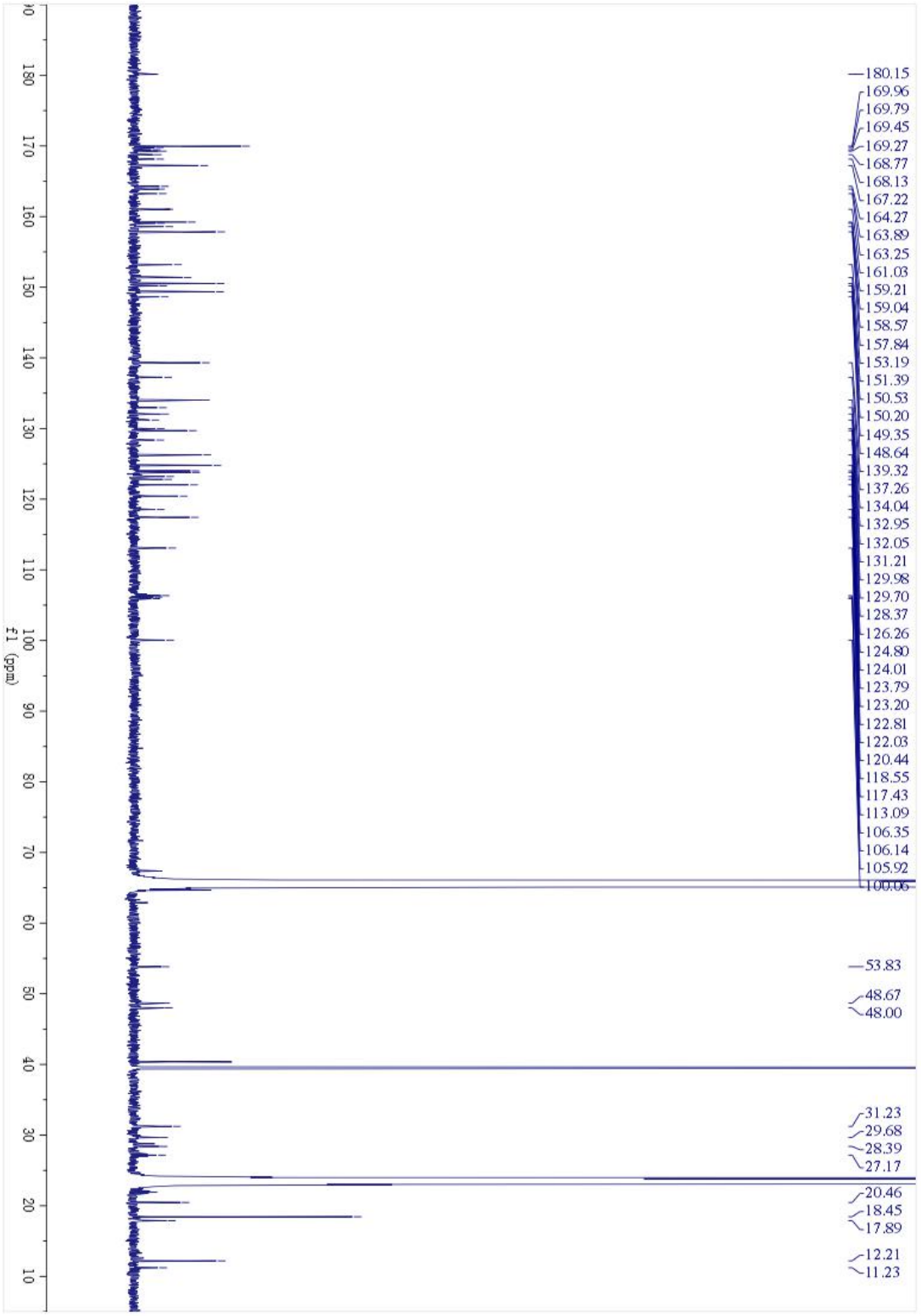


Fig. S8. NMR spectra of NOS-U. A, ^{1}H NMR (DMSO-d_6, 500MHz); B, ^{13}C NMR (DMSO-d_6, 500MHz); C, HSQC; D, ^{1}H-^{1}H COSY; E, HMBC; F, ROESY.

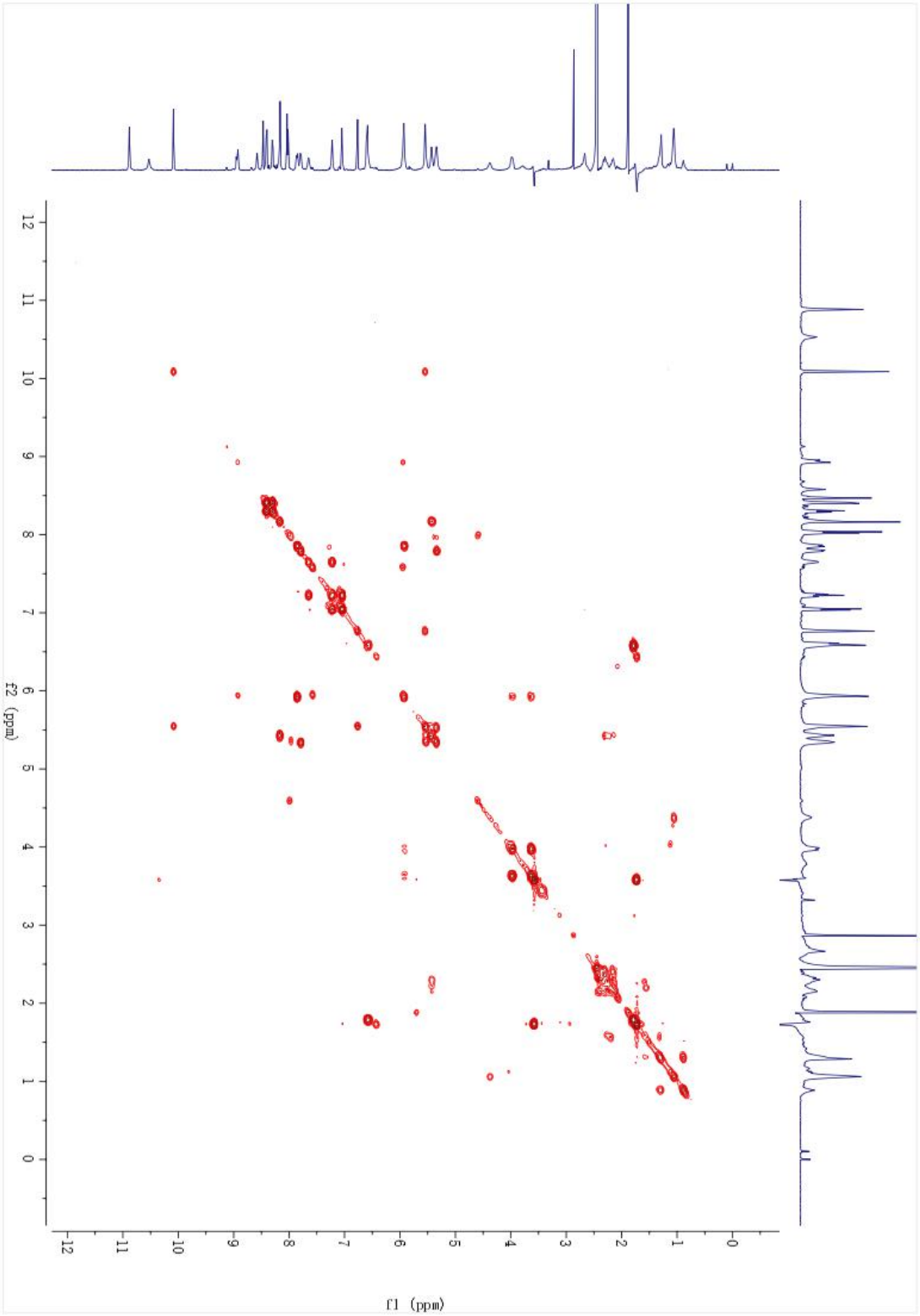
A



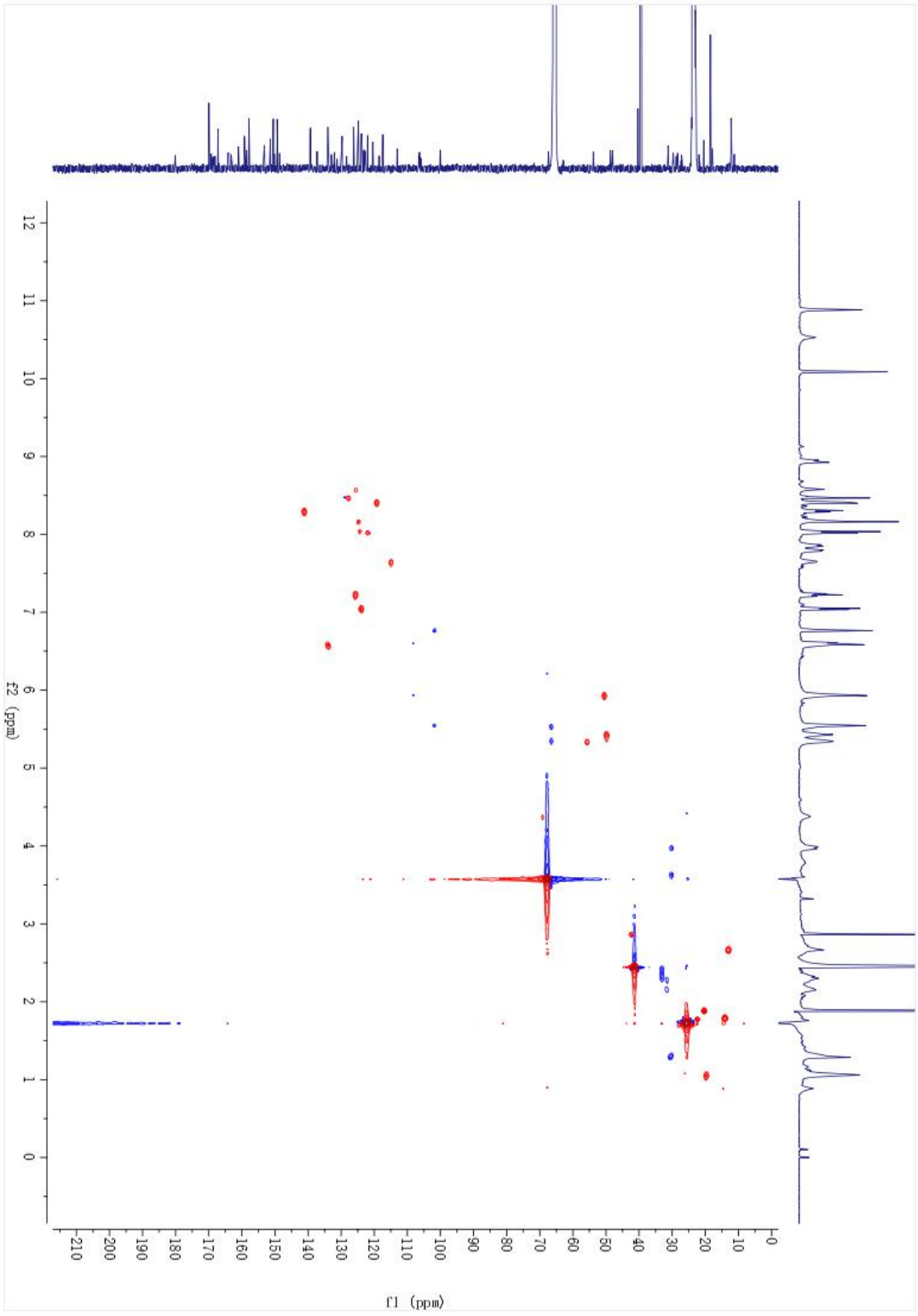
B


f1 (ppm)
180.15
169.96
169.79
169.45
169.27
168.77
168.13
167.22
164.27
163.89
163.25
161.03
159.21
159.04
158.57
157.84
153.19
151.39
150.53
150.20
149.35
148.64
139.32
137.26
134.04
132.95
132.05
131.21
129.98
129.70
128.37
126.26
124.80
124.01
123.79
123.20
122.81
122.03
120.44
118.55
117.43
113.09
106.35
106.14
105.92
53.83
48.67
48.00
31.23
29.68
28.39
27.17
20.46
18.45
17.89
12.21
11.23

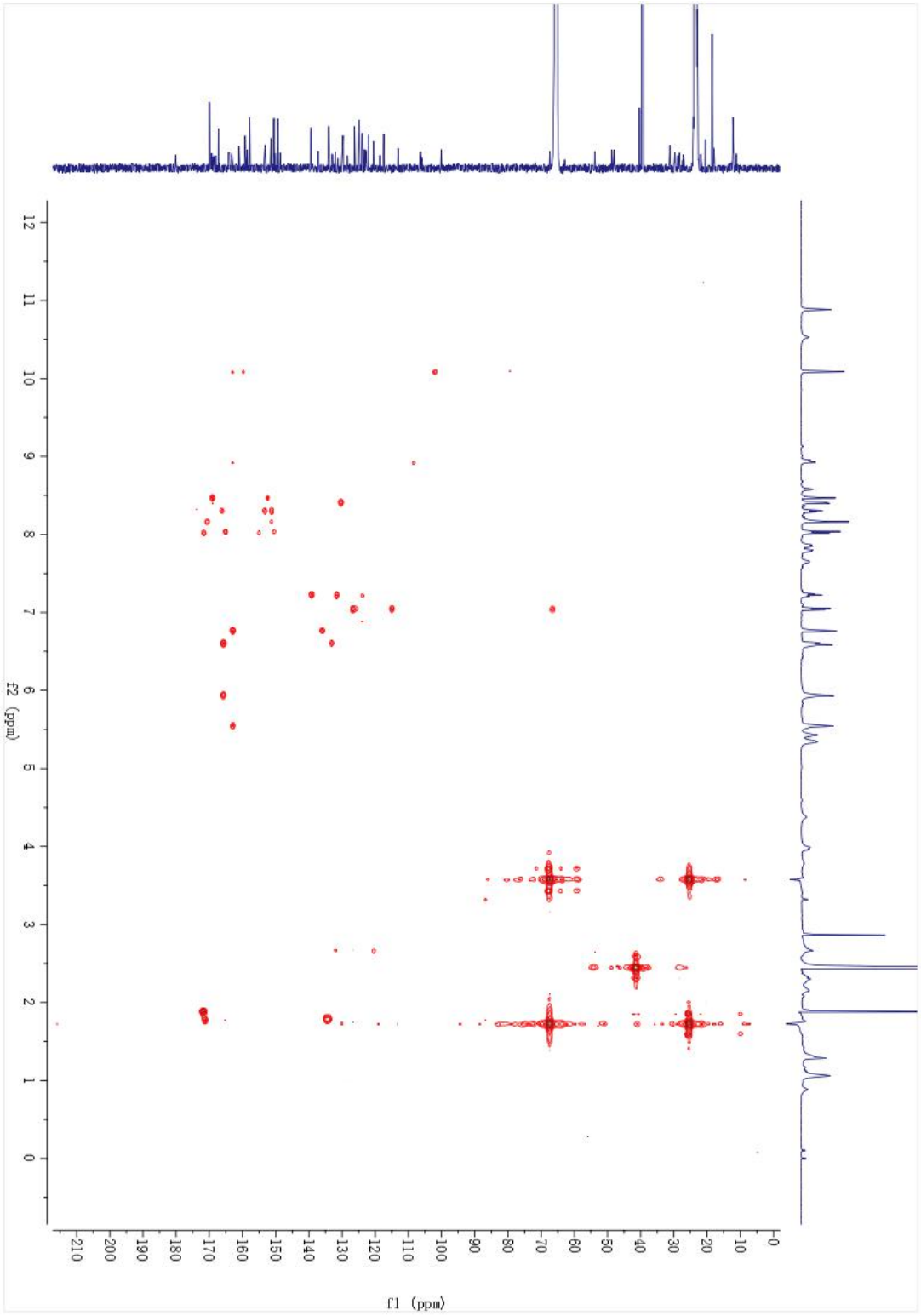
C



D



E



F

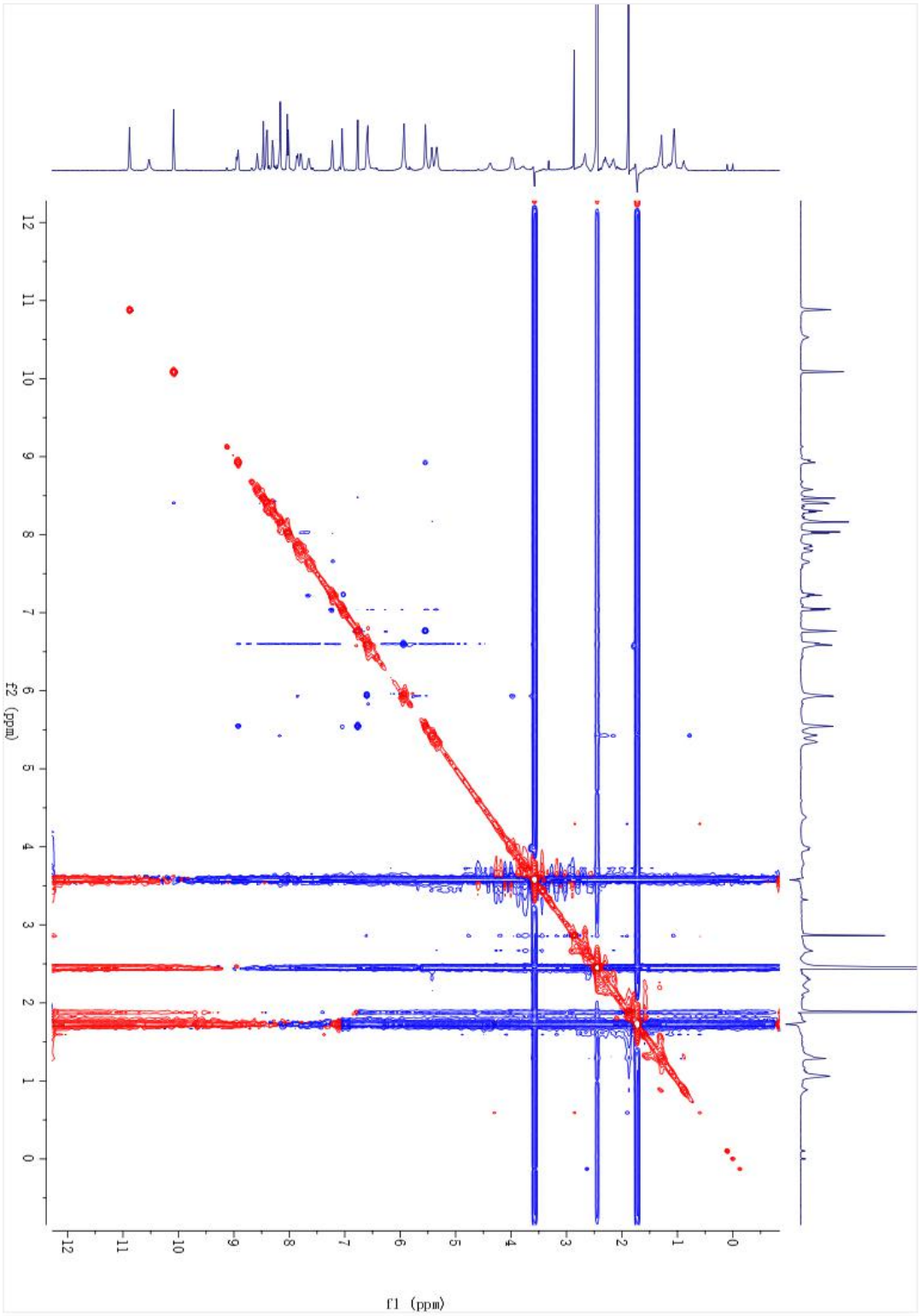


Fig. S9. SDS-PAGE of purified recombinant NocU. Lane 1, NocU (expected MW to be 45 kDa); Lane 2, protein marker.

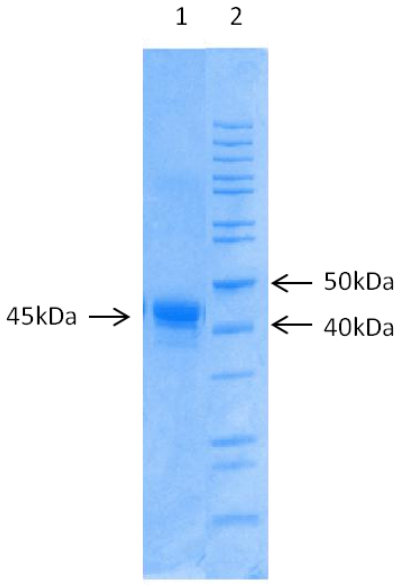


Fig. S10. UV-vis absorbance of NocU in reducing conditions with or without CO. I, the absorbance spectrum of the pure NocU in 100 mM Tris-HCl buffer (pH 7.5); II, absorptions of this solution after bubbling of CO for 1 min followed by reduction of dithionite.

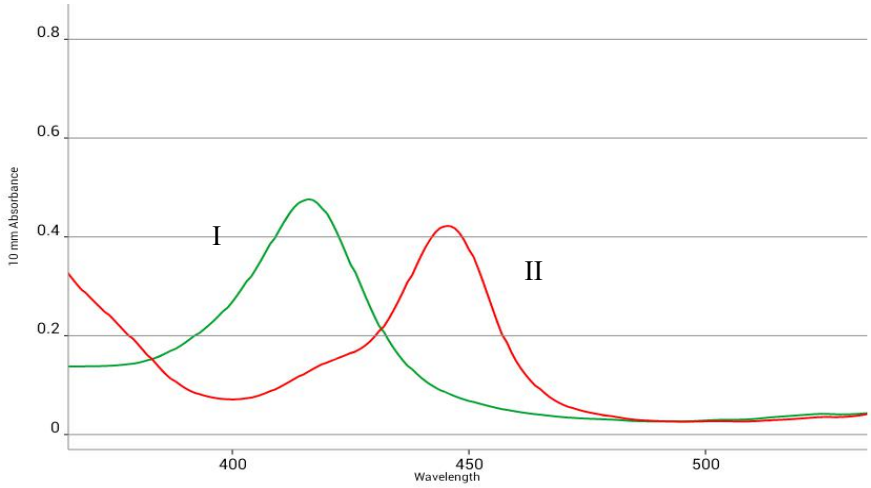
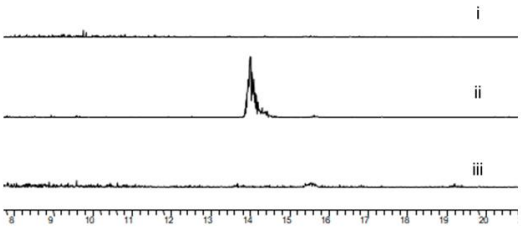
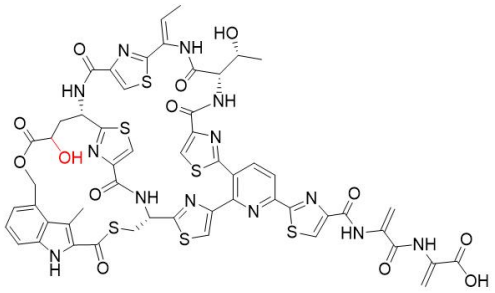


Fig. S11. Extracted ion chromatogram traces of NocU-catalyzed reactions. i, negative control: no enzyme reaction using NOS1260 as substrate, EIC^+ = 1276-1277. ii, positive control [3]: NocB reaction using NOS1260 as the substrate, EIC^+ = 1276-1277. iii, NocU reaction using NOS1260 as the substrate, EIC^+ = 1276-1277. The chemical structures of newly-obtained compound was predicted considering the proved function of NocB [12].





Supplementary References

1. Y. Ding, Y. Yu, H. Pan, H. Guo, Y. Li and W. Liu, *Mol. Biosyst.*, 2010, **6**, 1180-1185.
2. J. Wang, Z. Lin, X. Bai, J. Tao and W. Liu, *Org. Chem. Front.*, 2019, **6**, 1194-1199.
3. X. Bai, H. Guo, D. Chen, Q. Yang, J. Tao and W. Liu, *Org. Chem. Front.*, 2020, **7**, 584-589.
4. T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater and D. A. Hopwood, *Practical Streptomyces genetics*, John Innes Foundation, Norwich, 2000.
5. M. Bierman, R. Logan, K. O'Brien, E. T. Seno, R. N. Rao and B. E. Schoner, *Gene*, 1992, **116**, 43-49.
6. C. Zhang, K. Herath, H. Jayasuriya, J. G. Ondeyka, D. L. Zink, J. Occi, G. Birdsall, J. Venugopal, M. Ushio, B. Burgess, P. Masurekar, J. F. Barrett and S. B. Singh, *J. Nat. Prod.*, 2009, **72**, 841-847.
7. H. Jayasuriya, K. Herath, J. G. Ondeyka, C. Zhang, D. L. Zink, M. Brower, F. P. Gailliot, J. Greene, G. Birdsall, J. Venugopal, M. Ushio, B. Burgess, G. Russotti, A. Walker, M. Hesse, A. Seeley, B. Junker, N. Connors, O. Salazar, O. Genilloud, K. Liu, P. Masurekar, J. F. Barrett and S. B. Singh, *J. Antibiot.*, 2007, **60**, 554-564.
8. C. Zhang, D. L. Zink, M. Ushio, B. Burgess, R. Onishi, P. Masurekar, J. F. Barrett and S. B. Singh, *Bioorg. Med. Chem.*, 2008, **16**, 8818-8823.
9. J. E. Leet, W. Y. Li, H. A. Ax, J. A. Matson, S. Huang, R. Huang, J. L. Cantone, D. Drexler, R. A. Dalterio and K. S. Lam, *J. Antibiot.*, 2003, **56**, 232-242.
10. T. Sasaki, T. Otani, H. Matsumoto, N. Unemi, M. Hamada, T. Takeuchi and M. Hori, *J. Antibiot.*, 1998, **51**, 715-721.
11. Y. Yu, L. Duan, Q. Zhang, R. Liao, Y. Ding, H. Pan, E. Wendt-Pienkowski, G. Tang, B. Shen and W. Liu, *ACS Chem. Biol.*, 2009, **4**, 855-864.
12. X. Wu, P. Huang, Y. Xue, W. Liu, M. Ma and Y. Chen, *RSC Adv.*, 2016, **6**, 72399-72408.