Supplemental data

EXPERIMENTAL PROCEDURES

Purification of proteins—For the purification of CsdA, the cells from the 11.5 L culture were suspended in 150 ml of buffer A (20 mM Tris, 1 mM DTT, pH 7.5) and disrupted by sonication on ice. After removing the cell debris by centrifugation, the crude extract was treated by addition of ammonium sulfate at a concentration of 1.5 M. The mixture was kept on ice for 1 hour and centrifuged. The supernatant solution was filtrated and applied into 50 ml of a Butyl-Toyopearl (TOSOH, Japan) column equilibrated with buffer A containing 1.5 M ammonium sulfate. After that, the column was washed with the same buffer. The fractions containing CsdA confirmed by SDS-PAGE were pooled. Ammonium sulfate powder was added to the collected solution at 1.5 M. Then, the enzyme solution was loaded onto 30 ml of a Butyl-Toyopearl column equilibrated with buffer A containing 1.5 M ammonium sulfate. After the column was washed with 500 ml of the equilibration buffer, the enzyme was eluted with a linear gradient of 1.5-0.5 M ammonium sulfate in buffer A (1 L). The enzyme fractions containing CsdA confirmed by SDS-PAGE were collected and dialyzed against buffer A. The enzyme solution was then applied into a DEAE-Toyopearl (TOSOH, Japan) column (30 ml) equilibrated with buffer A. After the column was washed with 600 ml of buffer A, the adsorbed protein was eluted with 4 L of buffer A containing 150 mM NaCl. The fractions containing CsdA confirmed by SDS-PAGE were collected. Ammonium sulfate powder was added to the enzyme solution at 1.5 M. Then, the enzyme solution was applied to 30 ml of a Butyl-Toyopearl column equilibrated with buffer A containing 1.5 M ammonium sulfate. Without washing, the enzyme was directly eluted with 500 ml of buffer A. The fractions containing CsdA were pooled, dialyzed against buffer A containing 10 μM PLP, and concentrated using the AmiconUltra-15 centrifugal filter device (10K MWCO) (MILLIPORE, Japan). The final preparation of the enzyme was stored at -80 ^{o}C until use.

The cell pellet of *E. coli* expressing SufS from a 12 L culture was suspended in 150 ml of buffer A. After sonication on ice, the cell debris was removed by centrifugation. The supernatant was treated with 1 M ammonium sulfate and centrifuged. Then, SufS in the secondary supernatant was precipitated with 1.75 M ammonium sulfate. After another round of centrifugation, the pellet was resolubilized by buffer A containing 1 M ammonium sulfate. The enzyme solution was filtrated and applied into 50 ml of a Butyl-Toyopearl column equilibrated with buffer A containing 1 M ammonium sulfate. After washing with the same buffer, the column was eluted with 1 L of 0.9 M ammonium sulfate in buffer A and 1 L of buffer A. The fractions containing SufS were collected and dialyzed against buffer A. Then, the sample was loaded onto 100 ml of a DEAE-Toyopearl column and washed with buffer A containing 100 mM NaCl (500 ml). A linear gradient of 100-150 mM NaCl in buffer A (1 L) and 3 L of 150 mM NaCl in buffer A was used to elute the protein. The fractions containing SufS were pooled. Ammonium sulfate powder was added to the collected solution at 1 M. Then, the enzyme solution was loaded onto 50 ml of a Butyl-Toyopearl column equilibrated with buffer A containing 1 M ammonium sulfate. Without washing, the protein was eluted with 1 L of buffer A containing 0.8 M ammonium sulfate and a linear gradient of 0.7-0 M ammonium sulfate in buffer A (500 ml). The collected solution containing SufS was dialyzed against buffer A with 10 μM PLP and then purified by gel filtration with a Hiload 16/60 Superdex 200 column and AKTA explorer 10S (GE Healthcare Biosciences, Japan). The protein was eluted with 1 L of buffer A containing 200 mM NaCl. The pooled enzyme solution of SufS was dialyzed against buffer A with 10 μM PLP and concentrated. The final preparation of the enzyme was stored at -80 ^{o}C until use.

IscS was purified from the cells harboring pEF1. The cells from the 4 L culture were suspended in 40 ml of 50 mM potassium phosphate (KPB), 5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4, and sonicated on ice. Then, the mixture was centrifuged to remove the cell debris. The supernatant was filtrated and loaded onto 70 ml of a DEAE-Toyopearl column equilibrated with 10 mM KPB (pH 7.5). After washing with 400 ml

of an equilibration buffer, the proteins were eluted with a linear gradient of 0-0.15 M NaCl (1 L) and 0.15 M NaCl (200 ml) in 10 mM KPB (pH 7.5). The fractions containing IscS were collected. Ammonium sulfate powder was added to the collected solution at 0.9 M. Then, the enzyme solution was applied into 25 ml of a Phenyl-Toyopearl column equilibrated with 10 mM KPB and 0.9 M ammonium sulfate, pH 7.5. The IscS-containing solution passed through the column was concentrated and dialyzed against 10 mM KPB and 1 M ammonium sulfate, pH 7.5. Then, the sample was applied to a Phenyl-Toyopearl column equilibrated with 10 mM KPB and 1 M ammonium sulfate, pH 7.5. After washing with the same buffer, IscS was eluted with a linear gradient of 1-0 M ammonium sulfate in 10 mM KPB (pH 7.5). The fractions containing IscS were pooled and concentrated. During the concentration process, the solvent was exchanged with 10 mM KPB, 1 mM DTT, and 10 μM PLP, pH 7.5. The final preparation of the enzyme was stored at -80 ^{o}C until use.

The N-terminal His_6 tag-containing MoaD and MoeB were purified using His•Bind Kits (Novagen, Japan). The cells were suspended in 30 ml of a binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) and disrupted by sonication. After the cell debris was removed by centrifugation, the supernatant solution was filtrated and applied to a His-Bind Resin Chromatography column (17 ml) pretreated with 100 ml of a strip buffer (0.5 M NaCl, 20 mM Tris-HCl, 100 mM EDTA, pH 7.9), 100 ml of sterile deionized water, 100 ml of a charge buffer (50 mM $NiSO_4$), 100 ml of sterile deionized water, and 100 ml of the binding buffer. Then, the column was washed with 100 ml of the binding buffer and 200 ml of the wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole, pH 7.9), and the absorbed enzyme was eluted with a linear gradient of 1× wash buffer to 0.5× elute buffer (0.5 M NaCl, 20 mM Tris-HCl, 1.0 M imidazole, pH 7.9) (500 ml). For the purification of MoeB, the fractions containing enzyme were collected and dialyzed against 20 mM Tris-HCl and 1 mM DTT, pH 8.0. Then, the sample was concentrated by using an AmiconUltra-15 centrifugal filter device (10K MWCO). The final preparation of the enzyme was stored at -80 ^{o}C until use. For the purification of MoaD, the collected sample (90 ml) was concentrated to 3 ml using an AmiconUltra-15 centrifugal filter device (3K MWCO) and diluted with 100 ml of the binding buffer. The protein solution was then loaded onto 5 ml of a His-Bind Resin Chromatography column. After the same washing described before, MoaD was eluted with a linear gradient of 1× wash buffer to 0.5× elute buffer (250 ml). The fractions were pooled, dialyzed against 20 mM Tris-HCl, 1 mM DTT, pH 8.0, and concentrated using an AmiconUltra-15 centrifugal filter device (3K MWCO). The final preparation of the enzyme was stored at -80 ^{o}C until use.

All of the purified proteins were analyzed by SDS-PAGE (Fig. S1).

HPLC/ESI-MS analysis—HPLC/electrospray ionization mass spectrometry (ESI-MS) was performed with an API 3000 triple quadrupole instrument equipped with a nebulizer-assisted electrospray ionization interface (PE Sciex, Canada) under the same chromatographic conditions as described in the above-mentioned HPLC experiment. The effluent was split in a ratio of 1:19, resulting in 50 μl/min of it being admitted into the mass spectrometer. The ion spray, orifice, and focusing ring voltages were set to 5.0 kV, 40 V, and 200 V, respectively. Positive ion mass spectrometry (MS) spectra were acquired at 3 seconds per scan over a range of *m/z* 200-500.

ESI-MS/MS analysis—The peaks of 300-nm absorbing material eluting approximately between 5 and 7 min in the above-described HPLC experiment were collected and analyzed using ESI-MS/MS with the same mass spectrometer as in the above-mentioned HPLC/ESI-MS experiment. The channel electron multiplier voltage was set to 2.8 kV; the MS/MS spectra were acquired over a range of *m/z* 30-360.

UV-Vis spectroscopy—For ultraviolet-visible (UV-Vis) spectroscopy, the fractions of accumulated compound from the Capcell Pak C_{18} SG120 column were further purified using ion-exchange HPLC with an IEC QA-825 column (Showa Denko, Japan) equilibrated with water. Compound Z was eluted with a linear gradient of 0-1 M NaCl. The fractions of 300-nm absorbing material eluting between 20 and 22 min were collected and dispensed in 200-μl aliquots in 1.5-ml tubes. Next, 0.01 N HCl or 0.01 N NaOH was added to the aliquots in order to adjust the pH to 1, 5, or 12. The absorption spectrum of the sample was measured from 300

nm to 450 nm with a UV2450 spectrophotometer (Shimadzu, Japan).

RESULTS AND DISCUSSION

Identification of the compound accumulated in the ΔiscS mutant. To ascertain whether the 300-nm absorbing material accumulated in the Δ*iscS* and Δ*moaD* strains is compound Z, we performed HPLC/ESI-MS analysis of the acid extracts of these strains. The positive ion mass spectra obtained for the 300-nm absorbing material accumulated in the Δ*iscS* strain were identical to those accumulated in the Δ*moaD* strain (Fig. S4). Since the theoretical mass for compound Z with the formula $C_{10}H_{10}N_5O_7P$ is 343, the ions at *m/z* 344 and *m/z* 366 in the spectrum were attributed to be $[M + H]^+$ and $[M + Na]^+$ ions, respectively.

Furthermore, fractions of the compounds eluted at 5.75 min from the Δ*iscS* and Δ*moaD* mutants were collected and analyzed using ESI-MS/MS spectrometry. Two ions at *m/z* 246 and *m/z* 344 were detected in the samples of both the Δ*iscS* and Δ*moaD* mutants (Fig. S5A, B). The ion at *m/z* 344 corresponds to the parent $[M + H]^+$ ion of compound Z and that at *m/z* 246 has properties that are in accordance with those of a predicted daughter ion that is probably formed via cleavage of the 1,3,2-dioxaphosphorinane ring of compound Z (Fig. S5C).

Compound Z is known to exhibit characteristic UV-Vis absorption properties at different pH values (1). Therefore, we analyzed the 300-nm absorbing material accumulated in the acid extracts of the Δ*iscS* strain by UV-Vis spectroscopy. Fractions eluting between 5 and 7 min from a Capcell Pak C_{18} column were collected and further purified using an ion exchange column. The main peak detected at 21.15 min was collected, and the absorption spectra of this fraction were measured at pH 1, 5, and 12. At pH 1, the purified sample exhibited an absorption maximum at 315 nm and a shoulder at 370 nm (Fig. S6A). When the pH was adjusted to 5, it displayed an absorption peak at 310 nm and a broad absorption band centered at 350 nm (Fig. S6B). At pH 12, the sample exhibited an absorption maximum at 370 nm (Fig. S6C). These characteristic spectral features of the material accumulated in the Δ*iscS* mutant are similar to those of compound Z (1).

These data indicate that the 300-nm absorbing material eluted at 5.75 min in the HPLC analysis of acid extracts of the Δ*iscS* and Δ*moaD* mutants is compound Z. These results confirm the accumulation of compound Z in the *moaD*-deficient strain and provide the first demonstration of compound Z accumulation in an *iscS*-deficient bacterial strain.

Genetic complementation of the ΔiscS mutant. To further confirm the involvement of *iscS* in the biosynthesis of MPT, we performed a genetic complementation experiment. The cells containing the plasmid were grown in the presence of 100 μg/ml ampicillin and 1 mM IPTG at 37°C. As expected, the introduction of the pIscS plasmid expressing IscS into the Δ*iscS* strain resulted in absolutely no accumulation of compound Z, differently from that in the parent Δ*iscS* strain (Fig. S7).

FIGURE LEGENDS

Fig. S1. SDS-PAGE (15% polyacrylamide gel) analysis of the purified proteins. Lane 1, MoaD (10.9 kDa); lane 2, MoeB (28.9 kDa); lane 3, CsdA (43.2 kDa); lane 4, SufS (44.4 kDa); lane 5, IscS (45.1 kDa).

Fig. S2. Amino acid sequences of MoeB in various organisms were aligned with CLUSTALW. Two highly conserved cysteine residues were found (Cys142 and Cys187 in MoeB of *Escherichia coli*).

Fig. S3. Analysis of the sulfur transfer from cysteine desulfurases to MoaD by ^{35}S-labeled L-cysteine. Lane 1, CsdA and MoeB; lane 2, CsdA, MoeB, and MoaD; lane 3, CsdA and MoeB (C142A, C187A); lane 4, CsdA, MoeB (C142A, C187A), and MoaD; Lane 5, SufS and MoeB;

lane 6, SufS, MoeB, and MoaD; lane 7, SufS and MoeB (C142A, C187A); lane 8, SufS, MoeB (C142A, C187A), and MoaD; Lane 9, IscS and MoeB; lane 10, IscS, MoeB, and MoaD; lane 11, IscS and MoeB (C142A, C187A); lane 12, IscS, MoeB (C142A, C187A), and MoaD. The gel was stained with Coomassie Brilliant Blue (*A*), and the [^{35}S] radioactivity was visualized on a phosphor imaging plate (*B*).

Fig. S4. HPLC/ESI-MS of compound Z accumulated in the Δ*iscS* and Δ*moaD* strains. The positive ion mass spectra of the 300-nm absorbing material accumulated in the Δ*iscS* (*A*) and Δ*moaD* (*B*) strains are shown.

Fig. S5. ESI-MS/MS analysis of compound Z accumulated in the Δ*iscS* and Δ*moaD* strains. MS/MS spectra of the 300-nm absorbing material accumulated in the Δ*iscS* (*A*) and Δ*moaD* (*B*) strains are shown. A possible scheme for the cleavage of compound Z and the structural assignment of the major fragment ion under the ESI-MS/MS conditions are shown in *C*.

Fig. S6. UV-Vis absorption spectra of compound Z isolated from the Δ*iscS* strain. The absorption spectra of the Δ*iscS*-derived compound Z fraction eluting between 20 and 22 min from an IEC QA-825 column were measured at pH 1 (*A*), pH 5 (*B*), and pH 12 (*C*).

Fig. S7. Genetic complementation of the Δ*iscS* mutant by the expression of IscS. After oxidization with 1% I_2 and 2% KI, the acid extracts from the wild-type(pUC118), Δ*iscS*(pUC118), and Δ*iscS*(pIscS) were analyzed using HPLC as described in Experimental Procedures. The arrow indicates the accumulation of compound Z in the Δ*iscS*(pUC118) strain.

REFERENCE

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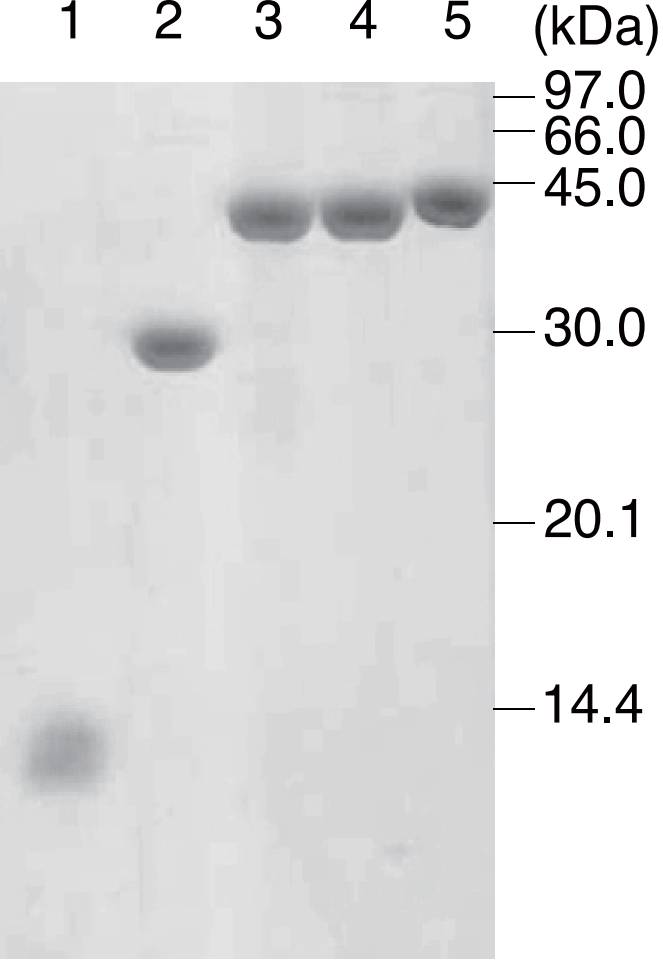


Fig. S1 Zhang *et al*.

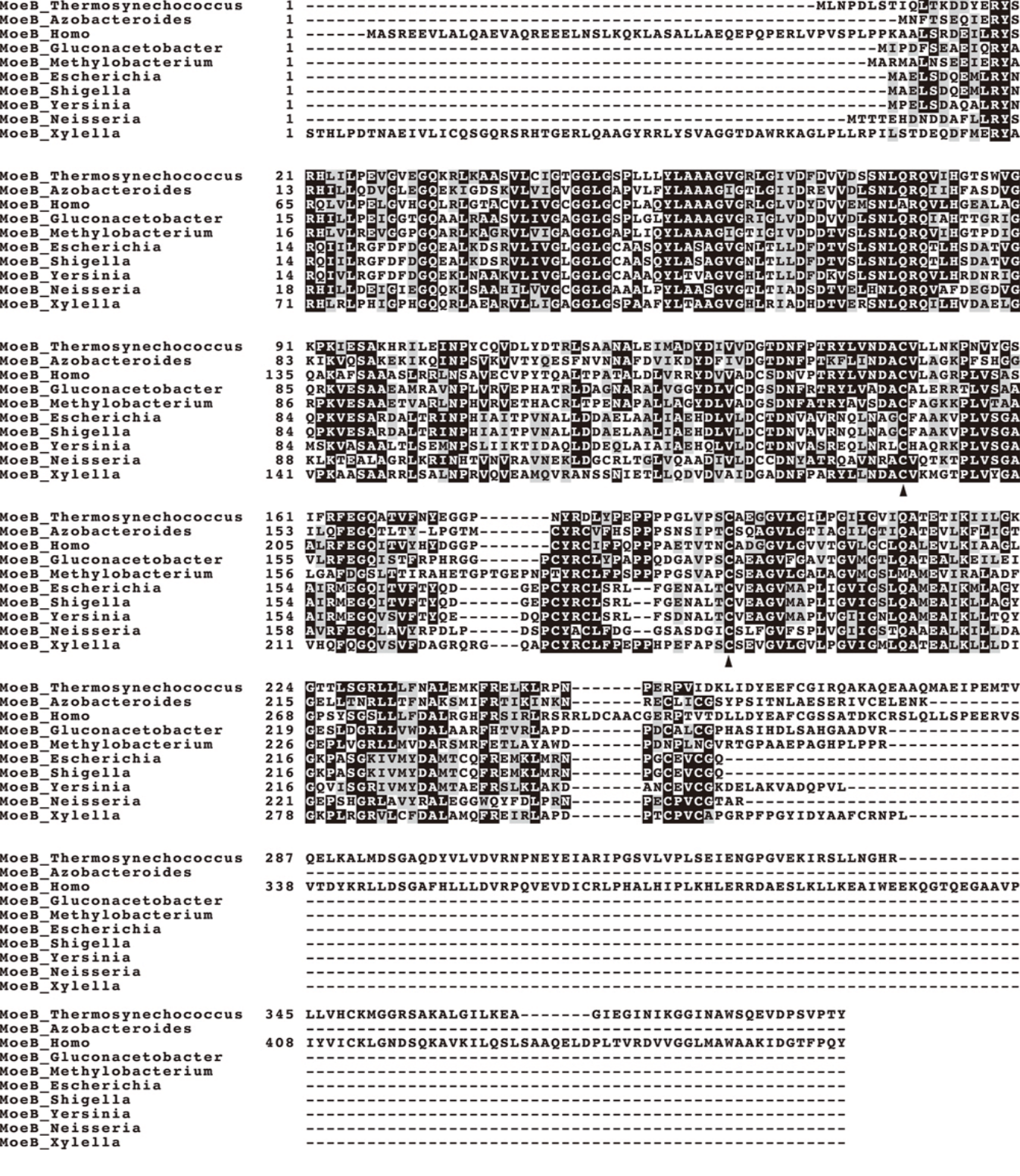
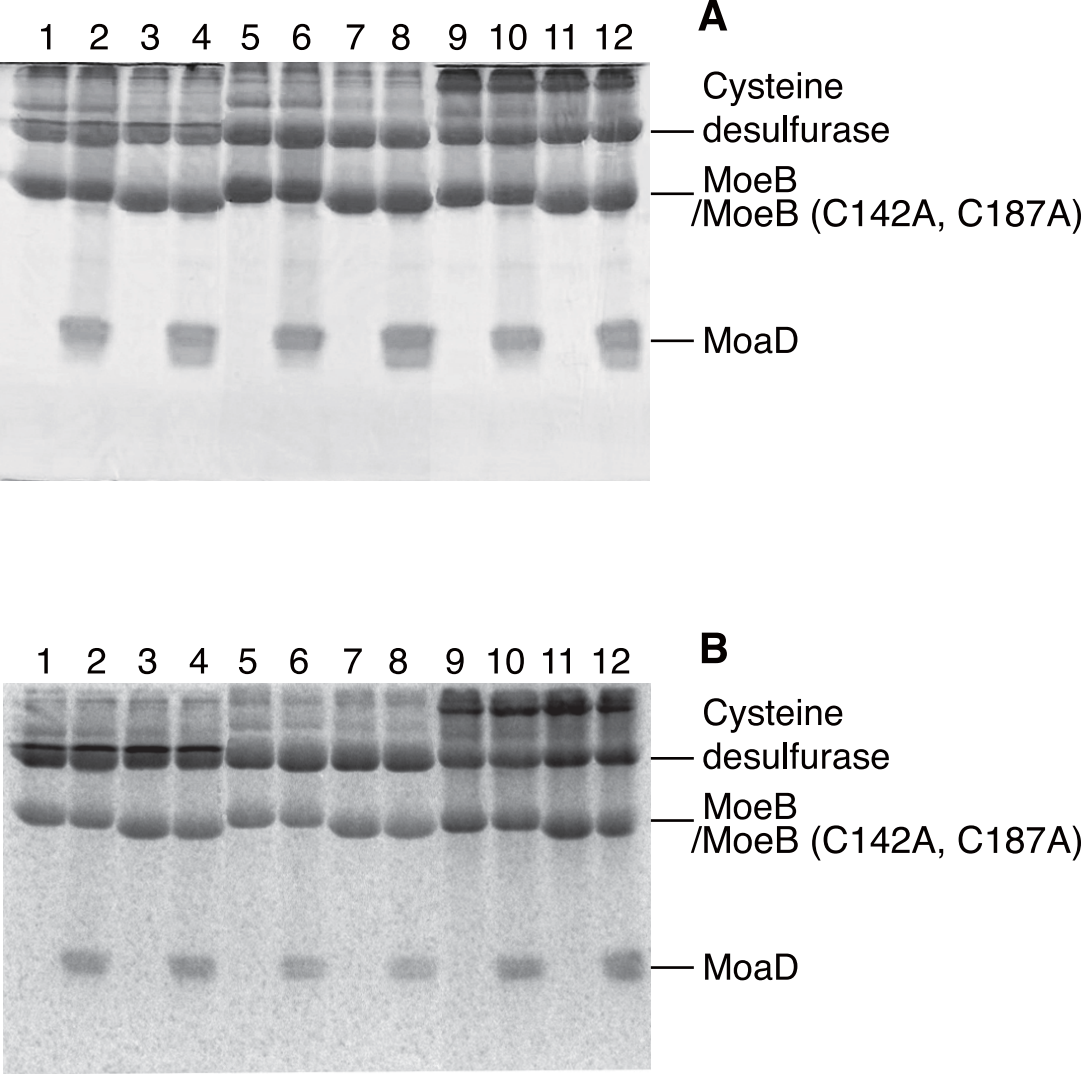


Fig. S2 Zhang *et al*.



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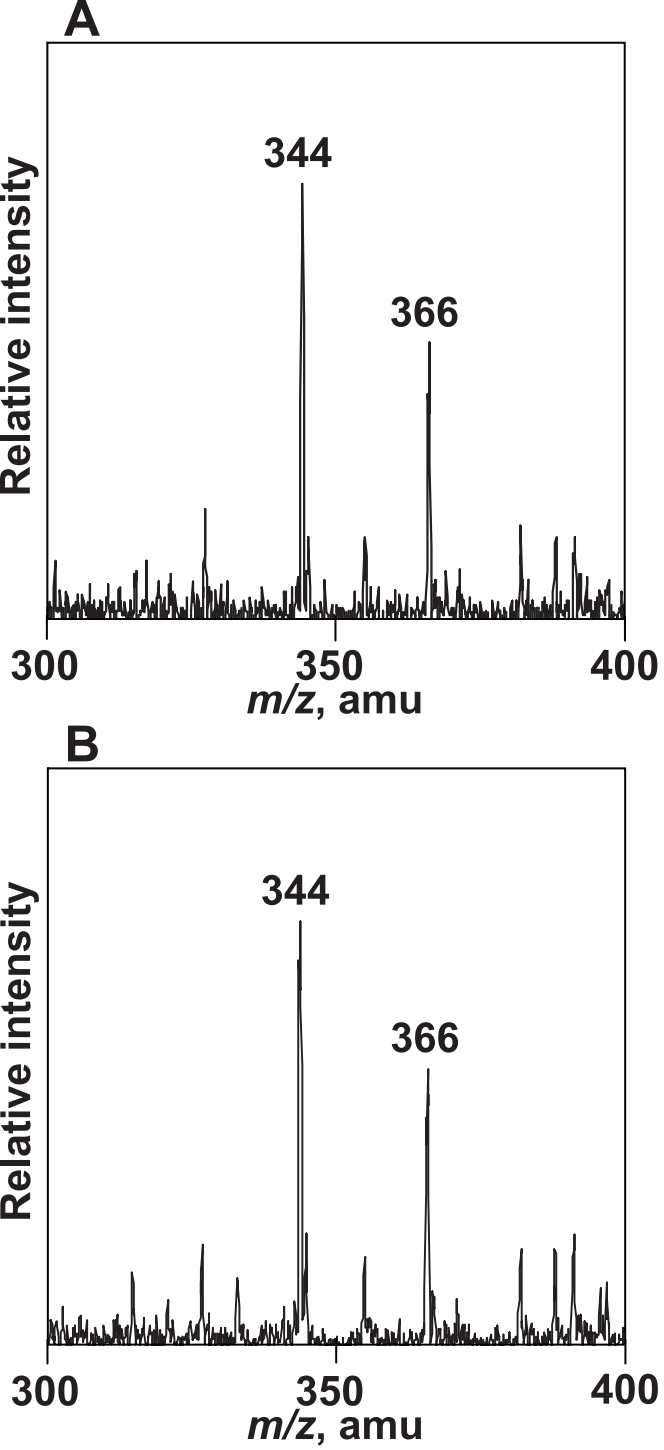
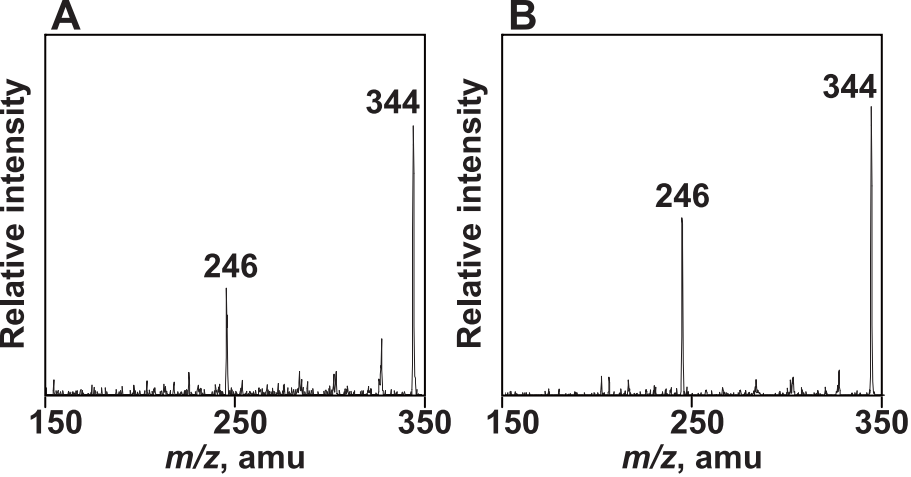


Fig. S4 Zhang *et al.*



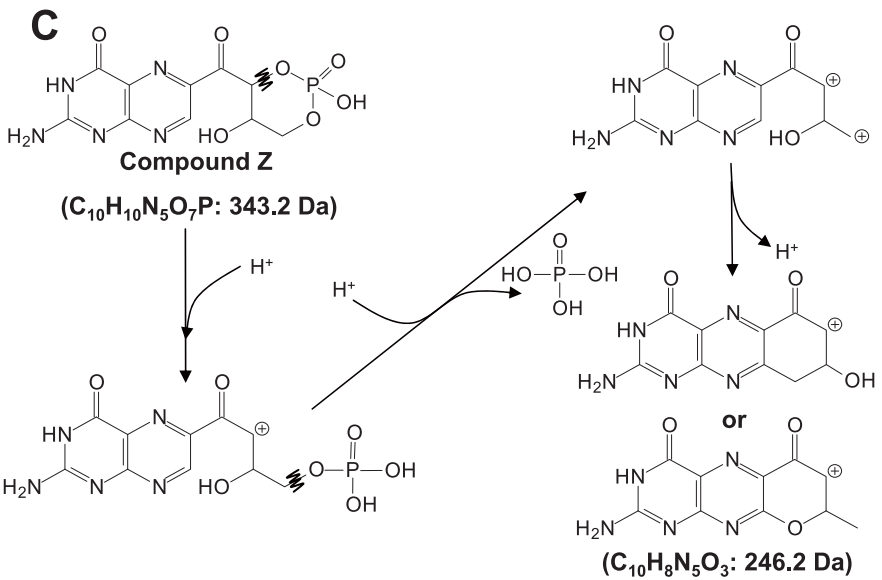


Fig. S5 Zhang *et al.*

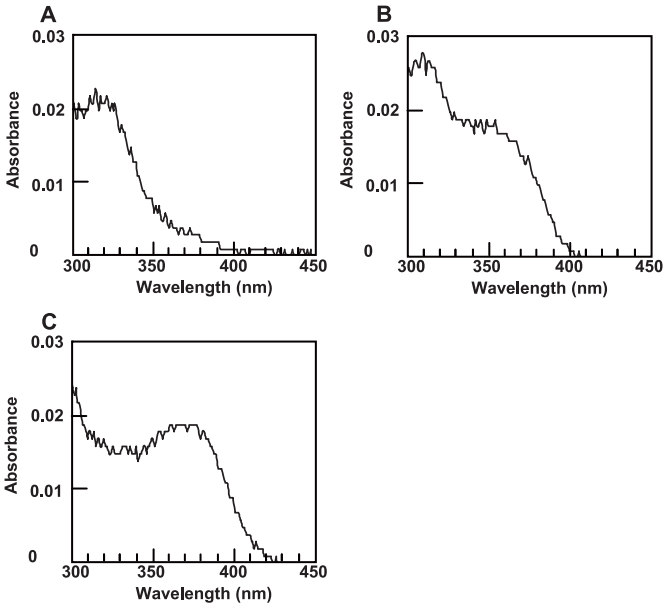


Fig. S6 Zhang *et al*.

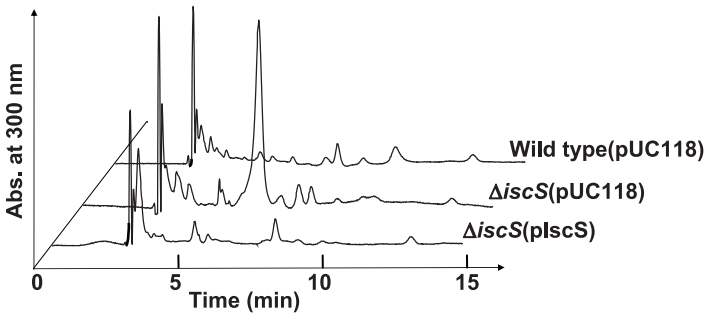


Fig. S7 Zhang *et al*.