Supplementary Information

Isolation and Structure Determination of Two New Nosiheptide-Type Compounds Provide Insights into the Function of the Cytochrome P450 Oxygenase NocV in Nocathiacin Biosynthesis

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Table of Contents

1. Supplementary Methods

1.1. General materials and methods

1.2. Construction of mutant and recombinant strains

1.3. Strain fermentation and compound isolation

1.4. Protein expression and purification

1.5. Characterization of *in vitro* enzymatic reactions

1.6. Bioassays for antibacterial activities against oral pathogens

2. Supplementary Results

Table S1. Strains and plasmids used in this study

Table S2. Primers used in this study

Table S3. ^{1}H and ^{13}C NMR data for NOS-V1 in DMSO-d6 (δ in ppm, *J* in Hz)

Table S4. ^{1}H and ^{13}C NMR data for NOS-V2 in DMSO-d6 (δ in ppm, *J* in Hz)

Fig. S1. UV spectra of NOS-V1, NOS-V2, NOS1260

Fig. S2. HR-ESI-MS analysis of NOS-V1

Fig. S3. Chemical structure of NOS-V1

Fig. S4. NMR spectra of NOS-V1

Fig. S5. HR-ESI-MS analysis of NOS-V2

Fig. S6. Chemical structure of NOS-V2

Fig. S7. NMR spectra of NOS-V2

Fig. S8. SDS-PAGE of purified recombinant NocB and NocV

Fig. S9. UV-vis absorbance of NocB/V in reducing conditions with or without CO

Fig. S10. Extracted ion chromatogram traces of NocB/V-catalyzed reactions

3. Supplementary References

1. Supplementary Methods

1.1. General materials and methods

Materials, bacterial strains, and plasmids. The bacterial strains and plasmids used in this study are listed in Table S1. Biochemicals and media were purchased from Sinopharm Chemical Reagent Co., Ltd. (China), Oxoid Ltd. (U.K.) or Sigma-Aldrich Co. LLC. (USA). Enzymes were purchased from Takara Biotechnology Co. Ltd. (China) unless otherwise stated. Restriction endonucleases were purchased from Thermo Fisher Scientific Co. Ltd. (USA). Chemical reagents were purchased from standard commercial sources.

DNA isolation, manipulation, and sequencing. Primers used for PCR amplifications in this study were listed in Table S2. DNA isolation and manipulation in *E. coli*, *Streptomyces actuosus* or *Nocardia* sp. were carried out according to standard methods[1]. PCR amplifications were carried out on an Applied Biosystems Veriti Thermal Cycler using either Taq DNA polymerase (Vazyme Biotech Co. Ltd, China) for routine genotype verification or PrimeSTAR HS DNA polymerase (Takara Biotechnology Co., Ltd. Japan) for high fidelity amplification. Primer synthesis was performed at Shanghai Sangon Biotech Co. Ltd. (China). DNA sequencing was performed at Shanghai Tsingke Biotech Co. Ltd. (China).

General chemical analysis. High performance liquid chromatography (HPLC) analysis was carried out on an Agilent 1260 HPLC system (Agilent Technologies Inc., USA) equipped with a DAD detector. Semi-preparative HPLC was performed on an Agilent 1100 system. HPLC electrospray ionization MS (HPLC-ESI-MS) was performed on a Thermo Fisher LTQ Fleet ESI-MS spectrometer (Thermo Fisher Scientific Inc., USA), and the data were analyzed using Thermo Xcalibur software. ESI-high resolution MS (ESI-HR-MS) analysis was carried out on an instrument consisting of a 1260 HPLC system or a 6538 UHD quadrupole time of flight (QTOF) high resolution mass spectrometry (Agilent Technologies, Santa Clara, USA). NMR

data were recorded on a Bruker AV-600 or Bruker AV-500 spectrometer (Bruker Co. Ltd, Germany).

1.2. Construction of mutant and recombinant strains

Construction of SL5001 via gene inactivation of *nosABC* in *S. actuosus* ATCC25421. To inactivate *nosABC*, the cosmid pSL4001 served as the template for PCR amplification[2]. A 2 kb DNA fragment obtained using the primers ABCcoprm-1 (containing the *Hin*dIII site) and ABCcoprm-2 and a 2 kb DNA fragment obtained using the primers ABCcoprm-3 and ABCcoprm-4 (containing the *Eco*RI site) were linked by overlapping PCR, yielding a 4 kb DNA fragment. After sequencing to confirm fidelity, the 4 kb DNA fragment was digested using *Hin*dIII and *Eco*RI and ligated into the *Hin*dIII/*Eco*RI site in pKC1139, giving the recombinant plasmid pSL5001, in which a 3 kp in-frame coding region of *nosABC* was deleted. Introduction of pSL5001 into *S. actuosus* ATCC25421 was carried out via *E. coli-Streptomyces* intergeneric conjugation, following a procedure described previously[1]. The colonies that were apramycin-resistant at 37℃ were identified as the integrating mutants, in which a single-crossover homologous recombination event occurred. These mutants were further cultured in liquid TSB medium for three rounds in the absence of apramycin. The genotypes of the resulting apramycin-sensitive strains were confirmed by PCR amplification and DNA sequencing, leading to identification of the mutant strain SL5001.

Construction of SL5002 via heterologous expression of *nocV* in *S. actuosus* SL5001. To overexpress *nocV*, the genome of *Nocardia* sp. ATCC202099 was used as the template for PCR amplification[3]. A 1.1 kb *nocV*-containing fragment was obtained using the primers NocV-DC-1-SE (containing the *Bam*HI site) and NocV-DC-1-RE. Another 1.1 kb *nocV*-containing fragment was obtained using the primers NocV-DC-2-SE and NocV-DC-2-RE (containing the *Xba*I site). After sequencing to confirm fidelity, these DNA fragments were co-ligated into the *Bam*HI/*Xba*I site in a pSET152 derivative containing the constitutive promoter *PermE** (from vector pLL6212), using a ClonExpress® One Step Cloning Kit (Vazyme Biotech Co. Ltd, China), yielding the

recombinant plasmid pSL5002, in which two copies of *nocV* were ligated and under the control of the *PermE** promoter. Introduction of pSL5002 into SL5001 was carried out via *E. coli-Streptomyces* intergeneric conjugation, generating the corresponding recombinant strain SL5002 for heterologous expression of *nocV*.

1.3. Strain fermentation and compound isolation

Culture and fermentation. For sporulation, the *S. actuosus* strains were grown on MS agar plates at 30ºC for 5 days. For production, 1×1 cm^2 MS agar consisting of *S. actuosus* spores were inoculated into a 500-mL flask containing 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. Then, 10 mL of seed culture was 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) in a 500-mL flask and incubated at 28℃ for 4 days.

Structural examination. Each fermentation broth was centrifuged for 15 min at 4,000 rpm. After removal of the supernatant, 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 or filtered 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.

Compound extraction and purification. A total of 30 L of *S. actuosus* fermentation broth was subjected to compound isolation according to the method described. 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 isolation and purification via 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 + 10 mM 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.

1.4. Protein expression and purification

Construction of protein expression plasmids. For heterologous expression of *nocB* and *nocV in E. coli*, the genome of *Nocardia* sp. ATCC20209 was used as the template for PCR amplification. A 1.2 kb DNA fragment containing *nocB* obtained using the primers NocB-pET37b-1 (containing the *Nde*I site) and NocB-pET37b-2 (containing the *Xho*I site) was ligated into pET37b, yielding pSL5003. A 1.1 kb DNA fragment containing *nocV* obtained using the primers NocV-pET37b-1 (containing the *Nde*I site) and NocV-pET37b-2 (containing the *Xho*I site) was ligated into pET37b, yielding pSL5004. The fidelity of *nocB* in pSL5003 and *nocV* in pSL5004 was confirmed by DNA sequencing.

Protein expression. The plasmids pSL5003 and pSL5004 were introduced into *E. coli* BL21(DE3) for heterologous expression of NocB and NocV, in the C-terminally 6 x His-tagged form, respectively. Cells were grown in LB medium supplemented with 50 μg/mL kanamycin at 37°C and 250 rpm until the cell density reached 0.5-0.6 at OD_{600}. To induce protein expression, isopropyl-β-D thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The cells were further incubated at 16°C for 32 hrs.

Protein purification. *E. coli* cells were harvested, re-suspended in lysis buffer (50

mM phosphate, 10 mM imidazole and 300 mM NaCl, pH 7.5). After sonication (8 × 10 sec pulsed cycle), the soluble fraction of the produced protein 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 NocB and NocV protein was concentrated and stored at -80 °C for *in vitro* assays.

1.5. Characterization of *in vitro* enzymatic reactions

CO binding difference spectra. Spectra of purified NocB and NocV in storage buffer were recorded from 300 to 600 nm. The ferric heme in NocB and NocV 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.

***In vitro* activity assays of NocB and NocV.** 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 NocB or NocV in Tris-HCl buffer (50 mM, pH 7.5). Three different types of ferredoxin-$NADP^+$ reductase and ferredoxin (from Cyanophyta, Spinach, or Micromonospora) were used in this study. The reactions were incubated at 30 °C for 3 hrs. To quench the reaction, an equal volume of methanol was added. NocB and NocV that were 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.

1.6. Bioassays for antibacterial activities against oral pathogens

Determination of minimum inhibitory concentrations (MICs). 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-V1 and NOS-V2. Experiments were carried out according to the methods described previously for thiostrepton (TSR) and siomycin (SIO)[4]. For culture of Gram-positive bacteria, brain-heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) was used and the bacteria were incubated in an anaerobic box (37℃, 5% CO_2) for 24 hrs. For culture of Gram-negative bacteria, BHI containing hemin (Sigma-Aldrich, St. Louis, MO, USA) and menadione (Sigma-Aldrich, St. Louis, MO, USA) (BHI-modified) were used, and the bacteria were incubated in an anaerobic box (37℃, 5% CO_2) for 48 hrs. Each tested sample was dissolved in DMSO to produce a stock solution (100 μg/mL), which was serially diluted into 100 μL of BHI or BHI-modified broth according to the corresponding strain in a 96-well microtiter plate to a final concentration ranging from 20 to 0 μg/mL in two-fold serial. Next, 100 μL of the testing strain (calculated according to 0.5 McFarland diluted 1:100 to a final concentration of 0.5-1×10^6 CFU/mL) was added into each well of the microtiter plate, followed by 24-48 hrs of incubation period under either aerobic or anaerobic condition accordingly (*S. mutans*, *L. acidophilus*, *A. viscosus* and *E. faecalis* for 24 hrs; *F. nucleatum* and *P. gingivalis* for 48 hrs). Sodium fluoride (NaF) and chlorhexidine (CHX) were chosen as Gram-positive controls; levofloxacin (LVX) and minocycline hydrochloride (MCC) were chosen as Gram-negative controls. The MIC value was defined as the lowest concentration that inhibited visible bacterial growth. All tests were carried out 3 times.

2. Supplementary Results

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) S17-1	Donor strain for conjugation between *E.coli* and *Streptomyces actuosus*	[1]
Streptococcus mutans UA159	Oral caries pathogenic bacteria, Gram-positive	ATCC
Lactobacillus acidophilus ATCC4356	Oral caries pathogenic bacteria, Gram-positive	ATCC
Actinomyces viscosus ATCC19246	Oral caries pathogenic bacteria, Gram-positive	ATCC
Enterococcus faecalis ATCC29212	Oral caries pathogenic bacteria, Gram-positive	ATCC
Fusobacterium nucleatum ATCC25286	Oral periodontopathic bacteria, Gram-negative	ATCC
Porphyromonas gingivalis ATCC33277	Oral periodontopathic bacteria, Gram-negative	ATCC
Streptomyces actuosus		
ATCC25421	Wild-type nosiheptide (NOS)-producing strain	ATCC, from H. G. Floss
SL5001	*nosABC* in-frame deletion mutant of ATCC 25421, NOS1260 producing strain	This study
SL5002	SL5001 derivative for heterologous expression of *nocV*, NOS-V1 and NOS-	This study

	V2 producing strain	
Plasmids		
pKC1139	*E. coli-Streptomyces* shuttle vector for gene inactivation	[5]
pSL5001	pKC1139 derivative containing a 4 kb fragment, construct for *nosABC* in-frame deletion	This study
pSET152	*E. coli-Streptomyces* shuttle vector for gene heterologous expression	[5]
pSL5002	pSET152 derivative containing a 2.2 kb fragment, construct for over-expressing *nocV*	This study
pET37b	*E. coli* protein expression vector	Novagen
pSL5003	pET37b derivative containing a 1.2kb fragment, construct for NocB expression	This study
pSL5004	pET37b derivative containing a 1.1kb fragment, construct for NocV 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**
ABCcoprm-1	GTCAAGCTTGGCGACGGCCTCGAGCAGCT (*Hin*dIII)
ABCcoprm-2	acaccccgttcgtaccGCCGGGCGCCGA
ABCcoprm-3	ggtacgaacggggtgtGGCGGGTGAAGG
ABCcoprm-4	CCTGAATTCTCGGCGACCCGCACAGCGAC (*Eco*RI)
NocV-DC-1-SE	gaaatcgataagcttggatccGGAGGGCATCCTGCGCCT (*Bam*HI)
NocV-DC-1-RE	acgctcatggcggttcccttTCCCGG
NocV-DC-2-SE	aagggaaccgccatgagcgtCGCACTCGCC
NocV-DC-2-RE	gggctgcaggtcgactctagaCAGCGACTTCTCCAGGTCGG (*Xba*I)
NocB-pET37b-1	taagaaggagatatacatatgATGACCCGCGCCGACGCC (*Nde*I)
NocB-pET37b-2	gtggtggtggtggtgctcgagTCGGGCGTCCGTTCGCTC (*Xho*I)
NocV-pET37b-1	taagaaggagatatacatatgATGAGCGTCGCACTCGCC (*Nde*I)
NocV-pET37b-2	gtggtggtggtggtgctcgagGCTGTAGGGCAGTGCCCG (*Xho*I)

Table S3. ^{1}H and ^{13}C NMR data for NOS-V1 in DMSO-d_6 (δ in ppm, J in Hz).

Substructure (No.)	δ_C	δ_H, mult. (J, Hz)
Thz 1 (2)	164.6 s	
Thz 1 (4)	149.3 s	
Thz 1 (5)	126.1 d	8.23 s
Thz 1 (C=O)	160.1 s	
Thr (1)	169.1 s	
Thr (2)	57.5 d	4.53 br s
Thr (3)	67.8 d	4.46 m (overlap)
Thr (4)	20.4 q	1.13 d (6.2)
Thr (NH)		7.94 d (8.1)
But (2)	129.9 s	
But (3)	127.9 d	6.37 m (overlap)
But (4)	13.5 q	1.74 d (7.1)
But (NH)		9.56 s
Thz 2 (2)	165.9 s	
Thz 2 (4)	148.3 s	
Thz 2 (5)	123.9 d	8.13 s
Thz 2 (C=O)	159.9 s	
Glu (1)	169.4 s	
Glu (2)	40.5 t	2.38 m, 2.15 m
Glu (3)	68.2 d	4.46 m (overlap)
Glu (4)	54.1 d	5.28 dd (9.0, 9.1)
Glu (NH)		8.73 d (9.1)
Thz 3 (2)	168.1 s	
Thz 3 (4)	151.8 s	
Thz 3 (5)	122.3 d	8.25 s
Thz 3 (C=O)	160.6 s	
Cys (2)	47.9 d	5.71 m
Cys (3)	29.9 t	3.73 m (overlap), 3.33 m (overlap)
Thz 4 (2)	168.6 s	

Thz 4 (4)	149.6 s	
Thz 4 (5)	126.1 d	8.49 s
Pyr (2)	150.2 s	
Pyr (3)	128.5 s	
Pyr (4)	141.2 d	8.39 d (8.0)
Pyr (5)	118.4 d	8.30 d (8.0)
Pyr (6)	149.8 s	
Thz 5 (2)	167.4 s	
Thz 5 (4)	150.3 s	
Thz 5 (5)	128.6 d	8.64 s
Thz 5 (C=O)	158.8 s	
Dha 2 (1)	160.4 s	
Dha 2 (2)	135.3 s	
Dha 2 (3)	103.4 t	6.37 m (overlap), 5.55 m (overlap)
Dha 2 (NH)		10.10 s
Dha 1 (1)	164.8 s	
Dha 1 (2)	137.8 s	
Dha 1 (3)	100.4 t	6.00 s, 5.55 m (overlap)
Dha 1 (NH)		9.75 s
Ind (2)	131.2 s	
Ind (3)	119.1 s	
Ind (3a)	125.1 s	
Ind (4)	129.9 s	
Ind (5)	122.5 d	7.02 d (7.1)
Ind (6)	124.9 d	7.19 dd (8.3, 7.0)
Ind (7)	113.8 d	7.40 d (8.3)
Ind (7a)	137.4 s	
Ind (3')	13.1 q	2.61 s
Ind (4')	65.7 t	5.55 m (overlap), 5.22 d (12.1)
Ind (NH)		11.70 s
Ind (C=O)	182.0 s	

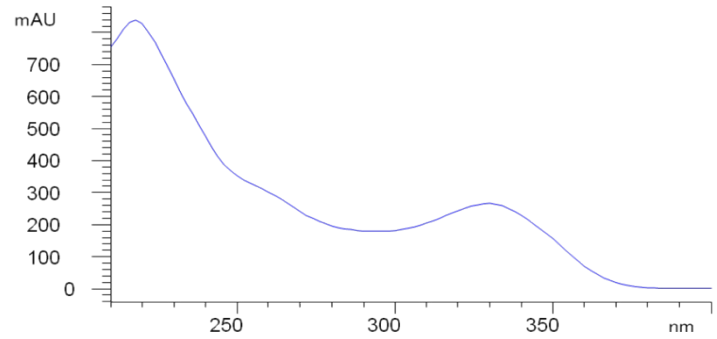
Table S4. ^{1}H and ^{13}C NMR data for NOS-V2 in DMSO-d_6 (δ in ppm, J in Hz).

Substructure (No.)	δ_C	δ_H, mult. (J, Hz)
Thz 1 (2)	164.1 s	
Thz 1 (4)	150.2 s	
Thz 1 (5)	126.6 d	8.81 s
Thz 1 (C=O)	158.4 s	
Thr (1)	166.8 s	
Thr (2)	55.4 d	4.14 dd (7.6, 3.7)
Thr (3)	65.7 d	2.51 brs (overlap)
Thr (4)	17.8 q	1.22 d (6.2)
Thr (NH)		7.22 d (7.6)
But (2)	129.1 s	
But (3)	128.9 d	6.40 m (overlap)
But (4)	13.9 q	1.57 d (6.9)
But (NH)		8.87 s
Thz 2 (2)	166.5 s	
Thz 2 (4)	147.1 s	
Thz 2 (5)	125.5 d	8.21 br s (overlap)
Thz 2 (C=O)	159.8 s	
Glu (1)	171.7 s	
Glu (2)	36.8 t	2.46 m (overlap), 2.46 m (overlap)
Glu (3)	78.8 d	4.06 m (overlap)
Glu (4)	52.2 d	5.88 d (9.3)
Glu (NH)		8.21 br s (overlap)
Thz 3 (2)	167.3 s	
Thz 3 (4)	148.5 s	
Thz 3 (5)	126.0 d	8.51 s
Thz 3 (C=O)	160.6 s	
Cys (2)	50.4 d	5.93 d (10.8)
Cys (3)	30.2 t	3.70 d (12.8), 3.45 d (13.3)
Cys (NH)		7.64 d (10.8)

Thz 4 (2)	169.3 s	
Thz 4 (4)	154.1 s	
Thz 4 (5)	121.5 d	8.10 s
Pyr (2)	151.5 s	
Pyr (3)	129.1 s	
Pyr (4)	140.9 d	8.43 d (8.1)
Pyr (5)	118.8 d	8.33 d (8.1)
Pyr (6)	149.5 s	
Thz 5 (2)	167.4 s	
Thz 5 (4)	150.1 s	
Thz 5 (5)	128.6 d	8.59 s
Thz 5 (C=O)	158.7 s	
Dha 2 (1)	161.5 s	
Dha 2 (2)	134.7 s	
Dha 2 (3)	104.6 t	6.40 m (overlap), 5.70 br s (overlap)
Dha 2 (NH)		10.08 s
Dha 1 (1)	164.8 s	
Dha 1 (2)	135.4 s	
Dha 1 (3)	106.9 t	6.00 br s, 5.70 br s (overlap)
Dha 1 (NH)		9.64 s
Ind (2)	132.7 s	
Ind (3)	113.2 s	
Ind (3a)	124.4 s	
Ind (4)	129.0 s	
Ind (5)	122.6 d	7.13 d (7.0)
Ind (6)	124.3 d	7.29 m
Ind (7)	115.7 d	7.69 d (8.3)
Ind (7a)	137.6 s	
Ind (3')	64.0 t	4.77 d (10.1), 4.06 m (overlap)
Ind (4')	67.0 t	5.76 d (12.4), 4.96 d (12.4)
Ind (NH)		11.62 s
Ind (C=O)	184.0 s	

Fig. S1. UV spectra of NOS-V1, NOS-V2, NOS1260. A, UV spectrum of NOS-V1; B, UV spectrum of NOS-V2; C, UV spectrum of NOS1260.

A



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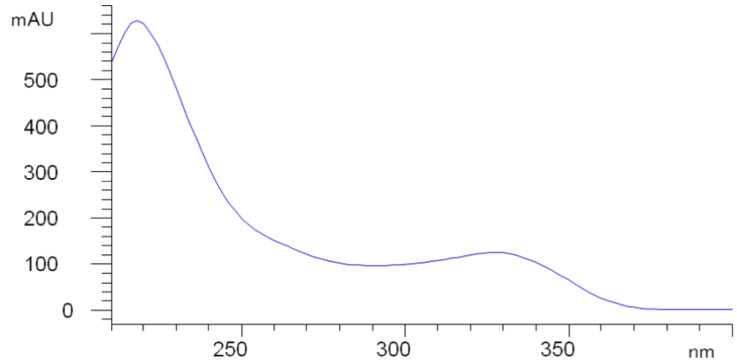

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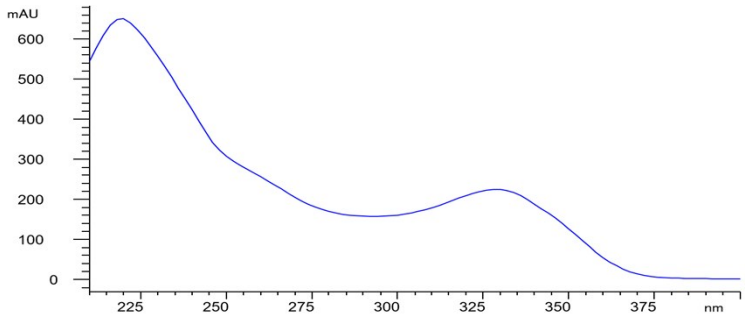


Fig. S2. HR-ESI-MS analyses of NOS-V1.

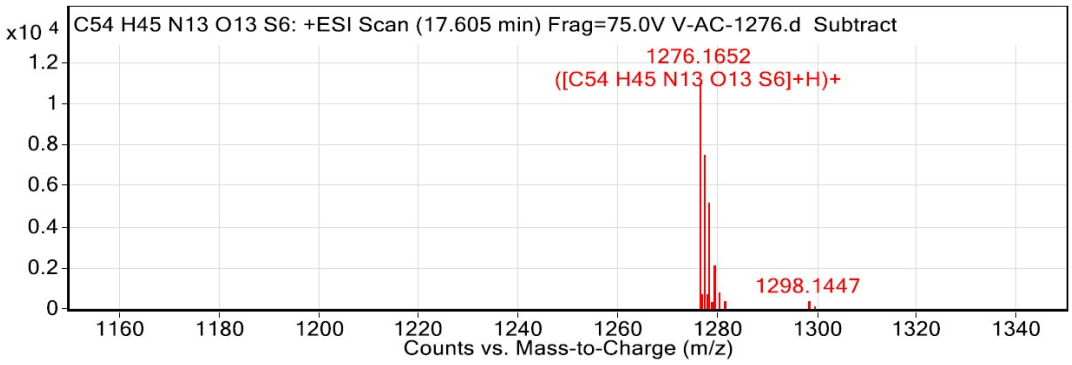


Fig. S3. Chemical structure of NOS-V1.

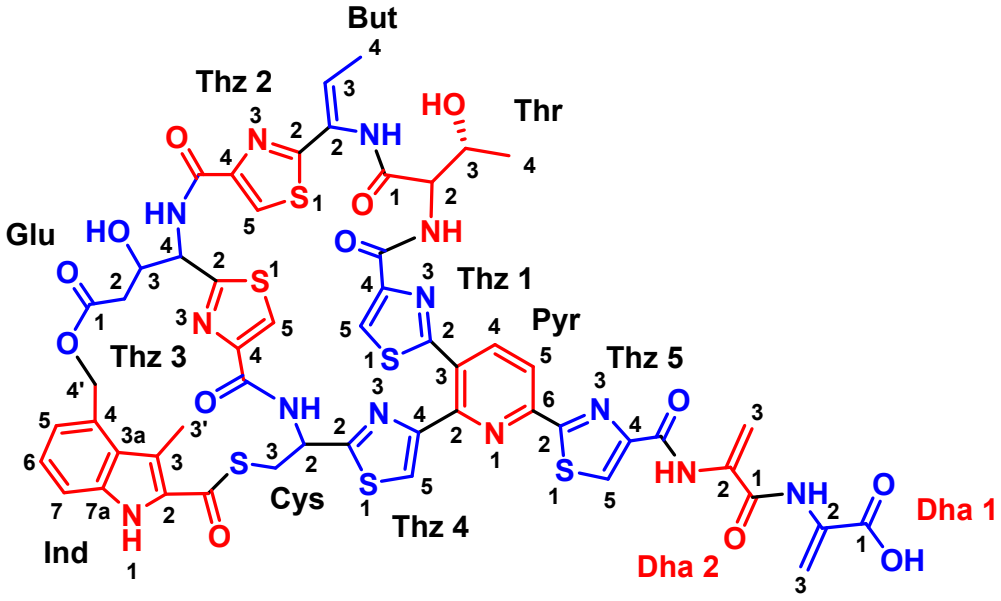
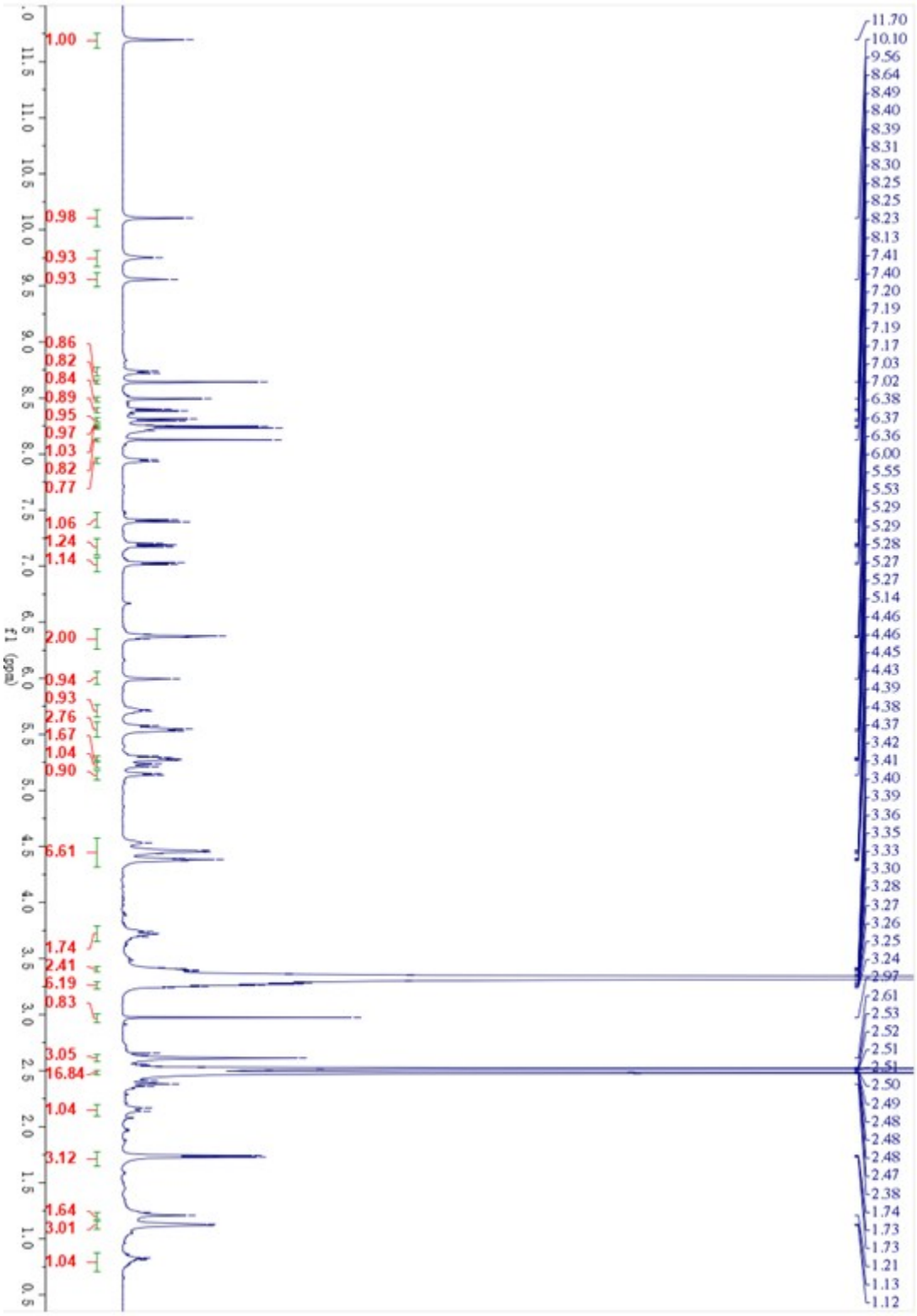
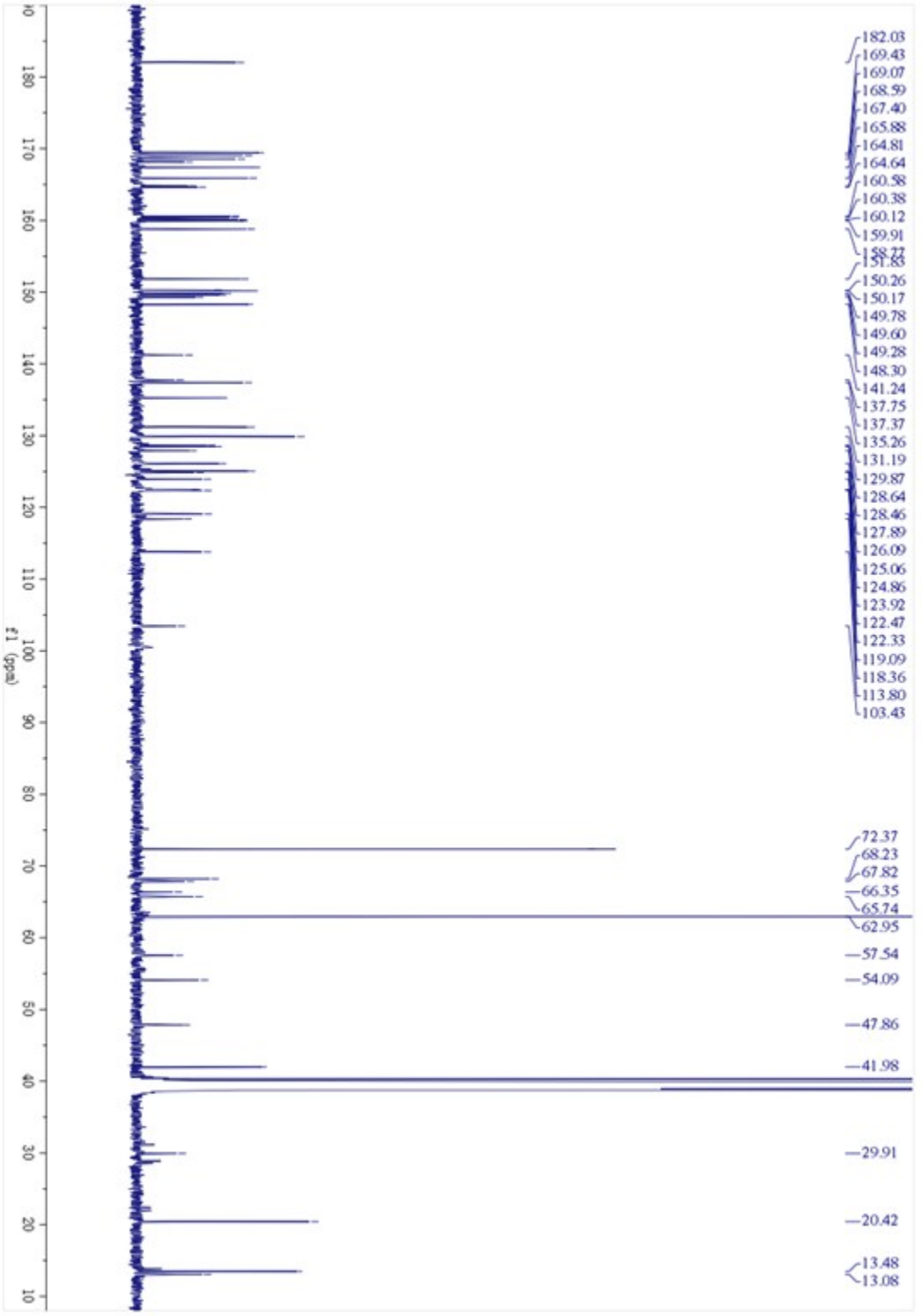


Fig. S4. NMR spectra of NOS-V1. A, ^{1}H NMR (DMSO-d_6, 500MHz); B, ^{13}C NMR (DMSO-d_6, 125 MHz); C, HSQC; D, ^{1}H-^{1}H COSY; E, HMBC; F, ROESY.

A



B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169.07
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167.40
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164.81
164.64
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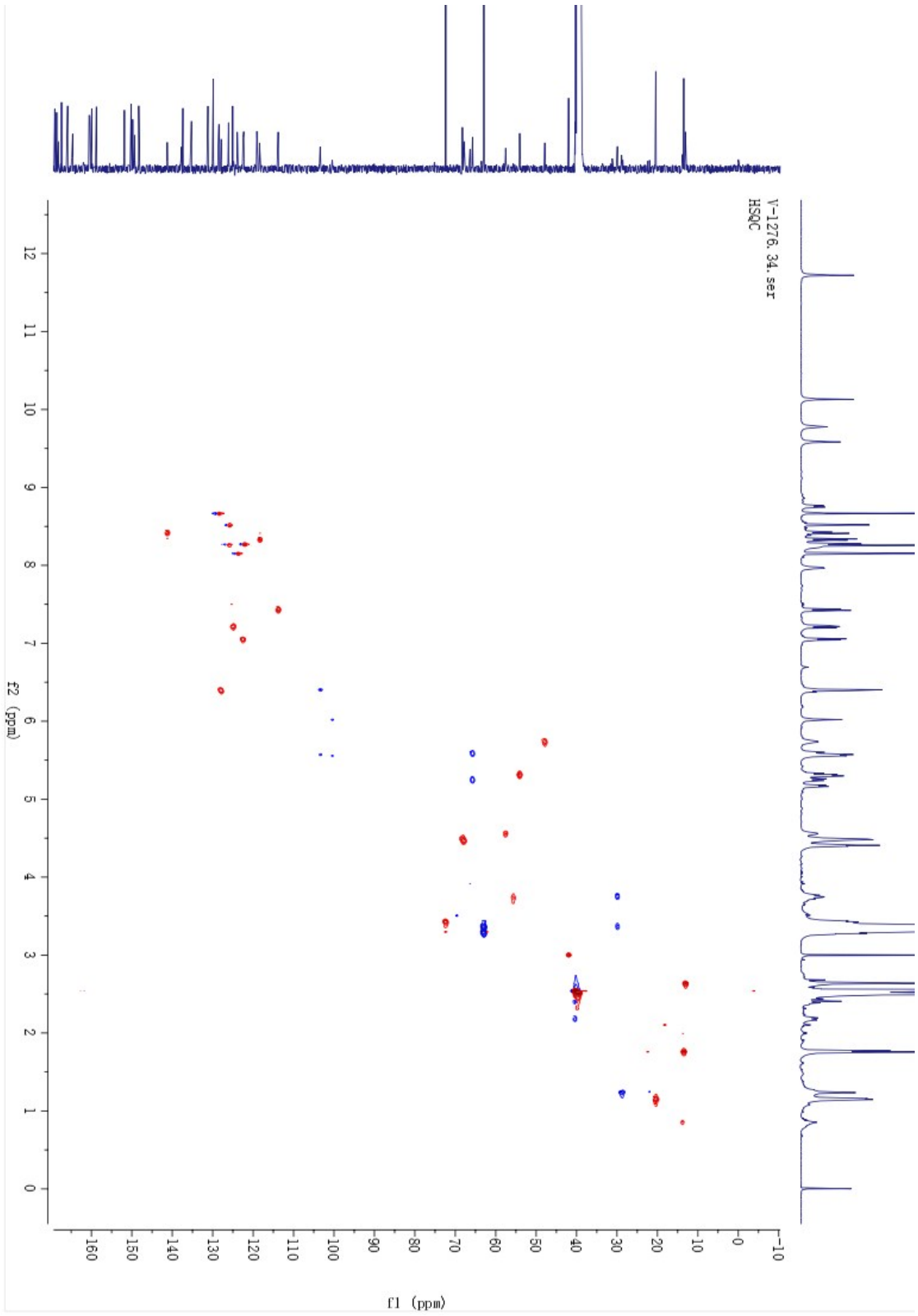
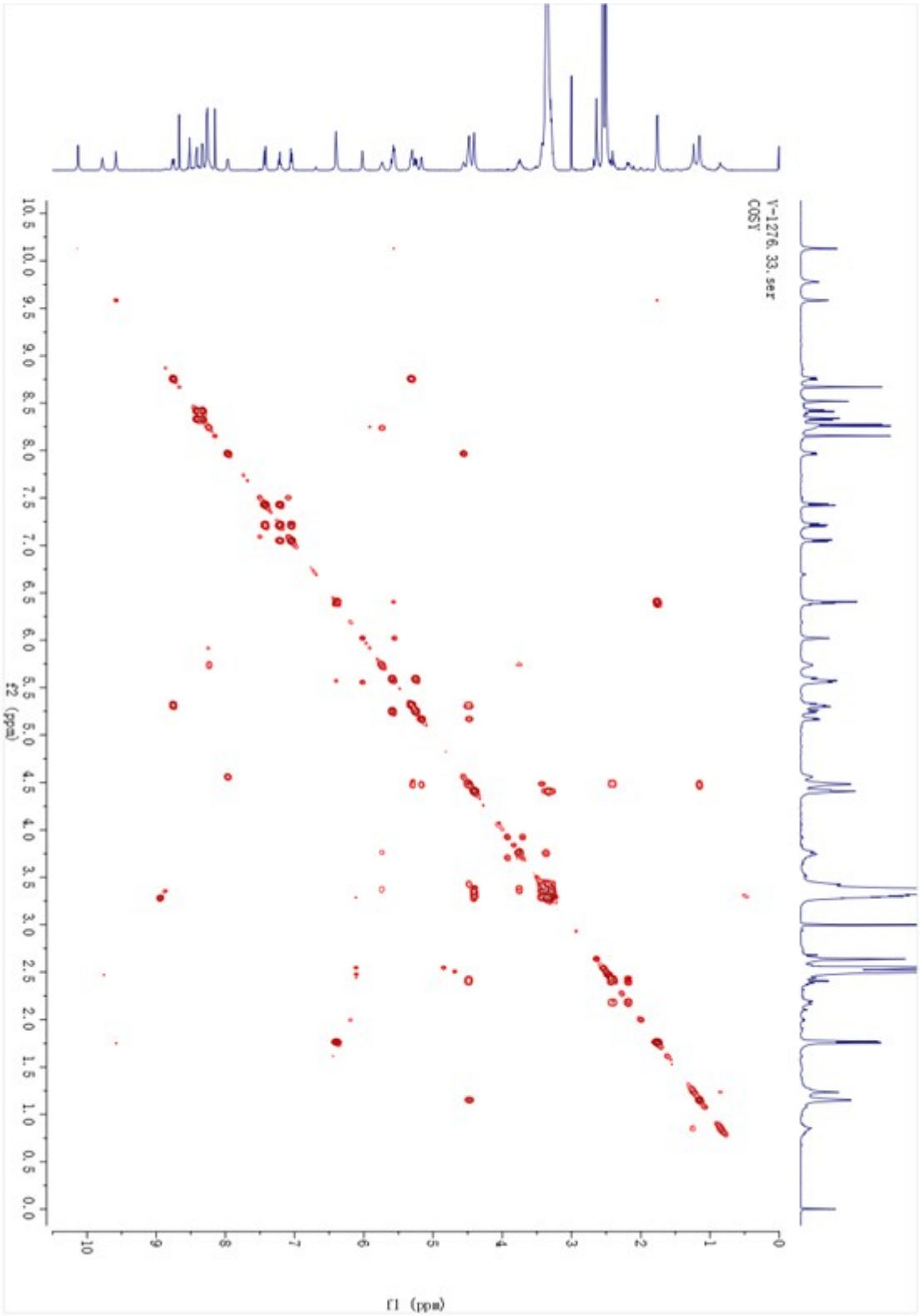
160.38
160.12
159.91
150.26
150.17
149.78
149.60
149.28
148.30
141.24
137.75
137.37
135.26
131.19
129.87
128.64
128.46
127.89
126.09
125.06
124.86
123.92
122.47
122.33
119.09
118.36
113.80
103.43
72.37
68.23
67.82
66.35
65.74
62.95
57.54
54.09
47.86
41.98
29.91
20.42
13.48
13.08

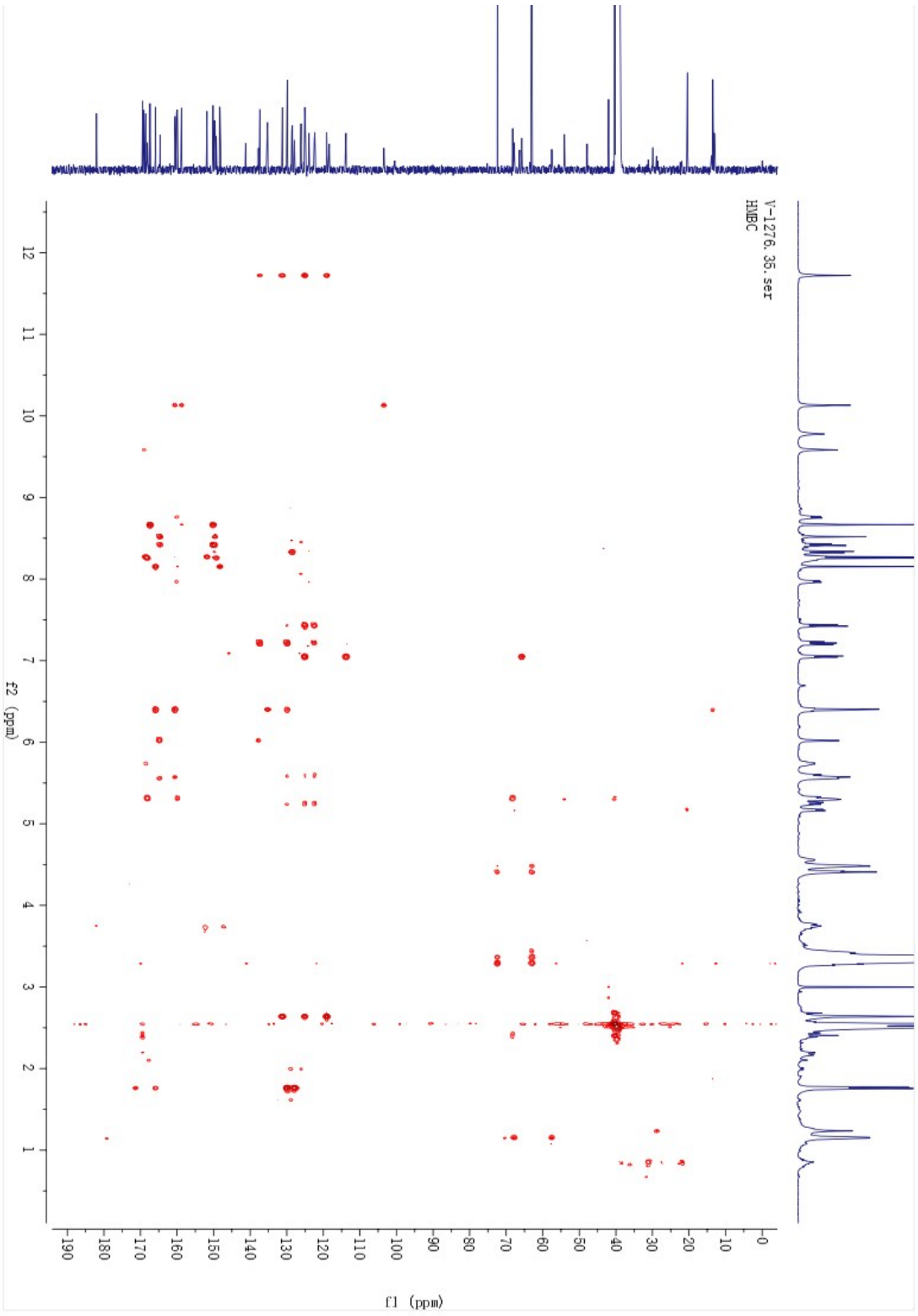
C



D



E



F

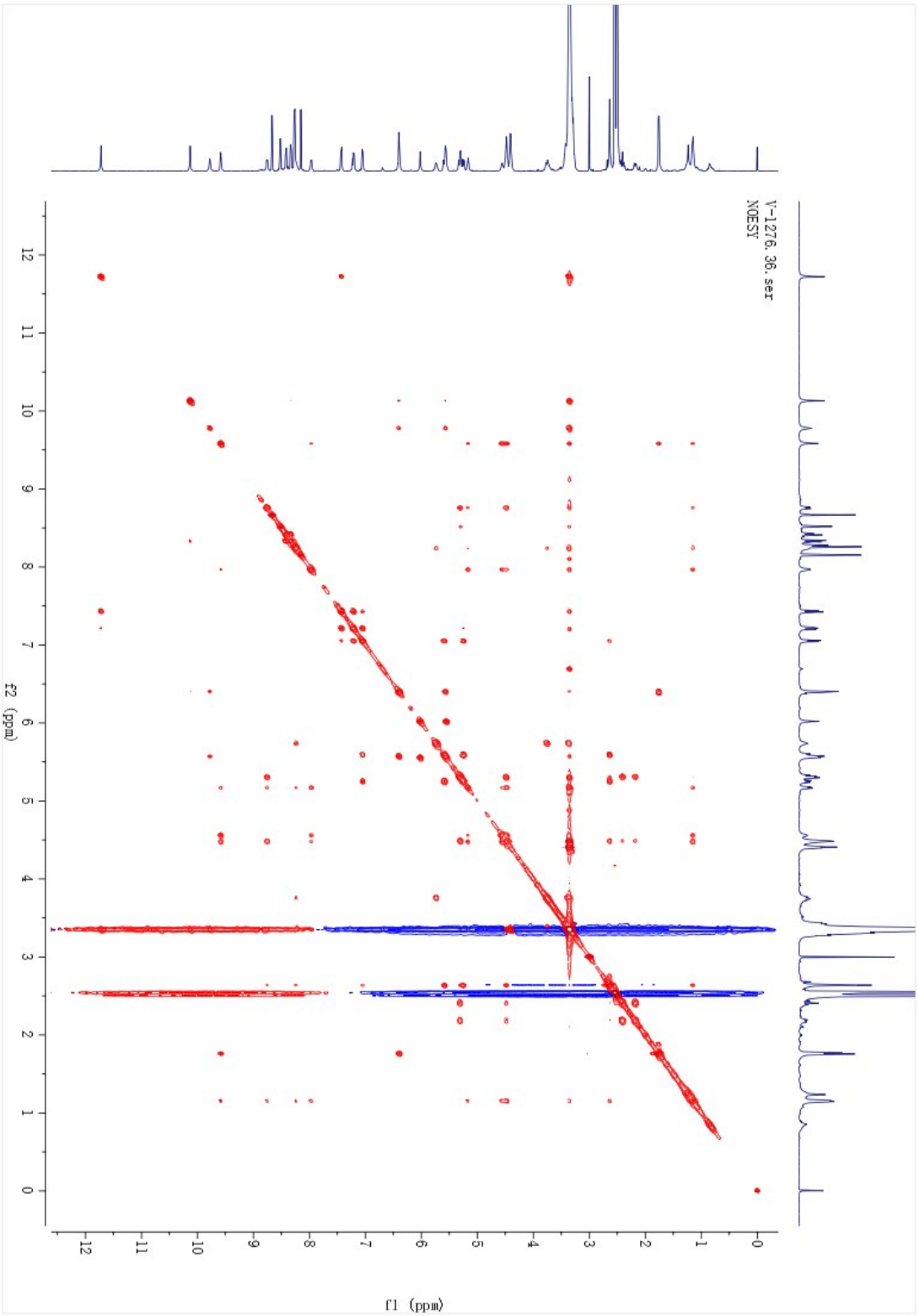


Fig. S5. HR-ESI-MS analysis of NOS-V2.

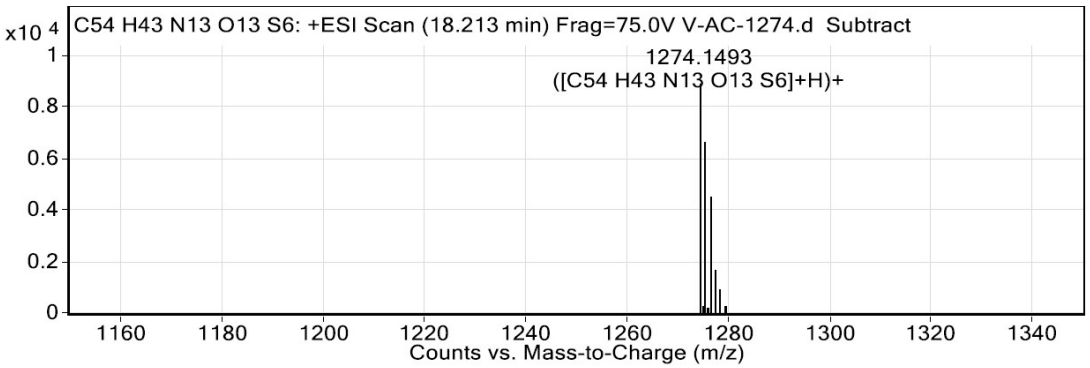


Fig. S6. Chemical structure of NOS-V2.

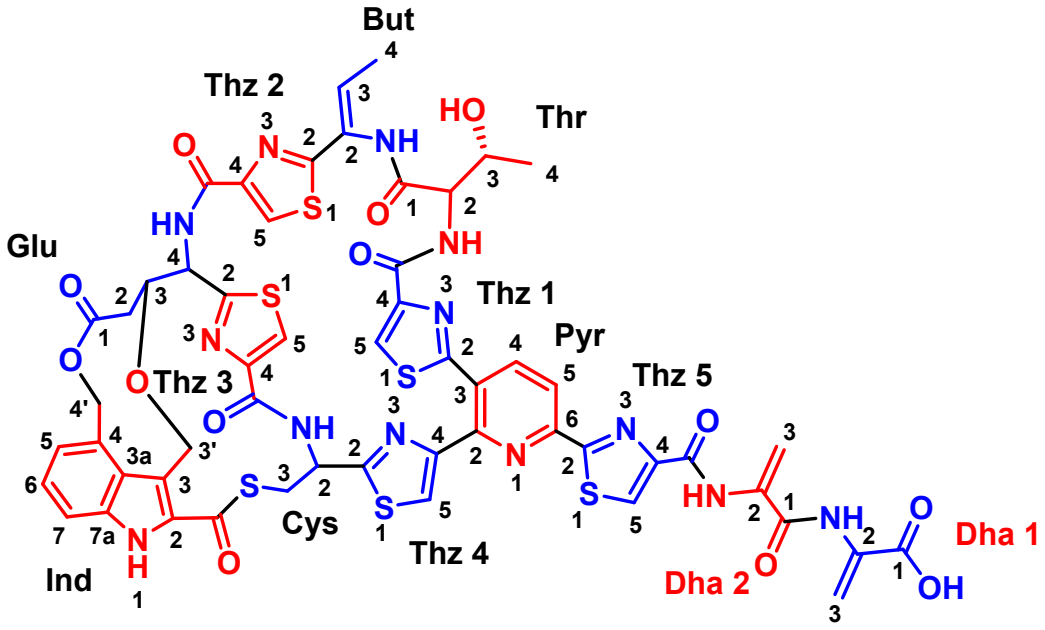
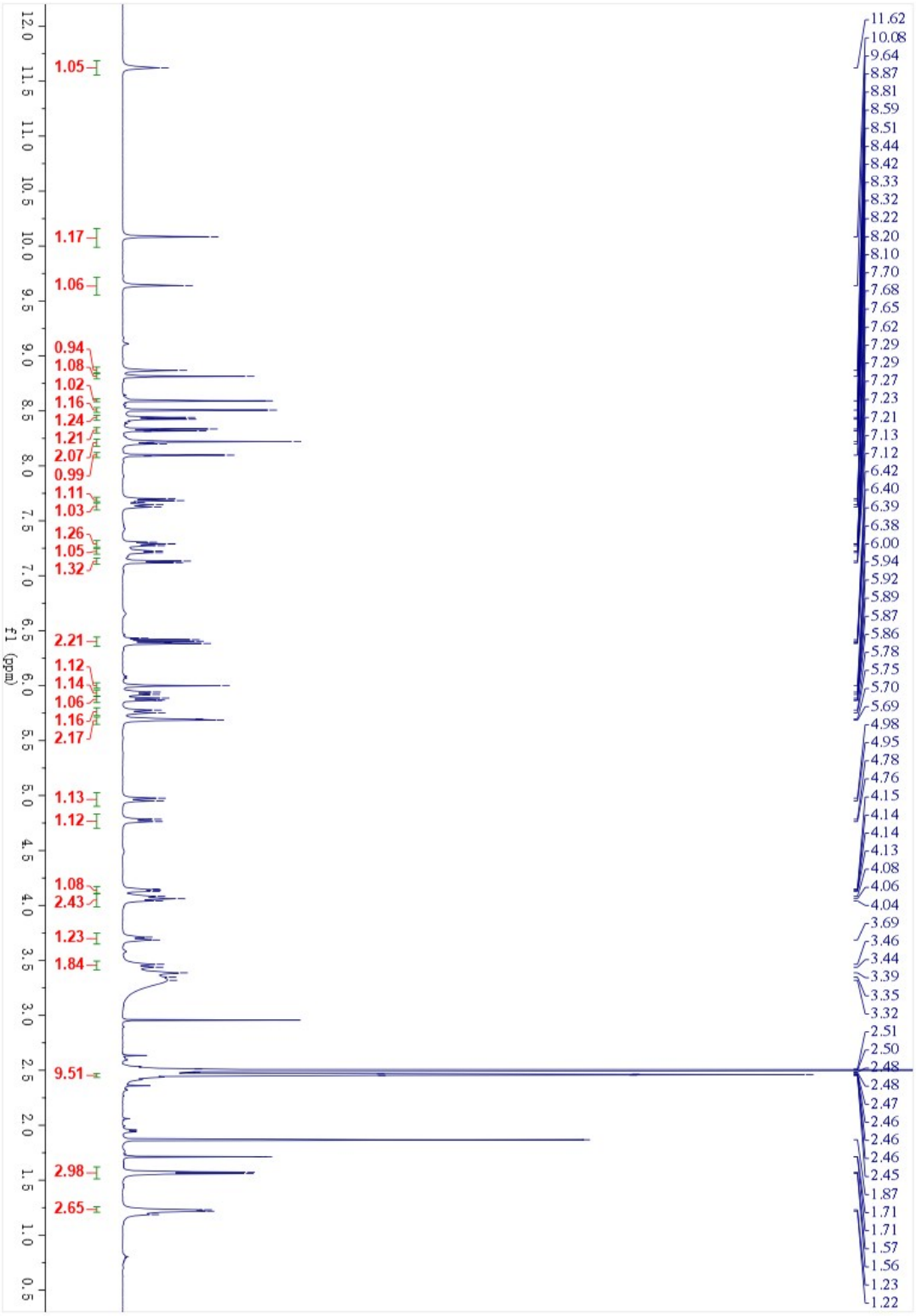
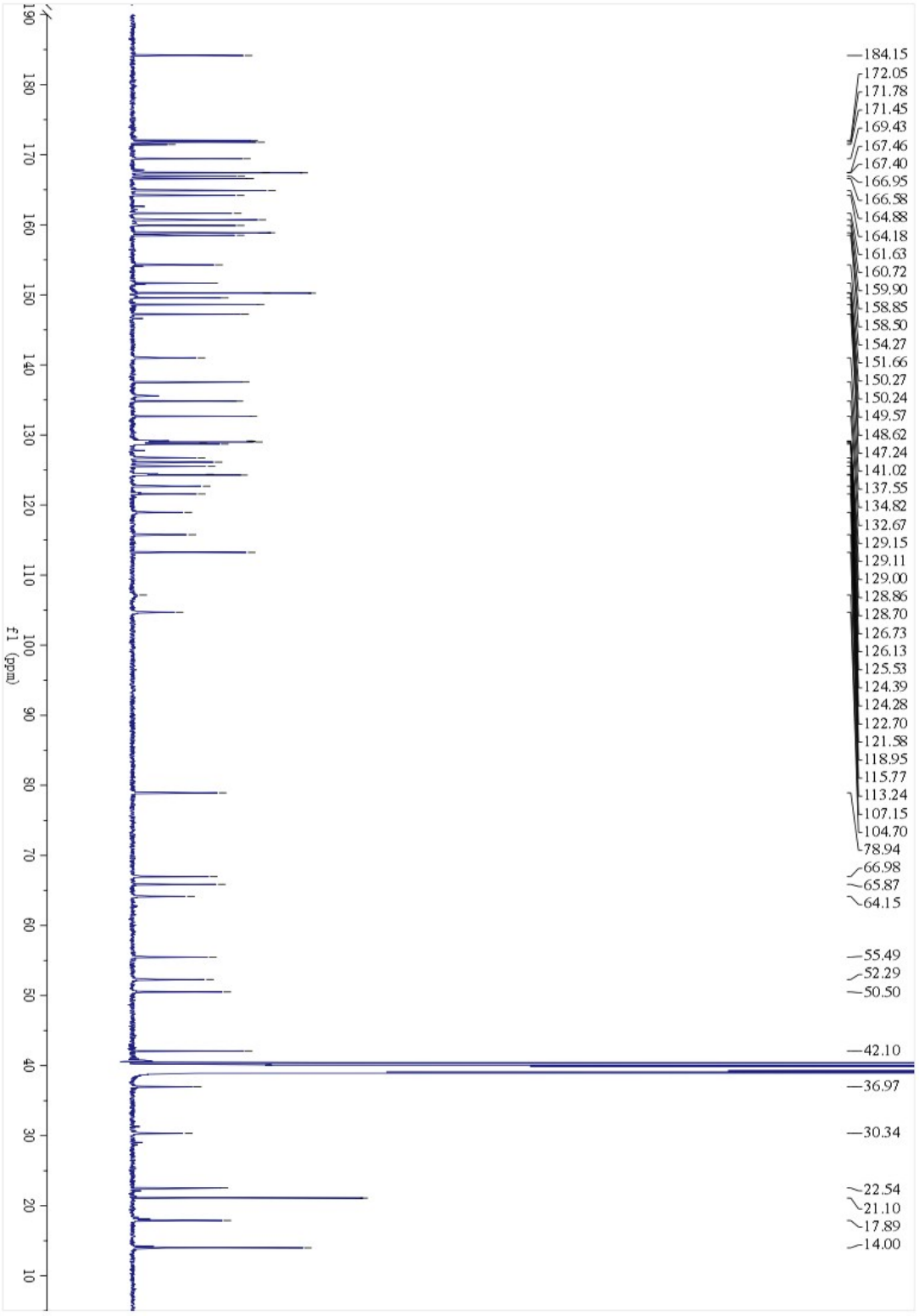


Fig. S7. NMR spectra of NOS-V2. A, ^{1}H NMR (DMSO-d_6, 500 MHz); B, ^{13}C NMR (DMSO-d_6, 125 MHz); C, HSQC; D, ^{1}H-^{1}H COSY; E, HMBC; F, ROESY.

A



B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171.45
169.43
167.46
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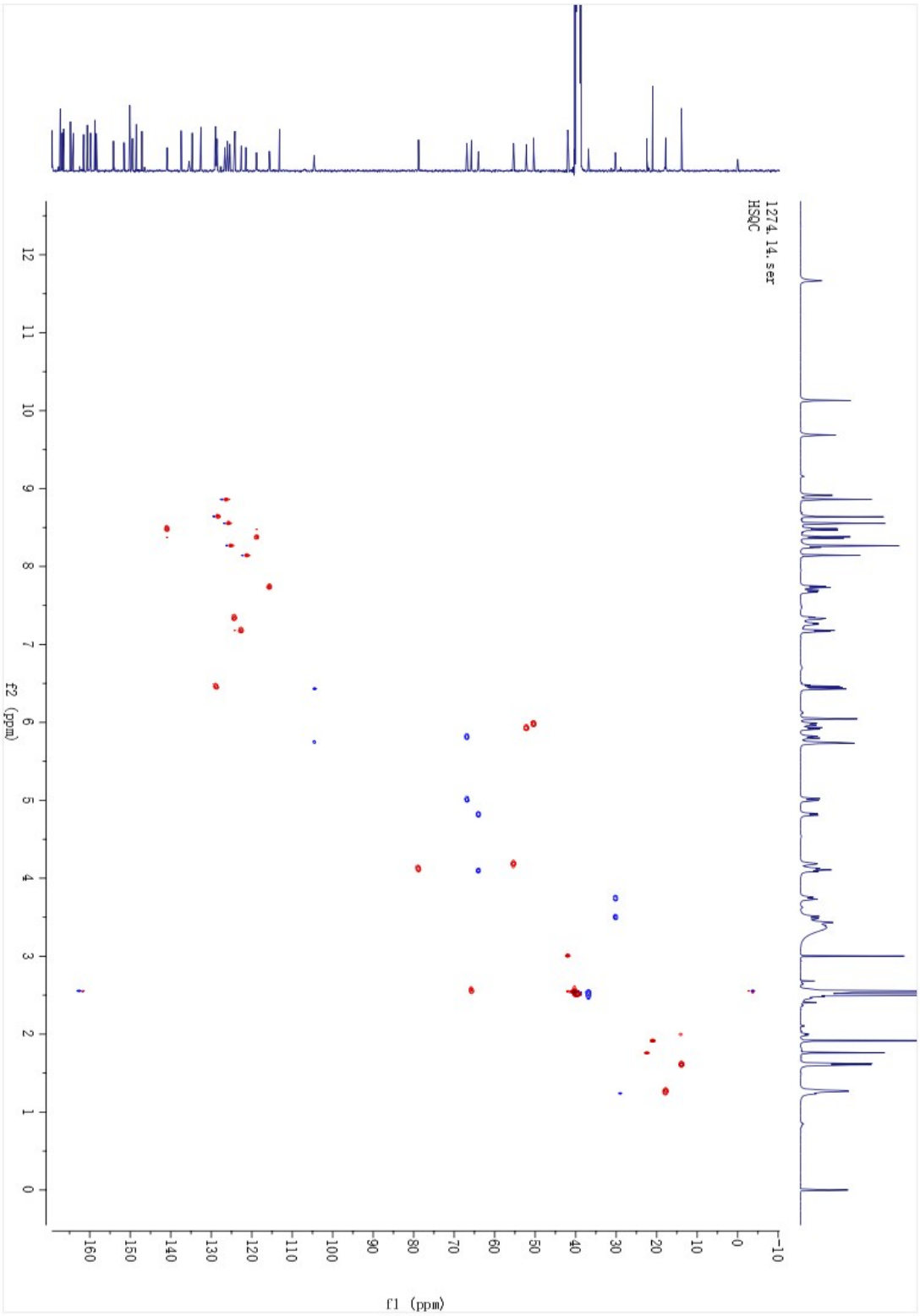
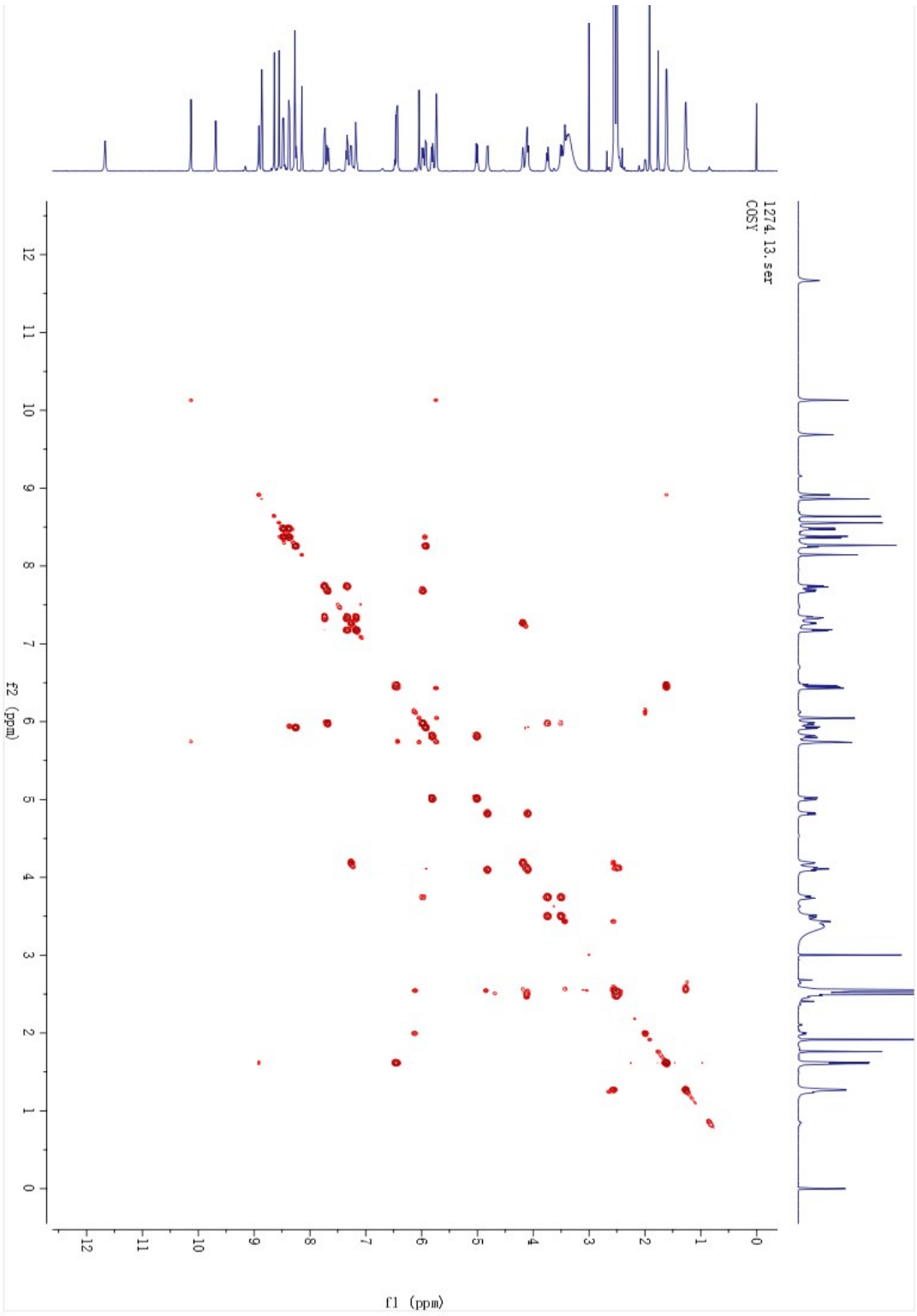
166.58
164.88
164.18
161.63
160.72
159.90
158.85
158.50
154.27
151.66
150.27
150.24
149.57
148.62
147.24
141.02
137.55
134.82
132.67
129.15
129.11
129.00
128.86
128.70
126.73
126.13
125.53
124.39
124.28
122.70
121.58
118.95
115.77
113.24
107.15
104.70
78.94
66.98
65.87
64.15
55.49
52.29
50.50
42.10
36.97
30.34
22.54
21.10
17.89
14.00

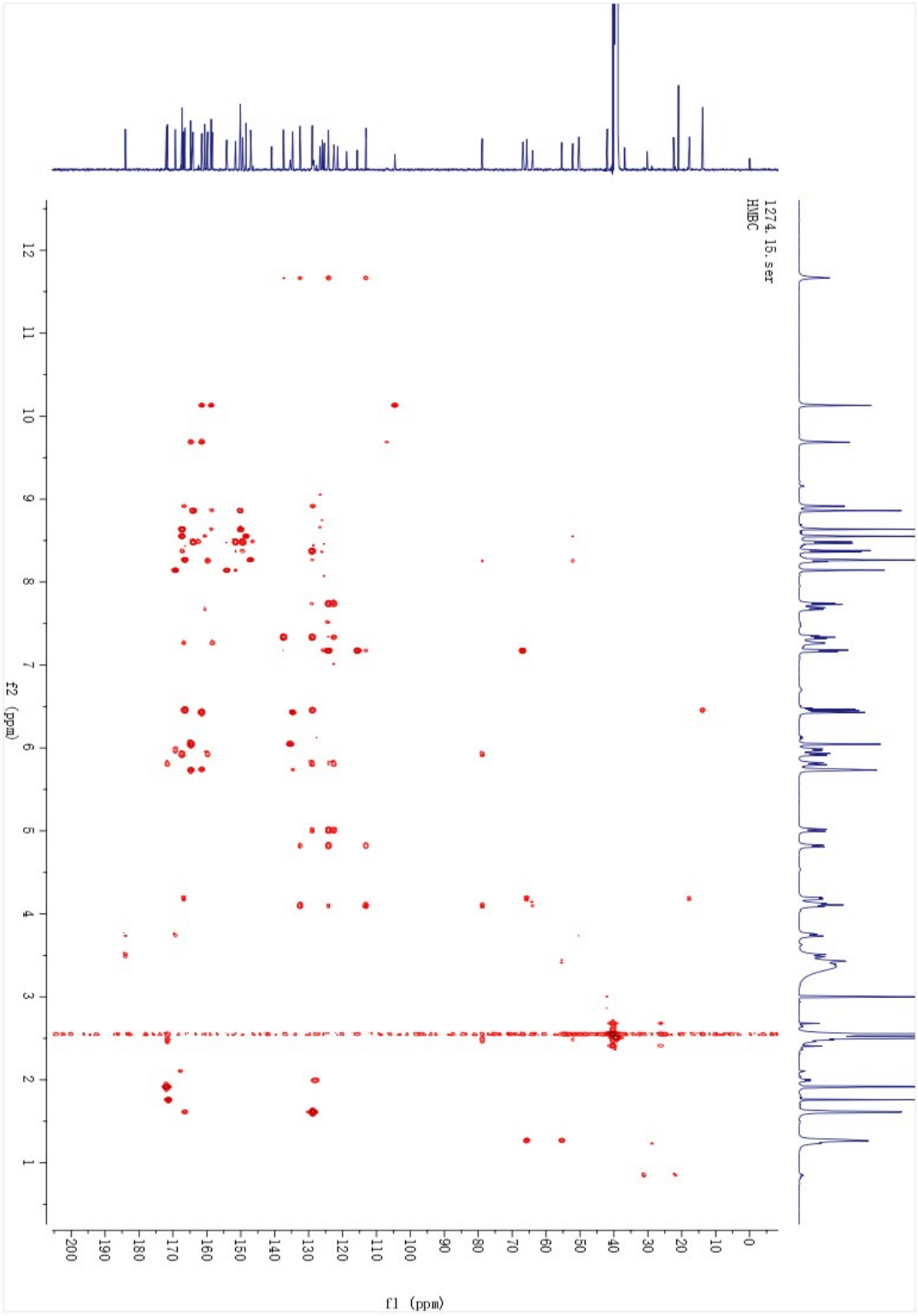
C



D



E



F

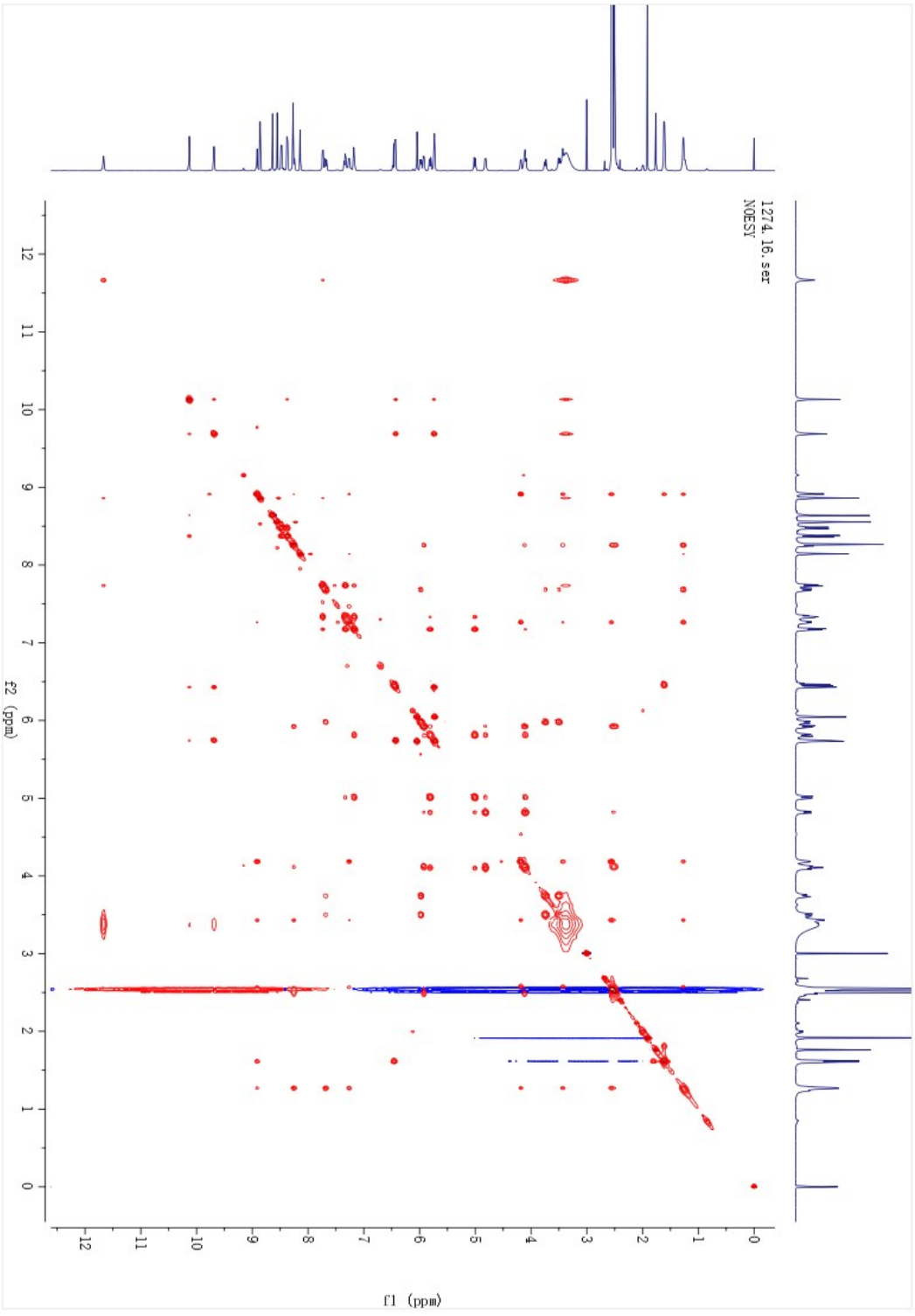
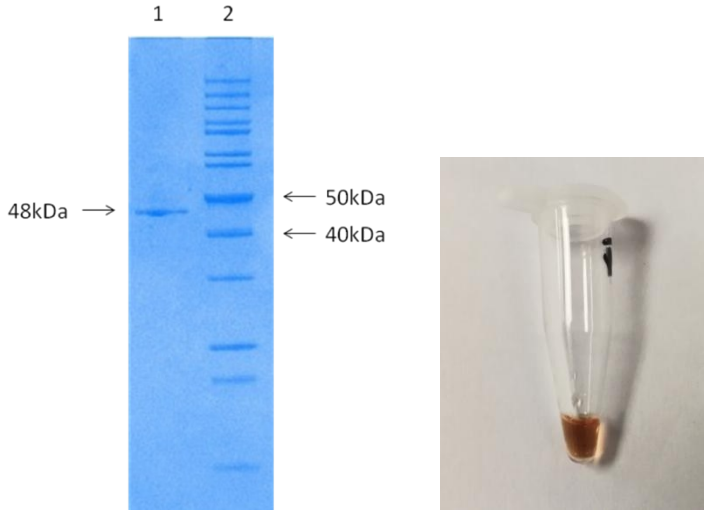


Fig. S8. SDS-PAGE of purified recombinant NocB and NocV. A, SDS-PAGE of NocB. Lane 1, NocB (expected MW to be 48 kDa); Lane 2, protein marker. B, SDS-PAGE of NocV. Lane 1, NocV (expected MW to be 40 kDa); Lane 2, protein marker.

A



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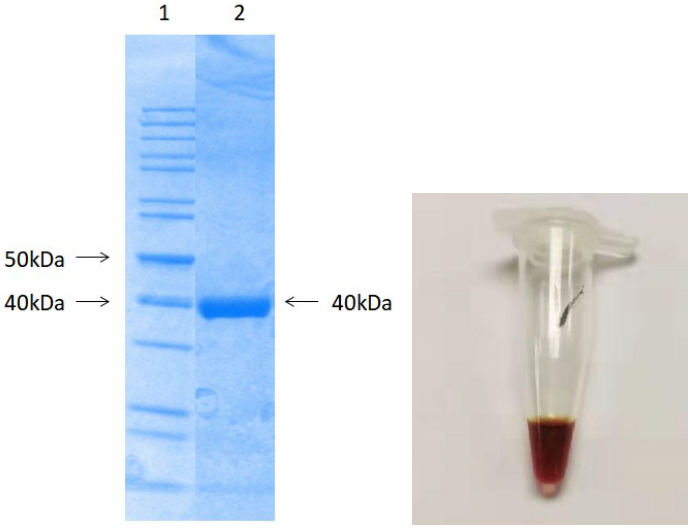
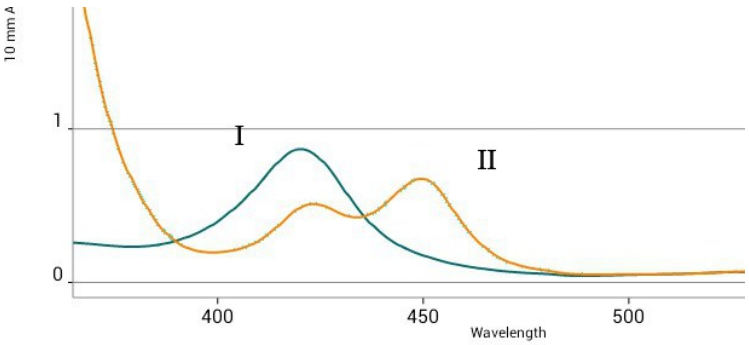


Fig. S9. UV-vis absorbance of NocB/V in reducing conditions with or without CO. A, CO binding difference spectrum of NocV. I, the absorbance spectrum of the pure NocV in 100 mM Tris-HCl buffer (pH 7.5); II, absorptions of this solution after bubbling of CO for 1 min followed by reduction of dithionite. B, CO binding difference spectrum of NocB. I, the absorbance spectrum of the pure NocB 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.

A



B

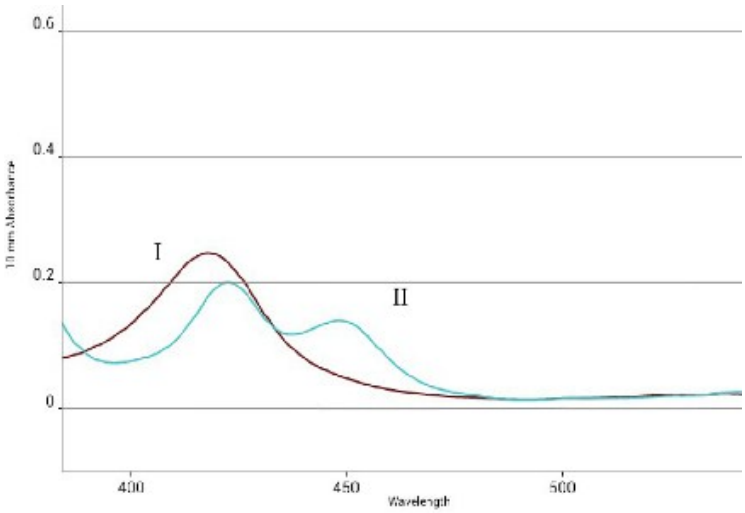
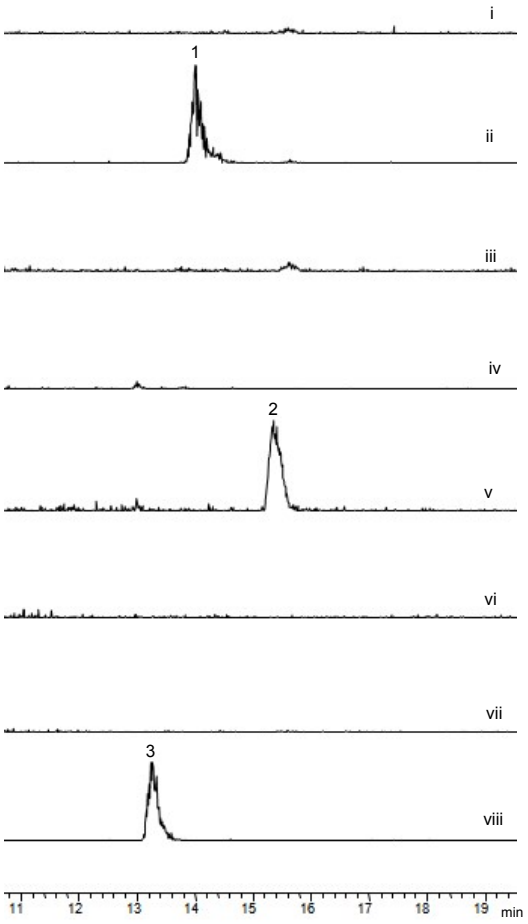
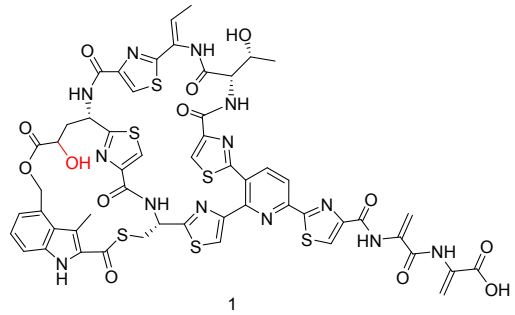
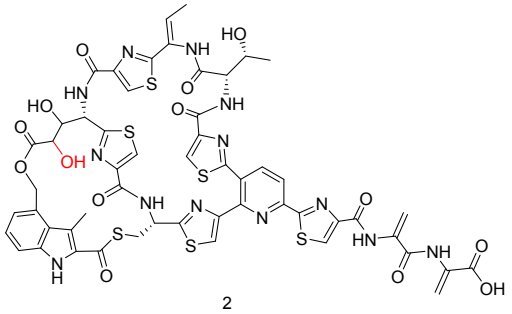
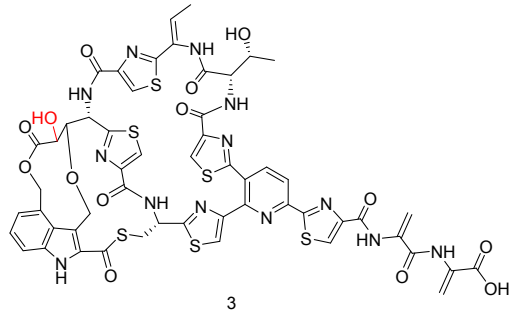


Fig. S10. Extracted ion chromatogram traces of NocB/V-catalyzed reactions. i, no enzyme reaction using NOS1260 as substrate, EIC+ = 1276-1277. ii, NocB reaction using NOS1260 as the substrate, EIC+ = 1276-1277. iii, NocV reaction using NOS1260 as the substrate, EIC+ = 1276-1277. iv, no enzyme reaction using NOS-V1 as the substrate, EIC+ = 1292-1293. v, NocB reaction using NOS-V1 as the substrate, EIC+ = 1292-1293. vi, NocV reaction using NOS-V1 as the substrate, EIC+ = 1292-1293. vii, no enzyme reaction using NOS-V2 as the substrate, EIC+ = 1290-1291. viii, NocB reaction using NOS-V2 as the substrate, EIC+ = 1290-1291. The chemical structures of compound **1**, **2**, and **3** were predicted considering the proved function of NocB[6].









3. Supplementary References

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6. Xuri Wu, Peiyu Huang, Yanjiu Xue, Weiying Liu, Min Ma and Yijun Chen. The catalytic characteristics of NocB in nocathiacin biosynthesis from *Nocardia sp. ATCC 202099*. *RSC Adv.*, 2016, **6**, 72399-72408.