Supporting Information

for

The S-adenosylmethionine (SAM)-dependent methyltransferase MftM is responsible for methylation of the redox cofactor mycofactocin

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Figure S1: Mass shifts in MS² fragments of 13C-labeled mycofactocin. Shown are MS² spectra of methylmycofactocinol-8. (A) MS² spectrum of unlabeled methylmycofactocinol-8 (12C62H99NO43, RT: 6.78 min, m/z 773.7906 [M+2H]²⁺, 4.91 ppm) (B) MS² spectrum of labeled methylmycofactocinol-8 (13C1412C68H99NO43, RT: 6.77 m/z 780.8140 [M+2H]²⁺, 4.74 ppm) from M. smegmatis wild-type cells fed with L-valine-13C5, L-tyrosine-13C9, and L-methionine-(methyl-13C). Highlighted in red are the deduced incorporations of 13C atoms into the fragments of MMFT-8H2. Arrows with dotted lines display the observed mass shifts between fragments of labeled and unlabeled precursors, fragments without mass shift were not highlighted. Apparently, thirteen heavy carbons were incorporated into the redox core structure of mycofactocin and one additional heavy carbon atom was incorporated into the second glucose unit. This can be deduced from a shift of +13.0430 m/z in fragments corresponding to MFT-1H2 and +14.0508 m/z for MMFT-2H2-like fragments, respectively, showing that the additional label (methyl group) is only found in the second glucose moiety. Fragmentation was performed at a normalized collision energy (NCE) of 20. Parts per million (ppm) values indicate relative mass deviations.
Figure S2. Representative metabolomics results for *M. smegmatis* wild-type and all tested mutant strains (Table 1) regarding the presence of methylmycofactocin. (A) Stacked extracted ion chromatograms (5 ppm window) for mycofactocinol-8 (C$_{61}$H$_{97}$NO$_{33}$, RT: 6.50 min, m/z 1532.5507 [M+H]$^+$). (B) Extracted ion chromatograms for methylmycofactocinol-8 (C$_{62}$H$_{99}$NO$_{43}$, RT: 6.81 min, m/z 1546.5664 [M+H]$^+$). Chromatograms were obtained from metabolome extracts of genotypes indicated on the right. The results show the absence of methylated mycofactocins in *M. smegmatis* ΔmftM (MSMEG_6237) in contrast to the wild-type. All other tested putative methyltransferase and the complementation mutant, ΔmftM-comp. Relative intensities were obtained through normalization of the peak intensities to the maximum intensity represented here. Experiments were conducted in quadruplicates.
Figure S3. Multiple sequence alignment (MSA) of representative MftM homologs and RebM. The latter was the closest characterized homolog found for MftM from \textit{M. smegmatis}, RebM from \textit{Lentzea aerocolonigenes}, a demethylrebeccamycin-d-glucose O-methyltransferase (36.19% identical). Shown are (A) the MSA in an overview graphic, (B) the N-terminal region of the MSA exhibiting a partial conservation of residues between MftM homologs which are absent in the RebM sequence, (C) the conserved S-adenosyl methionine binding domain (NCBI conserved protein domain family CDD 100107) present in all included sequences, and (D) the C-terminal region of the MSA exhibiting a partial conservation between all included MftM homologs and RebM.
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Figure S7. MS² fragmentation spectrum of mycofactocinol-7 from *Mycobacterium bovis BCG*. Shown is an MS² fragmentation spectrum of mycofactocinol-7 (C₅₅H₈₇NO₃₈, RT: 6.56 min, precursor m/z 1370.5052 [M+H]⁺ 5.3 ppm) from an *M. bovis* metabolome extract. Fragmentation was performed at 40 NCE. Parts per million (ppm) values indicate relative mass deviations.
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Figure S10. MS² fragmentation spectra of mycofactocin congeners found in *Saccharopolyspora erythraea*. Shown are MS² fragmentation spectra of (A) methylmycofactocinone-2 (C\textsubscript{26}H\textsubscript{37}NO\textsubscript{13}, RT: 7.47 min, precursor m/z 572.2371 [M+H]+, 5.8 ppm), (B) methylmycofactocinol-2 (C\textsubscript{26}H\textsubscript{39}NO\textsubscript{13}, RT: 7.10 min, precursor m/z 574.2514 [M+H]+, 3.5 ppm), and (C) methylmycofactocinone-3 (C\textsubscript{32}H\textsubscript{47}NO\textsubscript{18}, RT: 7.42 min, precursor m/z 734.2906 [M+H]+, 5.5 ppm) from *S. erythraea* metabolome extracts. Fragmentation was performed at 40 NCE. Parts per million (ppm) values indicate relative mass deviations.
Figure S11. Growth of *M. smegmatis* wild-type and mutant strains. Shown are the *M. smegmatis* wild-type, all included methyltransferase knock-out mutants, and the ΔmftM-comp complement strain in liquid culture on ethanol as the sole carbon source. Error bars represent standard deviation of four biological replicates.
Cellulase digest – Representative mycofactocin congeners

Figure S12. Representative mycofactocins in the metabolome of *Amycolatopsis methanolica* cultivated on ethanol as the sole carbon source before and after cellulase-treatment. Shown are stacked extracted ion chromatograms (5 ppm window) of representative mycofactocin congeners: premycofactocinol (C$_{13}$H$_{17}$NO$_{3}$, RT: 7.84 min, $m/z$ 236.1281 [M+H]$^+$), methylmycofactocinol-2 (C$_{26}$H$_{39}$NO$_{13}$, RT: 7.10 min, $m/z$ 574.2494 [M+H]$^+$), methylmycofactocinol-8 (C$_{62}$H$_{99}$NO$_{43}$, RT: 6.81 min, $m/z$ 1546.5664 [M+H]$^+$), premycofactocinone (C$_{13}$H$_{15}$NO$_{3}$, RT: 8.40 min, $m/z$ 234.1125 [M+H]$^+$), methylmycofactocinone-2 (C$_{26}$H$_{37}$NO$_{13}$, RT: 7.47 min, $m/z$ 572.2338 [M+H]$^+$), methylmycofactocinone-8 (C$_{62}$H$_{97}$NO$_{43}$, RT: 7.18 min, $m/z$ 1544.5507 [M+H]$^+$) in *A. methanolica* metabolome extracts before and after treatment with a cellulase mixture from *Trichoderma reesei*. The increase in intensity of the aglycones originates from mycofactocinols/-ones, while the increase of methylmycofactocin(-ol/-one)-2 originates from methylmycofactocin(-ol/-one)-n (with n > 2).
Figure S13. Representative mycofactocins in the metabolome of *Mycobacterium bovis* BCG cultivated on a medium containing ethanol before and after cellulase-treatment. Shown are stacked extracted ion chromatograms (5 ppm window) of representative mycofactocin congeners: premycofactocinol (C\textsubscript{13}H\textsubscript{17}NO\textsubscript{3}, RT: 7.84 min, m/z 236.1281 [M+H]\textsuperscript{+}) and mycofactocinol-7 (C\textsubscript{55}H\textsubscript{87}NO\textsubscript{38}, RT: 6.55 min, m/z 1370.4979 [M+H]\textsuperscript{+}) in *M. bovis* metabolome extracts before and after treatment with a cellulase mixture from *T. reesei*. The increase in intensity of the premycofactocinol originates from mycofactocinols.

Figure S14. Representative mycofactocins in the metabolome of *Mycobacterium tuberculosis* cultivated on a medium containing ethanol before and after cellulase-treatment. Shown are stacked extracted ion chromatograms (5 ppm window) of representative mycofactocin congeners: premycofactocinol (C\textsubscript{13}H\textsubscript{17}NO\textsubscript{3}, RT: 7.84 min, m/z 236.1281 [M+H]\textsuperscript{+}) and mycofactocinol-7 (C\textsubscript{55}H\textsubscript{87}NO\textsubscript{38}, RT: 6.55 min, m/z 1370.4979 [M+H]\textsuperscript{+}) in *M. bovis* metabolome extracts before and after treatment with a cellulase mixture from *T. reesei*. The increase in intensity of the premycofactocinol originates from mycofactocinols.
Figure S15. Representative mycofactocins in the metabolome of *Rhodococcus erythropolis* cultivated on ethanol as the sole carbon source before and after cellulase-treatment. Shown are stacked extracted ion chromatograms (5 ppm window) of representative mycofactocin congeners: premycofactocinol (C_{13}H_{17}NO_{3}, RT: 7.84 min, m/z 236.1281 [M+H]^+), methylmycofactocinol-2 (C_{26}H_{39}NO_{13}, RT: 7.10 min, m/z 574.2494 [M+H]^+), methylmycofactocinol-8 (C_{62}H_{99}NO_{43}, RT: 6.81 min, m/z 1546.5664 [M+H]^+), premycofactocinone (C_{13}H_{15}NO_{3}, RT: 8.40 min, m/z 234.1125 [M+H]^+), methylmycofactocinone-2 (C_{26}H_{37}NO_{13}, RT: 7.47 min, m/z 572.2338 [M+H]^+), methylmycofactocinone-8 (C_{62}H_{97}NO_{43}, RT: 7.18 min, m/z 1544.5507 [M+H]^+) in *R. erythropolis* metabolome extracts before and after treatment with a cellulase mixture from *T. reesei*. The increase in intensity of the aglycones originates from mycofactocins/-ones, while the increase of methylmycofactocin(-ol/-one)-2 originates from methylmycofactocin(-ol/-one)-n (with n > 2).
Figure S16. Representative mycofactocins in the metabolome of *Saccharopolyspora erythraea* cultivated on ethanol as the sole carbon source before and after cellulase-treatment. Shown are stacked extracted ion chromatograms (5 ppm window) of representative mycofactocin congeners: premycofactocinol (C_{13}H_{17}NO_{3}, RT: 7.84 min, m/z 236.1281 [M+H]^+), methylmycofactocinol-2 (C_{26}H_{39}NO_{13}, RT: 7.10 min, m/z 574.2494 [M+H]^+), premycofactocinone (C_{13}H_{15}NO_{3}, RT: 8.40 min, m/z 234.1125 [M+H]^+), methylmycofactocinone-2 (C_{26}H_{37}NO_{13}, RT: 7.47 min, m/z 572.2338 [M+H]^+), methylmycofactocinone-9 (C_{68}H_{107}NO_{48}, RT: 7.14 min, m/z 1706.6035 [M+H]^+) in *S. erythraea* metabolome extracts before and after treatment with a cellulase mixture from *T. reesei*. The increase in intensity of the aglycones originates from mycofactocinols/-ones, while the increase of methylmycofactocin(-ol/-one)-2 originates from methylmycofactocin(-ol/-one)-n (with n > 2).
Figure S17. Short-chain mycofactocin does not accumulate in the cells of *M. smegmatis* during early growth phase. Shown are (A) the growth of *M. smegmatis* WT on ethanol as the sole carbon source, (B) the abundance of methylmycofactocinol-2 ($\text{C}_{26}\text{H}_{39}\text{NO}_{13}$, RT: 7.10 min, $m/z$ 574.2494 [M+H]$^+$) and (C) the abundance of methylmycofactocinol-8 ($\text{C}_{62}\text{H}_{99}\text{NO}_{43}$, RT: 6.81 min, $m/z$ 1546.5664 [M+H]$^+$) at certain time points during the growth.
Figure S18. Mycofactocin aglycones do not accumulate in the exometabolome of *M. smegmatis* during early growth. Shown are the abundance of the mycofactocin aglycones AHDP-0 ($C_{13}H_{18}N_2O_2$, RT: 5.80 min, $m/z$ 235.1441 [M+H]⁺), premycofactocinone ($C_{13}H_{15}NO_3$, RT: 8.40 min, $m/z$ 234.1125 [M+H]⁺), and premycofactocinol ($C_{13}H_{17}NO_3$, RT: 7.84 min, $m/z$ 236.1281 [M+H]⁺) in the exometabolome of *M. smegmatis* cultivated on ethanol as the sole carbon source at 40 h (late lag phase) and 60 h (exponential growth). Significance was calculated using Student’s two-sided t-test ($n = 4$), N/A: t-test not applicable as premycofactocinone was not detected at $t = 40$ h.
Figure S19: Ratio of reduced to oxidized forms of non-methylated mycofactocins and methylated mycofactocins. Plotted is the ratio of peak areas of the main mycofactocin species MMFT-8H₂ over MFT-8 in comparison to the ratio of MFT-8H₂ over MFT-8 in the intracellular metabolome extracts of M. smegmatis WT at two different time points during growth on ethanol as the sole carbon source. The ratio was calculated as the mean of peak areas from four independent biological replicates, error bars represent the standard deviation. The p-values were calculated using Student’s two-sided t-test (n = 3).
Table S2. Primers used for the construction of *M. smegmatis* mutant strains.

| Primer pairs used for amplification of | Sequence 5’-3’ |
|----------------------------------------|----------------|
| Upstream region of *MSMEG_1650*        | GCTACACATGTTGTACAGTTGCTGACCGGA |
| Downstream region of *MSMEG_1650*      | GCTACATTTAAATCGTGTGACAGGGCCAC |
| Upstream region of *MSMEG_1771*        | GCTACACTAGTGGATCCGAGAAGATCATCC |
| Downstream region of *MSMEG_1771*      | GCTACATTTAAATTTCCAGCCAC |
| Upstream region of *MSMEG_5176*        | GCTACACTAGTGGATCCGAGAAGATCATCC |
| Downstream region of *MSMEG_5176*      | GCTACATTTAAATTTCCAGCCAC |
| Upstream region of *MSMEG_5354*        | GCTACACTAGTGGATCCGAGAAGATCATCC |
| Downstream region of *MSMEG_5354*      | GCTACATTTAAATTTCCAGCCAC |
| Upstream region of *MSMEG_6235*        | GCTACACTAGTGGATCCGAGAAGATCATCC |
| Downstream region of *MSMEG_6235*      | GCTACATTTAAATTTCCAGCCAC |
| *MSMEG_6237* gene and promoter region for the | GCTACGAATTCTGCACTGAGGGCGAGATTTC |
| complementation of Δ*MSMEG_6237* (ΔmftM) | GCTACAAGCTTGAGCAAGCGCGGGTTGAT |
| Upstream region of *MSMEG_6663*        | GCTACATTTAAATTTCCAGCCAC |
| Downstream region of *MSMEG_6663*      | GCTACATTTAAATTTCCAGCCAC |
Additional experimental procedures

Gene similarity searches and co-occurrence analysis

To find the closest characterized putative homolog of MftM, a PHMMer search was performed with the MSMEG_6237 protein sequence against the Protein Data Bank (PDB) and SwissProt databases with a sequence significance E-value cutoff of 0.01 using the HMMER web server in version 3.3.2. To identify potential recognition sites for the transcriptional regulator MftR (MSMEG_1420, WP_011727658.1), the MSMEG_6237 upstream region (~1000 to +100 bp with respect to the ATG start codon) was searched with the proposed conserved MftR recognition motif 5'-TNNGCANNNTGCCNNA-3'. As a control, the upstream region of mftA in the genome of M. smegmatis mc² 155 (accession NC_008596.1) was searched for potential binding sites of MftR with one hit at –74 to –55 bp upstream of the mftA “ATG” start codon.

To investigate the co-occurrence of mftC (a proxy for the presence of a MFT biosynthetic gene cluster), with mftM and mdo (mycofactocin-associated alcohol dehydrogenase) homologs, the nonredundant protein sequences database of the National Center for Biotechnology Information (NCBI) was searched for homologs of MftC (MSMEG_1423), MftM (MSMEG_6237), and Mdo (MSEMG_6242) from M. smegmatis using the NCBI BLASTP webserver with default parameters, a maximum hit number of 5000, and an E-value cutoff of 0.001. Hit lists were analyzed for sharp drops in sequence identity or query coverage and only sequences above this threshold were kept as potential orthologs. Accordingly, MftC BLAST hits were filtered at a Percent Identity of ≥ 36.09%, the Mdo hits at Percent Identity ≥ 59.09%, the MftM hits at Query Coverage ≥ 75% and Percent Identity ≥ 35%. The resulting output was filtered for hits originating from complete genomes only. Subsequently, each included entry/genome was manually checked for presence/absence of putative mftC, mdo, and mftM homologs. This approach delivered a total of 304 genomes encoding MftC, 104 genomes encoding MftM, and 99 genomes encoding Mdo orthologs (Supporting Information Table S1). Using the three filtered tables, Venn diagrams were constructed based on the NCBI taxid to connect the BLASTP output tables using the ggVennDiagram package for R. The p-values were calculated as follows: The individual probabilities of occurrence of mdo and mftM in genomes containing mftC were estimated from the dataset as 99/304 and 104/304, respectively, resulting in an expected probability (P) of co-occurrence of 11.1% (null hypothesis). Assuming a binomial (n, P) distribution, the p-value for k ≥ 98 co-occurrences in n = 304 trials is close to zero (p < 0.001).

A multiple sequence alignment between putative MftM homologs and the closest characterized homolog RebM was constructed using the amino acid sequences of the potential MftM homologs of the organisms biologically investigated in this study together with seven additional potential MftM homologs from the BLASTP search (above) and the RebM sequence from L. aerocolonigenes. For construction, the Clustal Omega algorithm version 1.2.2 was used as implemented in Geneious Prime 2020.2.3 (Biomatters) with default parameters.
Synthesis of PMFT\textsubscript{2} and PMFT standards

Our synthetic approach to PMFT\textsubscript{2} and PMFT (Scheme S1) is based on the key sequence to construct the lactam via (i) Michael addition of nitroalkane \textit{SI}-1 to 3,3-dimethylacrylate (\textit{SI}-2) to form the C3-C4 bond in PMFT(H\textsubscript{2}), (ii) followed by reduction of nitro group in the adduct \textit{SI}-3 and spontaneous cyclization to form lactam \textit{SI}-4A\textsuperscript{5}. Boc protection of the lactam \textit{SI}-4A produced \textit{SI}-5 and subsequent Davis’ oxidation introduced the alcohol at C2 position of the lactam in \textit{SI}-7\textsuperscript{6,7}. The intermediate \textit{SI}-7 was deprotected to give PMFT\textsubscript{2} (\textit{SI}-8) or subjected to Swern oxidation to \textit{SI}-9 which after deprotection afforded PMFT (\textit{SI}-10). A similar route to PMFT\textsubscript{2} was previously reported\textsuperscript{8}.

\begin{center}
\textbf{Scheme S1. Synthetic route to PMFT and PMFT\textsubscript{2}.}
\end{center}

\textbf{Experimental procedures and characterization data}

\textbf{General Information}

Purity of >95\% is established by the provided MS data and \textsuperscript{1}H / \textsuperscript{13}C NMR spectra for all reported compounds. All commercial chemical reagents and chromatography solvents were used as obtained unless otherwise stated: ethanol, toluene, ethyl acetate, hexanes, anhydrous sodium sulfate (Na\textsubscript{2}SO\textsubscript{4}). Anhydrous solvents were distilled over appropriate drying agents before being used. Analytical thin layer chromatography (TLC) was carried out on VWR silica gel 60 F\textsubscript{254} plates. Macherey-Nagel silica gel 60 (0.015 - 0.04 mm) was used for column chromatography. Visualization of TLC was achieved with UV light (254 nm) or by staining with aq. KMnO\textsubscript{4} solution. All NMR spectra were measured on a Bruker-Avance 250 MHz, 300 MHz, 400 MHz, and 500 MHz spectrometers. The residual solvent protons (\textsuperscript{1}H) or the solvent carbon (\textsuperscript{13}C) were used as internal standards. \textsuperscript{1}H-NMR data are listed as follows: chemical shift in ppm (δ) downfield from trimethylsilane (multiplicity, integration, coupling constant). NMR data are reported using the following abbreviation: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dq, doublet of quartets; m, multiplet. Infrared spectra were measured on the Shimadzu IR-Affinity-1 (FTIR) device. High resolution mass spectra were measured using Electrospray ionization (ESI) techniques.
methyl 5-(4-methoxyphenyl)-3,3-dimethyl-4-nitropentanoate (SI-3)

Previously published experimental procedure was adapted for this experiment. A flame dried round-bottom flask was charged with methyl 3-methylbut-2-enoate (SI-2) (4.5 g, 39.42 mmol, 1 equiv.) and tetra-n-butylammonium fluoride in THF 1 M (40 ml, 39.42 mmol, 1 equiv.). 1-methoxy-4-(2-nitrovinyl)benzene (SI-1, 7.14 g, 39.42 mmol, 1 equiv.) was added slowly to reaction mixture at room temperature. The reaction was stirred at 40 °C for 80 hours. The reaction was quenched with brine and organic products were extracted with ether. The dried organic extracts were concentrated under reduced pressure and purified by silica gel chromatography (eluent: 30% (v/v) EtOAc in petrol ether) to afford product SI-3 as a yellow oil (5.46 g, 47% yield). ¹H NMR (300 MHz, CDCl₃) δ 7.08 (2 H, d, J = 8.10 Hz) 6.81 (2 H, d, J = 7.7 Hz), 4.90 (dd, 1H, J₁ = 1.4 Hz, J₂ = 11.7 Hz), 3.76 (s, 3H), 3.71 (s, 3H), 3.00-3.27 (m, 2H), 2.44 (s, 2H), 1.24 (s, 3H), 1.19 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 171.2, 158.8, 129.7, 127.9, 114.2, 97.3, 55.2, 51.7, 43.2, 36.5, 33.5, 24.4 23.9. HRMS (ESI) m/z for C₁₅H₁₉NO₃Na [M+Na]⁺: calcd. 318.1317; found, 318.1318. IR (cast film): 2974, 2361, 2342, 2332, 1776, 1734, 1719, 1514, 1283, 1248, 1152 cm⁻¹.

5-(4-methoxybenzyl)-4,4-dimethylpyrrolidin-2-one (SI-4A)

Previously published procedure was adapted for this experiment. A flame dried round bottom flask was charged with Raney Nickel (2.0 g, 2600 Ni slurry in H₂O) and washed several times with dry methanol. Methyl 5-(4-methoxyphenyl)-3,3-dimethyl-4-nitropentanoate (SI-3, 1.4 g, 4.74 mmol, 1 equiv.) in dry MeOH (23 ml, 0.2 M) was added to Raney Nickel at room temperature. The mixture was bubbled with H₂ gas for 60 min at room temperature, and then heated in an oil bath at 60 °C under H₂-atmosphere for 12 h. The mixture was cooled down to room temperature. The suspension was filtered through a Celite plug and washed with dry methanol. The filtrate was concentrated under reduced pressure and purified by silica gel chromatography (eluent: 10% (v/v) MeOH in DCM) to afford 5-pentyl-4,4-dimethylpyrrolidin-2-one (SI-4A) as a white solid (0.56 g, 51% yield), and (eluent: 70% (v/v) EtOAc in petrol ether) to afford 5-(4-methoxybenzylidene)-4,4-dimethylpyrrolidin-2-one (SI-4B) as a white solid (0.37 g, 34% yield).

SI-4B was readily converted to SI-4A by Pd-catalyzed hydrogenation. A flame dried round bottom flask was charged with 5-(4-methoxybenzylidene)-4,4-dimethylpyrrolidin-2-one (SI-4B, 0.37 g, 1.6 mmol, 1.0 equiv.) and dry MeOH (16 ml, 0.1 M). The solution was bubbled with Argon for 15 min. 10% Pd/C (34 mg, 0.032 mmol, 0.02 equiv.) was added to the anhydrous solution in one portion under inert conditions. The solution was bubbled with H₂ gas at room temperature for 1 h and then the reaction was stirred for a further 12 h under H₂-atmosphere. The suspension was filtered through a Celite plug and washed with methanol. The filtrate was concentrated under reduced pressure to afford 5-pentyl-4,4-dimethylpyrrolidin-2-one (SI-4A) as a white solid (0.36 g, 96% yield).

(SI-4A) ¹H NMR (250 MHz, CDCl₃) δ 7.08 (d, 2 H, J = 8.6 Hz), 6.81 (d, 2 H, J = 8.8 Hz), 5.31 (s, 1H), 3.78 (s, 3H), 3.40 (dd, 1H, J₁ = 3.1 Hz, J₂ = 11.2 Hz), 2.80 (dd, 1H, J₁ = 3.1 Hz, J₂ = 13.5 Hz), 2.40 (m, 1H), 2.2 (s, 2H), 1.19 (s, 3H), 1.13 (s, 3H). ¹³C NMR (63 MHz, CDCl₃) δ 177.2, 159.4, 131.1, 130.7, 115.3, 66.2, 56.2, 47.1, 39.3, 36.8, 28.3, 23.8. HRMS (ESI) m/z for C₁₅H₁₉NO₃Na [M+Na]⁺ : calcd. 256.1313; found, 256.1305. IR (cast film): 3738, 2959, 2361, 1697, 1692, 1686, 1611, 1512, 1248, 1179, 1034 cm⁻¹.

(SI-4B) ¹H NMR (300 MHz, CDCl₃) δ 7.63 (br s, 1H), 7.11 (d, 2 H, J = 6.8 Hz), 6.87 (d, 2 H, J = 8.7 Hz), 3.80 (s, 3H), 2.38 (s, 2H), 1.34 (s, 6H). ¹³C NMR (63 MHz, CDCl₃) δ 175.7, 158.0, 146.7, 128.9, 128.5, 114.5, 99.2,
55.4, 45.2, 39.1, 29.1. HRMS (ESI) m/z for C_{14}H_{18}NO_{2} [M+H]^+: calcd. 232.1338; found, 232.1334. IR (cast film): 3251, 2358, 2341, 1782, 1749, 1716, 1514, 1367, 1303, 1247, 1149 cm\(^{-1}\).

**tert-butyl 2-(4-methoxybenzyl)-3,3-dimethyl-5-oxopyrrolidine-1-carboxylate (SI-5)**

In a dry 25 mL round bottom flask equipped with a stirring bar, the starting material (SI-4, 0.53 g, 2.73 mmol, 1.0 equiv.) was dissolved in dry dichloromethane (11 mL, 0.2 M) and charged with Boc\(_2\)O (1.24 g, 5.68 mmol, 2.5 equiv.), triethylamine (0.63 mL, 4.54 mmol, 2.0 equiv.) and DMAP (0.33 g, 2.73 mmol, 1.2 equiv.) at room temperature. The mixture was stirred at room temperature for 4 h. After that, Boc\(_2\)O (0.496 g, 2.72 mmol, 1 equiv.) and triethylamine (0.32 mL, 2.72 mmol, 1 equiv.) were added to the reaction mixture. The suspension was stirred for another 12 h at room temperature. The mixture was evaporated under reduced pressure. The crude product was purified by flash chromatography (elucent 40% (v/v) EtOAc in petrol ether) to yield the pure desired product SI-5 as a white solid (0.55 g, 73% yields). \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.12 (d, 2H, \(J = 8.6 \text{ Hz}\)), 7.02 (d, 2H, \(J = 8.6 \text{ Hz}\)), 4.02 (t, 1H, \(J = 6.5 \text{ Hz}\)), 3.78 (s, 3H) 2.74 - 2.96 (m, 2H), 2.43 (d, 1H, \(J = 17.0 \text{ Hz}\)), 2.10 (d, 1H, \(J = 17.0 \text{ Hz}\)), 1.41 (s, 9H), 1.11 (s, 3H), 1.07 (s, 3H). \(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta\) 173.5, 158.3, 150.1, 130.4, 129.9, 114.0, 82.7, 68.1, 55.3, 45.8, 36.2, 36.1, 29.4, 27.9, 23.2. HRMS (ESI) m/z for C\(_{19}\)H\(_{27}\)NO\(_4\)Na [M+Na]^+: calcd. 356.1838; found 356.1834. IR (cast film): 2968, 2358, 2341, 1782, 1749, 1716, 1514, 1367, 1303, 1247, 1149 cm\(^{-1}\).

**tert-butyl 4-hydroxy-2-(4-methoxybenzyl)-3,3-dimethyl-5-oxopyrrolidine-1-carboxylate (SI-7)**

A classical Davis’ oxidation protocol was followed. A freshly prepared solution of LDA (13.20 mmol, 5.28 ml of 2.5 M n-BuLi, 2.2 equiv.) and (13.20 mmol, 1.86 ml of diisopropylamine, 2.2 equiv.) in 10 mL anhydrous THF was slowly added to a solution of tert-butyl 2-(4-methoxybenzyl)-3,3-dimethyl-5-oxopyrrolidine-1-carboxylate (SI-5) (2.0 g, 6.0 mmol, 1 equiv.) in anhydrous THF (60 mL, 0.1 M) at -78°C. The mixture was allowed to warm up to 0°C over an hour. The mixture was cooled to -78°C and solution of Davis’ oxaziridine (SI-6, 2.35 g, 9.0 mmol, 1.5 equiv.) in THF (9 ml, 1.0 M) was added dropwise. The mixture was stirred at -78°C for 1 hour and then warmed to -40°C over 3 hours. After that, the reaction was warmed to room temperature for 30 min. The reaction was quenched with (1.0 M HCl) solution and extracted with Et\(_2\)O. The combined organic extracts were washed with brine and dried over Na\(_2\)SO\(_4\). Removal of volatiles under reduced pressure yielded the crude product which required repeated chromatographic purification on silica gel (3 columns with 40-50% (v/v) EtOAc in petrol ether as eluent) to obtain the sufficiently pure product (SI-7) as colorless oil (0.8 g, 38% yields). Yields of up to 80% have been obtained in other iterations of this experiments when only one chromatographic purification was performed, though the purity of the material obtained in this way was lower. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.18 (d, 2H, \(J = 8.5 \text{ Hz}\)), 6.83 (d, 2H, \(J = 8.6 \text{ Hz}\)), 3.95-4.10 (m, 1H), 3.78-3.81 (m, 4H), 3.35 (dd, 1H, \(J_1 = 4.5 \text{ Hz}, J_2 = 14.1 \text{ Hz}\)), 3.02 (d, 1H, \(J = 3.2 \text{ Hz}\)), 2.82 (m, 1H), 1.48 (s, 9H), 1.02 (s, 3H), 0.87 (s, 3H). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 174.5, 158.2, 150.0, 130.1, 113.9, 83.6, 87.7, 66.4, 55.2, 39.7, 35.3, 27.8, 26.4, 16.2. HRMS (ESI) m/z for C\(_{20}\)H\(_{29}\)NO\(_6\)Na [M+Na]^+: calcd. 372.1787; found, 372.1776. IR (cast film): 2970, 2361, 2342, 2332, 1775, 1719, 1514, 1283, 1248, 1152, 1038, 667 cm\(^{-1}\).

**3-hydroxy-5-(4-hydroxybenzyl)-4,4-dimethylpyrrolidin-2-one (SI-8)**

The tert-butyl 4-hydroxy-2-(4-methoxybenzyl)-3,3-dimethyl-5-oxopyrrolidine-1-carboxylate (SI-7, 60 mg, 12.3 mmol) was dissolved in anhydrous CH\(_2\)Cl\(_2\) (4.0 ml, 0.04 M) under argon, and the solution was cooled to 0°C. BBr\(_3\) (0.5 mL, 1 M in Hexane, 0.52 mmol, 3.0 equiv.) was then added dropwise. After stirring the reaction mixture at 0°C for 2 h (TLC Monitoring), saturated NH\(_4\)Cl solution was added and the mixture was extracted with EtOAc. Combined organic phase was washed with brine, dried over Na\(_2\)SO\(_4\) and
concentrated. The crude product was purified by column chromatography on silica (eluent 5% (v/v) MeOH in CH₂Cl₂) to yield the pure product SI-8 as a white solid (12 mg, 30% yields). ¹H NMR (300 MHz, d⁶-DMSO) δ 9.18 (br s, 1H), 7.43 (br s, 1H), 7.04 (d, 2H, J = 8.4 Hz), 6.67 (d, 2H, J = 8.4 Hz), 5.29 (d, 1H, J = 5.8 Hz), 3.62 (d, 1H, J = 5.8 Hz), 3.29 - 3.31 (m, 1H), 2.52 - 2.58 (m, 1H), 0.81 (s, 3H), 0.76 (s, 3H). ¹³C NMR (100 MHz, d⁶-DMSO) δ 175.8, 156.1, 130.3, 129.0, 115.6, 78.1, 60.6, 44.1, 35.0, 23.9, 14.9. HRMS (ESI) m/z for C_{13}H_{28}NO_{12}Na [M+Na]^+: calcd. 258.1106; found, 258.1095. IR (cast film) 2972, 2361, 2342, 1776, 1769, 1734, 1516, 1369, 1240, 1152 cm⁻¹.

tert-butyl 2-(4-methoxybenzyl)-3,3-dimethyl-4,5-dioxopyrrolidine-1-carboxylate (SI-9)

Swern oxidation protocol was followed. A flame-dried flask was charged with anhydrous DMSO (0.43 mL, 6.01 mmol, 3.0 equiv) in dry CH₂Cl₂ (0.1 M, 14 mL) and cooled to -78 °C with a dry ice/acetone bath. After 15 min, oxalyl chloride (0.26 mL, 3.00 mmol, 1.5 equiv.) was added to the mixture at the same temperature. The reaction was allowed to warm to room temperature over 30 min. The reaction mixture was quenched by adding 1 M HCl and extracted with EtOAc. The combined extracts were dried over Na₂SO₄ and concentrated. The crude product was purified by flash chromatography (eluent 40% (v/v) EtOAc in petrol ether) to yield the pure desired product (SI-9) as a white solid (0.5 g, 72% yield). ¹H NMR (300 MHz, CDCl₃) δ 7.03 (d, 2H, J = 8.6 Hz), 6.87 (d, 2H, J = 8.6 Hz), 4.37 (dd, 1H, dd, J₁ = 5.7 Hz, J₂ = 6.6 Hz), 3.75 (s, 3 H), 2.94 (dd, 1H, J₁ = 6.8 Hz, J₂ = 14.4 Hz), 2.76 (dd, 1H, J₁ = 5.5 Hz, J₂ = 14.3 Hz), 1.48 (s, 9 H), 1.26 (s, 3H), 1.22 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 201.8, 158.8, 157.2, 149.9, 130.7, 127.5, 114.3, 84.8, 63.9, 55.3, 47.1, 38.4, 27.9, 26.6, 17.9. HRMS (ESI) m/z for C₁₃H₁₈NO₃Na [M+Na]^+: calcd. 370.1630; found, 370.1630. IR (cast film) 2972, 2361, 2342, 1776, 1769, 1734, 1516, 1369, 1240, 1152 cm⁻¹.

Synthesis of 5-(4-hydroxybenzyl)-4,4-dimethylpyrrolidine-2,3-dione (SI-10)

tert-butyl 2-(4-methoxybenzyl)-3,3-dimethyl-4,5-dioxopyrrolidine-1-carboxylate (SI-9, 120 mg, 0.345 mmol) was dissolved in anhydrous CH₂Cl₂ (5.0 mL, 0.07 M) under argon, and the solution was cooled to 0 °C. BBr₃ (1 mL, 1 M in Hexane, 1.05 mmol, 3.0 equiv.) was added dropwise. After stirring the reaction mixture at 0 °C for 2 h (TLC Monitoring), saturated NH₄Cl solution was added and the mixture was extracted with EtOAc. Combined organic phase was washed with brine, dried over Na₂SO₄ and concentrated. The crude product was purified by column chromatography on silica (eluent 5% (v/v) MeOH in CH₂Cl₂) to yield the pure product SI-10 as a white solid (16.7 mg, 21% yield). ¹H NMR (500 MHz, d⁶-DMSO) δ 9.56 (br s, 1H), 9.25 (br s, 1H), 7.06 (d, 2H, J = 8.5 Hz), 6.70 (d, 2H, J = 8.5 Hz), 3.80 (dd, 1H, J₁ = 7.5 Hz, J₂ = 7.1 Hz), 2.77 (dd, 1H, J₁ = 7.1 Hz, J₂ = 14.1 Hz), 2.63 (dd, 1H, J₁ = 7.5 Hz, J₂ = 14.1 Hz), 1.02 (s, 3H), 0.95 (s, 3H). ¹³C NMR (75 MHz, d⁶-DMSO) δ 206.2, 160.8, 155.9, 130.1, 127.3, 115.2, 59.4, 45.3, 36.2, 22.4, 19.1. HRMS (ESI) m/z for C₁₃H₁₂NO₃Na [M+Na]^+: calcd. 256.0950; found, 256.0946. IR (cast film) 2972, 2924, 2361, 2344, 2332, 1769, 1734, 1717, 1707, 1516 cm⁻¹.
NMR Spectra

$^1$H-NMR of methyl 5-(4-methoxyphenyl)-3,3-dimethyl-4-nitropentanoate (SI-3) in CDCl$_3$ at 25°C.

$^{13}$C-NMR of methyl 5-(4-methoxyphenyl)-3,3-dimethyl-4-nitropentanoate (SI-3) in CDCl$_3$ at 25°C.
$^1$H-NMR of 5-(4-methoxybenzyl)-4,4-dimethylpyrrolidin-2-one (SI-4A) in CDCl$_3$ at 25 °C.

$^{13}$C-NMR of 5-(4-methoxybenzyl)-4,4-dimethylpyrrolidin-2-one (SI-4A) in CDCl$_3$ at 25 °C.
$^1$H-NMR of 5-(4-methoxybenzylidene)-4,4-dimethylpyrrolidin-2-one (SI-4B) in CDCl$_3$ at 25 °C.

$^{13}$C-NMR of 5-(4-methoxybenzylidene)-4,4-dimethylpyrrolidin-2-one (SI-4B) in CDCl$_3$ at 25 °C.
$^1$H-NMR of tert-butyl 2-(4-methoxybenzyl)-3,3-dimethyl-5-oxopyrrolidine-1-carboxylate (SI-5) in CDCl$_3$ at 25 °C.

$^{13}$C-NMR of tert-butyl 2-(4-methoxybenzyl)-3,3-dimethyl-5-oxopyrrolidine-1-carboxylate (SI-5) in CDCl$_3$ at 25 °C.

S29
$^1$H-NMR of tert-butyl 4-hydroxy-2-(4-methoxybenzyl)-3,3-dimethyl-5-oxopyrrolidine-1-carboxylate (S1-7) in CDCl$_3$ at 25 °C.

$^{13}$C-NMR of tert-butyl 4-hydroxy-2-(4-methoxybenzyl)-3,3-dimethyl-5-oxopyrrolidine-1-carboxylate (S1-7) in CDCl$_3$ at 25 °C.
$^1$H-NMR of PMFT$_2$ - 3-hydroxy-5-(4-hydroxybenzyl)-4,4-dimethylpyrrolidin-2-one (S1-8) in $d_6$-DMSO at 25 °C.

$^{13}$C-NMR of PMFT$_2$ - 3-hydroxy-5-(4-hydroxybenzyl)-4,4-dimethylpyrrolidin-2-one (S1-8) in $d_6$-DMSO at 25 °C.
$^1$H-NMR of tert-butyl 2-(4-methoxybenzyl)-3,3-dimethyl-4,5-dioxopyrrolidine-1-carboxylate (SI-9) in CDCl$_3$ at 25 °C.

$^{13}$C-NMR of tert-butyl 2-(4-methoxybenzyl)-3,3-dimethyl-4,5-dioxopyrrolidine-1-carboxylate (SI-9) in CDCl$_3$ at 25 °C.
$^1$H-NMR of PMFT - 5-[(4-hydroxybenzyl)]-4,4-dimethylpyrrolidine-2,3-dione (SI-10) in d$_6$-DMSO at 25 °C.

$^{13}$C-NMR of PMFT - 5-[(4-hydroxybenzyl)]-4,4-dimethylpyrrolidine-2,3-dione (SI-10) in d$_6$-DMSO at 25 °C.
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