SUPPLEMENTARY INFORMATION

Thiostrepton tryptophan methyltransferase expands the chemistry of radical SAM enzymes

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SUPPLEMENTARY RESULTS

Supplementary Figure 1 - (a) SDS PAGE analysis of TsrM. (b) Total mass analysis of TsrM by LC-MS. TsrM was applied onto a C-5 column and elution was performed by a gradient of acetonitrile in 0.6% formic acid. Mass spectrometry analysis was carried out in positive ion mode using a maXis UHR-TOF mass spectrometer (Bruker). (c) UV-visible spectra of TsrM before (solid line) and after (dashed line) reconstitution under anaerobic conditions. In inset, enzyme full spectrum.
Supplementary Figure 2 - HPLC analysis of TsrM incubated in the presence of SAM, tryptophan and sodium dithionite alone (a) or with methylcobalamin (b) after 12h of reaction. The reactions were analyzed by reverse phase HPLC coupled with UV (257 nm; blue line) or fluorescence detections (Ex/Em = 280/350 nm; red line). In-line UV-visible spectra of compound 2 (e), hydroxycobalamin (d) and methylcobalamin (e). (f) Mass spectrometry (LC-MS/MS LTQ Orbitrap) analysis of compound 1: Methyl-tryptophan [M+H]+ = 219.1136 (Theoretical mass of [M+H]+= 219.1133Da). (g) HPLC analysis (278 nm) of methylcobalamin (1) and hydroxycobalamin (2) standards. (h) UV-visible spectrum of methylcobalamin standard.
Supplementary Figure 3 - MS³ wideband Liquid chromatography–mass spectrometry LTQ-Orbitrap analysis of tryptophan (a) and methylated tryptophan (b) produced by TsrM

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Supplementary Figure 4 - $^{13}$C NMR spectra with proton decoupling of $^{13}$C labeled tryptophan (a), the full reaction with $^{13}$C labeled tryptophan and $^{13}$C labeled SAM (b) and the purified compound 2 (c). Spectra were recorded with 64K data points using a spectral width of 36 000 Hz. The spectra were recorded with 256 scans for tryptophan and 48000 scans for the reaction mixture and for the product using a repetition time of 2 s and a 30° pulse angle. An exponential weighting function was applied prior to Fourier transformation and a line-broadening of 3 Hz was applied for tryptophan and 10 Hz for the two other spectra. The downfield shift of the doublet corresponding to the C2 signal in tryptophan to a signal appearing as a triplet and overlapping with C7a together with the presence of a methyl group at 13ppm clearly demonstrate that a methyl group has been transferred on the C2 position of tryptophan.
Supplementary Figure 5 - UV-visible analysis of S-adenosyl-L-methionine (a) and the produced S-adenosyl-L-homocysteine (b). Mass spectrometry (LC-MS/MS LTQ Orbitrap) analysis of produced S-adenosyl-L-homocysteine (c): SAH [M+H]+ = 385.1289 Da (Theoretical mass [M+H]+ = 385.1288 Da)
Supplementary Figure 6 - Time course of methyl-tryptophan (♦) and SAH (■) production in presence (red traces) or absence (blue traces) of sodium dithionite by TsrM. TsrM (20 µM) was incubated under anaerobic conditions with DTT (5 mM), methylcobalamin (500 µM), tryptophan (1 mM) and SAM (1 mM) with or without sodium dithionite (2 mM).
Supplementary Figure 7 - Methylated tryptophan produced by TsrM without sodium dithionite. With d3-SAM, TsrM produces exclusively fully labeled tryptophan (b) ([M+H]+ = 222.132) while using tryptophan specifically deuterated on the five unexchangeable protons of the indole ring (c), methyltryptophan with a mass increment of 4 Da ([M+H]+ = 223.138) is produced. TsrM (20 µM) was incubated under anaerobic conditions with DTT (5 mM), methylcobalamin (500 µM) and (a) unlabeled tryptophan (1 mM) and SAM (1 mM), (b) unlabeled tryptophan (1 mM) and d3-SAM (1 mM) or (c) fully deuterated tryptophan on the indole ring (1 mM) and unlabeled SAM (1 mM).
Supplementary Figure 8 - Liquid chromatography–mass spectrometry LTQ-Orbitrap analysis of methly-tryptophan produced by TsrM incubated with (a) SAM or (b) d3-SAM. 1: Total ion current and 2: mass spectrometry analysis of the corresponding signal. TsrM (20µM) was incubated under anaerobic conditions with DTT (5mM), methyl-cobalamin (500µM) and unlabeled tryptophan (1mM) with SAM or d3-SAM (1 mM).
Supplementary Figure 9 - HPLC analysis of methyl-cobalamin incubated with (a) or without (b) DTT (5mM). The reactions were analyzed by reverse phase HPLC coupled with UV (275 nm) detection.
Supplementary Figure 10 - LC-MS/MS LTQ Orbitrap analysis of methylcobalamin produced by TsrM in the presence of SAM (a) or d3-SAM (b). TsrM (20µM) was incubated under anaerobic conditions with DTT (5mM), methylcobalamin (20µM), tryptophan (1mM) and SAM or d3-SAM (1 mM).
Supplementary Figure 11 - Time course of methyl-tryptophan (♦) and SAH (■) production by TsrM incubated under anaerobic conditions with SAM (1 mM), tryptophan (1 mM), DTT (5 mM) and 500 µM (red traces) or 20µM (blue traces) of methylcobalamin.
Supplementary Figure 12 - Time-dependent production of SAH by TsrM (■) or the Cx3Cx2C mutant enzyme (♦).
Supplementary Figure 13 - UV-visible analysis of methylcobalamin (100 µM) incubated with SAM (200µM) and tryptophan (200 µM) (a) or in the presence of TsrM (b). Reactions were performed in an anaerobic glove box and at each time point an aliquot was measured anaerobically. Spectra were recorded with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) housed in the anaerobic chamber. Measures were performed every 5 minutes from 0 to 30 min (dark to light blue) then every 10 minutes from 30 to 60 minutes (light blue to purple). The same color code applies for the same time points in the two panels.
**SUPPLEMENTARY TABLES**

Supplementary Table 1 - TsrM analysis after trypsic digestion and treatment with iodoacetamide by LC-MS/MS. Peptide analysis was performed by liquid chromatography–mass spectrometry/mass spectrometry connected to a LTQ-Orbitrap Discovery mass spectrometer. Experimental peptide masses (MH⁺ Obs) were compared with theoretical mass (MH⁺ Theo).

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Supplementary Table 2 - $^{13}$C Chemical Shifts (ppm) of $^{13}$C labeled tryptophan and $^{13}$C labeled 2-methyl tryptophan.

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SUPPLEMENTARY METHODS

Materials. Restriction enzymes were from Promega. Chemicals were obtained from Sigma Aldrich. Tryptophan fully deuterated on the indole ring was purchased from Cambridge Isotope Laboratories.

Bacterial strains, plasmids and DNA manipulations: Plasmids manipulations were performed in E. coli TOP10 (Invitrogen). Plasmids were purified with DNA purification kit (QIAprep, Qiagen). DNA fragments were extracted from agarose gel and purified with Qiaquick gel extraction kit (Qiagen). E. coli BL21 (DE3) star (Invitrogen) were used for overexpression of the tsrM gene.

Construction of the plasmid pASK-tsrM for gene expression in E. coli. A plasmid containing the tsrM gene (EMBL: ACN52303.1) was constructed using an engineered gene optimized for expression in E. coli (Invitrogen). The 1.8 kb BsaI/BsaI fragment containing the gene was ligated into pASK17+ plasmid to produce the recombinant plasmid pASK-tsrM expressing TsrM fused with an N-terminal strep-Tag. The entire cloned gene was sequenced to ensure that no error was introduced.

TsrM expression and purification. E. coli BL21 (DE3) star transformed with pASK-tsrM were used to inoculate fresh LB medium (9 L) supplemented with ampicillin (100 µg.mL-1) and bacterial growth proceeded at 37°C until the OD600 reached ~0.6. Protein expression was induced by adding 9 mL of anhydrotetracycline (0.2 mg/mL). After 24 hours of culture at 18°C, the cells were collected by centrifugation at 4,000 x g for 15 minutes. The cells were suspended in 10 mL of buffer (Tris 50 mM, KCl 300 mM, MgCl₂ 10 mM, NaCl 0.5mM, Glycerol 10% pH 7.5). The cells were disrupted by sonication on ice after adding 500 µL of triton X100 and 200µL of 2-mercaptoethanol and centrifuged at 45,000 x g at 4°C for 1.5 hour. The solution obtained was then loaded onto a Streptactin high capacity column (IBA) previously equilibrated with the same buffer. The column was washed with 5 column volumes. Proteins were eluted with 6mL of buffer containing desthiobiotin (0.6g/L) and DTT (3 mM) and further concentrated with Ultrafree cells (Millipore) with a molecular cut-off of 10 kDa. The purified protein was stored at -80°C and the purity was assayed on a 12% SDS PAGE.
**Mutation of the Cx₃Cx₂C motif in TsrM.** The plasmid pASK-tsrM that contained the tsrM gene was used as matrix. PCR amplification was carried out by using a two-step PCR method with the following primers: TsrM_C3_F (5'-AAT GGT GCT GTG TAT AAA GCC AGC TTT GCC GTT GAA-3') and TsrM_C3_R (5'-TTC AAC GGC AAA GCT GGC TTT ATA CAC AGC ACC ATT-3') in order to mutate the residues: C254, C259 & C262 into alanyl residues. After full gene sequencing, the TsrM mutant was expressed and purified using the same condition as the wild type enzyme.

**Methyltransferase activity.** All the in vitro reactions were carried out in an anaerobic chamber (Bactron IV) in a Tris-buffered solution (Tris 50 mM, KCl 300 mM, MgCl₂ 10 mM, NaCl 0.5mM, Glycerol 10% pH 7.5). The reactions mixture were composed of the following compounds depending of the reactions: TsrM (20µM), Tryptophan (Trp, 1mM), S-adenosyl L methionine (SAM, 1 mM), Methylcobalamin (MeCo, 500µM) and Dithiothreitol (DTT, 5mM). When used, sodium dithionite was at 2mM. Protein was reconstituted by adding 3mM of DTT at 12°C during 15 minutes. Then, Na₂S (400µM) and (NH₄)₂Fe(SO₄)₂ (400µM) were added and solution was incubated at 12°C during 4h. The protein was then desalted to remove unbounded iron and sulfide and the reaction was initiated, by addition of SAM and MeCo. Incubations were performed at 25°C under strict anaerobic conditions.

**HPLC analysis.** Samples (3µL) were diluted with 27µL of trifluoroacetic acid solution (TFA 0.1%). HPLC analysis was carried out on an Agilent 1200 series infinity with a reversed phase column (LiChroCART RP-18e 5µm). The column was equilibrated with 100% solvent A (H₂O, 0.1% TFA) and the following gradient was applied with the solvent B (80 % CH₃CN, 0.1% TFA): 0-1 min 100% A/0% B; 1-20 min, a linear gradient up to 60% A/40% B; 20-25 min, a linear gradient to 100% A/0% B. Flow rate was of 1 ml/min and detection was made at 257 & 278 nm with a diode array detector or by fluorescence (excitation at 278 nm and emission at 350 nm).

**Spectrophotometric measurement.** UV-visible absorption spectra were recorded on a Beckman DU 640 spectrophotometer.

**Protein analysis.** Protein concentration (by monomer) was determined by the Bradford protein assay (Bradford, M. M. (1976) Anal Biochem 72, 248-54). Protein-bound iron was determined according to published procedures (Fish, W. W. (1988) Methods Enzymol 158, 357-64). After acidic denaturation, the quantity of iron released in solution was assayed by
measure of the absorbance at 535 nm after incubation with the chelating agent: bathophenantroline disulfate. The total absorbance was corrected for the absorption at 680 nm. In parallel, a standard calibration curve ranging from 0 to 10 nmol Fe(II) was realized.

**Sulfur iron measurement.** Sulfur iron was determined according to Beinert (Beinert H. Anal Biochem. 1983 Jun;131(2):373-8). TsrM was incubated with Zn(OAc)$_2$ and NaOH. Reactions were further incubated with DMPD (N,N dimethyl phenylenediamin monochloryde) and FeCl$_3$. The released sulfur iron was spectrophotometrically assayed at 667 nm (corrected for the absorption at 850 nm).

**Iron binding characterization of TsrM before and after enzyme reconstitution.** TsrM (20 µM) was reconstituted during 4h under anaerobic conditions with a 20-fold molar excess of Na$_2$S and (NH$_4$)$_2$Fe(SO$_4$)$_2$. The protein was desalted on Sephadex G-25 (with Tris 50 mM, KCl 300 mM, MgCl$_2$ 10 mM, NaCl 0,5mM, Glycerol 10% pH 7.5 and 5 mM DTT). The colored fractions were concentrated on Amicon ultra 10K (Millipore) and spectra were recorded on a Beckman DU 640 spectrophotometer. For non-reconstituted enzyme, spectrum of TsrM was recorded directly after enzyme purification.

**LC-MS total protein mass analysis.** Enzyme sample was applied onto a C-5 column and elution was performed by a gradient of acetonitrile in 0.6% formic acid. Mass spectrometry analysis was carried out in positive ion mode using a maXis UHR-TOF mass spectrometer (Bruker).

**TsrM analysis after trypsic digestion and LC-MS/MS analysis.** TsrM was gel purified, reduced by dithiothreitol and alkylated with iodoacetamide. Samples were dried and incubated overnight at 37°C with 125 ng of sequencing grade trypsin (Promega). The tryptic digest was analyzed on an Ultimate 3000 LC system (Dionex) connected to a LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher). Protein identification was performed using X!tandem software (X!Tandem tornado 2010.12.01.1, http://www.thegpm.org) and a protein database containing TsrM and *E coli* BL21 proteome. In the search parameters were included: static alkylation of cysteine, methionine oxydation and variable methylation of all amino-acids except methionine. The search result were treated by the X!tandem parser developed and used on PAPPSO platform (X!Tandem pipeline version 3.1.2 http://pappso.inra.fr/bioinfo/xtandempipeline/) and peptides were validated if their E-value were lower than 0.05.
**Liquid chromatography–mass spectrometry /mass spectrometry analysis.** Liquid chromatography–mass spectrometry /mass spectrometry analysis were performed on Ultimate 3000 LC system (Dionex, Voisins le Bretonneux, France) connected to a LTQ-Orbitrap Discovery mass spectrometer (ThermoFisher) by nanoelectrospray ion source. Each reaction mixture was diluted 10 times and 4 µl were loaded at a flowrate of 20 µl min\(^{-1}\) onto precolumn Pepmap C18 (0.3 by 5 mm, 100 Å, 5 µm; Dionex). After 4 min, the precolumn was connected with the separating nanocolumn Pepmap C18 (0.075 by 15 cm, 100 Å, 3 µm). Mobile phase A was 2% CH\(_3\)CN, 0.1% aqueous FA, and mobile phase B was 80% CH\(_3\)CN, 20% H\(_2\)O, 0.1% FA. The flow rate was a constant at 300 nl min\(^{-1}\) and the mobile phase composition was as follows: 0% B for 4 min; linear increase over 31 min to 38% B to separate the tryptophan derivatives. Ionization was performed on liquid junction with a spray voltage of 1.3 kV applied to an uncoated capillary probe (PicoTip EMITER 10-µm tip inner diameter; New Objective). All ions were analyzed by full MS scan (m/z 120 to 1,500) with a resolution of 30000 on the Orbitrap mass analyzer in the positive mode. The tryptophan (m/z=205) and methyl-tryptophan (m/z=219) were respectively fragmented and detected in the LTQ linear ion trap mass analyzer after MS\(^3\) experiment. The normalized energy of collision was fixed to 45% for the consecutive MS\(^2\) and MS\(^3\) steps of fragmentation with the activation of the wideband option for the transition: 205>146 for tryptophan and 219>160 for methyl-tryptophan.

**\(^{13}\)C-methyl tryptophan synthesis.** Uniformly \(^{13}\)C-methyl tryptophan was synthesized by incubating labeled \(^{13}\)C-SAM (1 mM) with \(^{13}\)C-tryptophan (1mM) (Eurisotop), methylcobalamin (500µM), DTT (5mM) and TsR (20µM). \(^{13}\)C-SAM was enzymatically synthesized using uniformly labeled 13C-methionine and ATP as previously described (Benjdia et al. 2008 *J Biol Chem.*;283(26):17815-26).

**NMR analysis.** Samples were dissolved in 100 µL D\(_2\)O (99.99%) and transferred into 3 mm NMR tubes. The NMR spectra were obtained at 25\(^0\)C on a Bruker AVANCE III 600 MHz spectrometer equipped with a 5 mm 1H/13C/15N/31P QCI Z-Gradient Cryoprobe. The 13C NMR spectra with proton decoupling were recorded with 64K data points using a spectral width of 36 000 Hz. The spectra were recorded with 256 scans for tryptophan and 48000 scans for the reaction mixture and for the purified product using a repetition time of 2 s and a 30\(^0\) pulse angle. An exponential weighting function was applied prior to Fourier
transformation and a line-broadening of 3 Hz was applied for tryptophan and 10 Hz for the two other spectra.

**UV-visible analysis of the reaction under anaerobic conditions.** Reactions were carried-out in Tris-buffered solution (Tris 50 mM, KCl 300 mM, MgCl$_2$ 10 mM, NaCl 0.5mM, Glycerol 10\% pH 7.5) under anaerobic conditions. For each time point aliquots were removed and analysed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) housed in the anaerobic chamber.

**Standard compounds used in this study:**

![Trp](image)

**Trp.** The compound elutes at 14.8 min.

LC-MS ($m/z$): [M+H]$^+$ calc for C$_{11}$H$_{12}$N$_2$O$_2$, 205.0971 and found 205.0977

![2-Methyl-Trp](image)

**2-Methyl-Trp.** The compound elutes at 15.8 min.

LC-MS ($m/z$): [M+H]$^+$ calc: C$_{12}$H$_{14}$N$_2$O$_2$, 219.112, measured: 219.113

![2-Methyl(d3)-Trp](image)

**2-Methyl(d3)-Trp.** The compound elutes at 15.7 min.

LC-MS ($m/z$): [M+H]$^+$ calc: C$_{12}$H$_{14}$N$_2$O$_2$, 222.1316, measured: 222.1321
**SAM.** The compound elutes at 5 min.

LC-MS (m/z): [M+H]$^+$ calc for C$_{15}$H$_{22}$N$_6$O$_5$S, 399.1445, measured: 399.1446

**d3-SAM.** The compound elutes at 5 min.

LC-MS (m/z): [M+H]$^+$ calc: C$_{15}$H$_{19}$D$_3$N$_6$O$_5$S, 402.1633, measured: 402.1633

**SAH.** The compound elutes at 8 min.

LC-MS (m/z): [M+H]$^+$ calc: C$_{14}$H$_{20}$N$_6$O$_5$S, 385.1289, measured: 385.1291