Chemoenzymatic synthesis and utilization of a SAM analog with an isomorphic nucleobase

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Supplementary Information

1. Materials and Methods

Reagents and solvents were purchased from Sigma-Aldrich, Fluka, TCI, and Acros and were used without further purification unless otherwise specified. Solvents were purchased from Sigma-Aldrich and Fisher Scientific, and dried by standard techniques. NMR solvents were purchased from Cambridge Isotope Laboratories. M.*Taq*I (10 units/µl), *Taq*I (20 units/µl), *Sac*I (20 units/µl), pUC19 (1 µg/µl), SAM (32 mM solution in 5 mM H_2SO_4 and 10% EtOH), CutSmart buffer (pH 7.9), purple loading dye and *E. coli* BL21(DE3) cells were purchased from New England BioLabs. NAP 10 and Ni-NTA agarose columns were obtained from GE Healthcare and QIAGEN, respectively. Amicon Ultra 10 kDa spin concentrator were purchased from Merck Millipore. L-methionine and L-homocysteine were obtained from Sigma Aldrich. Stock solutions of L-Met (150 mM) were prepared in water. Stock solutions of SalL were prepared in 50 mM sodium phosphate buffer (pH 7.9 + 50%(v/v) glycerol).

All reactions were monitored with analytical TLC (Merck Kieselgel 60 F254). Column chromatography was carried out with Teledyne ISCO Combiflash Rf with silica gel particle size 40–63 μm. NMR spectra were obtained on Varian Mercury 400 MHz and Varian VX 500 MHz spectrometers. Mass spectra were obtained on an Agilent 6230 HR-ESI-TOF MS at the Molecular Mass Spectrometry Facility of the UCSD Chemistry and Biochemistry Department. HPLC analysis was performed with an Agilent 1200 series system Eclipse XDB-C18 5μm, 4.5 · 150 mm column. 0.01% TFA stock solutions were prepared by dissolving 1 ml of TFA (Acros, 99%) in 999 ml HPLC grade acetonitrile (Sigma) or MilliQ water and filtered using Millipore type GNWP 0.2 μm filters before use.

1.1. Abbreviations

ACN: acetonitrile; DCM: dichloromethane; EtOH: ethanol; MeOH: methanol; TFA: trifluoroacetic acid.

2. Synthetic procedures

2.1. General

Starting precursor th**A** was synthesized based on previously published procedure.[1]

ClDA was synthetized based on previously published procedures from commercial adenosine.[2, 3]

2.2. Synthesis of ClDthA

(2R,3R,4S,5S)-2-(4-aminothieno[3,4-d]pyrimidin-7-yl)-5-(chloromethyl)tetrahydrofuran-3,4-diol (ClDthA). To a solution of th**A** (20.0 mg, 0.07 mmol) in dry ACN (247μl), dry pyridine was added (12μl, 0.14 mmol). The solution was cooled down to 0 °C and thionyl chloride (25μl, 0.35 mmol) was added dropwise. The reaction was stirred at 0 °C for 4 h and at room temperature overnight. The resulting suspension was concentrated under vacuum and the resulting dark solid was dissolved in mixture of MeOH and water (10:1, 425μl). To the dark orange solution NH_4OH (35μl, 28–30% wt solution in water) was added and the mixture was stirred at room temperature for 30 minutes. The solvent was evaporated in vacuum. The resulting dark solid (Rf: 0.4 DCM/MeOH 9:1) was purified by column chromatography (DCM/MeOH 9:1) to afford **ClDthA** as a white solid (14.0 mg, 65%). ^{1}H-NMR (500 MHz, DMSO-d_6): 8.39 (s, 1H), 8.01 (s, 1H), 7.84 (br d, 2H), 5.47 (m, 2H), 5.18 (d, *J* = 5.50, 1H), 4.17 (m, 1H), 4.09 (m, 1H), 4.05 (m, 1H), 3.88 (dd, *J* = 11.7, 4.0 Hz, 1H), 3.77 (dd, *J* = 11.7, 5.3 Hz, 1H). ^{13}C-NMR (125 MHz, DMSO-d_6): 158.33, 154.36, 147.07, 131.86, 120.81, 120.02, 82.65, 78.02, 76.97, 72.25, 45.72. MS (ESI, DCM/MeOH 1:1): 302 (100, $[M+H]^+$). HRMS (ESI-TOFMS, +ve): calculated for $C_{11}H_{13}ClN_3O_3S$: 302.0361, found: 302.0363.

3. Enzymatic protocols

3.1. Expression and purification of SalL

A previously described construct of SalL in a pET28a variant with a sequence coding for an eightfold N-terminal his-tag was used for expression.[4] *E. coli* BL21(DE3) cells transformed with this plasmid were grown in terrific broth media supplemented with 50 mg/L kanamycin sulfate at 25°C/120 rpm. When the cell reached an optical density of 0.8 at 600 nm (OD_{600}), the temperature was reduced to 20 °C. After 30 min, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.25 mM, and the expression was allowed to continue overnight before the cells were harvested by centrifugation. Cell pellets were resuspended in buffer A (50 mM sodium phosphate, 500 mM ammonium sulfate, 20 mM imidazole, pH 7.9) and lysed by passing through a French Press. The lysate, clarified by centrifugation, was loaded onto a Ni-NTA agarose column equilibrated with buffer A. The column was subsequently washed with 10 column volumes washing buffer (buffer A with 50 mM imidazole), and the protein was eluted with 3 column volumes elution buffer (buffer A with 250 mM imidazole). The eluent was desalted using a NAP 10 column equilibrated with 50 mM sodium phosphate, 50%(v/v) glycerol (pH 7.9). Desalted SalL was concentrated to 467 µM (monomeric concentration) with an Amicon Ultra 10 kDa spin concentrator and stored at –20°C for further use.

3.2. Chemoenzymatic synthesis and purification of $S^{th}AM$

As a general procedure, aliquots (100µl) of $ClD^{th}A$ (200 µM; as $ClD^{th}A$ has the tendency to precipitate at low temperatures, the samples were heated to 50 °C for 3–5 min right before use) and L-methionine (15 mM) were incubated for 2 h, 4 h, 6 h and 16h at 37°C with SalL at different concentrations (3 and 9 µM) in CutSmart buffer and subsequently purified by reverse phase HPLC (example for 3 µM SalL and 2 h incubation shown in Figure S3). The best results were achieved when a reaction mixture containing 9 µM SalL was incubated for 2 h (Figure S4). A $ClD^{th}A$ standard (Figure S5) and a control reaction without SalL (Figure S6) were also analyzed.

For purification, the injection was subjected to a gradient (20 minutes, from 0 to 10% aqueous acetonitrile with 0.01% TFA, then 5 minutes from 10 to 70% aqueous acetonitrile with 0.01%). A flow rate of 1 ml/min was used and runs were carried out at 25 °C. Each run was monitored at 260 and 341 nm with calibrated references at 650 nm and slit set at 1 nm.

The fractions containing $S^{th}AM$ were lyophilized, dissolved in a small volume of water (20–50 µl) and analyzed by mass spectrometry (ESI +ve). Yields of ca. 60% were obtained (based on HPLC analysis). Aliquots of 30–50 µl of $S^{th}AM$ were stored in a 0.1 % solution of formic acid in water at –20 °C and their concentration was determined by

measuring absorbance at wavelength 341 nm and ε=7440 M^{-1} cm^{-1}. Ideally, fresh solutions should be used. In our experiments all samples were used within 6–8 weeks; full activity was retained during this period of time. It is likely S^{th}AM solutions can be stored for longer periods without noticeable degradation, but we have not exploited this in our experiments.

3.3. Enzymatic methylation-restriction experiments

3.3.1. General

1 µg pUC19 was linearized with 1 µl of SacI in CutSmart buffer for 1 h at 37 °C in a total reaction volume of 10 µl. SacI was heat inactivated for 20 min at 65 °C. Subsequently, 1 µl M.TaqI and S^{th}AM to a final concentration of 100 µM were added (as S^{th}AM has the tendency to precipitate at low temperatures, the samples were heated to 50 °C for 3-5 min right before use), and the mixture was incubated for 1 h at 65°C CutSmart buffer (pH 7.9). 1 µl of Proteinase K was added to the reaction and the reaction was incubated for 1h at 55°C. Proteinase K was heat inactivated for 10 min at 95°C (certain methylation-restriction experiments have included the use of Proteinase K, although its absence does not influence the reaction outcome). An aliquot of 10 µl containing 1 µl *Taq*I in CutSmart buffer was added and the reaction was further incubated for 1 h at 65 °C. Heat inactivation of the restriction enzyme was done at 80 °C for 20 min. A positive control using SAM was performed under the same conditions. A negative control in the absence of M.*Taq*I for both S^{th}AM and SAM and a control in the absence of M.*Taq*I and cofactor in the presence and absence of *Taq*I were performed under the same reaction conditions as the actual reaction. A purple loading dye was added to all the samples and the reaction was analyzed using a 1% agarose gel (123 V for 30 min).

3.3.2. Time-course methylation-restriction assay

4.2 µg of linearized pUC19 was combined with 100 µM S^{th}AM and was incubated at 65 °C with 4.2 µl M.*Taq*I in CutSmart buffer at pH 7.9 in a total reaction volume of 120 µl. Aliquots of 20 µl were taken after 10, 30, 45, 105 and 120 min and to each of these a 10 µl aliquot of 1 µM of *Taq*I in CutSmart buffer was added and the reaction was further incubated for 1 h at 65 °C. The restriction enzyme was inactivated at 80 °C for 20 min. A control time course experiment using SAM was performed under the same conditions (lanes 6–10). A control in the absence of M.*Taq*I and cofactor and in the presence and absence of *Taq*I were performed under the same conditions as the 2h methylation reactions. A purple loading dye was added to all the samples and the reaction was analyzed using a 1% agarose gel (123 V for 30 min).

3.3.3. One-pot synthesis/methylation/restriction

1 µg pUC19 was linearized with 1 µl of *Sac*I in CutSmart buffer for 1 h at 37 °C in a total reaction volume of 10 µl. *Sac*I was heat inactivated for 20 min at 65 °C. ClDA or ClD^{th}A (final concentration 100 µM; as ClD^{th}A has the tendency to precipitate at low temperatures, the samples were heated to 50 °C for 3-5 min right before use), L-Met (15 mM), SalL (3 µM), and 1 µL M.*Taq*I were added and the mixture was incubated in CutSmart buffer for 1 h at 37°C and for another 30 min at 65°C. 10 µL containing 1 µL *Taq*I in CutSmart buffer were added and the reaction was further incubated for 1h at 65°C. Heat inactivation of the restriction enzyme was done at 80 °C for 20 min. Positive controls in the presence of ClDA or ClD^{th}A and 100 µM SAM but in absence of SalL were performed. Negative controls in the presence of ClDA or ClD^{th}A but in the absence of SalL, L-Met or of M.*Taq*I were also executed. One negative control in the absence of ClDA or ClD^{th}A was performed and another where only DNA was added. All these control experiments were treated under the same conditions as the actual reaction. A purple loading dye was added to all the samples and the reaction was analyzed using a 1% agarose gel (123 V for 30 min).

4. Supplementary figures

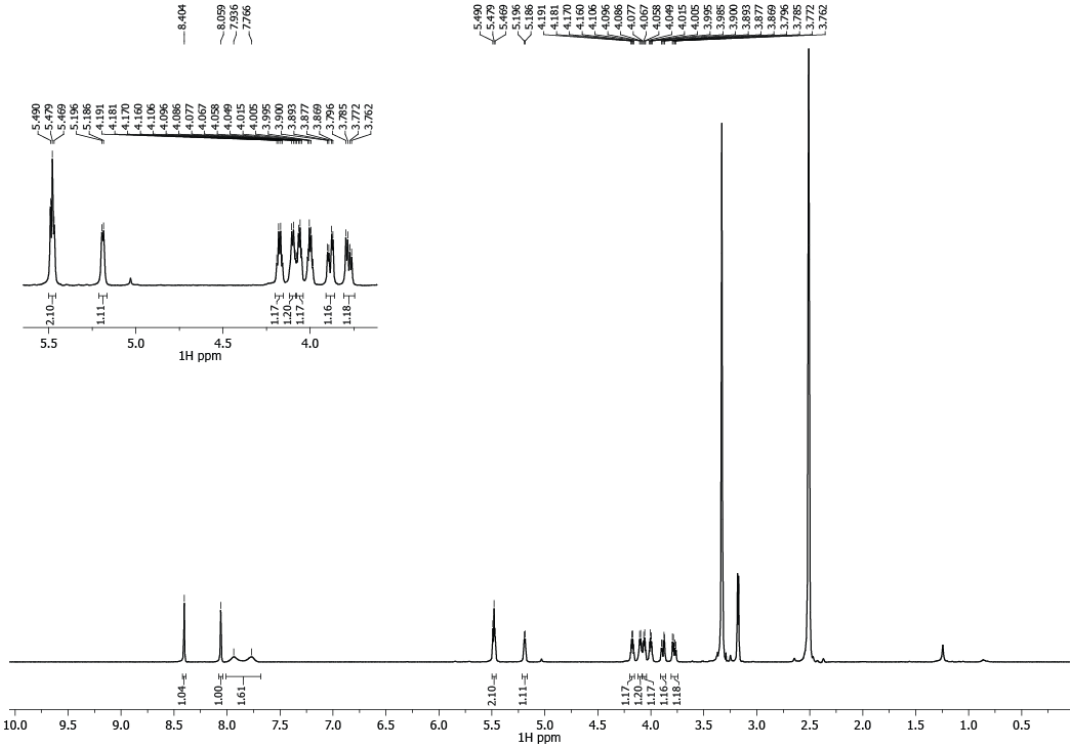

Figure S1. ^{1}H-NMR of ClDthA in DMSO-d_6.

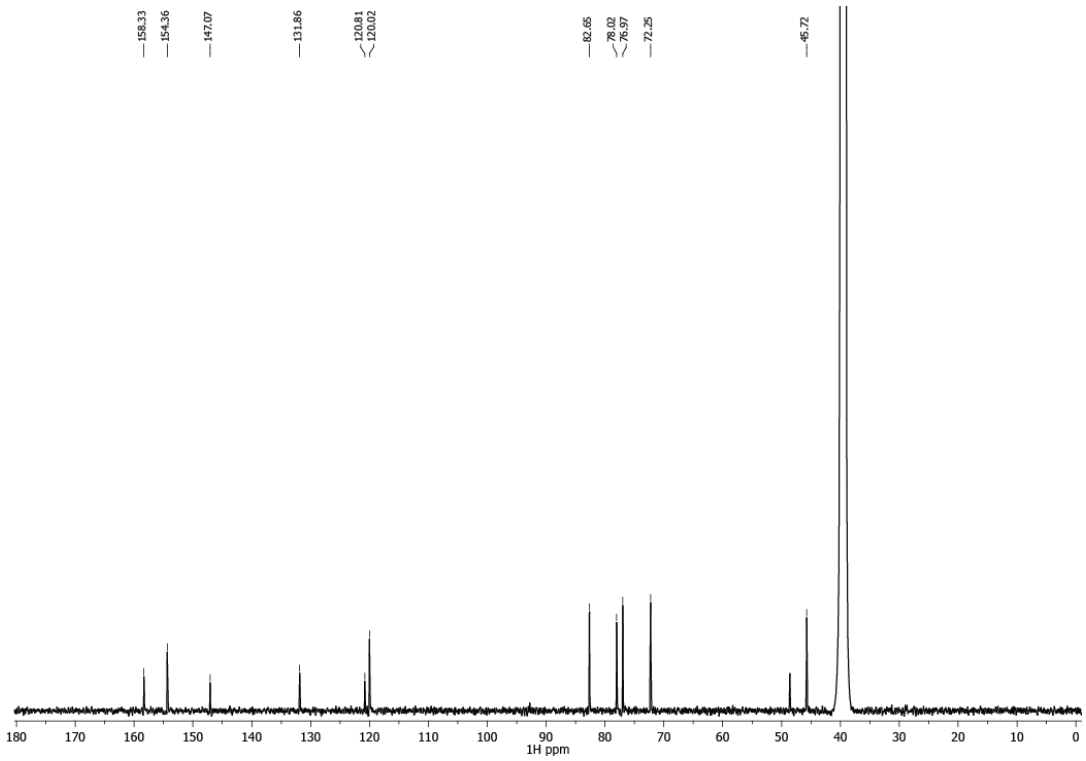

Figure S2. ^{13}C-NMR of ClDthA in DMSO-d_6.

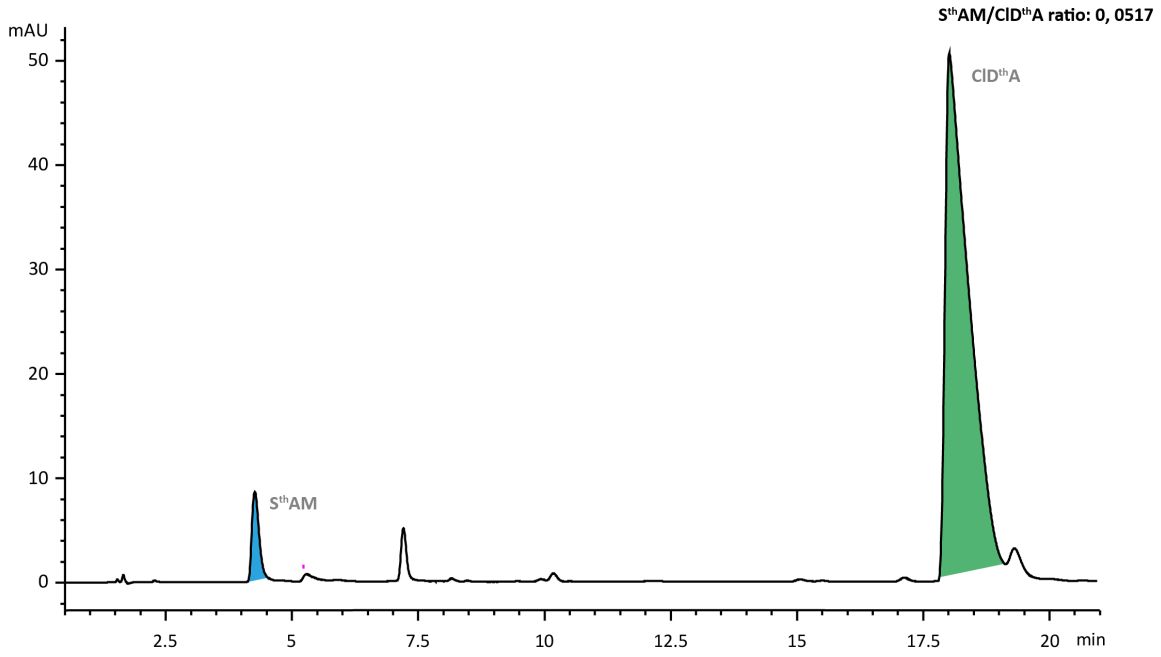

Figure S3. UV-trace of a HPLC run of a 2 h chemoenzymatic SthAM synthesis using 3 µM SalL monitored at 341 nm.

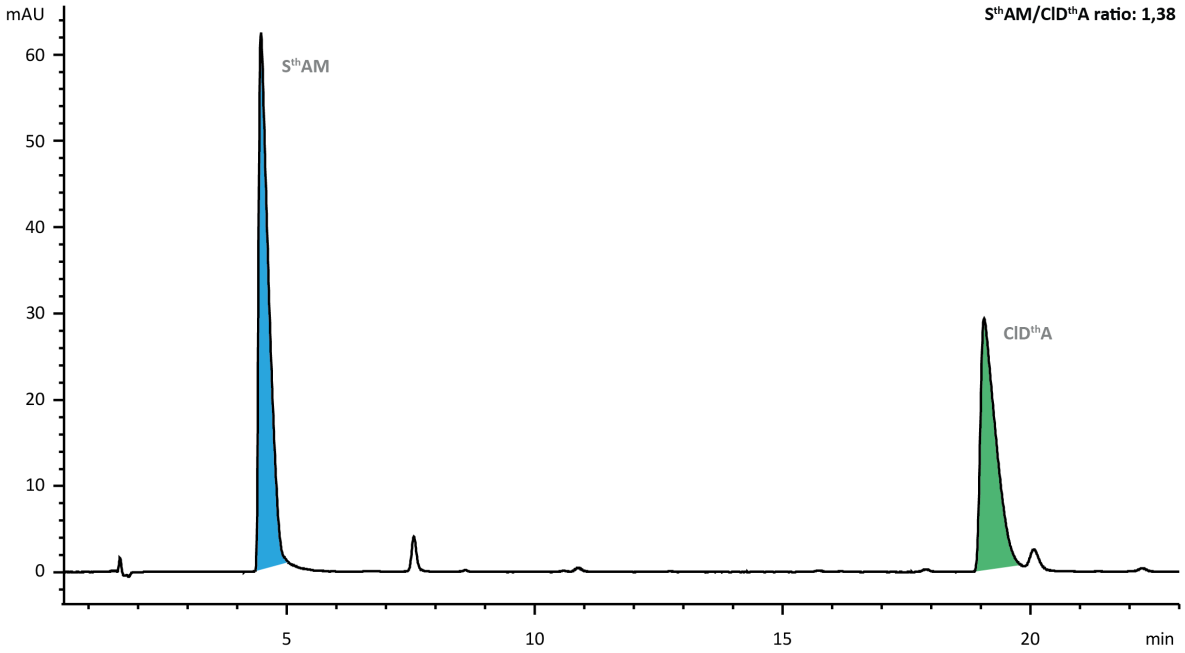

Figure S4. UV-trace of a HPLC run of a 2 h chemoenzymatic SthAM synthesis using 9 µM SalL monitored at 341 nm.

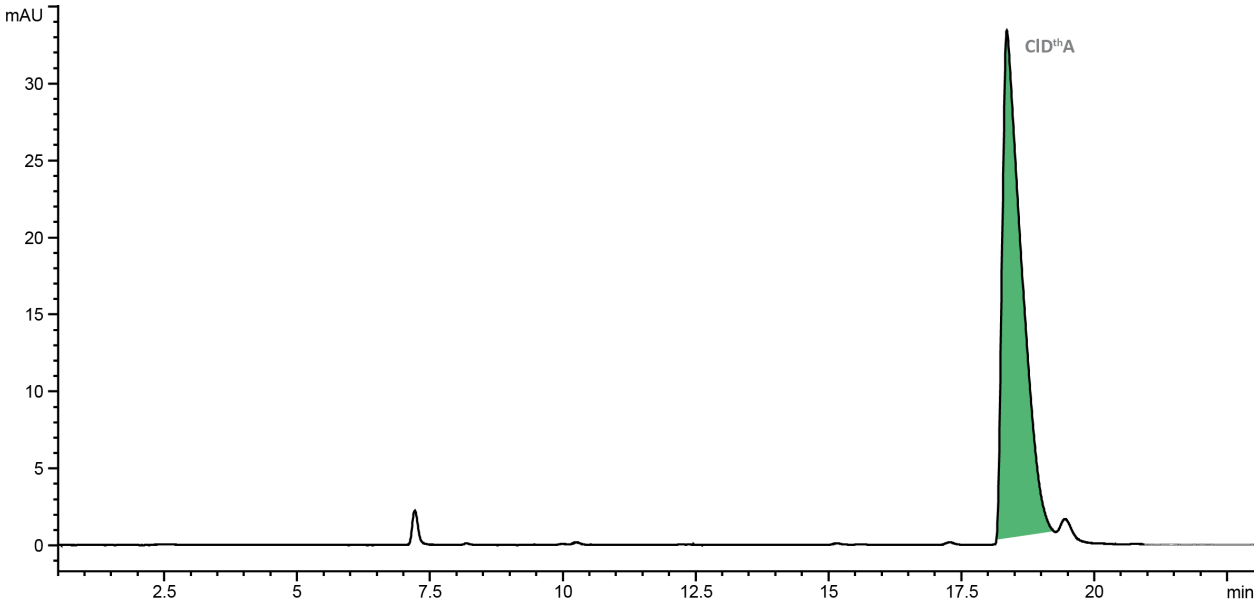

Figure S5. UV-trace of a HPLC run of ClDthA monitored at 341 nm.

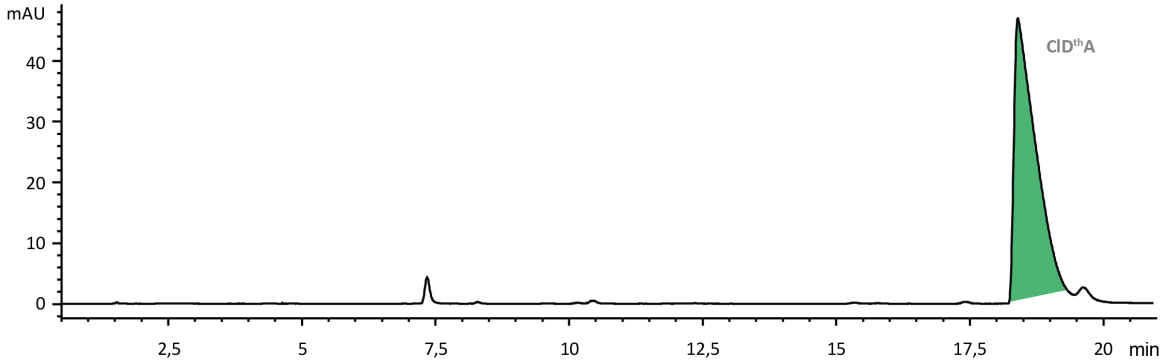

Figure S6. UV-trace of a HPLC run of a 2 h chemoenzymatic SthAM synthesis in the absence of SalL monitored at 341 nm.

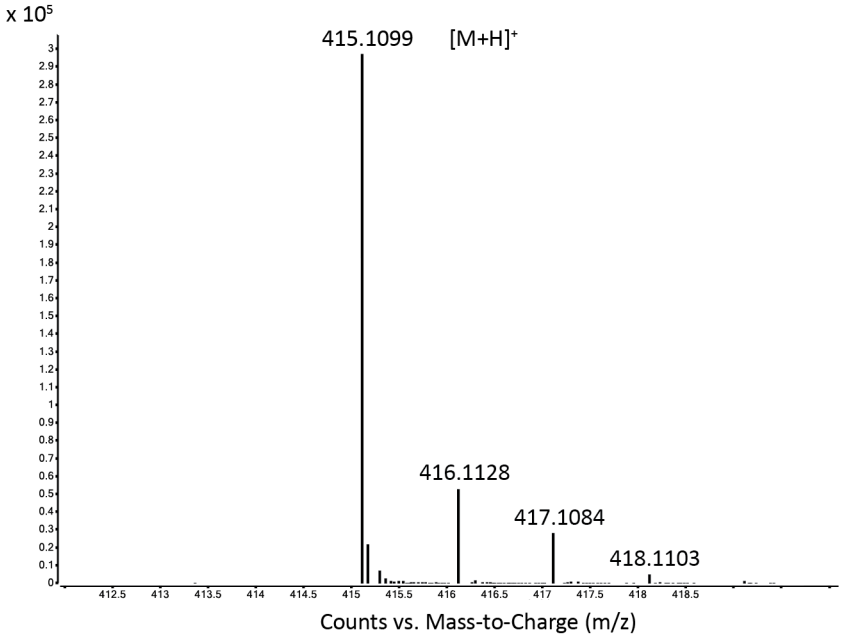

Figure S7. HR-ESI spectrum (+ve) of SthAM. Calculated for $C_{16}H_{24}N_4O_5S_2$: 415.1104, found: 415.1099.

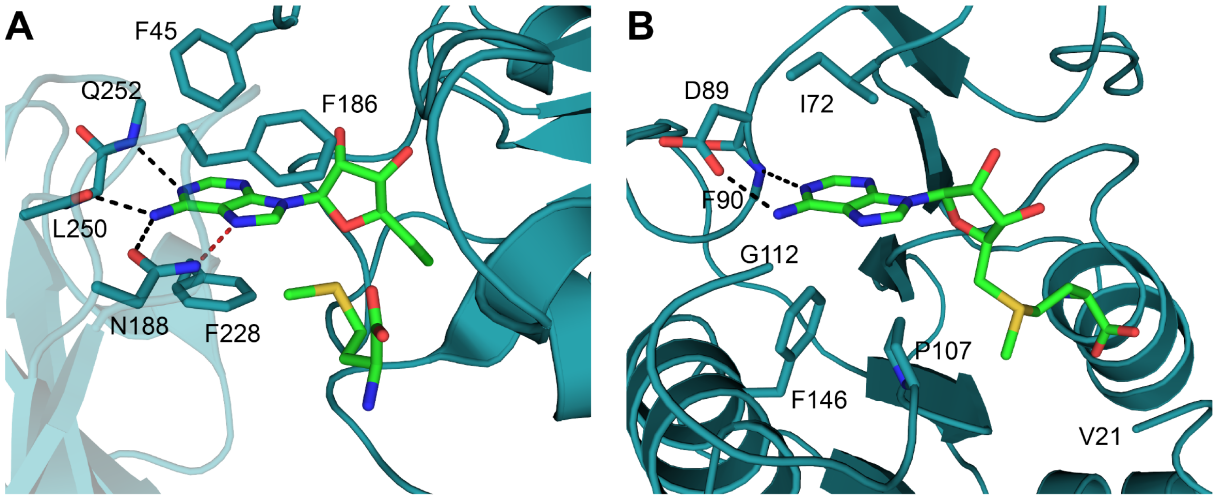

Figure S8. Cofactor binding of SalL (PDB code: 2Q6I)[4] and M.*Taq*I (PDB code: 2ADM)[5]

A) ClDA and methionine (green sticks) are bound at the interface of two SalL monomers (solid and semi-transparent, respectively). The nucleobase of ClDA shows hydrophobic interactions with several side chains (F45, F186, F228) and forms hydrogen bonds (dashed lines) to the backbone of L250 and Q252 as well as the side chain of N188. Presumably, only one of these hydrogen bonds cannot form upon binding of ClDthA (red line), whereas the other interactions are not affected by the alteration. B) The binding of the adenine portion of SAM (green sticks) to M.*Taq*I is also characterized by hydrophobic interactions (to the side chains of I72, P107, and F146) and hydrogen bonds (to the backbone amide of F90 and the side chain of D89). However, the hydrogen bond network

is limited to the part of the nucleobase that is common in both, SAM and S^{th}AM. The portion that differs between the two analogs points towards a region of the enzyme that is disordered: both, the N-terminus (M1-R20) and a loop (E113-H120) of M.*Taq*I, show no electron density in the structure.

5. Supplementary notes and references

1. D. Shin, R. W. Sinkeldam and Y. Tor, *Journal of the American Chemical Society*, 2011, **133**, 14912-14915.
2. A. Micoli, A. Turco, E. Araujo-Palomo, A. Encinas, M. Quintana and M. Prato, *Chemistry*, 2014, **20**, 5397-5402.
3. D. P. C. McGee and J. C. Martin, *Canadian Journal of Chemistry*, 1986, **64**, 1885-1889.
4. A. S. Eustaquio, F. Pojer, J. P. Noel and B. S. Moore, *Nat Chem Biol*, 2008, **4**, 69-74.
5. G. Schluckebier, M. Kozak, N. Bleimling, E. Weinhold and W. Saenger, *Journal of Molecular Biology*, 1997, **265**, 56-67.