Supporting Information

Discovery and Biosynthesis of Guanipiperazine from a NRPS-like Pathway

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Reference
Experimental Procedures

General experimental procedures.

The NMR spectra were collected on Bruker Avance 400 for 400 MHz for ¹H and 100 MHz for ¹³C nuclei. UV spectrum was recorded on a Nanodrop 2000 spectrometer with a 10 mm cuvette. LC-MS analysis was performed on an Agilent 6530 TOF LC/MS mass spectrometer with a Poroshell 120 EC-C18 column (4.6×50 mm, 2.7 µm). MPLC was conducted on Biotage Isolera One MPLC system using a Biotage SNAP Cartridge Ultra C18 60 g column and semipreparative RP-HPLC was performed on an Agilent 1200 HPLC with an Agilent Eclipse XDB-C18 column (5 µm, 250×9.4 mm). The HPLC analysis was run on an Agilent 1200 infinity HPLC equipped with a Poroshell 120 EC-C18 column (4.6×50 mm, 2.7 µm). Sephadex LH-20 was purchased from GE Biotechnology. All chemicals used in the study were of analytical grade. The enzymes for PCR were purchased form Vyazyme.

PCR procedures for targeted genes.

Fragments were respectively amplified by the condition: step 1: 95°C for 3 min; step 2: 95°C for 15 s; step 3: 72°C for 15 s; step 4: 72°C by 30s/kb; step 5: step 2 to step 4 for 35 cycles; step 6: 72°C 10 min, then the productions were puried by gel.

Construction of plasmids for heterologous expression.

gupB, gupA-B, gupA-C, and gupA-D were respectively amplified with the primer pairs gupB-152-F/R, gupA-B-152-F/R, gupA-C-152-F/R, and gupA-D-152-F/R, and then were cloned into the EcoRI and BcaI sites of pSET152 to generate pHG9101, pHG9102, pHG9103, and pHG9104, respectively.

Heterologous expression, fermentation, extraction and HPLC detection.

The plasmids pHG9101-pHG9106 were transferred into the E. coli ET12567 (pUZ8002) strain individually, and further introduced into Streptomyces lividans TK24 by conjugation. The recombinant strains were grown on ISP4 agar medium supplemented with 50 µg/mL of apramycin for sporulation. After growing at 30 °C for 10 days, the medium was soaked with methanol. The afforded methanol extract was directly used for HPLC analysis. Each sample (10 µL) was injected into the column and first eluted with a linear gradient of 10% methanol to 90% methanol in water for 13 min, and finally with 100% methanol for 3 min at a flow rate of 0.5 mL/min.

Construction of in-frame deletion in plasmid pHG9104.

The gene inactivation was performed by pKOV-Amp and pDF25 to generate the in-frame deletion mutants. Briefly, the up-and downstream homology arms were amplified with primers upF/R and downF/R listed in Table S2 using genomic DNA as template, respectively. The purified PCR products were ligated to linearized pKOV-Amp to generate plasmids pHG9105-pHG9106. After confirmed by DNA sequencing, plasmids were used for in-frame deletion of each gene in E. coli, following the previous reported procedure. The genotype of mutated cosmids were confirmed by PCR and DNA sequencing. The afforded mutated cosmids were transformed into S. lividans TK24 through conjugation as mentionbed above. The resulting mutant strains were used for further fermentations.

Isolation of compounds 1 and 2.

The strain harboring plasmids were cultivated on ISP4 solid medium for 10 days, 4 liters medium were carried out for fermentation, then were extracted by methanol, extract was fractionated by MPLC over ODS column eluted with a linear gradient MeOH-H₂O system from 10% MeOH to 100% MeOH to give ten fractions. The fractions were subjected for TLC analysis with the system containing dichloromethane and MeOH(5:1). The same fractions were combined and concentrated, and further fractionated by Sephadex LH-20 column using MeOH to give several fractions. The target fraction was purified by semi-HPLC with a gradient solvent system from 30% to 70% MeOH in H₂O system over 25 min with 2.0 mL/min flow rate to give 1 and 2 (1 mg, t₀: 20.5 min).

Isolation of compounds 3, 4 and 6.

The strains harboring plasmids were cultivated on ISP4 solid medium for 10 days, 3 liters medium were carried out for fermentation, then were extracted by methanol, extract was fractionated by MPLC over ODS column eluted with a linear gradient MeOH-H₂O system from 10% MeOH to 100% MeOH to give ten fractions. The fractions were subjected for TLC analysis with the system containing dichloromethane and MeOH(5:1). The same fractions were combined and concentrated, and further fractionated by Sephadex LH-20 column using MeOH to give several fractions. The target fraction was purified by semi-HPLC, 3 was purified with a gradient solvent system from 50% to 90% MeOH in H₂O system over 20 min with 2.0 mL/min flow rate, 4 was purified with an isocratic solvent system 12% ACN in H₂O system over 13 min with 2.0 mL/min flow rate, 6 was purified with a isocratic solvent system 13% ACN in H₂O system over 15 min with 2.0 mL/min flow rate.

Procedure for MICs of 1 and 2.

Saccharomyces cerevisiae were cultured in a 5 mL scale at 30 °C for overnight, and then 1 mL culture medium was mixed with 100 mL YPD (1% Yeast Extract, 2% Peptone, 2% Glucose), 100 μL mixture was added into the hole of 96-well plate. The guanipiperazine was prepared with a 10 nM concentration by DMSO. Gradient Guanipiperazine was added into 96-well...
plate controlled by the equal volume DMSO, after cultured at 30°C for 12h, OD600 was collected on a Nanodrop 2000 spectrometer. For each sample, three parallel groups were set, and the average value were adopted for analysis. Firstly, a gradient from 0.1 mM to 2 mM with 0.1 mM ladder concentration was used, as for compound 1, the inhibitory concentration was range to 0.9–1.1 mM, as for compound 2, the inhibitory concentration was range to 1.1–1.3 mM. For a meticulous measure, a ladder with 0.01 mM concentration was adopted, which finally prove the inhibitory concentrations for 1 and 2 were 1.01 and 1.26 mM, equivalent to 4.5 and 5.6 μg/mL, respectively.

**Chemical complementation of compound 3 and 4 into ΔgupB mutant.**

The ΔgupB mutant were cultured in a 200 ml scale at 30°C in fermentation medium. After 24 hours cultivation, compounds 3 or 4 (1.5 mg, 2 μmol) dissolved in DMSO were supplemented into fermentation broth individually and cultured for another 12 hours. The metabolic extract was analyzed by LC-MS using the method mentioned above.

**Protein Expression and Purification.**

DNA fragments containing target genes including GupA, GupB, GapC, GapD, Ctg_5604, and Ctg_7941 were amplified from genomic DNA of *S. chrysomelica* NA4264 with primers listed in Table S2. The purified PCR products were ligated with linearized pET28a (linearized by NdeI and HindIII) to afford pHG9107-pHG9112. The pHG9107-pHG9112 were further introduced into *E. coli* BL21(DE3), respectively. The transformants were cultivated in 400 mL LB medium supplemented with 50 μg/mL kanamycin at 37 °C (220 rpm) until OD600 value reached around 0.6. The culture was cooled to 4 °C and induced with 0.1 mM IPTG, continued to cultivate at 16°C (220 rpm) for 20 h. After centrifugation at 8000 rpm for 10 min, cells were resuspended in lysis buffer (100 mM Tris, pH 8.0, 15 mM imidazole, 300 mM NaCl, 10 % glycerol) and lysed on ice by sonication. After centrifugation at 15000 rpm for 30 min, the supernatant was filtered and purified by ÄKTA FPLC system equipped with a 5 mL Histrap HP column (GE lifesciences). The proteins were pooled and desalted by a PD10 column (GE Healthcare) with 100 mM phosphate buffer (pH 8.0) and 10% glycerol and stored at -80°C.

**Adenylation activities of A domain**

To evaluate the adenylation activity of A domain in GupB, we conducted a coupled continuous assay for inorganic pyrophosphate using a unique fluorogenic pyrophosphate sensor (Sigma-Aldrich Pyrophosphate Assay Kit MAK168). Briefly, the presence of pyrophosphate results in the production of a fluorescent product (λex=316, λem=456 nm) proportional to the pyrophosphate present. The A domain specificity assay against different amino acid substrates was conducted in a 50 μL reaction volume containing 50 mM Tris-HCl, pH 7.5, 60 μM NRPS protein, 12.5 mM MgCl2, 2.0 mM TCEP, 2 mM amino acid, 4 mM ATP. The control group was treated using the boiled protein. After reacting for 30 min at 25 °C, an equal volume of the master reaction mix were added to each of the sample, and the reaction was incubated at room temperature. After 20 min, the fluorescence intensity (λex=316/λem=456 nm) was determined by a microplate reader. After deducting the data of the control group, the relative fluorescence intensity was calculated.

**In vitro biochemical assay of GupA, GupB.**

The holo-GupB was activated with a mixture (50 μL) containing 50 μM apo-GupB, 0.8 mM CoA, 2 mM TCEP, 12.5 mM MgCl2, 20 μM Sfp for 45 min. Co-reaction of GupA and holo-GupB was carried out in a 100 μL reaction system containing 50 mM Tris-HCl buffer (pH 7.5), 25 μM apo-GupA, 2 mM l-Tyr, 4 mM ATP, 1 mM NADH, 2 mM TCEP, 12.5 mM MgCl2, 8 μM GupA, 1 mM F420, 1.6 μM FGD, 2.5 mM glucose-6-phosphate. After incubation at 30 °C for 2 h, the reaction was quenched by adding 100 μL methanol. Then the mixture was centrifuged at 15,000 g for 10 min and the supernatant was analyzed by LC-MS. The LC-MS analysis was performed using a 18 min solvent gradient from 10% to 70% (0–18 min) methanol in water supplied with 0.1 TFA at a flow rate of 0.5 mL/min.

**In vitro biochemical assay of GupC.**

The GupC catalyzed reaction was carried out in a 100 μL reaction system containing 50 mM MES buffer (pH 5.5), 134 μM substrate, 1.5 mM NADPH, 8 μM FDR, 10 μM FDX and 27 μM GupC. After incubation at 30°C for 2 h, the reaction was quenched by adding 100 μL methanol. The LC-MS analysis was performed using a 18 min solvent gradient from 10% to 50% (0–18 min) methanol in water supplied with 0.1 TFA at a flow rate of 0.5 mL/min.

**In vitro biochemical assay of CupD.**

The reaction mixture (100 μL) contained 17 μM CupD, 1.5 mM NADPH, 8 μM FDR, 10 μM FDX, 1 mM GTP, 70 μM compound 6, and 50 mM PIPES buffer (pH 6.5). After incubation at 30°C for 4 h, the reaction was quenched by adding 100 μL methanol. Then the mixture was centrifuged at 15,000 g for 10 min and the supernatant was analyzed by LC-MS. The LC-MS analysis was performed using a 18 min solvent gradient from 10% to 50% (0–18 min) methanol in water supplied with 0.1 TFA at a flow rate of 0.5 mL/min.

**Sequence similarity network (SSN) analysis.**

R domain was used as the search query in antiSMASH database and our own, 1,230 NRPS BGCs containing a terminal R domain were retrieved. After manually removing the multi-modular NRPS BGCs, we were left with 608 single-module NRPS-like BGCs. For SSN analysis, an initial sequence identity 10% was used from the local blast analysis (all vs all). Cytoscape
3.7.0 were used for network visualization. Both self loops and duplicate loops were deleted. Finally, the sequence identity 40% was chosen for figure generation.

**Physical data for isolated compounds**

**Compound 1:** light brown powder; NMR data see Table S4; HRESIMS \( m/z \) 446.1937 [M+H]+ (calcd for C_{23}H_{23}N_{7}O_{3}, 446.1935); UV (MeOH): \( \lambda_{\text{max}} \) (log \( \varepsilon \)) =229 nm (2.93), 314 nm (1.78).

**Compound 2:** white powder; NMR data see Table S5, HRESIMS \( m/z \) 446.1939 [M+H]+ (calcd for C_{23}H_{23}N_{7}O_{3}, 446.1935); UV (MeOH): \( \lambda_{\text{max}} \) (log \( \varepsilon \)) =229 nm (2.92), 280 nm (2.63).

**Compound 3:** white powder; NMR data see Table S6, HRESIMS \( m/z \) 293.1283 [M+H]^+ (calcd for C_{18}H_{16}N_{2}O_{2}, 293.1285); UV (MeOH): \( \lambda_{\text{max}} \) (log \( \varepsilon \)) =229 nm (3.21), 277 nm (3.13).

**Compound 4:** Light yellow oil; NMR data see Table S7, HRESIMS \( m/z \) 299.1754 [M+H]^+ (calcd for C_{18}H_{22}N_{2}O_{2}, 299.1754); UV (MeOH): \( \lambda_{\text{max}} \) (log \( \varepsilon \)) =218 nm (3.22), 277 nm (2.76).

**Compound 6:** Light yellow oil; NMR data see Table S8, HRESIMS \( m/z \) 297.1596 [M+H]^+ (calcd for C_{18}H_{20}N_{2}O_{2}, 297.1598); UV (MeOH): \( \lambda_{\text{max}} \) (log \( \varepsilon \)) =228 nm (3.29), 279 nm (2.75).
Table S1. Deduced gene functions of in the gup BGC.\(^a\)

| ORF   | Amino acids\(^b\) | Blastp homologue        | Identity/coverage [%] | Protein ID\(^c\) |
|-------|-------------------|-------------------------|-----------------------|-----------------|
| GupA  | 162               | F\(_{429}\)H\(_2\)-dependent reductase | 24/52                 | O06553.1        |
| GupB  | 1074              | non-ribosomal peptide synthetase | 97/99 | GCD40661.1 |
| GupC  | 417               | cytochrome P450          | 94/90                 | KOG65251.1      |
| GupD  | 409               | cytochrome P450          | 79/99                 | GT86359.1       |
| Ctg_7941 | 428             | ferredoxin reductase     | 93/99 | KOT90529.1 |
| Ctg_5604 | 106             | ferredoxin              | 100/100               | KOT40977.1      |

\(^a\) The sequence has been deposited in GenBank with the accession number MW051641.\(^b\) Numbers are in amino acids. \(^c\) Given in numbers are NCBI accession numbers.

Table S2. Bacterial plasmids and strains.

| Plasmid/Strain | Relevant characteristics                                                                 | Source |
|----------------|--------------------------------------------------------------------------------------------|--------|
| Plasmid        |                                                                                           |        |
| pSET152        | Apr\(^r\), integrated vector for heterologous expression                                  | 5      |
| pUZ8002        | Kan\(^r\), including tra for conjugation                                                  | 5      |
| pKOV-Amp       | plasmid used for gene disruption, temperature sensitive                                    | 6      |
| pDF25          | plasmid used for gene disruption, temperature sensitive                                    | 7      |
| pET28a(+)      | Kan\(^r\), protein expression vector                                                      | Novagen|
| pHG9101        | The plasmid for heterologous expression of gupB                                           | This study |
| pHG9102        | The plasmid for heterologous expression of gupA-B                                         | This study |
| pHG9103        | The plasmid for heterologous expression of gupA-C                                          | This study |
| pHG9104        | The plasmid for heterologous expression of gupA-D                                          | This study |
| pHG9105        | pKOV derived plasmid for disruption of gupB                                               | This study |
| pHG9106        | pKOV derived plasmid for disruption of gupC                                               | This study |
| pHG9107        | pET28a(+) derived plasmid for expressing N-terminal His-tag GupA                          | This study |
| pHG9108        | pET28a(+) derived plasmid for expressing N-terminal His-tag GupB                          | This study |
| pHG9109        | pET28a(+) derived plasmid for expressing N-terminal His-tag GupC                          | This study |
| pHG9110        | pET28a(+) derived plasmid for expressing N-terminal His-tag GupD                          | This study |
| pHG9111        | pET28a(+) derived plasmid for expressing N-terminal His-tag Ctg_7941                     | This study |
| pHG9112        | pET28a(+) derived plasmid for expressing N-terminal His-tag Ctg_5604                     | This study |
| E. coli strains|                                                                                           |        |
| DH5α           | General cloning host                                                                      | 8      |
| DH10B          | General cloning host                                                                      | 8      |
| BL21 (DE3)     | Heterologous host for protein expression                                                  | NEB    |
| ET12567        | Methylation-deficient host used for E. coli-Streptomyces intergeneric conjugation         | 9      |
| (pUZ8002)      |                                                                                           |        |
| Strains        | Model actinomycete used for gene heterologous expression.                                 | 10     |
| HG9101         | S. lividans TK24 carrying pHG9101                                                         | This study |
| HG9102         | S. lividans TK24 carrying pHG9102                                                         | This study |
| HG9103         | S. lividans TK24 carrying pHG9103                                                         | This study |
| HG9104         | S. lividans TK24 carrying pHG9104                                                         | This study |
| HG9105         | S. lividans TK24 carrying ΔgupB/pHG9104                                                   | This study |
| HG9106         | S. lividans TK24 carrying ΔgupC/pHG9104                                                   | This study |
Table S3. Primers used in this study.

| Name         | Sequence                                                                 |
|--------------|--------------------------------------------------------------------------|
| gupB-152-F  | GCTGCATGACATACGTACTAGTccccacgctacctggacg                               |
| gupB-152-R  | CTATGACATGATTACGAATTtcatcggtgtgcttcgt                                   |
| gupa-B-152-F| GCTGCATGACATACGTACTAGTgacggttaaaggccagatgt                               |
| gupa-B-152-R| CTATGACATGATTACGAATTtcatcggtgtgcttcgt                                   |
| gupa-C-152-F| GCTGCATGACATACGTACTAGTgacggttaaaggccagatgt                               |
| gupa-C-152-R| CTATGACATGATTACGAATTtcatcggtgtgcttcgt                                   |
| gupa-D-152-F| GCTGCATGACATACGTACTAGTgacggttaaaggccagatgt                               |
| gupa-D-152-R| CTATGACATGATTACGAATTtcatcggtgtgcttcgt                                   |
| ΔgupB-UF    | GGTCGACTCTAGGAGATCC cacctcatacgtaccggg                                 |
| ΔgupB-DR    | CCGTGCAGTTAGAGATCC gatccctcgtccgt                                        |
| ΔgupB-DF    | AGCCTGTCCTCGACGCCcagcgcggtgcgcc                                      |
| ΔgupB-UR    | GTCCCGGGCAGGTCAGAGGttcccgggccggctgcggctg                                |
| ΔgupB-CF    | GCCGCCGCCGCAGGCACCCGAGGAGGtacggtggccggccggc                              |
| ΔgupB-DR    | CCGTGCAGTTAGAGATCC gatccctcgtccgt                                        |
| ΔgupB-DF    | AGCCTGTCCTCGACGCCcagcgcggtgcgcc                                      |
| ΔgupB-DR    | CCGTGCAGTTAGAGATCC gatccctcgtccgt                                        |
| ΔgupB-DF    | AGCCTGTCCTCGACGCCcagcgcggtgcgcc                                      |
| ΔgupB-PO    | atgacccacaggctgccg                                                    |
| ΔgupB-NE    | gtcacccagccctgccg                                                   |
| ΔgupC-PO    | tcatcgctcgtgcgccg                                                   |
| ΔgupC-NE    | cgctcctcgtgcgccg                                                   |
| ΔgupC-PO    | tcatcgctcgtgcgccg                                                   |
| ΔgupC-NE    | gtcgttgtagacgctgcgcc                                                |
| ΔgupC-NE    | ggcgtgccggccgcgcc                                                  |
| GupA-pET28a-F | GTGCAGACCGCCGCAGCGCAGCTAGTtacctggccg                                   |
| GupA-pET28a-R | GTGACGAGCGCCGCCAGCGCAGCTAGTtacctggccg                                   |
| GupB-pET28a-F | GTTCGACTCTAGAGATCC gatccctcgtccgt                                        |
| GupB-pET28a-R | GTTCGACTCTAGAGATCC gatccctcgtccgt                                        |
| GupC-pET28a-F | GTTCGACTCTAGAGATCC gatccctcgtccgt                                        |
| GupC-pET28a-R | GTTCGACTCTAGAGATCC gatccctcgtccgt                                        |
| GupD-pET28a-F | GTTCGACTCTAGAGATCC gatccctcgtccgt                                        |
| GupD-pET28a-R | GTTCGACTCTAGAGATCC gatccctcgtccgt                                        |
| Ctg_7941-pET28a-F | AAGAAGAGGATATACATAGTgacggttgccgagcgt                                |
| Ctg_7941-pET28a-R | AAGAAGAGGATATACATAGTgacggttgccgagcgt                                |
| Ctg_5604-pET28a-F | AAGAAGAGGATATACATAGTgacggttgccgagcgt                                |
| Ctg_5604-pET28a-R | AAGAAGAGGATATACATAGTgacggttgccgagcgt                               |
Table S4. $^1$H and $^{13}$C NMR data of I in DMSO-$d_6$.

| No. | $\delta_C$  | $\delta_H$ (mult, $J$, Hz)                |
|-----|-------------|------------------------------------------|
| 2   | 42.9, CH$_2$| 2.62 (t, 1.08), 2.40 (t, 7.72)           |
| 3   | 52.1, CH    | 2.92 (dd, 7.72, 1.44)                    |
| 4   | 34.3, CH$_2$| 2.52 (t, 1.24), 2.83 (dd, 11.76, 1.88)   |
| 5   | 126.7, C    |                                          |
| 6   | 121.6, CH   | 6.83 (s)                                 |
| 7   | 146.8, C    |                                          |
| 8   | 144.4, C    |                                          |
| 9   | 112.9, C    |                                          |
| 10  | 118.8, CH   | 7.20 (s)                                 |
| 12  | 47.9, CH$_2$| 3.05 (dd, 8.72, 3.84), 3.45 (d, 8.76)     |
| 13  | 48.7, CH    | 3.59 (m)                                 |
| 14  | 37.7, CH$_2$| 3.20 (ddd, 15.32, 5.44, 2.16)            |
| 15  | 135.3, C    |                                          |
| 16  | 132.6, CH   | 7.57 (dd, 8.0, 2.0)                      |
| 17  | 125.5, CH   | 7.27 (dd, 8.0, 2.0)                      |
| 18  | 160.2, C    |                                          |
| 19  | 124.1, CH   | 6.92 (dd, 8.4, 1.8)                      |
| 20  | 132.0, CH   | 6.79 (dd, 8.4, 1.8)                      |
| 21  | 151.2, C    |                                          |
| 23  | 117.5, C    |                                          |
| 24  | 155.2, C    |                                          |
| 26  | 153.9, C    |                                          |
| 28  | 152.6, C    |                                          |
| 30-NH|            | 6.68 (s)                                 |
**Table S5.** $^1$H and $^{13}$C NMR data of 2 in DMSO-$_d_6$.\(^a\)

| No. | $\delta$C | $\delta$H ( mult, $J$, Hz) |
|-----|---------|--------------------------|
| 2   | 43.0, CH$_2$ | 2.64 (d, 13.4), 2.38 (m) |
| 3   | 52.1, CH   | 2.92 (dd, 3.84, 1.56)    |
| 4   | 34.6, CH$_2$ | 2.57 (dd, 13.6, 6.2), 2.83 (dd, 13.6, 5.8) |
| 5   | 135.3, C   |                          |
| 6   | 121.8, CH  | 6.27 (s)                 |
| 7   | 153.7, C   |                          |
| 8   | 138.5, C   |                          |
| 9   | 111.7, CH  | 7.09 (d, 8.0)            |
| 10  | 122.6, CH  | 6.62 (dd, 8.0, 1.8)      |
| 12  | 47.2, CH$_2$ | 3.01 (dd, 14.4, 4.16), 3.42 (dd, 14.4, 8.64) |
| 13  | 48.8, CH   | 3.57 (brs)               |
| 14  | 37.8, CH$_2$ | 3.17 (dd, 14.4, 4.88), 3.23 (dd, 14.4, 5.64) |
| 15  | 135.9, C   |                          |
| 16  | 132.7, CH  | 7.55 (dd, 8.0, 1.8)      |
| 17  | 123.8, CH  | 6.93 (dd, 8.0, 1.8)      |
| 18  | 159.7, C   |                          |
| 19  | 125.3, CH  | 7.24 (dd, 8.4, 2.2)      |
| 20  | 131.9, CH  | 7.64 (dd ,8.4, 2.2)      |
| 21  | 158.0, C   |                          |
| 23  | 121.4, C   |                          |
| 24  | 155.2, C   |                          |
| 26  | 158.5, C   |                          |
| 28  | 158.7, C   |                          |

**Table S6.** $^1$H NMR data of 3 in DMSO-$_d_6$.\(^a\)

| No. | $\delta$H ( mult, $J$, Hz) |
|-----|---------------------------|
| 2   | 8.43 (s)                  |
| 4   | 3.94 (s)                  |
| 6   | 6.60 (d, 8.4)             |
| 7   | 7.04 (d, 8.4)             |
| 9   | 7.04 (d, 8.4)             |
| 10  | 6.60 (d, 8.4)             |
| 12  | 8.43 (s)                  |
| 14  | 3.94 (s)                  |
| 16  | 6.60 (d, 8.4)             |
| 17  | 7.04 (d, 8.4)             |
| 19  | 6.60 (d, 8.4)             |
| 20  | 7.04 (d, 8.4)             |
Table S7. $^1$H NMR data of 4 in DMSO-$d_6$.

$$
\begin{align*}
\text{No.} & & \delta_H (\text{ mult, } J, \text{ Hz}) \\
2 & & 2.98 (m) \\
3 & & 3.70 (m) \\
4 & & 3.20 (m) \\
6 & & 6.76 (d, 8.4) \\
7 & & 7.11 (d, 8.4) \\
9 & & 7.11 (d, 8.4) \\
10 & & 6.76 (d, 8.4) \\
12 & & 2.98 (m) \\
13 & & 3.70 (m) \\
14 & & 3.20 (m) \\
16 & & 6.76 (d, 8.4) \\
17 & & 7.11 (d, 8.4) \\
19 & & 7.11 (d, 8.4) \\
20 & & 6.76 (d, 8.4)
\end{align*}
$$

Table S8. $^1$H NMR data of 6 in DMSO-$d_6$.$^a$

$$
\begin{align*}
\text{No.} & & \delta_H (\text{ mult, } J, \text{ Hz}) \\
2 & & 2.63 (d, 7.4), 2.36 (m) \\
3 & & 2.93 (brd) \\
4 & & 2.50 (dd, 6.20, 2.28) \\
& & 2.76 (dd, 5.68, 1.88) \\
6 & & 5.89 (s) \\
9 & & 7.21 (dd, 8.0, 2.4) \\
10 & & 6.62 (d, 8.0) \\
12 & & 3.04 (dd, 13.3, 5.6) \\
& & 3.45 (d, 13.3) \\
13 & & 3.53 (brs) \\
14 & & 3.17 (dd, 14.7, 3.6) \\
& & 3.23 (dd, 14.7, 5.4) \\
16 & & 7.55 (dd, 8.4, 2.2) \\
17 & & 6.89 (dd, 8.4, 2.2) \\
19 & & 7.62 (dd, 8.4, 2.2) \\
20 & & 6.38 (dd, 8.4, 2.2)
\end{align*}
$$

$^a$
Figure S1. The *gup* homologous gene clusters obtained from NCBI database.

Figure S2. Chemical complementation of compounds into TK24/gupACD strain.

Figure S3. SDS-PAGE analysis of reconstituted proteins. A) GupA (calculated molecule weight 19.90kDa); B) GupB (calculated molecule weight 115.70kDa); C) GupC (calculated molecule weight 47.53kDa); D) GupD (calculated molecule weight 47.56kDa); E) Ctg_5604 (Fdx, calculated molecule weight 13.80kDa); F) Ctg_7941 (Fdr, calculated molecule weight 47.09kDa).
Figure S4. LC-MS analyses of the purified ferredoxin (ctg_5604). A) The m/z values of average protein masses of the 14+ charge states (MS1) are shown. B) The molecular weight of ctg_5604.

Figure S5. FGD-catalyzed reaction. FGD utilizes the oxidized form of cofactor F_{420} to convert glucose-6-phosphate (G6P) 6-phosphogluconolactone, thus yielding the reduced cofactor F_{420}H_{2}{\textsuperscript{11}}.
Figure S6. HPLC analysis (monitored at 280 nm) of *in vitro* GupB and GupA enzymatic reactions utilize iii) FAD iv) FMN v) F420 as reducing agents.

Figure S7. HPLC analysis (monitored at 280 nm) of *in vitro* GupD enzymatic reactions.
Figure S8. $^1$H NMR spectrum of 1 in DMSO-$d_6$ at 400 MHz.

Figure S9. $^{13}$C NMR spectrum of 1 in DMSO-$d_6$ at 100 MHz.
Figure S10. DEPT 135 spectrum of 1 in DMSO-$d_6$.

Figure S11. $^1$H-$^1$H COSY NMR spectrum of 1 in DMSO-$d_6$. 
Figure S12. HSQC NMR spectrum of 1 in DMSO-$d_6$.

Figure S13. HMBC NMR spectrum of 1 in DMSO-$d_6$. 
Figure S14. NOESY spectrum of 1 in DMSO-$d_6$.

Figure S15. $^1$H NMR spectrum of 2 in DMSO-$d_6$ at 400 MHz.
Figure S16. $^{13}$C NMR spectrum of $2$ in DMSO-$d_6$ at 100MHz.

Figure S17. DEPT 135 spectrum of $2$ in DMSO-$d_6$. 
**Figure S18.** $^1$H-$^1$H COSY NMR spectrum of 2 in DMSO-$d_6$.

**Figure S19.** HSQC NMR spectrum of 2 in DMSO-$d_6$. 
Figure S20. HMBC NMR spectrum of 2 in DMSO-$d_6$.

Figure S21. NOESY spectrum of 2 in DMSO-$d_6$. 
Figure S22. $^1$H NMR spectrum of 3 in DMSO-$d_6$ at 400 MHz.

Figure S23. $^1$H NMR spectrum of 4 in DMSO-$d_6$ at 400 MHz.
Figure S24. 1H NMR spectrum of 6 in DMSO-d6 at 400MHz.

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