Description of Supplementary Files

File Name: Supplementary Information
Description: Supplementary Figures, Supplementary Tables, Supplementary Methods and Supplementary References
Supplementary Methods

Instrumentation

High-performance liquid chromatography (HPLC) was performed using a Thermo Scientific Dionex Ultimate 3000 system with an evaporative light scattering detector (Alltech, 2000ES) equipped with a DIKMA Diamonsil C18 column (3.5 μm, 150 x 2.1 mm). High resolution mass spectra (HRMS) were acquired using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc.) equipped with a Dionex Ultimate 3000 HPLC system (Thermo Fisher), or an LTQ XL Orbitrap Mass Spectrometer equipped with an Accela 600 U-HPLC system (Thermo Fisher Scientific Inc.). NMR spectra were recorded using Varian Inova 500 MHz NMR spectrometer. UV-vis spectroscopy analysis was performed on a 1900 double beam UV-vis spectrometer (Yoke Instrument Co. Ltd., Shanghai, China). PCR was performed on a Bio-Rad T100™ Thermal Cycler using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme Biotech) or KOD DNA polymerase (TOYOBO).

Chemicals and Biochemicals

All chemical reagents and anhydrous solvents were purchased from commercial sources and used without further purification unless otherwise specified. Most chemicals and biochemicals were purchased for Sigma-Aldrich. Enzymes were from Takara Biotechnology (Dalian, China) or from Vazyme Biotech(Nanjing, China). Biochemicals and culture media were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). S-adenosyl-L-methionine (SAM) and kanamycin were purchased from Sangon Biotech Co. Ltd (Shanghai, China). Fe(NH₄)₂(SO₄)₂•6H₂O and Na₂S were from Adamas Reagent Co. Ltd (Shanghai, China).

DNA Isolation, Manipulation, and Sequencing.

DNA isolation and manipulation in E. coli were carried out according to the standard methods R1. PCR amplifications were carried out on a Bio-Rad T100™ Thermal Cycler using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co. Ltd, China). Primer synthesis and DNA sequencing were performed at the Suzhou Genewiz Biotech Co. Ltd.

Construction of plasmids for overexpressing N-terminally hexa-His-tagged NosI, NosJ, NosK, and NosM

PCR products containing nosJ, nosI, nosK, and nosM were each amplified from Streptomyces actuosus genomic DNA using the corresponding primer pairs (nosJ-For and nosJ-Rev for nosJ, nosI-For and nosI-Rev for nosI, nosK-For and nosK-Rev for nosK, and nosM-For and nosM-Rev for nosM) respectively (see Supplementary Table 2 for the sequence). PCR products were each cloned into the NdeI/XhoI sites of pET28a using the homologous recombination method (ClonExpress II One
Step Cloning Kit Vazyme Biotech Co. Ltd, China) to produce the recombinant constructs pNosl-ET28a, pNosJ-ET28a, pNosK-ET28a, and pNosM-ET28a for expressing nosI, nosl, nosK, and nosM respectively.

Production and Aerobic Purification of NosJ, NosI, NosK,

*E. coli* BL21 (DE3) cells were transformed via electroporation with the expression plasmids, whereas *E. coli* BAP1 was used for expression of holo-NosJ. A single colony transformant was used to inoculate a 5 mL culture of LB supplemented with 100 μg/mL kanamycin. The culture was grown at 37 °C for 12 h and was used to inoculate 1 L of LB medium containing 100 μg/mL kanamycin. Cells were grown at 37 °C and 220 rpm to an OD_{600}~0.6-0.8, and then IPTG was added to a final concentration of 0.1 mM. After additional 18 h of incubation at 22 °C, the cells were harvested by centrifugation at 4000 x g for 15 min at 4 °C. The pellet was used directly for protein purification or stored at −80 °C before use.

Aerobic Protein purification

NosI, NosJ and NosK were purified aerobically. The cell pellet collected by centrifugation was re-suspended in 20 ml lysis buffer (50 mM MOPS, 200 mM NaCl, and 10% glycerol, pH 8.0), and was lysed either by using a high pressure homogenizer (FB-110X, Shanghai Litu Mechanical Equipment Engineering Co., Ltd, China) or by sonication on ice. Cell debris was removed via centrifugation at 21000 x g for 30 min at 4 °C. The supernatant was incubated with 4 ml Ni-NTA resin pre-equilibrated with the lysis buffer, and then subjected to affinity purification on a column. The desired elution fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit, and the concentrated protein solution was desalted using a DG-10 column (Bio-Rad) pre-equilibrated with the elution buffer (50 mM MOPS, 25 mM NaCl, and 10% (v/v) glycerol, pH 8.0). The protein fraction was collected and concentrated, analyzed by SDS-PAGE (10% Tris-glycine gel), and was used directly for in vitro assay or stored at −80 °C upon further use. Protein concentration was determined using a Bradford assay kit (Promega) using bovine serum albumin (BSA) as a standard.

Expression and Purification of the Precursor Peptide NosM

Expression and purification of NosM was performed using a protocol similar to that reported previously. The nosM-expressing plasmid was introduced into *Escherichia coli* BL21 (DE3) by electroporation, and a single colony transformant was grown in a shaker in 4 ml of LB medium supplemented with 100 mg/mL kanamycin for 12-15 hours at 37°C. This culture was used to inoculate 2 L of LB/Kanamycin (100 mg/mL) medium in 5L flask shaker, and the culture was grown at
37 °C/200rpm until the OD_{600} reached 0.7-0.9. IPTG was added to a final concentration of 0.5 mM and the culture was grown at 37°C for an additional 3 h. Cells were harvested by centrifugation at 4000 x g for 15 min at 4°C, and the pellet was resuspended in 20 mL of Buffer A (20 mM NaH₂PO₄, 500 mM NaCl, 0.5 mM imidazole, 10% glycerol, pH 7.5 at 25°C) and lysed by sonication. The sample was centrifuged at 21000 x g for 1hour at 25°C. The pellet was resuspended in 20 mL of denaturing Buffer B (6 M guanidine hydrochloride, 20 mM NaH₂PO₄, 500 mM NaCl, 0.5 mM imidazole pH 7.5 at 25°C). The insoluble portion was removed by centrifugation (21000 x g for 1hour) and the supernatant was clarified through 0.45-mm syringe filters. The 6 x His NosM peptide was purified by immobilized metal affinity chromatography (IMAC) approach at room temperature using a HiTrap chelating HP nickel affinity column (GE Healthcare). The desired NosM peptide was eluted using 3 x CV (column volume) of Elution Buffer (4M guanidine hydrochloride, 20 mM Tris, pH 7.5 at 25°C, 100 mM NaCl, 1M imidazole) before washing by 20 x CV of Washing Buffer (4M guanidine hydrochloride, 20 mM Tris, pH 7.5 at 25°C, 100 mM NaCl, 50 mM imidazole). Desalting was performed by solid phase extraction (SPE) using BioSelect™ reversed-phase C4 column (214SPE3000, Vydac). Collected fractions were lyophilized to afford white fluffy solid, which was stored at -20°C upon further use.

**Preparation of the Reconstituted NosN**

Production, purification and reconstitution of NosN were performed according to the previously reported protocol.³ Protein purification was performed in an anaerobic glove box (Coy Laboratory Product Inc., USA) with less than 5 ppm of O₂. The pellet was resuspended in 30 mL of lysis buffer (40mM Tris, 200 mM NaCl, 10mM imidazole, 10% glycerol, pH 8.0) and was lysed by sonication on ice. Cell debris was removed via centrifugation at 21000 x g for 1 hr at 4 °C. The supernatant was passed through a column containing 4 mL of high-affinity Ni-NTA resin (Qiagen Co. Ltd) pre-equilibrated with lysis buffer, and the column was then washed using 50 mL wash buffer (40 mM Tris, 200mM NaCl, 40 mM imidazole, 10% glycerol, pH 8.0). The protein fractions were collected using 10 mL of elution buffer (40 mM Tris, 200 mM NaCl, 500 mM imidazole, 10% glycerol, pH 8.0). The desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit and analyzed by SDS-PAGE (12% Tris-glycine gel).

For reconstitution of the NosN [4Fe-4S] cluster, dithiothreitol (DTT) was added to the purified protein solution to a final concentration of 5 mM. Fe(NH₄)₂(SO₄)₂ solution was then added slowly to a final concentration of 800 µM. After 15min incubation at the room temperature, Na₂S solution was added carefully to a final concentration of 800 µM. After further incubation on ice for 7-10 h, the resulting dark solution was subjected to desalt on a PD-10 (GE) column pre-equilibrated with the elution buffer (40mM Tris, 25mM NaCl, 10 mM DTT and 10% (v/v) glycerol, pH 8.0). The eluted protein fraction
was collected and concentrated, and was used directly for in vitro assay or stored at -80°C upon further use.

**Construction of the nosI Mutant**
To inactivate nosI, a 2.2 kb upstream fragment and 1.8 kb downstream fragment were amplified separately from the *S. actuosus* genomic DNA by PCR using the primer pairs nosI-L-For and nosI-L-Rev, and nosI-R-For and nosI-R-Rev, respectively (Supplementary Table 1). The resulting fragments were purified using a PCR purification kit (CWbiotech Co.Ltd, China) and then cloned into the EcoRI/HindIII site of pKC1139 using ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co. Ltd, China) to give the in-frame deletion construct pFDU1711. This plasmid was then introduced into *S. actuosus* via *E. coli*-Streptomyces conjugation. The nosI in-frame deletion mutant (designated as mFDU1711) was screened and its genotype was confirmed by PCR and DNA sequencing.

**Construction of the nosK mutant**
To inactivate nosK, a 2.2 kb upstream fragment and 2.1 kb downstream fragment were amplified separately from the *S. actuosus* genomic DNA by PCR using the primer pairs nosK-L-For and nosK-L-Rev, and nosK-R-For and nosK-R-Rev, respectively (Supplementary Table 1). The resulting fragments were purified using a PCR purification kit (CWbiotech Co.Ltd, China) and then cloned into the EcoRI/HindIII site of pKC1139 using ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co. Ltd, China) to give the in-frame deletion construct pFDU1713. This plasmid was then introduced into *S. actuosus* via *E. coli*-Streptomyces conjugation. The nosK in-frame deletion mutant (designated as mFDU1713) was screened and its genotype was confirmed by PCR and DNA sequencing.

**In trans Complementation of nosI in *S. actuosus***
A ~1.3kb PCR product containing nosI was amplified by PCR using the primer nosI-C-For and nosI-C-Rev (Supplementary Table 1), and the resulting DNA fragment was cloned into pMD19-T to yield pNosI-MD19. The 1.3 kb HindIII/XbaI fragment was recovered from pNosI-MD19, which was co-ligated with a 0.4 kb EcoRI/HindIII fragment harboring the promoter PermE* into the EcoRI/XbaI site of pSET152 to yield pFDU1712, in which the 1.3kb fragment containing nosI is under the control of the PermE* promoter. pFDU1712 was then introduced into mFDU1711 using *E. coli*-Streptomyces conjugation to yield the recombinant strain mFDU1712.

**In trans Complementation of nosK in *S. actuosus***
A ~0.8 kb PCR product containing nosK was amplified by PCR using the primer nosK-C-For and
nosK-C-Rev (Supplementary Table 1), and the resulting DNA fragment was cloned into pMD19-T to yield pNosK-MD19. The 0.8 kb HindIII/XbaI fragment was recovered from pNosK-MD19, which was co-ligated with a 0.4 kb EcoRI/HindIII fragment harboring the promoter PermE* into the EcoRI/XbaI site of pSET152 to yield pFDU1714, in which the 0.8 kb fragment containing nosK is under the control of the PermE* promoter. pFDU1714 was then introduced into mFDU1713 using E. coli-Streptomyces conjugation to yield the recombinant strain mFDU1714.

**Nosiheptide Production and Analysis**

Fermentation and production of nosiheptide was performed similarly to a previously reported procedure.6 100 μl spore suspension the S. actuosus wild type or mutant strains was inoculated into a 2L flask containing 500 ml of seed medium (sucrose 2.0%, corn steep liquor 3.0%, peptone 0.5% and CaCO3 0.5%) and grown for 36 h at 28 °C, 220 rpm. The culture was then transferred into 10 x 500 ml fermentation medium (L-glutamate 0.5%, L-arginine 0.1%, L-aspartate 0.1%, K2HPO4·7H2O 0.05%, MgSO4·7H2O 0.1%, Na2SO4 0.26%, ZnSO4·7H2O 0.001%, FeSO4·7H2O 0.002%, CaCO3 0.3%, MnSO4·H2O 0.002%, and glucose 4%, the pH was adjusted to 7.25 before autoclaving) for continuous cultivation at 28 °C, 220 rpm for 7 days. The mycelia cake was collected by centrifugation (4000 x g for 25 min), and was soaked with 3 L acetone overnight, whereas the culture was adjusted to a pH of ~4 before extracted by n-butanol. The organic solvent was removed on a rotary evaporator, and the residue was combined and mixed with 100 ml 100 mM sodium citrate (pH 4.0), and the resulting mixture was extracted three times with an equal volume of ethyl acetate. The organic layer was then combined and taken to dryness on a rotary evaporator, and the residue was redissolved in methanol and analyzed by HPLC. The column (Agilent 1100 with a Zorbax SB-C18 column (9.4 mm × 25 cm) was equilibrated with 85% solvent A (H2O containing 0.1% TFA) and 15% B (CH3CN), and developed with the following program: 0 to 3 min, constant 85% A/15% B; 3 to 6 min, a linear gradient from 85% A/15% B to 60% A/40% B; 6 to 12 min, constant 60%A/40% B; 12 to 19 min, a linear gradient from 60% A/40% B to 45% A/55% B; 19 to 22 min, a linear gradient from 45% A/55% B to 15% A/85% B; 22 to 28 min, constant 15% A/85% B; and 28 to 32 min, a linear gradient from 15% A/85% B to 85% A/15% B.

**Synthesis**

Synthesis of MIA (3), MIA-SNAC (9), and DMIA (12) were detailed in a recent Communication.3
Preparation of Methyl N-acetyl-S-(3-methyl-indole-2-carbonyl)cysteinate (11)

Methyl cysteinate hydrochloride salt (MCA) (1.03 g, 6 mmol) was added to a pyridine-toluene solution (2 mL pyridine in 20 mL toluene), and acetic anhydride (0.56 mL, 7.2 mmol) was subsequently added in a dropwise manner at 0°C. After a further stirring for 2 h, the reaction mixture was poured into 30 ml cold water, and toluene was removed by stratification. The aqueous layer was adjusted to pH 7 ~ 8 by saturated NaHCO₃, and the resulting solution was then stirred at RT for another 30 min. The mixture was then adjusted to pH 5 ~ 6 by 1M HCl, extracted with AcOEt (20 mL×3), and the combine organic layer was dried by anhydrous Na₂SO₄. The crude methyl N-acetylcysteinate (MACA, 0.85 g) was obtained as yellow oil, which was used for the next step without further purification.

MIA (0.2 g, 1.15 mmol) and MACA (0.35 g, 2 mmol) was dissolved in 20 mL dry DCM. EDCI (0.25 g, 1.3 mmol), HOBt (0.20 g, 1.5 mmol) and Et₃N (0.4 mL) were sequentially added to the solution, and the resulting mixture was stirred at RT overnight. The reaction mixture was poured into 10 ml cold water, extracted with DCM, and the organic layer was washed with water and brine, and dried with anhydrous MgSO₄. After removal of the solvent, the crude was purified by silica gel column chromatography to afford pure compound 11 (0.20 g, yield 53%). MS (ESI) [M+H]⁺: 335.1060 (calculated), 335.1038 (found). ¹H NMR (400 MHz, DMSO-d₆) δ 11.63 (s, 1H), 8.56 (d, J = 7.9 Hz, 1H), 7.71 (d, J = 8.1 Hz, 1H), 7.43 (d, J = 8.3 Hz, 1H), 7.31 (t, J = 7.6 Hz, 1H), 7.09 (t, J = 7.5 Hz, 1H), 4.53 (td, J = 8.3, 5.3 Hz, 1H), 3.68 (s, 3H), 3.65 (d, 2H), 2.59 (s, 3H), 1.87 (s, 3H).

Preparation of (R)-pantothenic acid (S2)

Preparation of (R)-pantothenic acid (S2) was performed by following a previous report. Briefly, a suspension of calcium (R)-pantothenate (S1) (25.0 g, 52.6 mmol) in 1M HCl (125 mL) was stirred vigorously until the starting material was completely dissolved. The solution was saturated by the addition of NaCl and the resulting suspension was extracted with EtOAc (6 x 250 mL). The combined
organic layers were dried over Na$_2$SO$_4$ and concentrated in vacuo to yield (R)-pantothenic acid as a colorless oil (9.5 g, 82%). The crude product was used directly without further purification.

**Preparation of (R)-3-(2,2,5,5-tetramethyl-1,3-dioxane-4-carboxamido)propanoic acid (S3)**

Preparation of S3 was performed by following a previous report.$^8$ To a solution of (R)-pantothenic acid (S2) (9.5 g, 43.4 mmol) in acetone (250 mL), 2-methoxyprop-1-ene (13.5 mL, 141 mmol) and pTsOH·H$_2$O (0.45 g, 2.39 mmol) were added subsequently. The mixture was stirred for 3~4 hours at room temperature and then concentrated in vacuo to give a yellow solid, which was washed with water and acetone. After dried at room temperature, a pale yellow solid was obtained (4.9 g, 44%). $^1$H NMR (DMSO-d$_6$, 400MHz): $\delta$ = 0.89 (s, 3H, CH$_3$), 0.92 (s, 3H, CH$_3$), 1.38 (s, 6H, -O-C-CH$_3$), 2.41 (t, 2H, J = 6.8 Hz, -CH$_2$-COO-), 3.19 (d, 1H, J = 11.6 Hz, -CH$_2$-O-), 3.21~3.37 (m, 2H, -CH$_2$-N-), 3.64 (d, 1H, J = 11.6 Hz, -CH$_2$-O-), 4.04 (s, 1H, -O-CH-CONH-), 7.44 (t, 1H, J = 5.6 Hz, -NH-), 12.26 (s, 1H, -COOH).

**Preparation of S4**

To a solution of acid S3 (100 mg, 0.39 mmol) in CH$_2$Cl$_2$ (2 mL) was added cysteamine hydrochloride (57 mg, 0.50 mmol), PyBOP (304 mg, 0.59 mmol) and diisopropylethylamine (0.3 mL, 1.9 mmol). The reaction was allowed to stir for 12 h. The solvent was washed with water (3 x 3 mL). The organic layer were dried over Na$_2$SO$_4$ and concentrated in vacuo to yield a colorless oil. The crude product was used directly without further purification.

**Preparation of S5**

To a solution of crude compound S4 (0.30 mmol) in CH$_2$Cl$_2$ (5 mL) was added MIA (50 mg, 0.282 mmol), HoBt 40.6 mg, 0.30 mmol), EDCI (57.46 mg, 0.30 mmol) and TEA (30.3 mg, 0.30 mmol). The reaction was allowed to stir for 12 h. The solvent was washed with water (3 x 10 mL).
The organic layer were dried over Na$_2$SO$_4$ and concentrated in vacuo to yield yellow oil. The crude product was purified using preparative TLC plate (V(Petroleum) / V(ethyl acetate) = 1:3) to give compound 5 (35mg, 24.5%). $^1$H NMR (DMSO-d$_6$, 400MHz): $\delta$ = 0.83 (s, 3H, CH$_3$), 0.91 (s, 3H, CH$_3$), 1.36 (s, 6H, -O-C-CH$_3$), 2.27–2.33 (m, 3H, -CH$_2$-CONH-, -S-CH$_2$-), 2.59 (s, 3H, indol- CH$_3$), 2.66–2.67 (m, 1H, -S-CH$_2$-), 2.99–3.03 (m, 1H, -S-CH$_2$-CH$_2$-), 3.14–3.19 (m, 3H, -CH$_2$-O-, -CH$_2$NHCOCO-CH$_2$O-), 3.21–3.25 (m, 1H, -S-CH$_2$-CH$_2$-), 3.62 (d, 1H, $J$ = 11.6 Hz, -CH$_2$-O-), 4.02 (s, 1H, -O-CH-CONH-), 7.08 (t, 1H, $J$ = 7.6 Hz, indol-H), 7.27–7.31 (m, 1H, indol-H), 7.34–7.41 (m, 1H, indol-H), 7.43 (t, 1H, $J$ = 3.6 Hz, -CONH-), 7.70 (t, 1H, $J$ = 8.8 Hz, indol-H), 8.22 (t, 1H, $J$ = 5.2 Hz, -O-CH-CONH-), 11.59 (s, 1H, indol-NH-). HRMS (ESI) m/z [M+H]$^+$: calculated 476.2213, found 476.2219.

**Preparation of Compound 5 (in the main text)**

Compound S$^5$ was dissolved in AcOH / H$_2$O (2:1, 2 mL) and the mixture was stirred for 5 h. The solvent was evaporated and the residue was purified using preparative TLC plate (V(Petroleum) / V(ethyl acetate) = 1:3) to give the final product. $^1$H NMR (DMSO-d$_6$, 400MHz): $\delta$ = 0.77 (s, 3H, CH$_3$), 0.80 (s, 3H, CH$_3$), 1.22–1.27 (m, 3H, -CH$_2$OH, -NH-CO-CH$_2$-), 1.51 (d, 1H, $J$ = 6.8 Hz, -CH-OH), 2.27–2.33 (m, 3H, -CH$_2$NHCOCO-CH-OH, -CH$_2$NHCOCO-), 2.60 (s, 3H, indol-CH$_3$), 2.67–2.69 (m, 1H, -CH$_2$NHCOCO-), 3.14–3.18 (m, 4H, -S-CH$_2$-, -CH$_2$OH), 3.69 (s, 1H, -CH-OH), 7.07–7.10 (m, 1H, indol-H), 7.28–7.31 (m, 1H, indol-H), 7.43 (d, 1H, $J$ = 8.0 Hz, indol-H), 7.42 (t, 1H, $J$ = 8.0 Hz, -CONH-), 7.69–7.71 (m, 1H, indol-H, -NH-CO-CH-OH), 8.21 (t, 1H, $J$ = 4.4 Hz, -S-CH$_2$CH$_2$NHCOCO-), 11.60 (s, 1H, indol-NH-). HRMS (ESI) m/z [M+H]$^+$: calculated 436.1896, found 436.1906.

**Synthesis of CoA thioesters**

Under the protection of N$_2$, 50 µmol MIA or DMIA, 30 mg PyBOP and 15 mg K$_2$CO$_3$ were
dissolved in 2 ml THF in a 15 mL serum bottle, and 39 µm CoA tri-lithium salt dissolved in 1 ml ddH₂O was added to the solution. The reaction mixture was stirred at room temperature for 3 hrs, and was then filtered using 0.45µm filter membrane. The resulting CoA thioester was purified from the resulting solution using semi-preparative HPLC, which was carried out on a Thermo Scientific Dionex Ultimate 3000 system with UV detection at 254 nm and a flow rate of 2.5 mL·min⁻¹. The C18 semi-preparative column (Kromasil C18, 10 x 250 mm, 5 µm) was equilibrated with 85% solvent A (H₂O containing 10 mM NH₄OAc) and 15% B (Methanol), and developed with the following program: 0 to 20 min, a linear gradient from 85% A/15% B to 15% A/85% B; 20 to 22 min, a linear gradient from 15% A/85% B to 85% A/15% B. CoA thioesters were produced as a white powder after lyophilization of the desired elution collected around 11-12 min. ESI-MS ([M+H]+) MIA-CoA: 925.1753 (calc.), 925.1761 (found); DMIA-CoA: 939.1901 (calc.), 939.1889 (found)
**Supplementary Figure 1.** HR-MS/MS analysis of MIA-AMP (4), showing the collision induced dissociation (CID) fragments and the MS/MS spectrum.
**Supplementary Figure 2.** HR-MS/MS analysis of MIA-Pan (5) produced in the NosI reaction with MIA, ATP and pantetheine, showing the collision induced dissociation (CID) fragments and the MS/MS spectrum.
Supplementary Figure 3. HR-MS spectrum of NosJ_{28-38} obtained by GluC-digestion of NosJ, which contains a Ppant arm on Ser36.
**Supplementary Figure 4.** Homology modeling of NosJ using SWISS Model\textsuperscript{9} based on the structure of human RGS12 (PDB: 2EBZ \_A). (A) The sequence alignment of NosJ with RGS12 (24% identity, 31% similarity). The secondary structure predicted by JPred4\textsuperscript{10} is shown above the NosJ sequence (blue font, H indicates helix). This analysis is basically consistent with that predicted by SWISS Model, and the only difference lies between Pro30 and Leu34 (highlighted in yellow), which is likely because Pro30 disrupts the \( \alpha \)-helical structure. (B) The NosJ homology structure. The Ppant arm is attached to Ser36, the non-hydrogen atoms of which are shown in spheres. The Local quality estimate (C) and the QMean score (D) of the SWISS Model prediction are also shown.

A

\[ \text{NosJ} \quad \text{DLQELAGLLQEDDPRRRLDSLETVVLYSFAQRGLTPLELDPRRRLDSL} \quad \text{2ebz} \quad \text{-QLQIFNLKDNSYFTRFLKSPL--YQECILAEVGRALPDSQVPS} \]

B

C

D

![Diagram of NosJ structure](image)

![Graph showing Local Quality Estimate and Z-score](image)
Supplementary Figure 5. HR-MS/MS analysis of DMIA-Pan (6), showing the collision induced dissociation (CID) fragments and the MS/MS spectrum.
**Supplementary Figure 6.** Bioinformatic analysis of NosK analogous enzymes, showing (A) the Maximum-likelihood phylogeny, and (B) the sequence similarity network (SSN). The accession numbers of the associated organisms of these NosK analogous enzymes are summarized in Supplementary Table 3.
**Supplementary Figure 7.** NosK is essential for nosophite biosynthesis, showing the HPLC traces of the culture extracts from *S. actuosus* wild type strain, the *nosK*-knockout mutant, and the *nosK*-knockout mutant containing a *nosK*-expressing plasmid. DMIA production was not observable in HPLC chromatogram and only observed in LC-MS analysis (see Fig. 5a in the main text).
**Supplementary Figure 8.** MS spectrum of NosM treated with NosK and DMIA-NosJ, showing that NosM was not modified. The sequence of NosM is GSSHHHHHHS SGLVPRGSHM DAAHLSDLDALEISEFLD ESRLEDSEVV AKVMSASCTT CECCCSSSS.
**Supplementary Table 1.** PCR primers used in this study.

| Primer | Sequence | Function |
|--------|----------|----------|
| nosJ-For | GTGCCGCGCGCGACCCATATG ACCATCTAGCGACCGAACACACCC | NosJ overexpression |
| nosJ-Rev | GTGGTGTCGAGTCGACGAAGCGCTGGAGAACCACGCTTGG | NosJ overexpression |
| nosI-For | GTGCCGCGCGCGACCCATATG GGTGACATGGGCCGACCAGCG | NosI overexpression |
| nosI-Rev | GTGGTGTCGAGTCGACGAAGCGCTGGAGAACCACGCTTGG | NosI overexpression |
| nosK-For | GTGCCGCGCGCGACCCATATG GACGCGACAGCGATGGGC | NosK overexpression |
| nosK-Rev | GTGGTGTCGAGTCGACGAAGCGCTGGAGAACCACGCTTGG | NosK overexpression |
| nosM-For | GTGCCGCGCGCGACCCATATG GACGCGACAGCGATGGGC | NosM overexpression |
| nosM-Rev | GTGGTGTCGAGTCGACGAAGCGCTGGAGAACCACGCTTGG | NosM overexpression |
| nosI-L-For | CATGATTACGATTTCGACACACCCCGCGCTCCTCGCTCCTTC | nosI in-frame deletion |
| nosI-L-Rev | GTCCAGCGCGCGCGATTTTTAGCATGACGCGACACCGCCACTTTGGGCGC | nosI in-frame deletion |
| nosI-R-For | GTGGCCGTCGCGCCCTACTCGCCGTTCAGCCGACCGCGAAGCCCA | nosI in-frame deletion |
| nosI-R-Rev | GCCAGTGCCAAGCTCAGCGCGCTCAGCGCGCGCGCGCGC | nosI in-frame deletion |
| nosI-C-For | TCCAAGCTT GTGGGTGACATGGGCCGACCAGCG | ΔnosI complementation |
| nosI-C-Rev | TGGTCTAGA TCAGACTCCTTCCCCGTGCCTCCTTAG | ΔnosI complementation |
| nosK-L-For | CATGATTACGATTTCGACACACCCCGCGCTCCTCGCTCCTTC | nosK in-frame deletion |
| nosK-L-Rev | CTCCAGGGACAGCGTTTTTGTCGACGCGACACCGCCACTTTGGGCGC | nosK in-frame deletion |
| nosK-R-For | GATCTGGCGGTCACACCGTGCTCAGCGCGCTCAGCGCGCTCAGCG | nosK in-frame deletion |
| nosK-R-Rev | GCCAGTGCCAAGCTCAGCGCGCTCAGCGCGCGCGCGCGC | nosK in-frame deletion |
| nosK-C-For | TCCAAGCTT GTGGGTGACATGGGCCGACCAGCG | ΔnosK complementation |
| nosK-C-Rev | TGGTCTAGA TCAGACTCCTTCCCCGTGCCTCCTTAG | ΔnosK complementation |
**Supplementary Table 2** Bacteria strains and plasmids used in this study.

| Stain/Plasmid        | Relevant genotype/comments                                                   | Reference |
|----------------------|-----------------------------------------------------------------------------|-----------|
| **Strain**           |                                                                             |           |
| *Escherichia coli*   |                                                                             |           |
| DH5α                 | Host for general cloning                                                    | Invitrogen|
| E. coli BL21 (ED3)   | Host for protein expression                                                 | Invitrogen|
| E. coli BL BAP1      |                                                                             | (1)       |
| **Streptomyces**     |                                                                             |           |
| *actuosus* ATCC 25421 | Nosihpeptide producing strain                                              | ATCC      |
| mFDU1711             | nosI in-frame deletion mutant of *S. actuosus*                             | This study|
| mFDU1712             | nosI complemented to mFDU1711                                              | This study|
| mFDU1713             | nosK in-frame deletion mutant of *S. actuosus*                             | This study|
| mFDU1714             | nosK complemented to mFDU1713                                              | This study|
| **Plasmid**          |                                                                             |           |
| pET28a               | Vector for expression of N-terminal 6 X His-tagged protein in *E. coli*     | Novagen   |
| NosN-ET28a           | pET28a derivative, containing the a 1.2 kb PCR product encoding NosN       | (3)       |
| NosI-ET28a           | pET28a derivative, containing the a 300 bp PCR product encoding NosI       | This study|
| NosJ-ET28a           | pET28a derivative, containing the a 1.3 kb PCR product encoding NosJ       | This study|
| NosK-ET28a           | pET28a derivative, containing the a 0.8 kb PCR product encoding NosJ       | This study|
| pNosl-MD19           | pMD19-T derivate, containing 1.3kb PCR product encoding Nosl               | This study|
| pNosK-MD19           | pMD19-T derivate, containing 0.8 kb PCR product encoding NosK              | This study|
| mFDU1711             | nosI in-frame deletion construct                                           | This study|
| mFDU1712             | nosI complementation construct                                             | This study|
| mFDU1713             | nosK in-frame deletion construct                                           | This study|
| mFDU1714             | nosK complementation construct                                             | This study|
**Supplementary Table 3** The accession number and organisms of the NosK homologues enzymes discussed in Supplementary Figure 7.

| Organism | Candidate | Annotation |
|----------|-----------|------------|
| Phylum Actinobacteria | ACR48340 | Putative hydrolase |
| Class Streptomycetales | EPJ36918 | Putative hydrolase |
| *Streptomyces actinosus* | WP_020274905 | Putative hydrolase |
| *Streptomyces afghaniensis* | ACRN52292 | TsrU |
| *Streptomyces laurentii* | ACRN80646 | StoU |
| *Streptomyces laurentii* | BAU84771 | TsrB |
| *Streptomyces sp. IB2014-011-1* | ON154807 | Pimeloyl-[acyl-carrier protein] methyl ester esterase |
| Class Pseudonocardiales | WP_016697821 | hypothetical protein |
| *Actinoalloteichus spihtensis* | WP_026422078 | hypothetical protein |
| *Actinokineospora inagensis* | WP_026422080 | alpha/beta hydrolase |
| Class Corynebacteriales | ADM01086 | NocK |
| *Nocardia sp. ATCC 202099* | HSAD_RH0R | Putative hydrolase |
| Phylum Firmicutes | EEP73620 | hypothetical protein |
| Class Clostridia | OLZ11957 | hypothetical protein |
| *Sulfobacillus thermosulfidooxidans* | OLZ17640 | hypothetical protein |
| *Sulfobacillus thermosulfidooxidans* | OLZ22421 | hypothetical protein |
| *Sulfobacillus thermosulfidooxidans* DSM 9293 | SMC03285 | Pimeloyl-ACP methyl ester carboxylesterase |
| *Sulfobacillus thermosulfidooxidans* | WP_037911918 | alpha/beta hydrolase |
| *Sulfobacillus thermosulfidooxidans* | WP_020374264 | alpha/beta hydrolase |
| Class Bacilli | WP_048013654 | hypothetical protein |
| *Bacillus marisflavi* | KML03011 | hypothetical protein |
| *Bacillus marisflavi* | KML33639 | hypothetical protein |
| *Bacillus marisflavi* | WP_048005255 | hypothetical protein |
| *Pontibacillus halophilus* | WP_026800584 | alpha/beta hydrolase |
| *Paenibacillus senegalensis* | WP_010269203 | alpha/beta hydrolase |
| *Halobacillus sp. BAB-2008* | ELK46610 | alpha/beta hydrolase |
| *Exiguobacterium antarcticum* | WP_026831393 | alpha/beta hydrolase |
| *Exiguobacterium sp. MH3* | WP_023468379 | hypothetical protein |
| *Exiguobacterium sp. RIT341* | WP_052019012 | hypothetical protein |
| *Pontibacillus yanchengensis Y32* | KGP73418 | alpha/beta hydrolase |
| Phylum Chordata, Class mammalia | BPHL_HUMAN | Valacyclovir hydrolase |
| *Homo sapiens* | | |

| Phylum Actinobacteria | ACR48340 | Putative hydrolase |
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| *Streptomyces afghaniensis* | ACRN52292 | TsrU |
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| Class Pseudonocardiales | WP_016697821 | hypothetical protein |
| *Actinoalloteichus spihtensis* | WP_026422078 | hypothetical protein |
| *Actinokineospora inagensis* | WP_026422080 | alpha/beta hydrolase |
| Class Corynebacteriales | ADM01086 | NocK |
| *Nocardia sp. ATCC 202099* | HSAD_RH0R | Putative hydrolase |
| Phylum Firmicutes | EEP73620 | hypothetical protein |
| Class Clostridia | OLZ11957 | hypothetical protein |
| *Sulfobacillus thermosulfidooxidans* | OLZ17640 | hypothetical protein |
| *Sulfobacillus thermosulfidooxidans* | OLZ22421 | hypothetical protein |
| *Sulfobacillus thermosulfidooxidans* DSM 9293 | SMC03285 | Pimeloyl-ACP methyl ester carboxylesterase |
| *Sulfobacillus thermosulfidooxidans* | WP_037911918 | alpha/beta hydrolase |
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| *Pontibacillus yanchengensis Y32* | KGP73418 | alpha/beta hydrolase |
| Phylum Chordata, Class mammalia | BPHL_HUMAN | Valacyclovir hydrolase |
| *Homo sapiens* | | |
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