Supporting Information

Heterocyclic aromatic *N*-oxidation in the biosynthesis of phenazine antibiotics from *Lysobacter antibioticus*

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1. Bacterial strains, plasmids, general DNA manipulations and media

Lysobacter antibioticus OH13 (CGMCC No.7561) and mutants were grown in nutrient broth (NB, 3 g beef extract paste, 1 g yeast extract, 5 g peptone, 10 g sucrose, pH 7.0-7.2, in one liter distilled water; without sucrose, NBWS) or on nutrient agar (NA, 1 L NB with 17 g agar; without sucrose, NAWS) at 30 °C with shaking at 200 rpm. *Escherichia coli* XL1-Blue was used as the host for general plasmid DNA propagation, and the cloning vector was pJQ200SK. Where appropriate, media were supplemented with kanamycin (50 μg/ml) or gentamycin (25 μg/ml). Plasmid preparation and DNA extraction were carried out with Qiagen kits (Valencia, CA), and all other DNA manipulations were carried out according to standard methods. The LaPhz genes were deposited in GenBank, with accession numbers of KU900196-900205.

2. Large-scale fermentation and isolation of phenazine compounds

L. antibioticus OH13 was cultivated for 24 h in a 250 ml flask containing 50 ml NB medium. The seed culture was then transferred into 200 ml production medium in 1 liter flask (total 50 flasks). The flasks were placed in incubators for 24 h with shaking at 200 rpm at 30 °C. Then the cultures were extracted with equal volumes of ethyl acetate for three times, and the organic phase was evaporated to dryness under vacuum to afford the crude extract (600 mg). The crude extract was separated by Sephadex LH-20 column chromatography with MeOH as elution solvent, yielding 5 fractions as monitored by thin layer chromatography, which was carried out with glass precoated silica gel GF254 plates (Qingdao

Haiyang Chemical Company Ltd.). The bioactive fraction 5 was further purified by reversed-phase high-performance liquid chromatography with 45% CH_3CN in H_2O (XDB-C18, 5 μm, 9.4×250 nm) under 274 nm (Fig. S1). Six compounds were obtained, namely compounds **1**-**6**, the amount of which was approximately 16, 20, 2, 3, 3, and 5 mg, respectively.

3. NMR and MS analysis of phenazines.

NMR spectra were obtained on a Bruker Avance III 600 or Brucker Avance DRX-400 NMR-spectrometer operating at 600 or 400 MHz for ^1H and 100 MHz for ^{13}C in the indicated solvents. Chemical shifts are expressed in ppm (δ units) relative to TMS signal as internal reference. ESI mass spectra for compound **2** were obtained on a Mariner System 5304 mass spectrometer and for other compounds were carried out on LTQ-Orbitrap XL (Table S1). The most abundant compound **2** appeared as orange pigment. HRESI-MS gave an *m*/*z* 243.0794 for [M ($C_{13}H_{10}N_2O_3$) + H]$^+$ (calculated, 243.0725). The ^1H-NMR and ^{13}C-NMR spectroscopic data (Table S1), plus 2-D NMR data (spectra in Supporting Information), confirmed compound **2** to be 6-methoxy-1-phenazinol-N10-oxide.[1] Compound **1** was isolated as dark red pigment. ESI-MS data (*m*/*z* 259.2) and NMR data (Table S1) revealed compound **1** to be identical to myxin (1-hydroxy-6-methoxyphenazine-N5,N10-dioxide).[1,2] Compound **3** was isolated as purple pigment. ^1H NMR (600 MHz, $CDCl_3$) gave the follows: δ 14.10 (s, 2H), 8.04 (d, *J* = 9.0 Hz, 1H), 7.73 (dd, *J* = 9.0, 7.8, 2H), 7.18 (d, *J* = 7.8 Hz, 2H). These data were consistent with that of iodinin.[3,4] Compound **4** was isolated as yellow powder. ^1H NMR (400 MHz, $CDCl_3$) gave δ 8.25 (d, *J* = 9.2 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.64 (dd, *J* = 8.8, 7.6 Hz, 1H), 7.61 (dd, *J* = 9.2, 7.6Hz, 1H), 7.01 (d, *J* = 7.6 Hz, 2H), 4.16 (s, 3H), 4.08 (s, 3H). ESI-MS gave an *m/z* of 257.1 for [M+H] $^+$ ($C_{14}H_{12}N_2O_3$). Compound **4** was identified as 1,6-dimethoxy-phenazinol-5-oxide.[2] Compound **5** was isolated as yellow powder. ^1H NMR (400 MHz, CDCl3) δ 8.01 (d, *J* = 8.8 Hz, 2H), 7.76 (dd, *J* = 8.8, 7.6 Hz, 2H), 7.11 (d, *J* = 7.6 Hz, 2H), 4.19 (s, 6H). ESI-MS gave an *m/z* of 241.1 for [M+H]$^+$ ($C_{14}H_{12}N_2O_2$). Compound **5** was identified as 1,6-dimethoxyphenazine.[1] To our knowledge, compounds **4** and **5** have not been reported as natural products, although they had been chemically synthesized.[1,2] Compound **6** was isolated as yellow powder.

^{1}H NMR (400 MHz, CDCl3) δ 8.18 (s, 1H), 7.94 (d, *J* = 9.2 Hz, 1H), 7.83 - 7.74 (m, 3H), 7.26 (d, *J* =7.6Hz, 1H), 7.11 (d, *J* = 7.6 Hz, 1H), 4.19 (s, 3H). ESI-MS gave an *m/z* of 227.3 for $[M+H]^+$ ($C_{13}H_{10}N_2O_2$). Compound **6** was identified as 1-hydroxy-6-methoxyphenazine.[1]

4. Construction of plasmids for gene inactivation in *Lysobacter antibioticus* OH13

pJQ200SK-*LaPhzC* was constructed by ligating a 544-bp internal fragment of *LaPhzC* into pJQ200SK at *Bam*HI/*Xba*I sites. This fragment was amplified from the genome using primers LaPhzC-F/ LaPhzC -R (Table S2). The construct was transferred into OH13 wild type by electroporation (voltage, 1.8 kv). Homologous recombination of the *LaPhzC* fragment with the corresponding sequence in the genome of OH13 generated disruption mutants (Fig. S4A). The mutants were selected on NAWS medium containing gentamycin (25 μg/mL) and verified by PCR. Diagnostic PCR was performed with primers pJQ-F/ LaPhzC-VR and LaPhzC-VF/pJQ-R to identify mutants that resulted from a homologous recombination. A 936-bp fragment was amplified from the individual colonies by using primers LaPhzC-VF and pJQ-R, whereas a 614-bp fragment was obtained when primers pJQ-F and LaPhzC-VR were used (Table S2 and Fig. S4B). The PCR products were sequenced, which confirmed the colonies were true *LaPhzC* disruption mutants.

To construct vectors for gene deletion of *LaPhzD* and *LaPhzB* in OH13, two flanking regions of the target genes were amplified by PCR using primers LaPhzD-F1/R1 and LaPhzD-F2/R2, LaPhzB-F1/R1 and LaPhzB-F2/R2, respectively (Table S2). These two flanking regions were digested and cloned into the corresponding sites of pJQ200SK. The final constructs pJQ200SK- *LaPhzD* or pJQ200SK- *LaPhzB* were transformed into the wild-type OH13 by electroporation. The gentamycin-resistant colonies were screened by PCR using primers LaPhzD-F1/ LaPhzD-R2 or LaPhzB-F1/ LaPhzB-R2, to identify the single crossover mutants. Then, the single crossover mutants were applied on NAWS medium containing 6.5% sucrose, which would screen for deletion mutants through a second homologous recombination of the flanking regions (Fig. S5A). The mutants were verified by PCR using primers LaPhzD-deletion-MF/ LaPhzD-deletion-MR or LaPhzB-deletion-MF/ LaPhzB-deletion-MR. The PCR product of the wild type

was 626-bp by using primers LaPhzD-deletion-MF/ LaPhzD-deletion-MR, whereas the *LaPhzD* deletion mutants gave a product of 286-bp (Fig. S5B). The product of the wild type was 1000-bp with primers LaPhzB-deletion-MF/ LaPhzB-deletion-MR, whereas the *LaPhzB* deletion mutants gave a product of 655-bp (Fig. S5C).

To disrupt *LaPhzNO1*, a 366-bp internal fragment was amplified from the genomic DNA of OH13 with primers LaPhzNO1-F/LaPhzNO1-R (Table S2). To construct the *LaPhzS* disruption vector, a 535 bp internal fragment was amplified form the target gene by using primers LaPhzS-F/LaPhzS-R (Table S2). The fragments were digested with *Xba*I/*Xho*I and cloned into the corresponding sites of pJQ200SK. The vectors were introducd into OH13 by electroporation. *Lysobacter* transconjugants were selected on NAWS plates with gentamycin (25 μg/mL). Diagnostic PCR was performed to identify mutants that resulted from a homologous recombination (Fig. S6). A 1435-bp fragment was amplified from the *LaPhzNO1* mutants by using primers LaPhzNO1-PF and pJQ-R (Fig. S6A), and this product produced two fragments of 1129-bp and 306-bp upon *Xba*I digestion (Fig. S6C). A 1143-bp fragment was obtained when primers LaPhzS-PF and pJQ-R were used for *LaPhzS* mutants (Fig. S6B), and this product produced two fragments of 837-bp and 306-bp upon *Xba*I digestion (Fig. S6C).

5. Preparation and HPLC analysis of phenazines from OH13 and mutants

OH13 wild type strain and mutants were cultured in NBWS medium (supplemented with 25 μg/ml gentamicin for disruption mutant) at 30 °C in a rotary shaker at 200 rpm for 24 h. To extract the phenazines, 5 ml ethyl acetate was added into equal volume cultures with stirring until the aqueous phase became colorless. 2 ml extract was transferred to an Eppendorf tube and fully evaporated. Finally, the remaining residues were dissolved in 200 μl methanol, and this methanol extract was collected by centrifugation (12000 rpm at 4 °C for 10 min). To analyze the metabolites, 20 μl methanol extract was injected into RP HPLC (COSMOSIL C18, 250 mm by 4.6 mm) under 274 nm. The mobile phase was 20% to 45% CH_3CN in H_2O from 0 to 5 min, 45% to 50% CH_3CN in H_2O from 5 min to 19 min, 50% to 60% CH_3CN in H_2O from 19 to 20min, 60% to 100% CH_3CN in H_2O from 20 to 23 min, 100% CH_3CN

from 23 to 27 min, 100% to 20% CH_3CN in H_2O from 27 to 28 min, 20% CH_3CN in H_2O from 28 to 30 min (H_2O containing 0.01% formic acid).

6. Antibacterial activity assays

The gram negative bacterium *E. coli* XL1-Blue and gram positive bacterium *Bacillus subtilis* were used as indicator strains. The antibacterial activity of six phenazine compounds were also tested with the followed bacteria, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas oryzae* pv. *oryzicola*, *Xanthomonas oryzae* pv. *oryzae*, *Pseudomonas syringae* pv.*glycinea*, *Erwinia amylovora*, *Dickeya chrysanthemi*, *Erwinia pyrifoliae Kim*, *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *lachrymans*, *Pectobacterium carotovora*, *Claribacter michiganes* subsp. *sepedonicum*, *Staphylococcus aureus*. These bacterial suspensions with OD_{600nm} 1.0 were 100-fold diluted and then spread on NAWS plates which support the production of phenazine compounds. For *L. antibioticus* wild type and mutants, 3 μl droplets of strains ($OD600_{nm}$=1.0) were individually added on the plates containing the indicator bacteria. Compounds dissolved in chloroform were applied on 5mm filter paper disks. After chloroform evaporating off, the paper disks were placed on the surface of media which were spread with test bacteria. For each of the petri dish, compound **1** was applied with10 μg**,** and compounds **2-6** were applied with 20 μg respectively. The paper disk in the center was added with the same volume of evaporating off chloroform as negative control. Cultures were incubated at 30°C for 1 or 2 days, and then growth inhibition zones surrounding the strains or compounds were observed.

7. Expression of *LaPhzNO1* and *LaPhzS* in *E. coli*

LaPhzNO1 and *LaPhazS* genes were amplified from the genomic DNA of OH13 using primers LaPhzNO1-PF/LaPhzNO1-PR and LaPhzS-PF/LaPhzS-PR (Table S2). The products were digested by *Bam*HI/*Eco*RI and *Eco*RI/*Hind*III respectively and cloned into corresponding sites of plasmid pET-28a. The sequences were confirmed by DNA sequencing. The expression vector was introduced into *E. coli* BL21 (DE3). To purify the LaPhzNO1 and LaPhzS proteins, BL21- pET-28a-LaPhzNO1 or BL21- pET-28a-LaPhzS overnight culture was added to 100 ml fresh LB medium containing kanamycin (50 μg/ml)

and continued in a shaker (200 rpm) at 37 °C until the cell density (OD_{600nm}) reached 0.6. To induce the expression of protein, IPTG (final concentration 0.1mM) was added to the culture, and the cells were allowed to grow (200 rpm) at 25 °C for another 12 hours. To extract proteins, the cells were harvested and resuspended in 20 ml of PBS buffer (500 mM NaCl, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 7.8). The cell suspension was sonicated on ice, and the soluble fraction of protein extracts was loaded onto a Ni-NTA column, which was previously calibrated with the PBS buffer containing 5 mM imidazole. The column was washed three times with the buffer containing 10 mM imidazole, and the LaPhzNO1 protein (60.2 kDa) and LaPhzS (44.1 kDa) were purified by using an imidazole step-gradient as described by the manufacturer's protocol. Purified LaPhzNO1 and LaPhzS were checked by SDS-PAGE separately (Fig. S8). The pure fractions were pooled and dialyzed in PBS buffer.

8. Enzyme activity assays

In the reactions with phenazines as substrates, the assay mixture (500 μl) contained 100 μM NADH or NADPH, 100 μM FMN or FAD, 50 μM substrate, 1 μM LaPhzNO1 or LaPhzS in 0.1 M PBS buffer (0.1 M K_2HPO_4, 0.1 M KH_2PO_4, pH 7.0). The reactions were incubated at 30 ℃ for different time, then quenched and extracted with 500 μl EtOAc. The solvent was evaporated to dryness in a fumehood. The residue was dissolved in 200 μl methanol followed by centrifuging at 13,200 × g for 10 min. The supernatant (20 μl) was subjected to reversed-phase HPLC analysis or LC-MS. HPLC condition: COSMOSIL C18, 250 mm by 4.6 mm, 20% to 45% CH_3CN in H_2O from 0 to 5 min,45% to 50% CH_3CN in H_2O from 5 min to 19 min,50% to 60% CH_3CN in H_2O from 19 to 20min, 60% to 100% CH_3CN in H_2O from 20 to 23 min,100% CH_3CN from 23 to 27 min,100% to 20% CH_3CN in H_2O from 27 to 28 min,20% CH_3CN in H_2O from 28 to 30 min (H_2O containing 0.01% formic acid).

In the reactions with other heterocyclic aromatic compounds as substrates, the assay mixture (250 μl) contained 100 μM NADPH, 50 μM substrate, 1 μM LaPhzNO1 in 0.1 M PBS buffer (0.1 M K_2HPO_4, 0.1 M KH_2PO_4, pH 7.0). Tested substrates included 8-hydroxyquinoline (8-HQ), 6-hydroxyquinoline (6-HQ), quinoline, quinoxaline, quinine and 2-phenylpyridine (Fig. S13). The reactions were incubated at 30°C

for 30 min, then quenched and extracted with 250 µl EtOAc. The EtOAc extract was evaporated to dryness in a fume hood. Prior to analysis, each of the samples was dissovled in 20 µl EtOAc, and 1 µl was used for GC-MS analysis in split mode (1:30). The GC column was 30 meter DB-5 column (J&W, Fisher Scientific) and started with temperature at 35 °C for 2.5 min, ramped to 200 °C at 10 degrees/min, and held at 200 °C for 1.5 min. Acquisition range for MS was 50 to 400 Daltons, at 1 sec for each of acquisitions.

Table S1. ^{1}H (600MHz, DMSO-d_6) and ^{13}C (100 MHz, $CDCl_3$) data for compound **1**, ^{1}H (400 MHz, CDCl3) and ^{13}C (100 MHz, $CDCl_3$) data for compound **2**. δ_H and δ_C (ppm), mult. (*J* in Hz).

	1		**2**	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
position				
1	153.7, C		152.1, C	
2	114.9, CH	7.30, d (7.8)	112.9, CH	7.09, d (8.0)
3	131.7, CH	7.81, m	132.4, CH	7.70, dd (8.4, 8.0)
4	108.9, CH	7.81, m	120.1, CH	7.82, d (8.4)
4a	135.9, C		145.2, C	
5a	129.8, C		138.9, C	
6	153.7, C		155.7, C	
7	109.7, CH	7.12, d (7.8)	108.1, CH	7.09, d (8.0)
8	132.5, CH	7.71, dd (7.8, 9.0)	131.2, CH	7.68, dd (8.8, 8.0)
9	110.6, CH	8.08, d (9.0)	109.6, CH	8.13, d (8.8)
9a	138.6, C		133.5, C	
10a	125.8, C		125.0, C	
MeO-6	57.2, CH_3	3.98, s	56.8, CH_3	4.17, s
OH-1		15.00, s		13.66, s

Table S2. Sequence of primers used in this study

PJQ-F	ATCGAATTCCTGCAGCCC
PJQ-R	TTTGCCGTTACGCACCAC
LaPhzC-F	**CGGGATCCCG**TCCAGCGACGAGGAAACG
LaPhzC-R	**GCTCTAGAGC**ACTCGTCGGGCTTGGTGG
LaPhzC-VF	TGCAGATGAGCATCGTCCTG
LaPhzC-VR	CGGCTCTTCGTAGGGATTG
LaPhzD-F1	**CGGGATCCCG**ACGAAGCCCTGCTGCTACCC
LaPhzD-R1	**CCCAAGCTTGGG**CGAAGCGGCATTCTTGACC
LaPhzD-F2	**CCCAAGCTTGGG**ACGCCGTCGCCGATTTCT
LaPhzD-R2	**GCTCTAGAGC**CGTGCGCCCTTCGATGAA
LaPhzD-deletion-MF	AAGATCCAGCCTTATGCG
LaPhzD-deletion-MR	CAGCAACGGGGTAGTCAT
LaPhzB-F1	**CGGGATCCCG**CGGAGTCGGACTTGCTGTGG
LaPhzB-R1	**CCCAAGCTTGGG**CGCCCTTGCGGCTCATAT
LaPhzB-F2	**CCCAAGCTTGGG**GATTCCGGCGATCAAGCG
LaPhzB-R2	**GCTCTAGAGC**CAGCAATCCAACCAGGTCTCG
LaPhzB-deletion-MF	CGGTGAAACCCGACCTGC
LaPhzB-deletion-MR	GGCGTCTGCCCACTTCTT
LaPhzNO1-F	**GCTCTAGAGC**CCAGCAGGTCCAGTTTGTGC
LaPhzNO1-R	**CCCTCGAGGG**TCTCGGCCATGAGCGTGT
LaPhzNO1-PF	**CGCGGATCC**GTGATCGACAATGACAAGAC
LaPhzNO1-PR	**CCGGAATTC**TCAGGCGACCATCGACGAGG
LaPhzS-F	**GCTCTAGAGC**CACATCGAGCCAACCGAACTT
LaPhzS-R	**CCCTCGAGGG**TCCGCAATACGCTATCCACAGA
LaPhzS-PF	**CCGGAATTC**ATGACCACCGCTACGCAAAC
LaPhzS-PR	**CCCAAGCTT**TCACTGAACGTGCGAGCGAT

Figure S1. HPLC profile of the metabolite extract from *Lysobacter antibioticus* OH13 wild type and chemical structure of the six phenazines as determined by spectroscopic analyses. The detection wavelength was 274 nm.

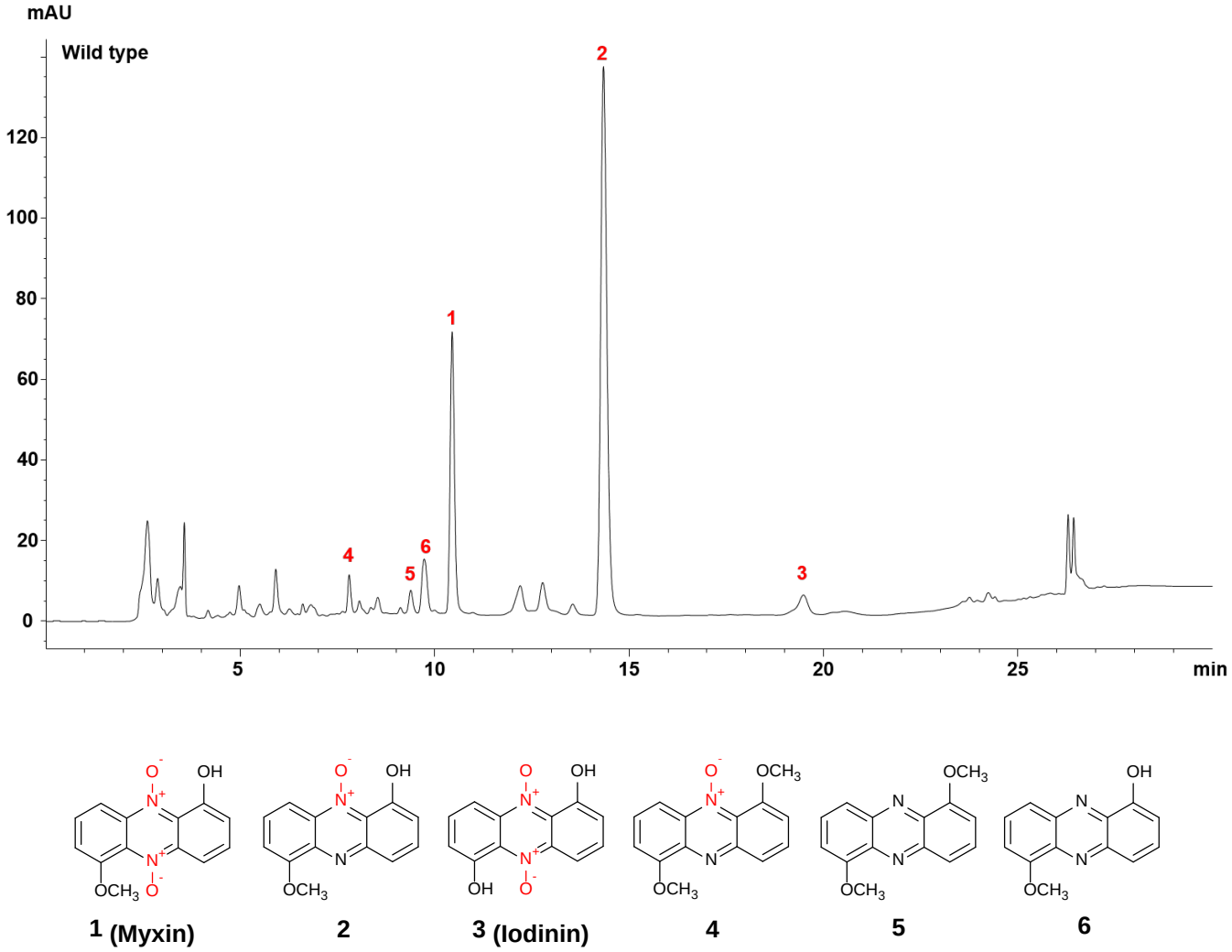


Figure S2. Antibacterial activity of six phenazine compounds from OH13. The compounds dissolved in chloroform were applied onto the 5 mm filter paper disks. After chloroform was evaporated, the paper disks were placed on the NAWS plates innocuated with bacteria. The paper disk in the center of the plates was added with the same volume of chloroform as control. For each of the plates, the top paper disk was added with compound **1** (10 μg)**,** and in counterclockwise direction each of the following disks was added with compounds **2-6** (20 μg) respectively. The tested bacteria were *Xanthomonas campestris* pv. *campestris*, *Xanthomonas oryzae* pv. *oryzicola*, *Xanthomonas oryzae* pv. *oryzae*, *Pseudomonas syringae* pv.*glycinea*, *Erwinia amylovora*, *Dickeya chrysanthemi*, *Erwinia pyrifoliae Kim*, *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *lachrymans*, *Pectobacterium carotovora*, *Claribacter michiganes* subsp. *sepedonicum*, *Bacillus subtilis*, *Staphylococcus aureus*.

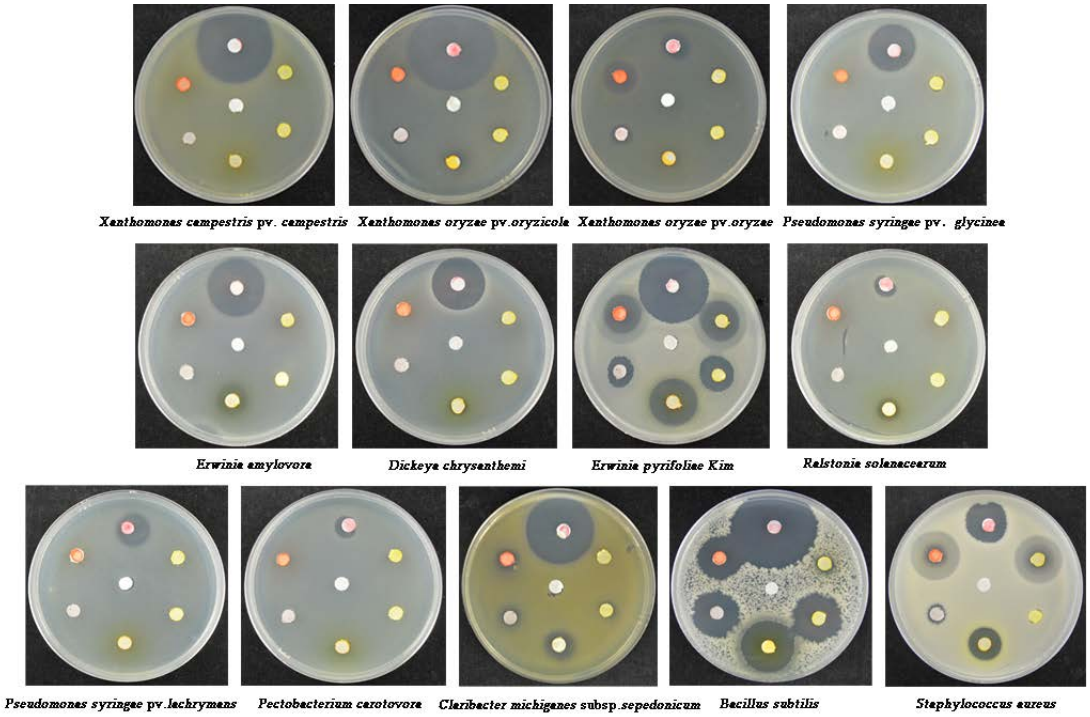
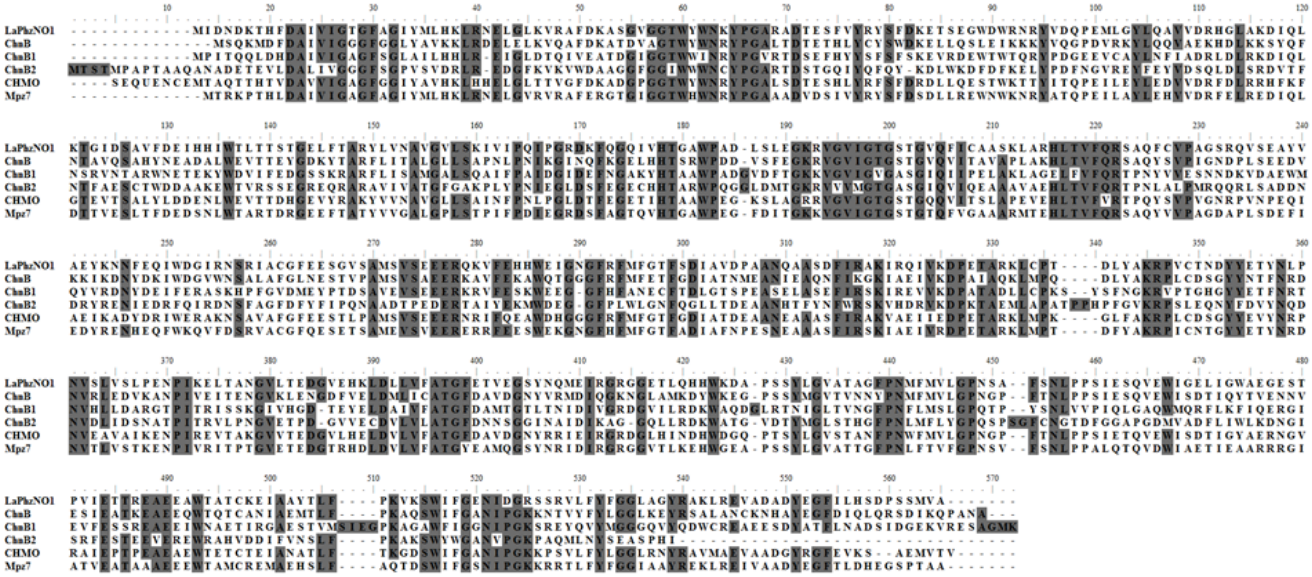


Figure S3. Multiple alignment of the amino acid sequence of LaPhzNO1 and LaPhzX. (A) LaPhzNO1 from *L. antibioticus* OH13, ChnB from *Acinetobacter* sp. SE19 (AAG10021.1), ChnB1 from *Brevibacterium* sp. HCU (AAG01289.1), ChnB2 from *Brevibacterium* sp. HCU (AAG01290.1), CHMO from *Rhodococcus* sp. HI-31 (PDB ID 3GWD), and Mpz7 from *Streptomyces* sp. SpC080624SC-11 (KF808339). (B) LaPhzX from *L. antibioticus* OH13, ActVA-Orf6 from *Streptomyces Coelicolor* A3 (2) (1N5T_B), and TcmH from *Streptomyces glaucescens* (AIS01206.1).

A



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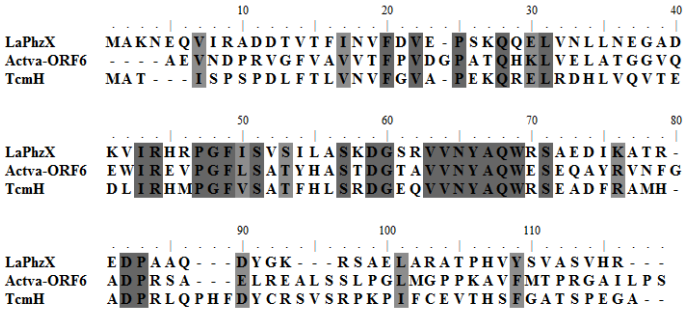


Figure S4. Generation of *LaPhzC* disruption mutant. (A) Scheme for gene disruption in *L. antibioticus* OH13. (B) Diagnose of the *LaPhzC* disruption mutant by PCR assays. M, Marker DL2000; *LaPhzC* mutant (left), using primers LaPhzC-VF and pJQ-R, with the expected size of 936-bp; *LaPhzC* mutant (right), using primers pJQ-F and LaPhzC-VR, with the expected size of 614-bp.

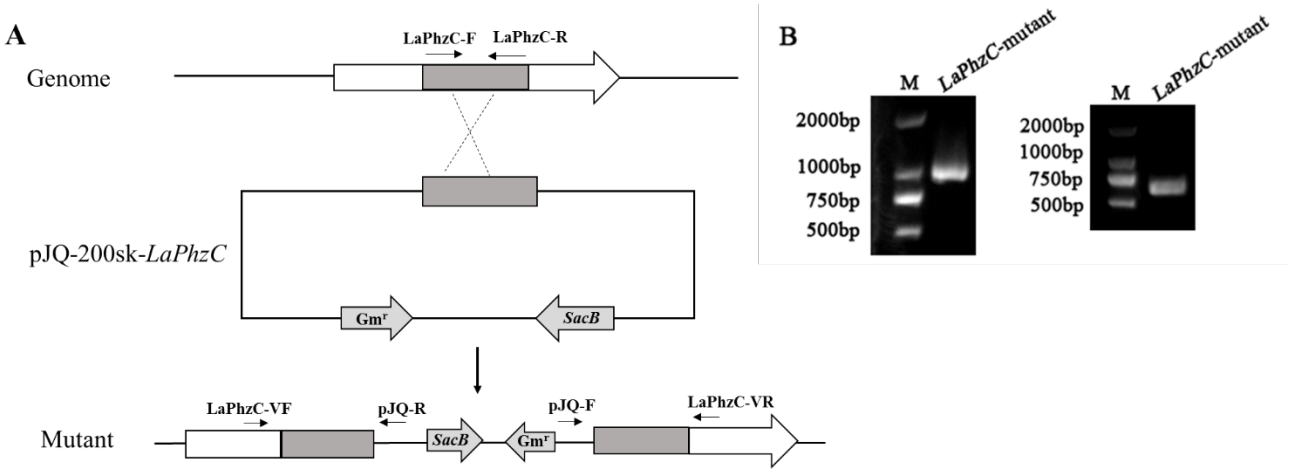


Figure S5. Generation of deletion mutants for *LaPhzD* and *LaPhzB*. (A) Scheme for gene deletion in *L. antibioticus* OH13. (B) Screening of *LaPhzD* deletion mutants by PCR with primers LaPhzD-deletion-MF/LaPhzD-deletion-MR. M, Marker DL2000; W, wild type DNA as the PCR template, with the expected size of 626-bp; V, the vector pJQ200SK- *LaPhzD* as the PCR template, with the expected size of 286-bp; -, negative control; 1, 2, 3, gene *LaPhzD* deletion mutants, with the expected size of 286-bp. (C) Screening of the *LaPhzB* deletion mutants by PCR using primers LaPhzB-deletion-MF/ LaPhzB-deletion-MR. M, Marker DL2000; W, wild type DNA as the PCR template, with the expected size of is 1000-bp; V, the vector pJQ200SK-*LaPhzB* as the PCR template, with the expected size of 655-bp; -, negative control; 1, 2, 3, 4 gene *LaPhzB* deletion mutants, with the expected size of 655-bp.

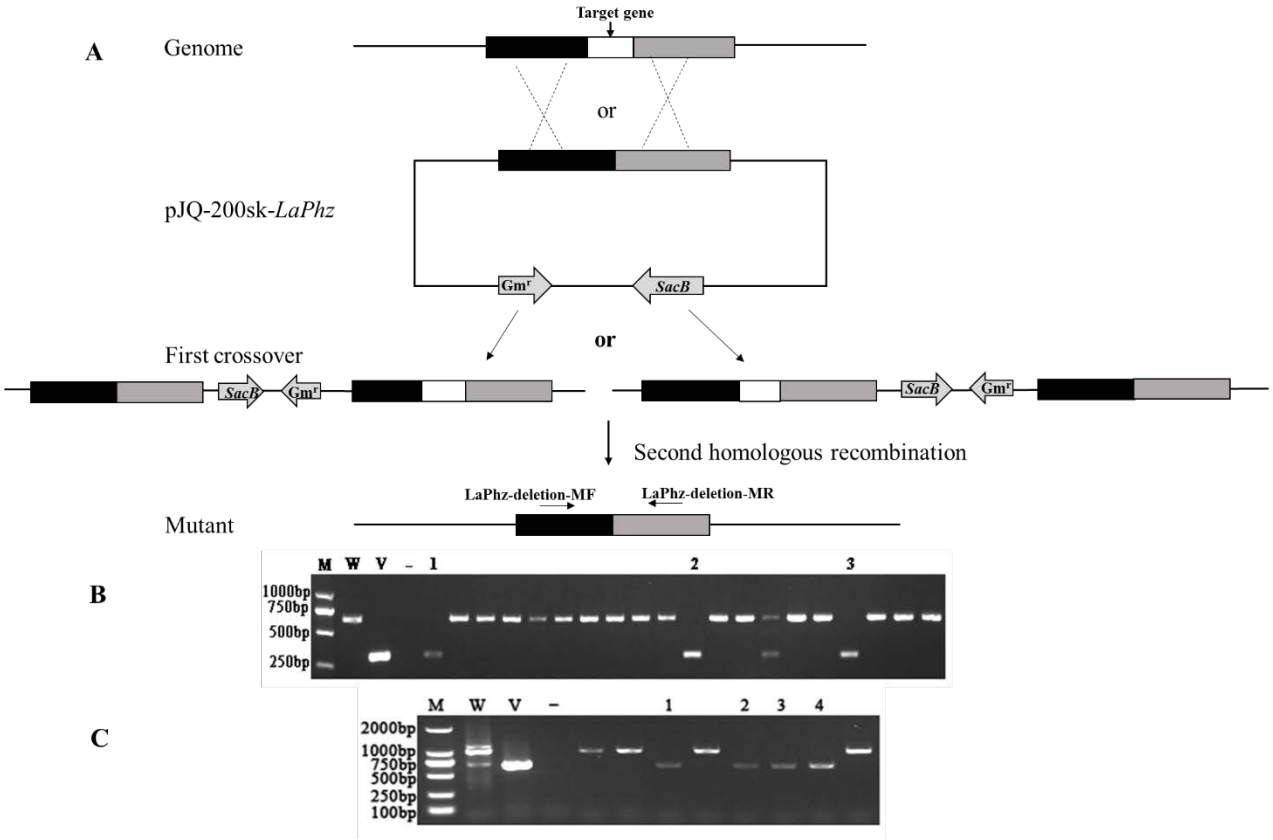


Figure S6. Generation of disruption mutants for *LaPhzNO1* and *LaPhzS*. Confirmation of gene disruption mutants by PCR and digestion. (A) Diagnose of gene *LaPhzNO1* disruption mutant by PCR assays. M, Marker DL2000; W, wild type; -, negative control; 1-2, *LaPhzNO1*-mutants, using primers LaPhzNO1-PF and pJQ-R, with the expected size of 1435-bp. (B) Diagnose of the gene *LaPhzS* disruption mutant by PCR assays. M, Marker DL2000; W, wild type; -, negative control; 1-2, *LaPhzS*-mutants, using primers LaPhzS-PF and pJQ-R, with the expected size of 1143-bp. (C) Confirmation of mutants *LaPhzNO1* and *LaPhzS* by digestion of PCR products. 1, digestion of PCR product from (A), with the expected size of 306-bp and 1129-bp; 2, digestion of PCR product from (B), with the expected size of 306-bp and 837-bp.

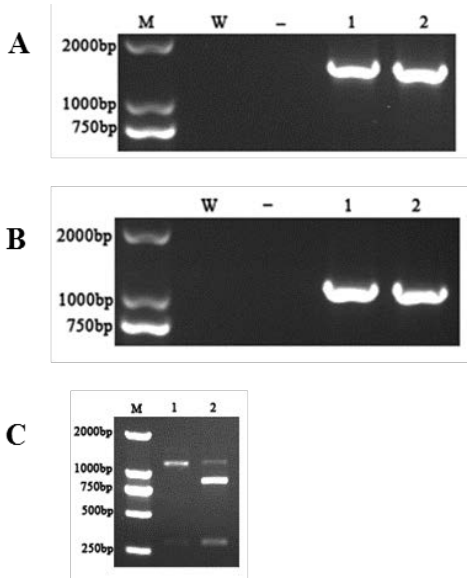


Figure S7. Antibacterial activity of *L. antibioticus* OH13 and *LaPhz* mutants, with *E. coli* (A) and *B. subtilis* (B) as the test organisms. Wild type, *L. antibioticus* OH13; Δ*LaPhzC*, Δ*LaPhzD* and Δ*LaPhzB*, mutants of phenazine core genes; Δ*LaPhzNO1* and Δ*LaPhzS*, mutants of phenazine modification genes.

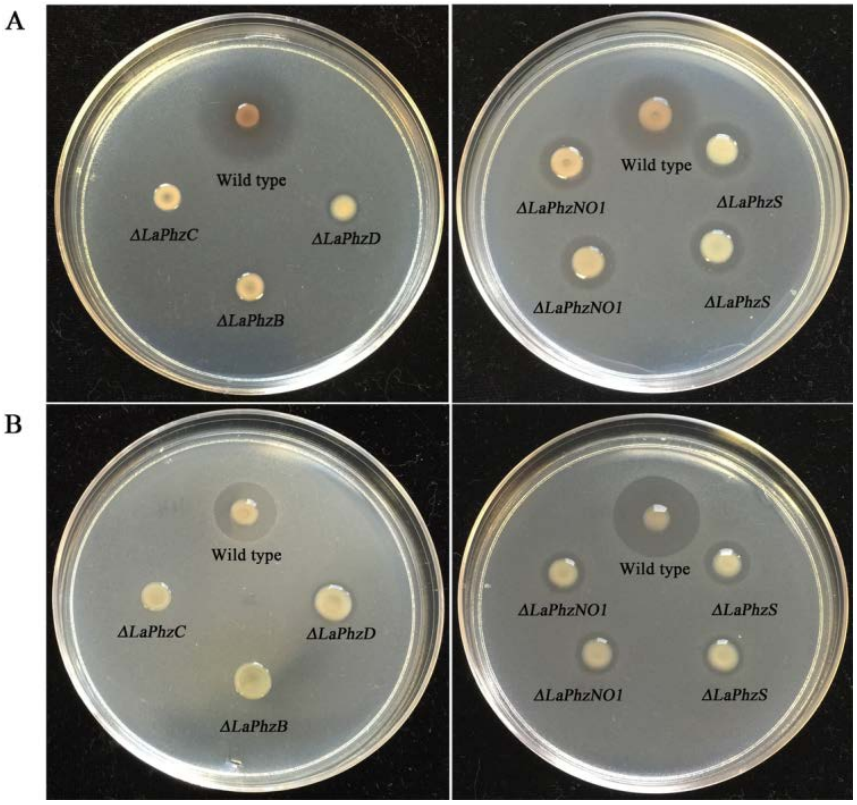
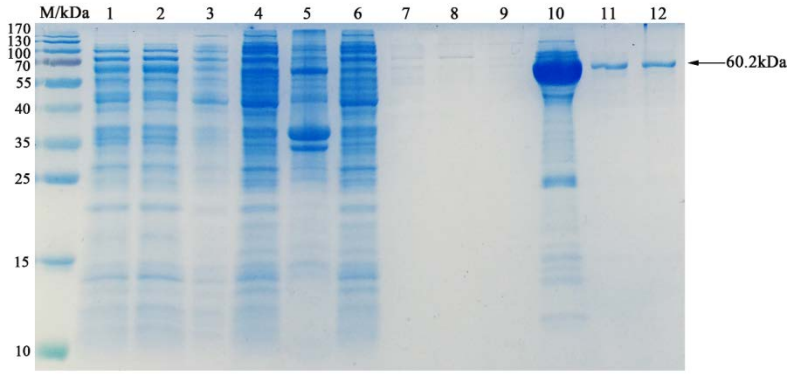


Figure S8. SDS-PAGE of LaPhzNO1 (A) and LaPhzS (B) expressed in *E. coli*. **A.** M, markers; 1 and 2, negative controls, total proteins from *E. coli* containing the empty pET-28a vector before and after IPTG induction; 3, total proteins from *E. coli* containing pET-28a-LaPhzNO1, before IPTG induction; 4, total soluble proteins after sonication from *E. coli* containing pET-28a-LaPhzNO1; 5, total precipitated proteins after sonication from *E. coli* containing pET-28a-LaPhzNO1; 6, flow-through fraction from a Ni-NTA column; 7-9, wash-off fractions from the Ni-NTA column; 10-12, imidazole-eluted fractions of the Ni-NTA column. The expected size of LaPhzNO1 is 60.2 kDa. **B.** M, markers; 1, total proteins before IPTG induction; 2, total precipitated proteins after sonication; 3, flow-through fraction of the Ni-NTA column; 4-7, imidazole-eluted fractions of the Ni-NTA column. The expected size of LaPhzS is 44.1 kDa.

A.



B.

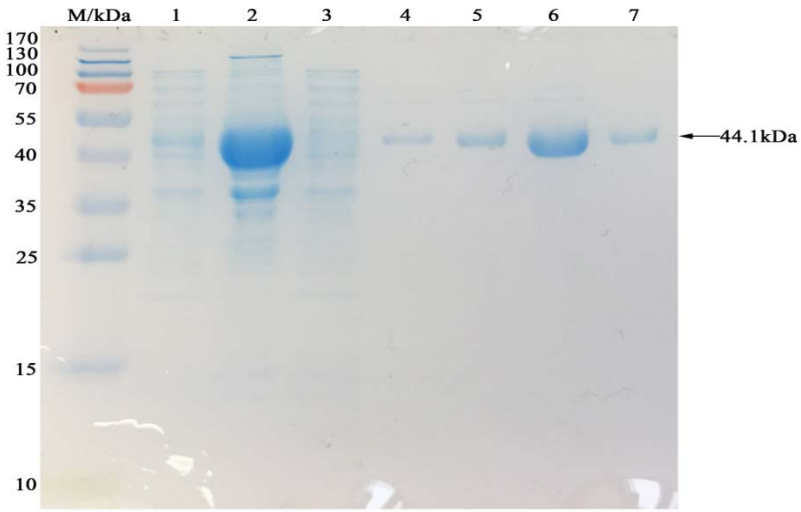


Figure S9. LC-MS analysis of *in vitro* reactions of LaPhzNO1, with compound **6** as substrate and in the presence of NADPH and FAD. Extracted ion chromatography of the mass (*m*/*z* 227.67) for substrate compound **6** is shown for the control reaction (top) and the LaPhzNO1 reaction (second). The peak at 10.39 min and 14.87 min in the LaPhzNO1 reaction gave *m*/*z* 227.67 and *m*/*z* 243.47 (compound **2**) respectively in LCQ-MS analysis (bottom two figures).

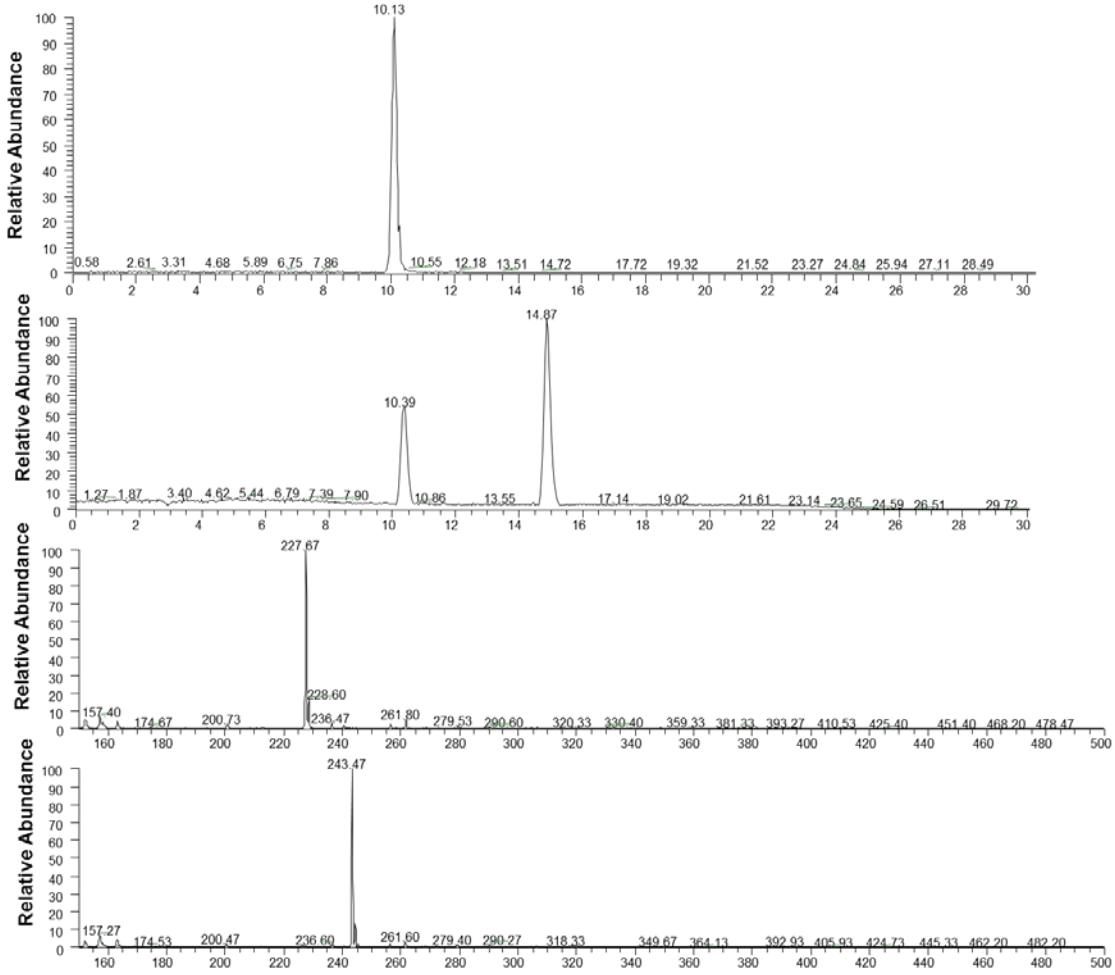
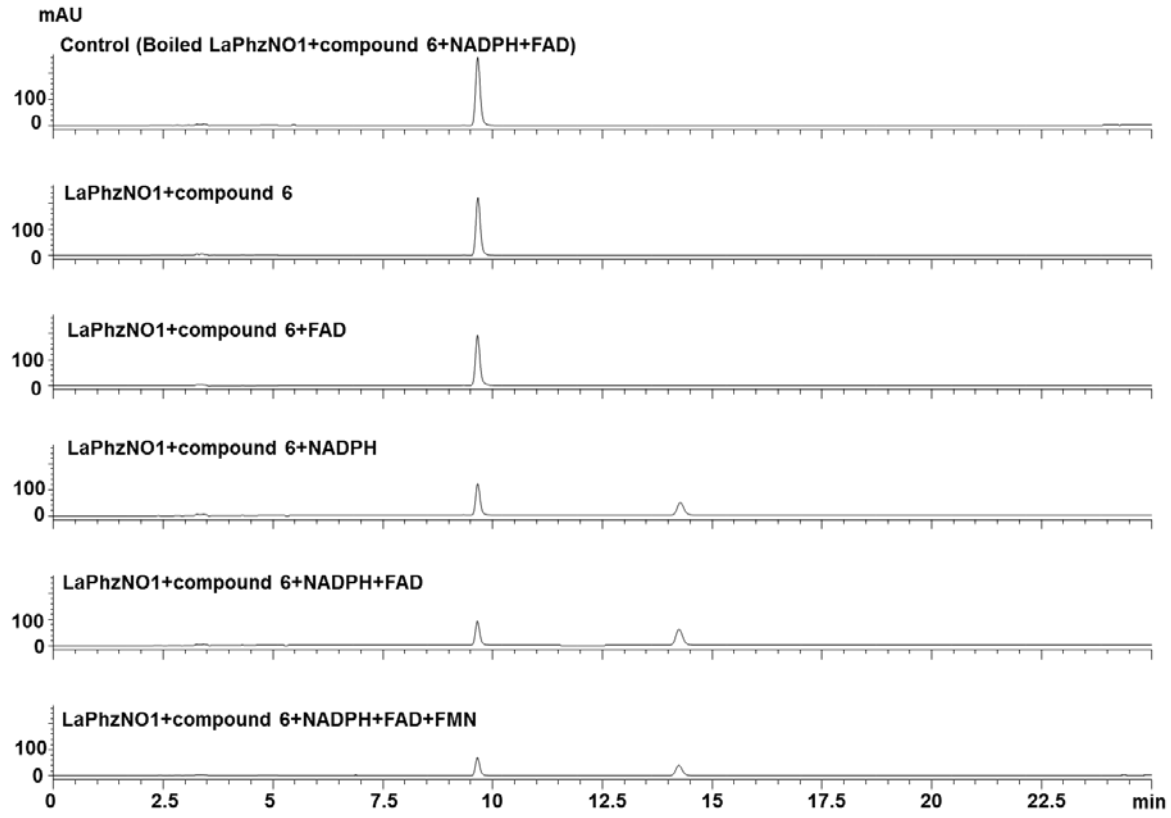


Figure S10. Test of the cofactor dependency of the LaPhzNO1 catalyzed N-oxidation. The reactions were incubated at 30 °C for 30 min.



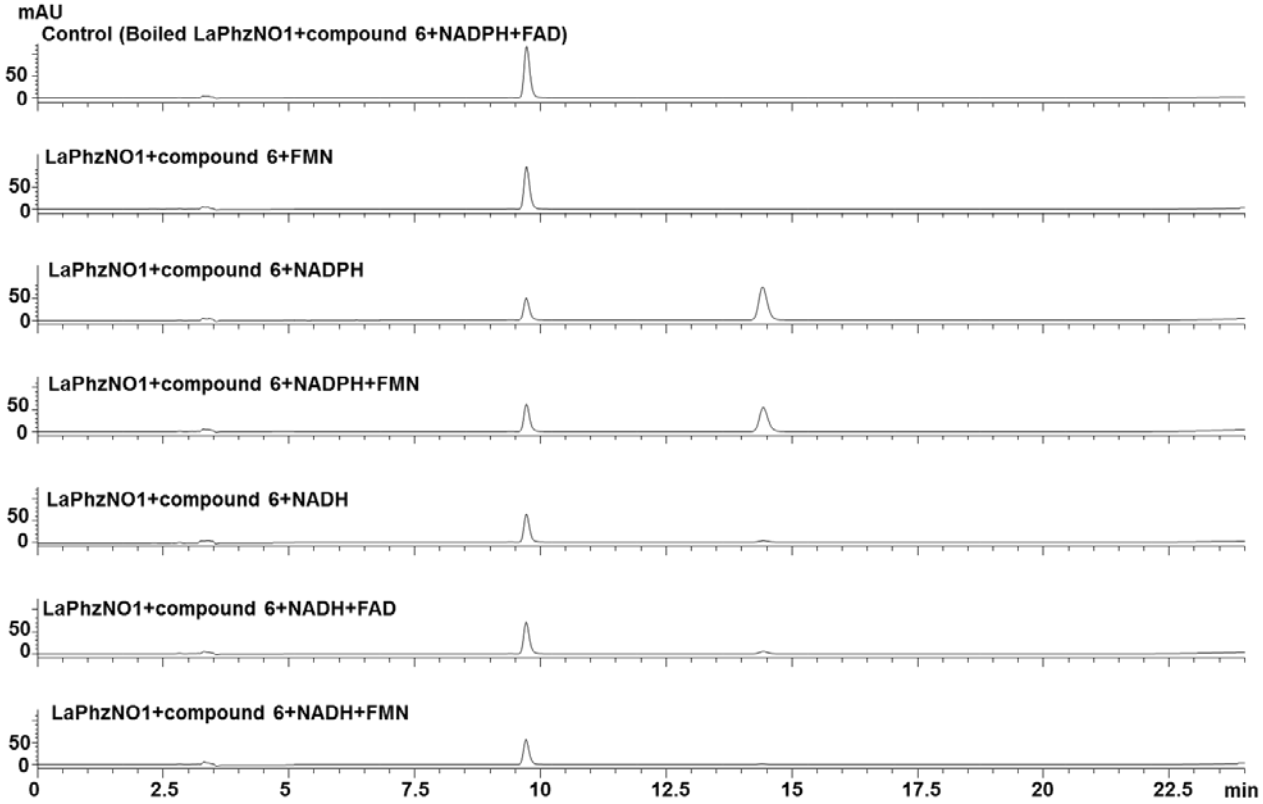
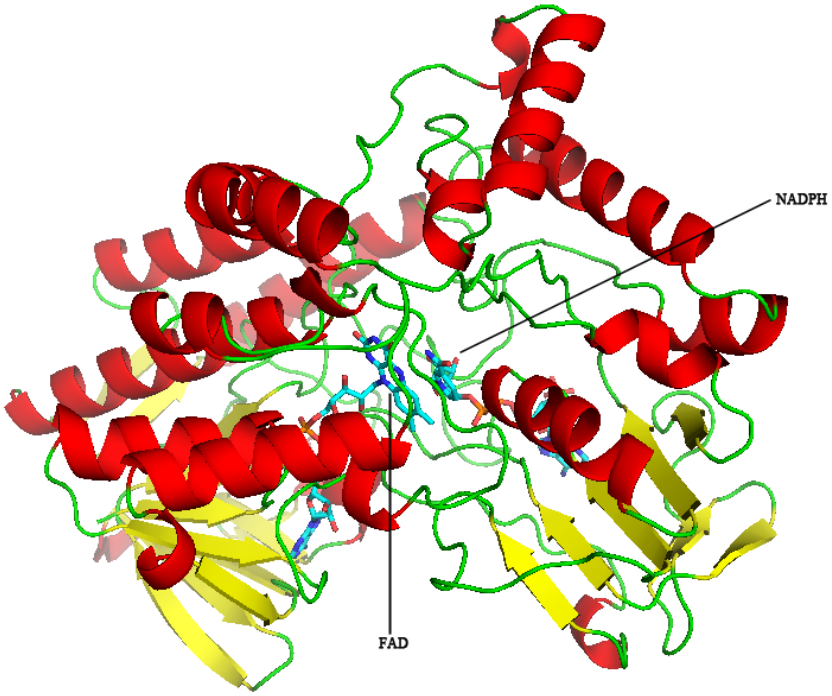

mAU
Control (Boiled LaPhzNO1+compound 6+NADPH+FAD)
LaPhzNO1+compound 6+FMN
LaPhzNO1+compound 6+NADPH
LaPhzNO1+compound 6+NADPH+FMN
LaPhzNO1+compound 6+NADH
LaPhzNO1+compound 6+NADH+FAD
LaPhzNO1+compound 6+NADH+FMN
50
0
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
min

Figure S11. (A) Structure modeling of LaPhzNO1 based on the crystal structure of cyclohexanone monooxygenase (PDB 3GWD, **DOI**: 10.2210/pdb3gwd/pdb).[5] **(B)** Visible spectrum of LaPhzNO1 and the supernatant of heat-denatured LaPhzNO1, with peak 1 at 442 nm and peak 2 at 382 nm. The supernatant remained the yellow color of LaPhzNO1, suggesting a non-covalent binding of the flavin cofactor to LaPhzNO1.



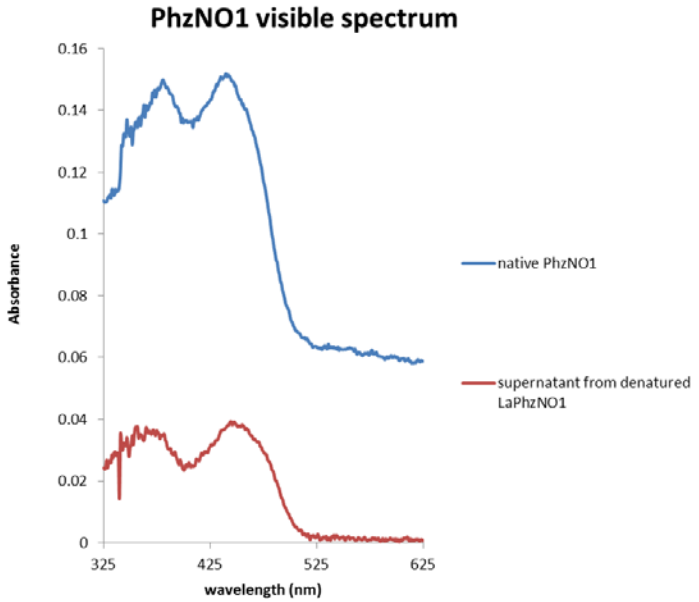


Figure S12. A proposed mechanism for N-oxidation of phenazines, which is catalyzed by the NADPH-dependent flavin-monooxygenase LaPhzNO1.

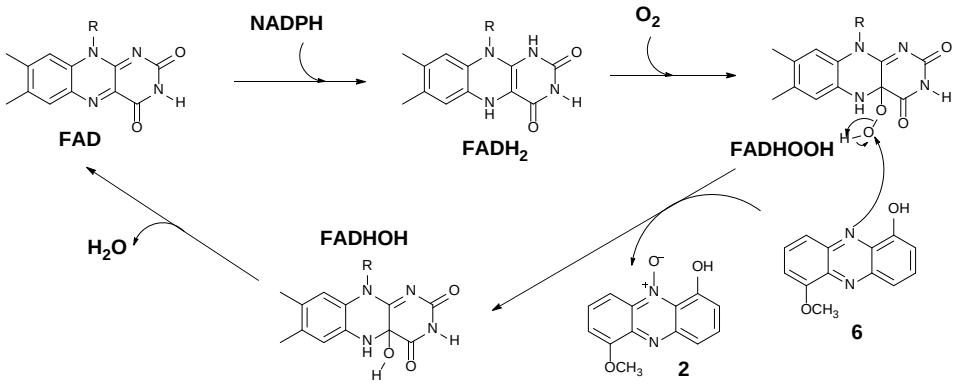
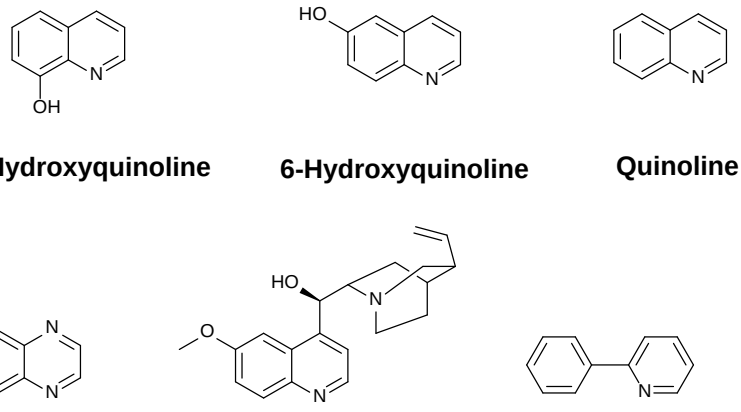
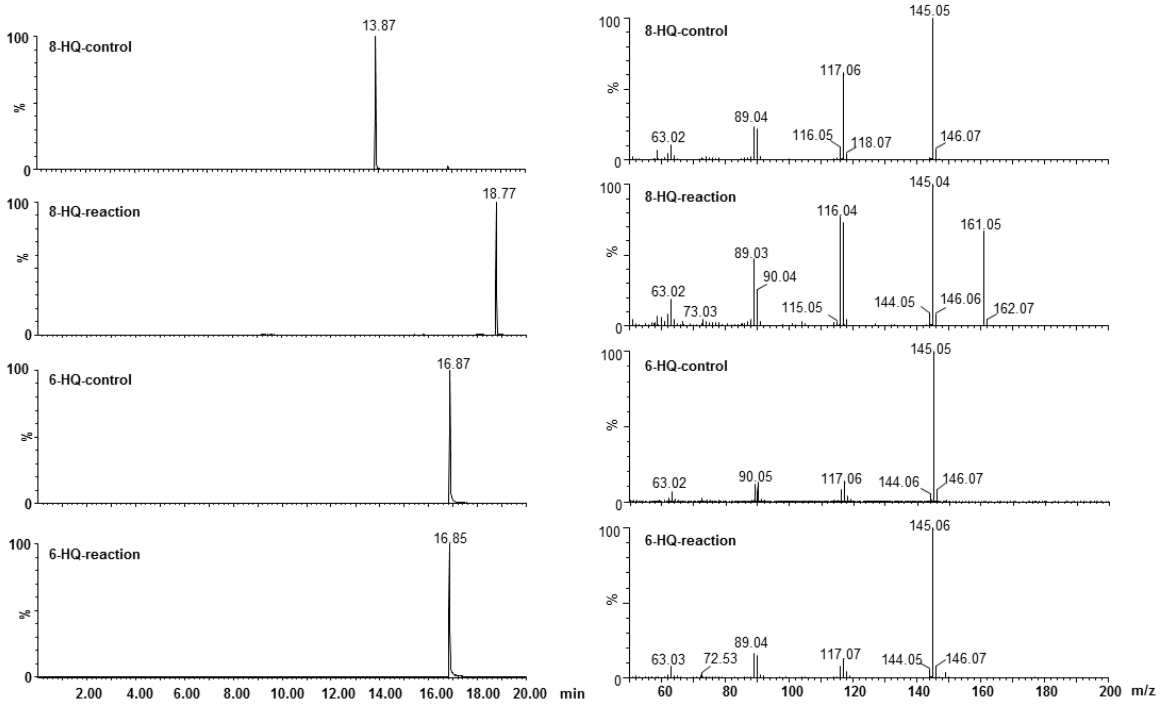
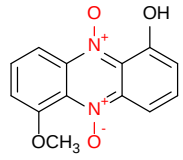


Figure S13. Test of LaPhzNO1 substrate selectivity using non-phenazine *N*-containing aromatic compounds as substrates, showing the GC-MS analysis of reactions with 8-hydroxyquinoline (8-HQ) and 6-hydroxyquinoline (6-HQ) as substrate. Total ion chromatograms (left) and the corresponding mass spectra (right) showed 8-HQ (13.87 min, *m*/*z* 145.05) was converted to its oxide (18.77 min, *m*/*z* 161.05), whereas 6-HQ (16.87 min, *m*/*z* 145.05) was not converted by the enzyme.

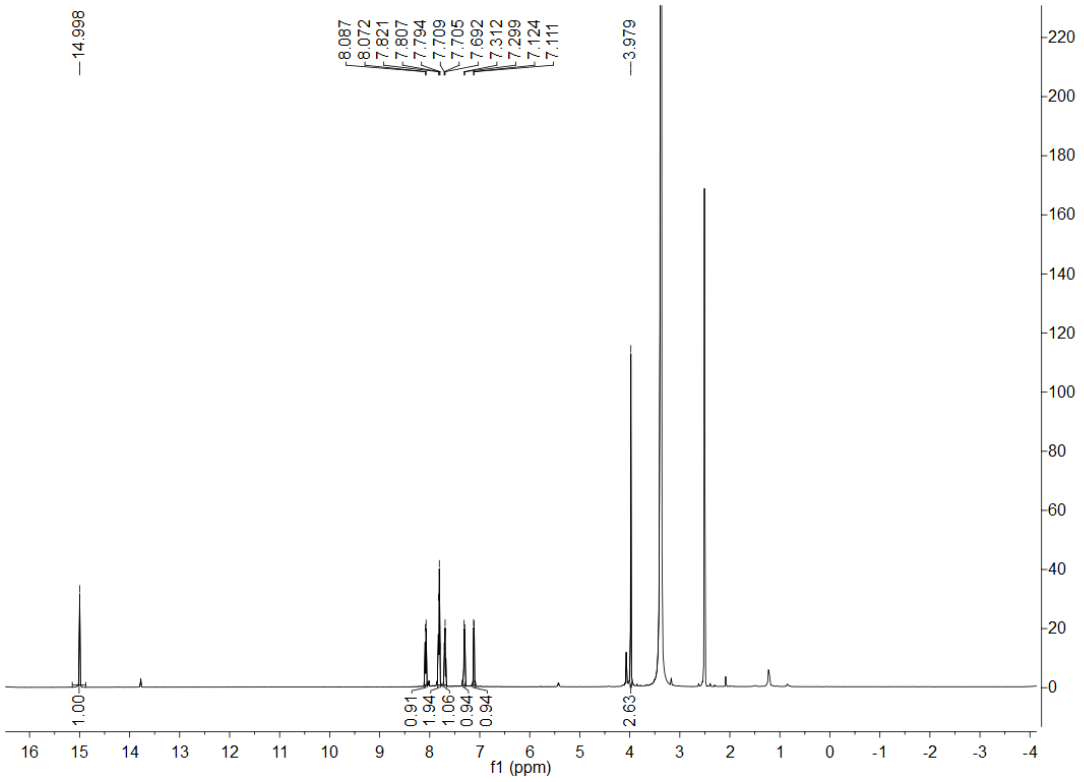


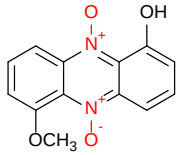


Below are NMR spectra for Compounds **1**-**6** shown in Figure 1 and S1.

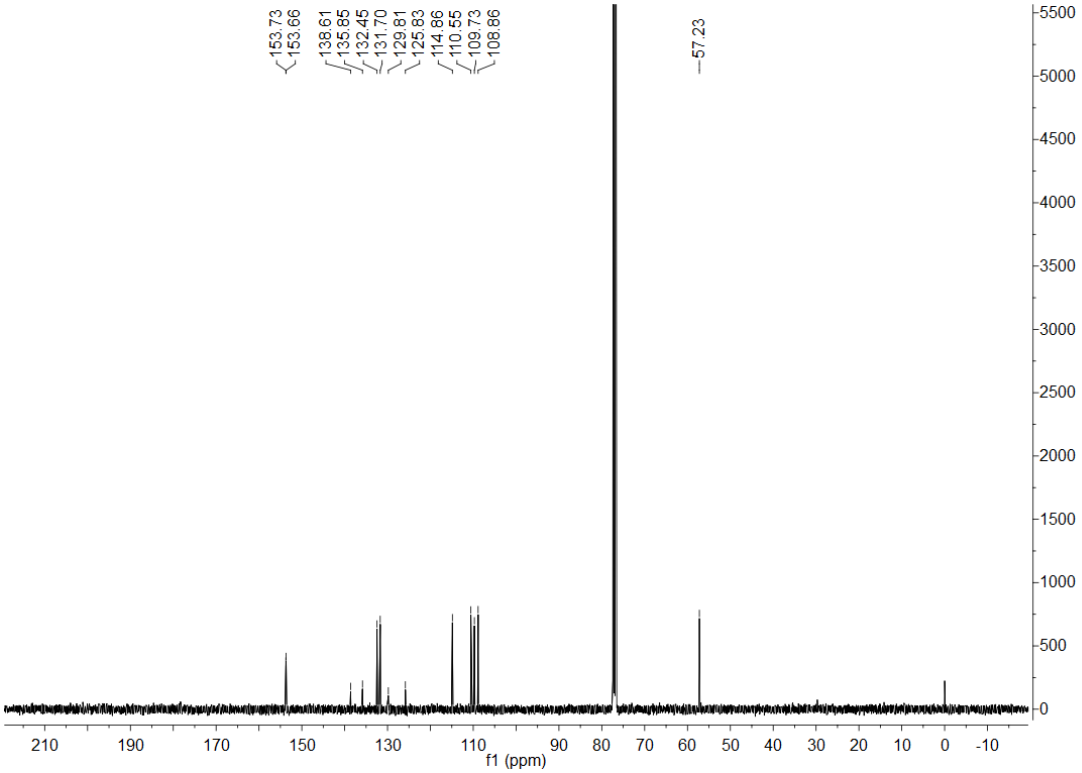


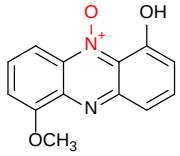
1



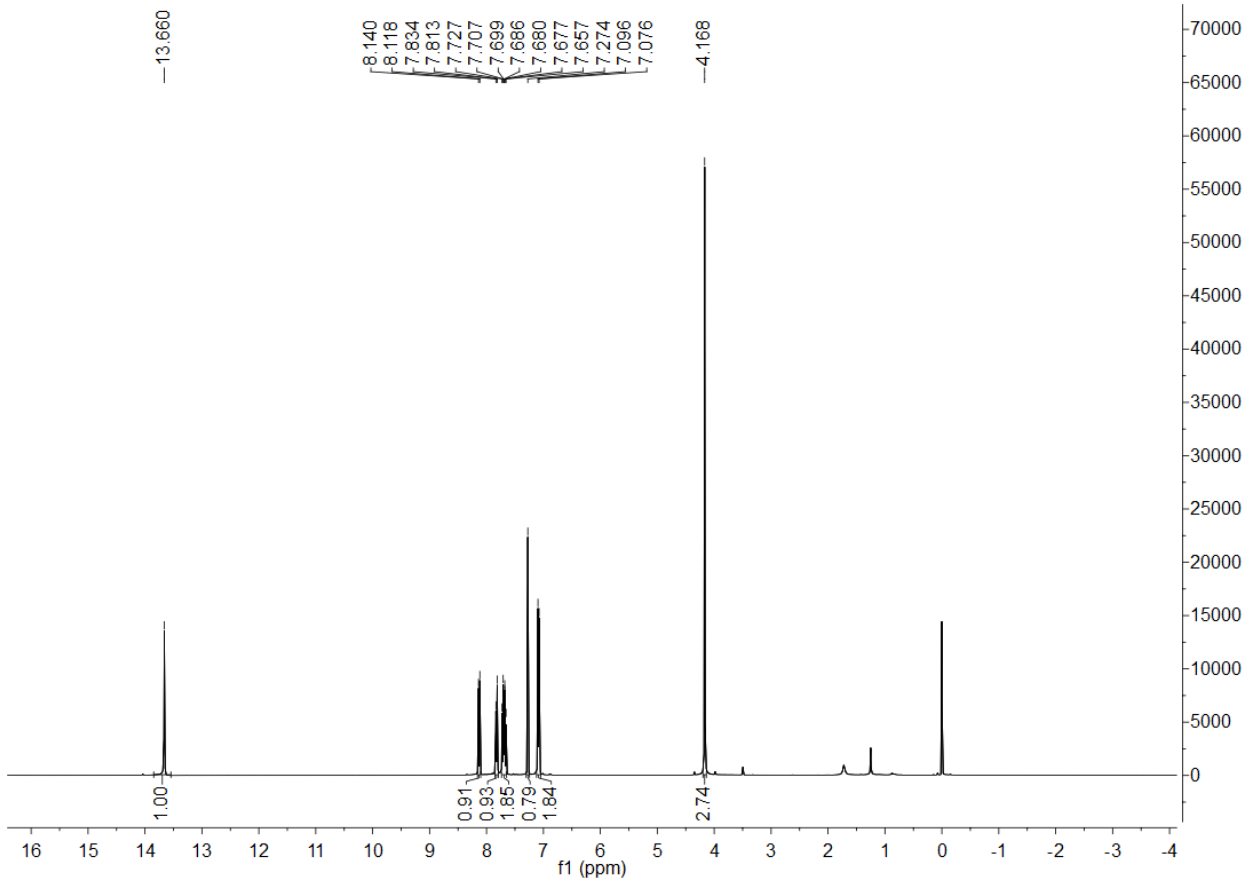

O
OH
N+
N+
OCH3
O-

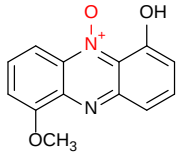
1


153.73
153.66
138.61
135.85
132.45
131.70
129.81
125.83
114.86
110.55
109.73
108.86
57.23
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

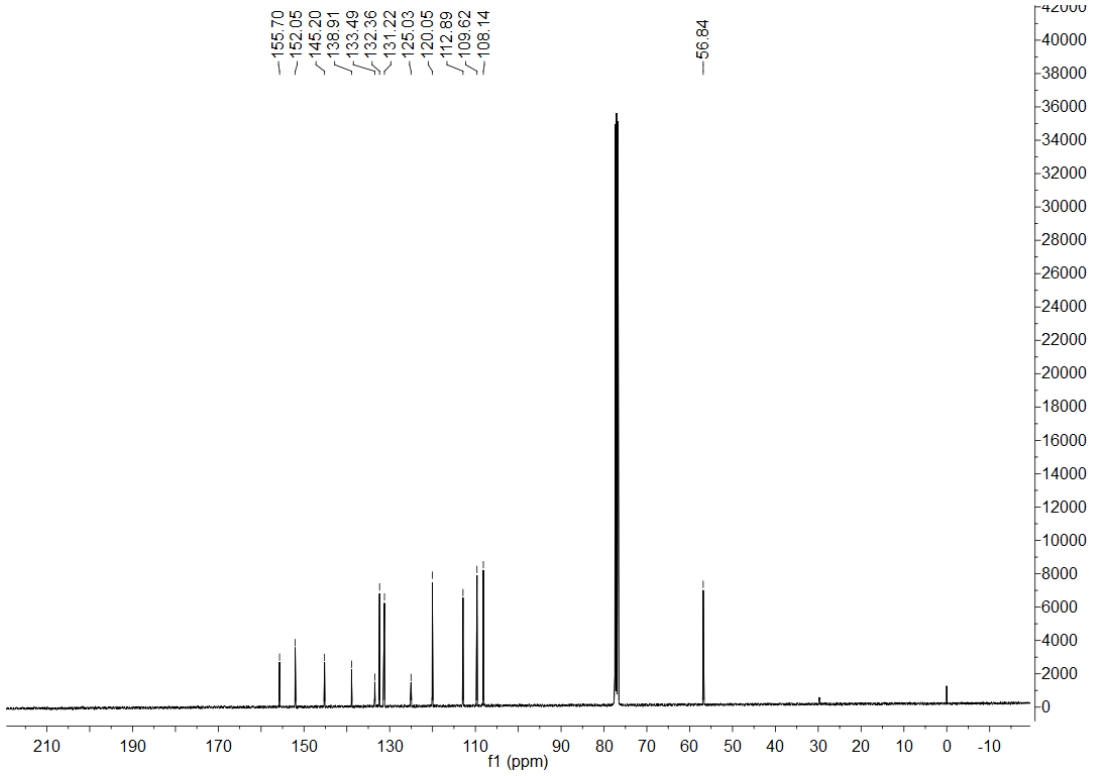

O
OH
N+
N
OCH3

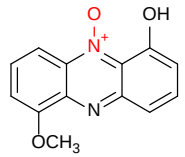
2


13.660
8.140
8.118
7.834
7.813
7.727
7.707
7.699
7.686
7.680
7.677
7.657
7.274
7.096
7.076
4.168
1.00
0.91
0.93
1.85
0.79
1.84
2.74
f1 (ppm)

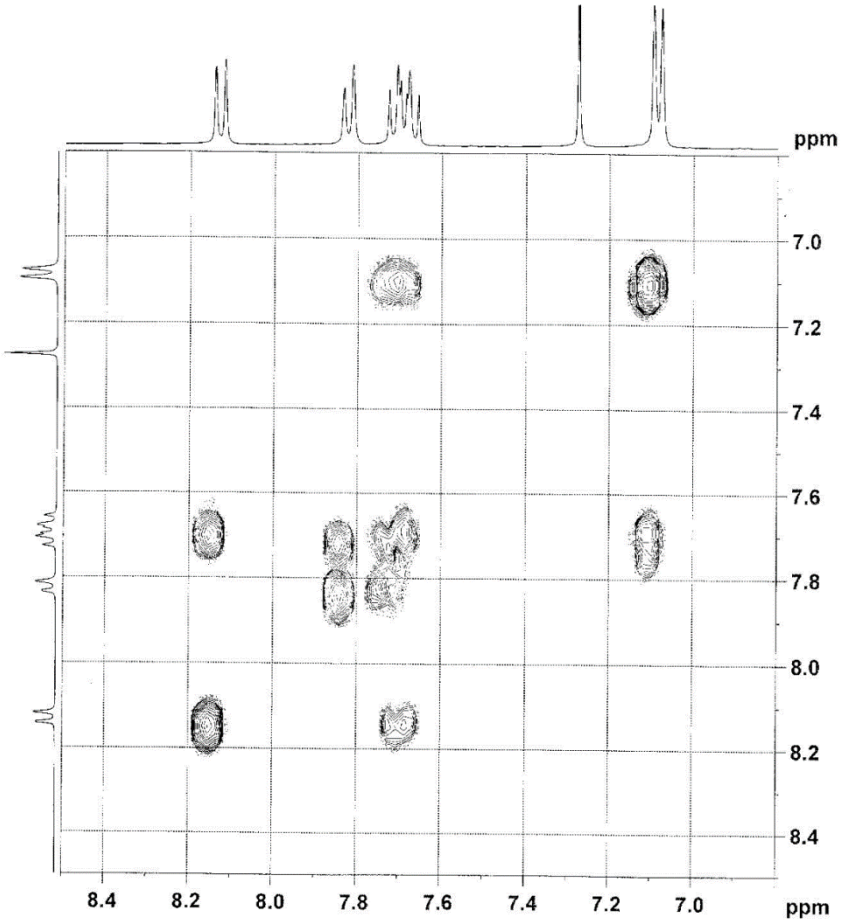

O
OH
N+
N
OCH3

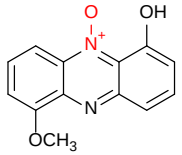
2


155.70
152.05
145.20
138.91
133.49
132.36
131.22
125.03
120.05
112.89
109.62
108.14
56.84
42000
40000
38000
36000
34000
32000
30000
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

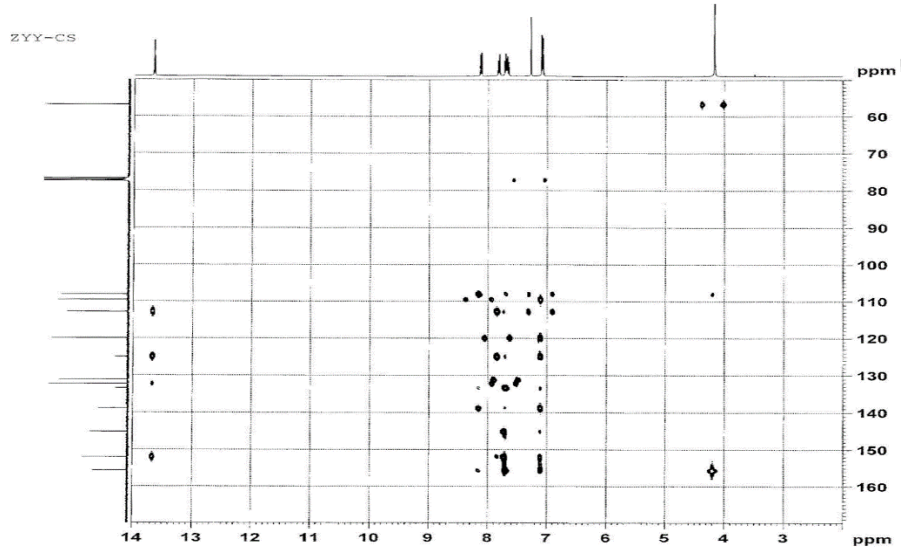


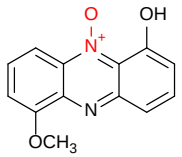
2



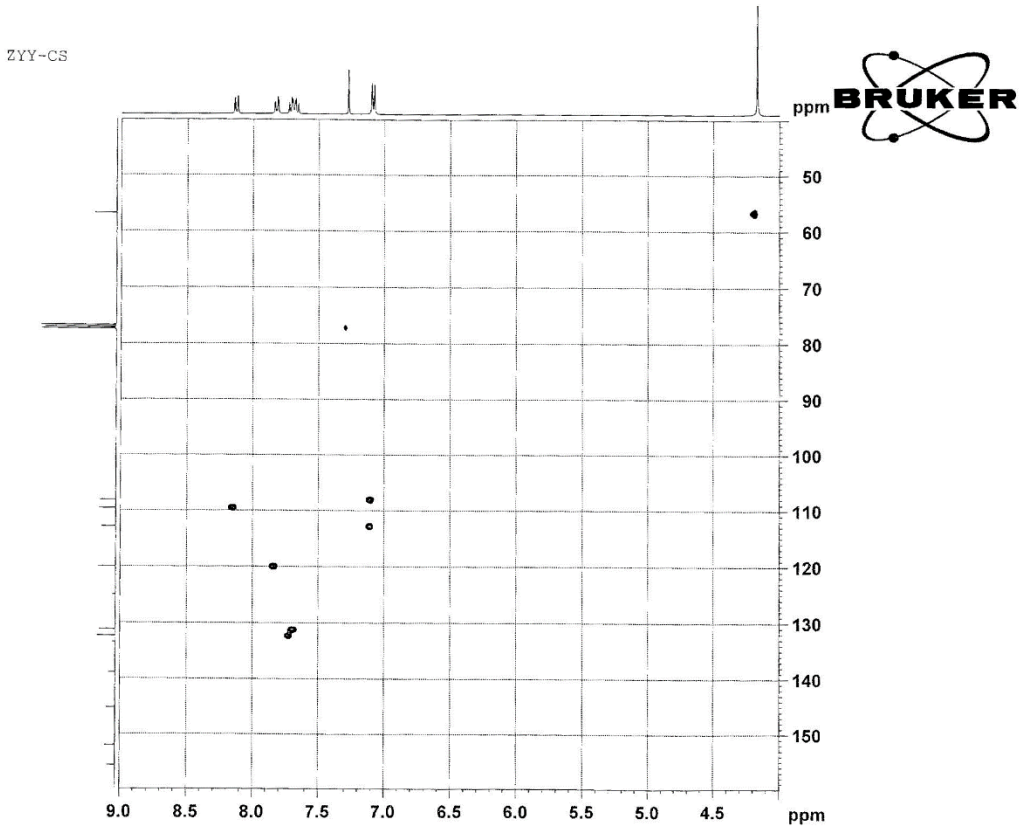

O
OH
N+
N
OCH3

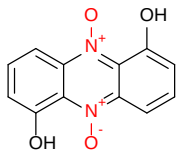
2


ZYY-CS
ppm
60
70
80
90
100
110
120
130
140
150
160
14
13
12
11
10
9
8
7
6
5
4
3
ppm

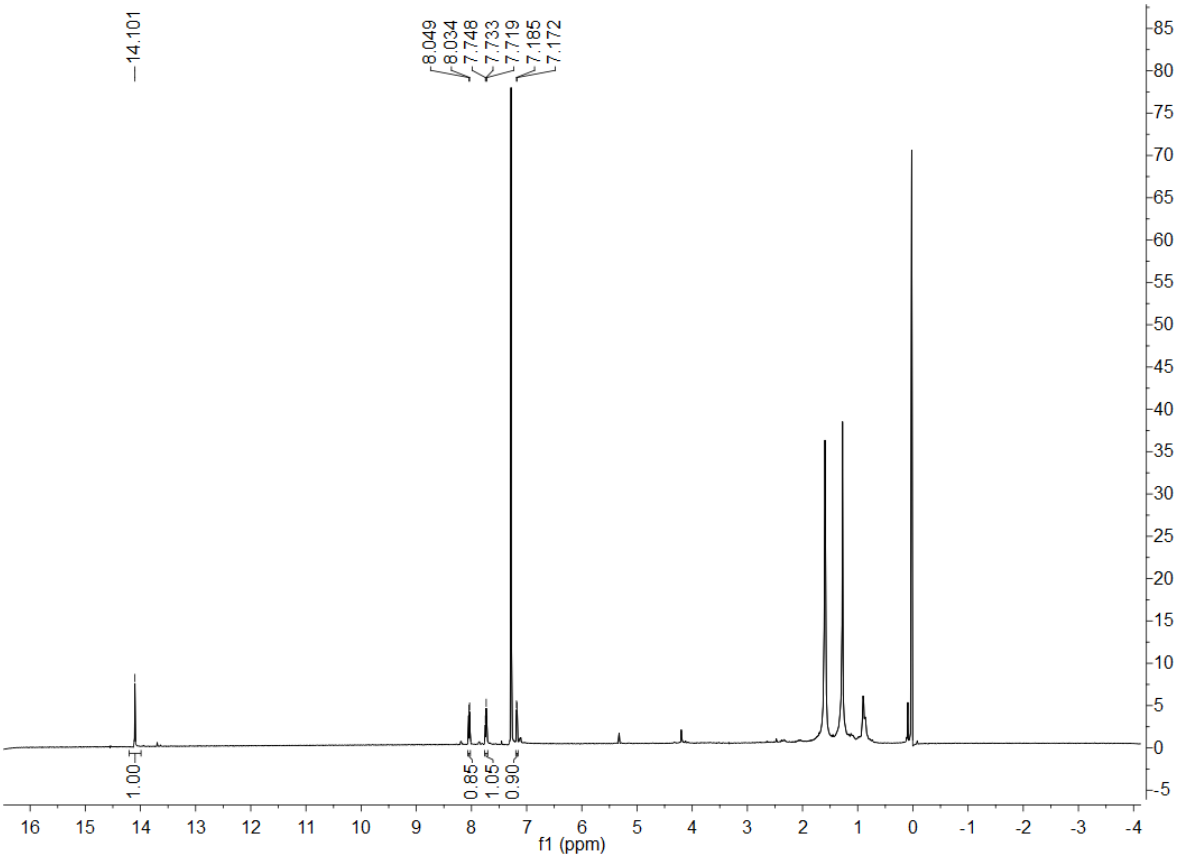

O
OH
N+
N
OCH3

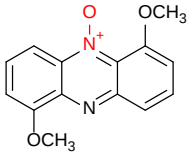
2


ZYY-CS
ppm
BRUKER
50
60
70
80
90
100
110
120
130
140
150
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
ppm

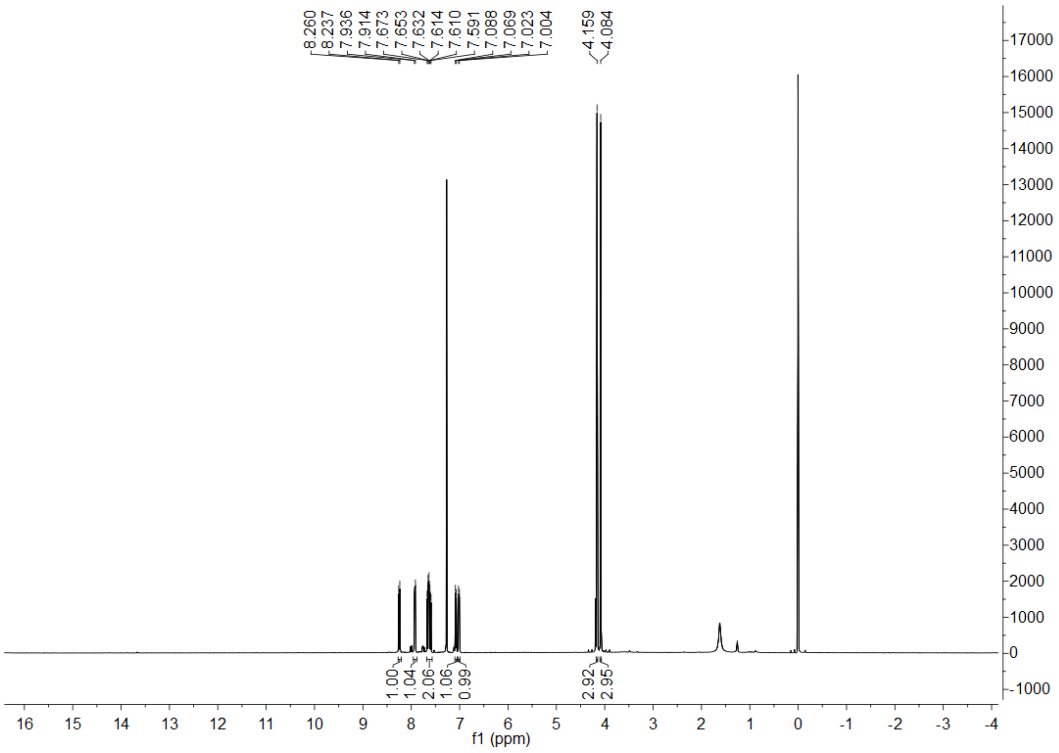

O
OH
N+
N+
O-
OH

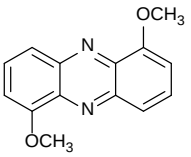
3


14.101
8.049
8.034
7.748
7.733
7.719
7.185
7.172
1.00
0.85
1.05
0.90
f1 (ppm)

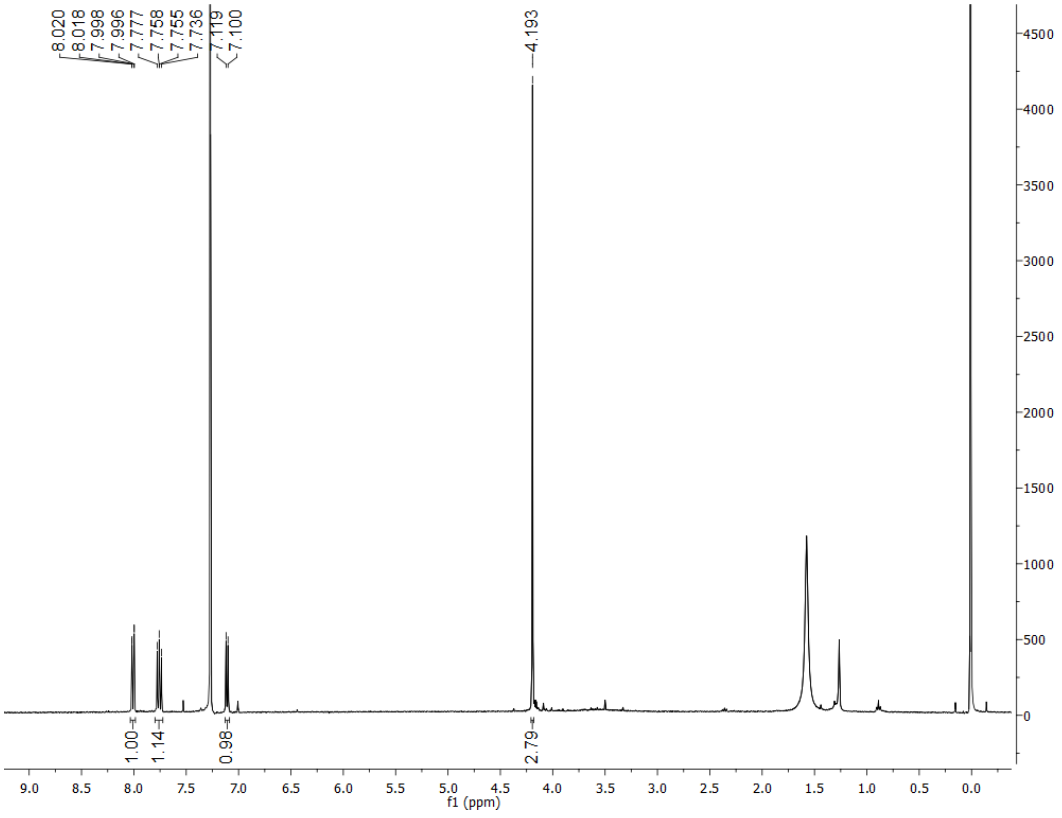

O
N+
OCH3
N
OCH3

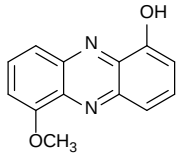
4


8.260
8.237
7.936
7.914
7.673
7.653
7.632
7.614
7.610
7.591
7.088
7.069
7.023
7.004
4.159
4.084
1.00
1.04
2.06
1.06
0.99
2.92
2.95
f1 (ppm)

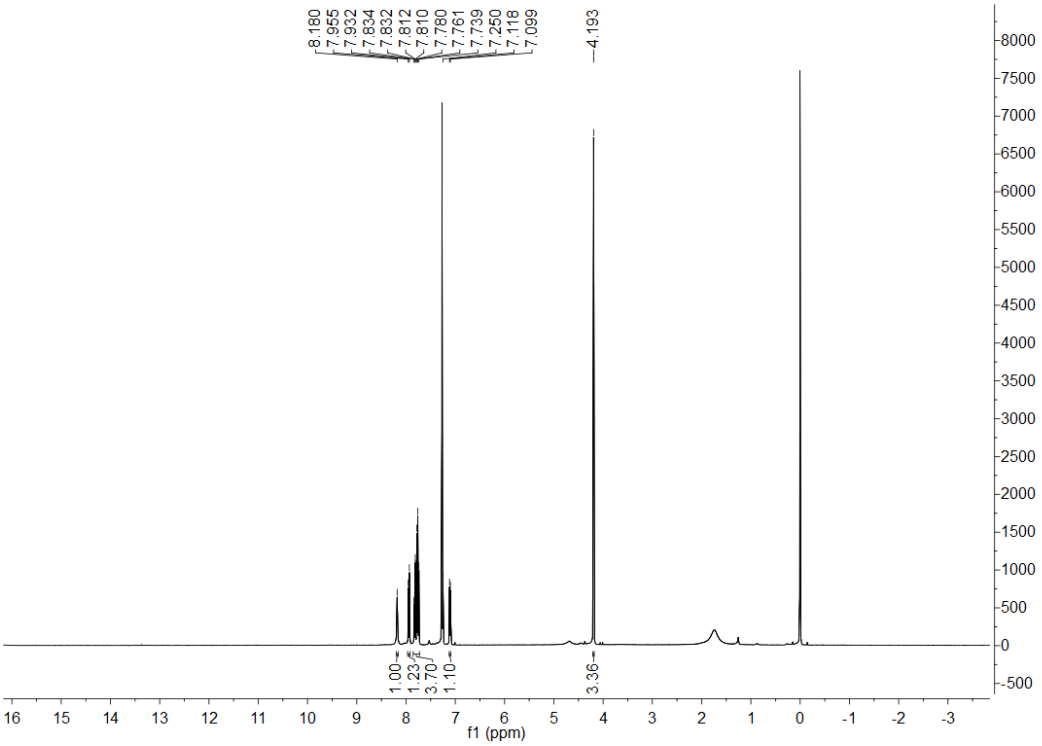

OCH3
N
N
OCH3

5


8.020
8.018
7.998
7.996
7.777
7.758
7.755
7.736
7.119
7.100
4.193
1.00
1.14
0.98
2.79
f1 (ppm)


OH
N
N
OCH3

6


8.180
7.955
7.932
7.834
7.832
7.812
7.810
7.780
7.761
7.739
7.250
7.118
7.099
4.193
1.00
1.23
3.70
1.10
3.36
f1 (ppm)

References

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