Supporting information

Selective and Catalytic Conversion of Hydroxymethyl Cytosine to Formyl Cytosine by a Synthetic Model of TET Enzymes

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Materials and methods

Chemicals

Most of the chemicals that were used as starting materials in this project were bought from Sigma-Aldrich, SRL chemicals, TCI chemicals, BLD Pharma & SPECTROCHEM Private Limited. Dry DMF was purchased from Sigma-Aldrich. Dry THF was freshly distilled by using metallic sodium. DCM was dried over P_2O_5. The progress of the reactions was monitored using Thin Layer Chromatography (Merck Silica Gel 60 F-254, 0.25 nm).

Instrumentation

Nuclear Magnetic Resonance (NMR) Spectroscopy

Bruker Advance III FT-NMR spectrometer was used to record the NMR spectra. ^{1}H spectra were recorded at 400 or 500 MHz and ^{13}C NMR spectra were recorded at 101 or 126 MHz. Residual solvent signals were used as internal standards and chemical shifts were reported in parts per million (ppm).

High-Resolution Mass Spectrometry (HRMS)

Bruker MicrOTOF-Q-H mass spectrometer instrument was used to record HRMS-ESI data. Most commonly LC-MS grade Water and Acetonitrile were used for running the compounds.

High Performance Liquid Chromatography (HPLC)

HPLC experiments were performed on a Waters Alliance System (Milford, MA) with a 2695 separation module and a 2996 photodiode-array detector. The assays were carried out in 1.8 mL sample vials, with sample injection performed using a built-in autosampler. EMPOWER software was used to control the HPLC apparatus (Waters Corporation, Milford, MA). A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) and a Cosmosil Packed HPLC column (4.6 ID x 100 mm; 3C18-MS-II) was used for the separation and analysis.

Chromatography

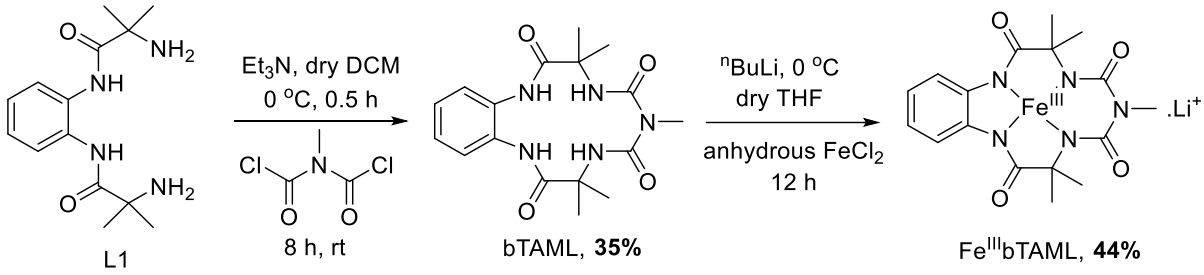
Column chromatography was done manually by loading silica gel of 100-200 mesh (RANKEM) on glass columns or an automated flash chromatography system (Teledyne Combiflash, Nextgen 300) by using 12 g or 24 g stainless steel columns by loading 230-400 mesh silica (RANKEM). The progress of the reactions was monitored using Thin Layer Chromatography (Merck Silica Gel 60 F-254, 0.25nm).

Immuno-Dot Blot

Blotting of the DNA sample was done on a Positively Charged Nylon Membrane (Invitrogen by Thermo Fisher Scientific, AM10104). Visualization of the blot was done by using the Clarity™ Western ECL Substrate (Bio-Rad, 170-5061) and the ChemiDoc MP system (Bio-Rad). Quantification of signals of the blot was done using the image-processing software ImageJ (NIH). Details of the antibody are provided in Figure S20.

Experimental section

Synthetic scheme of Fe^{III}bTAML Complex



Scheme S1. Synthetic scheme of Fe^{III}bTAML Complex.[1]

N, N'-(1,2-phenylene)bis(2-amino-2-methylpropanamide) (L1) was synthesized as has been described before.[2]

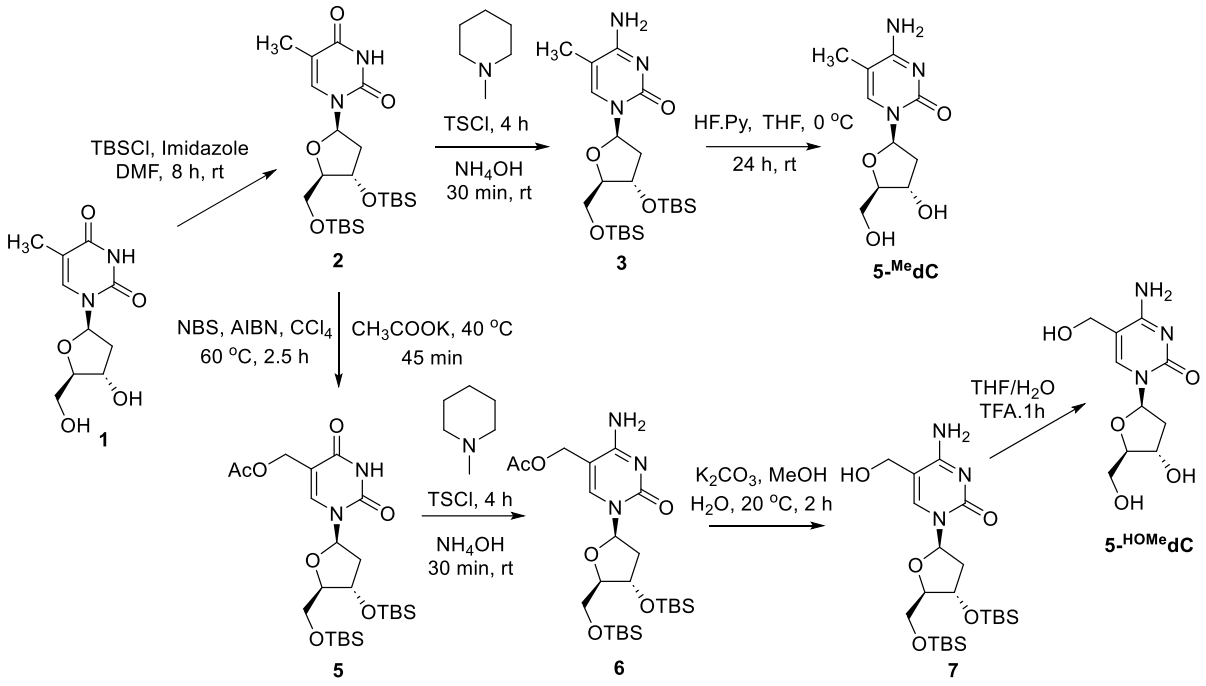
Synthesis of bTAML ligand

Compound **L1** (200 mg, 0.7 mmol) was added to 10 ml dry DCM with dry Et_3N (0.21 ml, 4.1 mmol) and the resultant solution was stirred for half an hour at room temperature at inert condition in an Ar atmosphere. N, N-dichloroformylmethylamine (0.037 ml, 0.7 mmol) was diluted into another 10 ml dry DCM. Then both solutions were added dropwise through a syringe pump into a 250 ml round-bottomed flask containing 100 ml dry DCM at 0 °C. Then the solution was kept stirring overnight at room temperature. The reaction was monitored by TLC in 75% EtOAc - 35% Hexane using UV. It was then purified by column chromatography in a 3:1 (EtOAc:Hexane) mixture to get a white solid of **bTAML** with a 35 % (75 mg) yield. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.30 (s, 2H), 7.64 (dd, J = 6.0, 3.6 Hz, 2H), 7.22 (dd, J = 6.1, 3.5 Hz, 2H), 7.12 (s, 2H), 2.53 (s, 3H), 1.66 (s, 12H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.8, 157.2, 126.5, 125.9, 59.5, 30.8, 25.5. **ESI-HRMS** (M+H) Calculated: 362.1828, found: 362.1814.

Synthesis of Fe^{III}bTAML complex

To a solution of bTAML ligand (50 mg, 0.138 mmol) in 10 ml of dry THF was added n-BuLi (0.28 ml of 2 M solution in hexane, 0.57 mmol) at 0 °C under Ar. Solid anhydrous $FeCl_2$ (21.1 mg, 0.17 mmol) was then added as a solid into this solution under positive Ar flow. The reaction was allowed to proceed under Ar at room temperature for 12 hours after which it was opened to air to yield a dark orange-brown precipitate. The precipitate was filtered through a frit and was dissolved in methanol to afford an orange solution. The orange solution was evaporated in a vacuum to get a solid. Further purification was achieved by column chromatography using basic alumina with CH_2Cl_2:MeOH = 70:30 as an eluent to get an orange solid of 44 % yield. **ESI-HRMS** (negative ion mode) Calculated $[C_{17}H_{19}FeN_5O_4]^-$: 413.0781, found: 413.0772.

Synthetic scheme of 5-^{Me}dC and 5-^{HOMe}dC



Scheme S2. Synthetic scheme of 5-^{Me}dC and 5-^{HOMe}dC.

We have synthesized the 5-^{Me}dC and the 5-^{HOMe}dC by the following procedure where all the steps are confirmed by both NMR and mass spectrometry.[3,4]

1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tertbutyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H) dione (2)

Compound 1 (4 g, 16.4 mmol) was dissolved in 10 ml of DMF and stirred with imidazole (5.6 g, 82.56 mmol) and TBSCl (6.2 g, 41.2 mmol) at room temperature for 8 hours. After completion of the reaction, the product was extracted with EtOAc. The compound was purified with EtOAc:Hexane (30:70) column chromatography to get compound 2 with an 8 g yield (99 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (s, brs, 1H), 7.49 (s, 1H), 6.36 (t, 1H), 4.43 (m, 1H), 3.96 (m, 1H), 3.89 (m, 1H), 3.78 (m, 1H) 2.27 (m, 1H), 2.03 (m, 1H), 1.94 (s, 3H), 0.94 (18H), 0.11 (12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.9, 150.3, 135.5, 110.8, 87.9, 84.8, 72.3, 63.0, 41.4, 25.9, 25.8, 18.4, 18.0, 12.5, -3.6, -4.6, -4.8, -5.4, -5.4. **ESI-HRMS** (M+H) Calculated: 471.2711, found: 471.2725.

4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-methylpyrimidin-2(1H)-one (3)

To an ice-cold solution of 2 (2 g, 4.2 mmol), N-methyl piperidine (0.64 mL, 5.08 mmol) and Et_3N (0.658 mL, 9.32 mmol) in acetonitrile (10 mL) were added TsCl (1.70 g, 9.32 mmol). The reaction mixture was stirred at that

temperature for 4 h after which NH_4OH (2 mL) was added and stirred for another 30 min. The reaction mixture was subsequently diluted with EtOAc (40 mL), extracted twice with sat NaCl, dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed (50-70% EtOAc in hexane) to obtain compound 3 (1.65 g) as white floppy solids (82%). δ ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.30 (d, J = 25.5 Hz, 1H), 7.69 (d, J = 15.0 Hz, 1H), 7.45 (d, J = 18.9 Hz, 1H), 6.17 (q, J = 7.6 Hz, 1H), 3.75 (dd, J = 22.8, 3.7 Hz, 2H), 3.63 – 3.52 (m, 1H), 2.52 (s, 2H), 2.08 (dd, J = 10.1, 6.4 Hz, 1H), 1.78 (s, 3H), 0.90 – 0.84 (m, 18H), 0.08 (d, J = 3.8 Hz, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 150.9, 136.5, 109.8, 84.1, 72.6, 70.9, 61.8, 61.4, 26.3, 26.1, 18.2, 18.2, 12.7, -2.5, -4.3. **ESI-HRMS** (M+H) Calculated: 470.2870, found: 470.2862.

4-amino-1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-5-methylpyrimidin-2(1H)-one (5-^{Me}dC)

To an ice cold and stirring solution of 7 (0.280 g, 0.59 mmol) (in polypropylene falcon tube) in THF (4 mL) was added HF.Py complex solution (300 µL, 12 mmol). The reaction mixture was stirred at that temperature for 2 h and then slowly allowed to come to rt and stirred for 24 hrs. By this time TLC shows complete conversion of the starting compound. Solid $NaHCO_3$ was added to the reaction and stirred at rt for 1 h. The reaction mixture was then filtered, and the filtrate was concentrated under reduced pressure. Then purified by column chromatography with 6% DCM-MeOH to yield a solid of 70 mg (51 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.27 (s, 1H), 7.70 (s, 1H), 6.17 (t, J = 6.9 Hz, 1H), 5.23 (d, J = 4.2 Hz, 1H), 5.02 (t, J = 5.2 Hz, 1H), 4.24 (p, J = 3.6 Hz, 1H), 3.76 (q, J = 3.6 Hz, 1H), 3.62 – 3.52 (m, 2H), 2.13 – 2.00 (m, 2H), 1.78 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.2, 150.9, 136.6, 109.8, 87.7, 84.2, 70.9, 61.8, 12.7. **ESI-HRMS** (M+) Calculated: 241.1063, found: 241.1048.

(1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tertbutyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)methyl acetate (5)

To a solution of 2 (1 g, 2.11 mmol) in dry CCl_4 (16 mL) was added recrystallized NBS (0.45 g, 2.5 mmol) and AIBN (8.3 mg, 0.05 mmol) at 60 °C. The reaction was refluxed for 45 min. Then, the second portion of recrystallized NBS (0.45 g, 2.5 mmol) and AIBN (8.3 mg, 0.05 mmol) was added. After 1.5 h, the reaction mixture was cooled to room temperature and diluted with $CHCl_3$ (100 mL). The organic solution was washed with brine (200 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was dissolved in DMF (6 mL). To the solution was added CH_3COOK (346 mg, 4.22 mmol). The resulting slurry was stirred at 40 °C for 45 min and diluted with EtOAc (70 mL). The organic solution was washed with H_2O (100 mL) and brine (200 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo. Flash column chromatography (PE/EtOAc 4:1) afforded the 5 with an 8 % yield (92 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.38 (s, 1H), 7.83 (s, 1H), 6.32 – 6.26 (m, 1H), 4.87 – 4.77 (m, 2H), 4.41 (dt, J = 5.4, 2.3 Hz, 1H), 3.97 (q, J = 2.8 Hz, 1H), 3.88 – 3.75 (m, 3H), 2.33 (ddd, J = 13.2, 5.8, 2.6 Hz, 1H), 2.05 (s, 3H), 0.91 (d, J = 9.4 Hz, 18H), 0.11 – 0.08 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8, 162.7, 150.1, 141.0, 109.2, 88.1, 85.5, 72.3, 63.0, 59.1, 41.5, 25.9, 20.9, 18.4, 18.0, -4.7, -4.8, -5.4, -5.5. **ESI-HRMS** (M+H) Calculated: 529.2765, found: 529.2758.

(4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-5-yl)methyl acetate (6)

To a solution of 5 (190 mg, 0.359 mmol), N-methylpiperidine (42.8 mg, 0.431 mmol), and Et_3N (0.11 mL, 0.791 mmol) in dry CH_3CN (5 mL) was added TsCl (150 mg, 0.791 mmol) under an inert atmosphere at 0 °C. The reaction was stirred for 4 h. 28% NH_4OH (1 mL) was added at 0 °C, and the reaction mixture was stirred at 20 °C for 30 min and diluted with EtOAc (400 mL). The organic solution was washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Flash column chromatography (DCM/MeOH, 40:1) afforded the 6 (113 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (s, 1H), 6.31 – 6.21 (m, 1H), 4.93 – 4.77 (m, 2H), 4.35 (dd, J = 5.8, 2.8 Hz, 1H), 3.98 – 3.93 (m, 1H), 3.87 (dd, J = 11.3, 2.8 Hz, 1H), 3.77 (dd, J = 11.6, 2.6 Hz, 2H), 3.70 (s, 1H), 2.50 – 2.40 (m, 1H), 2.07 (s, 3H), 0.90 (d, J = 12.7 Hz, 18H), 0.07 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 164.8, 155.4, 100.8, 87.9, 86.3, 71.8, 67.1, 62.8, 60.6, 42.3, 25.9, 25.6, 20.9, 18.4, 18.0, -4.6, -4.9, -5.4. **ESI-HRMS** (M+H) Calculated: 528.2925, found: 528.2935.

4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-(hydroxymethyl)pyrimidin-2(1H)-one (7)

To a solution of 6 (50 mg, 0.09 mmol) in MeOH (2 mL) were added K_2CO_3 (40 mg, 0.18 mmol) and H_2O (0.2 mL). The reaction was stirred at 20 °C for 2 h and concentrated in vacuo. The residue was dissolved in EtOAc (30 mL) and washed with H_2O (30 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated in vacuo. Flash column chromatography (DCM/MeOH 30:1) afforded the 7 (48 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H), 6.20 (t, J = 6.4 Hz, 1H), 4.48 – 4.39 (m, 2H), 4.35 (dt, J = 6.4, 3.4 Hz, 1H), 3.94 (q, J = 3.0 Hz, 1H), 3.81 (ddd, J = 40.2, 11.3, 2.9 Hz, 2H), 2.40 (ddd, J = 13.3, 5.8, 3.5 Hz, 1H), 1.97 (dt, J = 13.2, 6.5 Hz, 1H), 0.91 (d, J = 8.6 Hz, 18H), 0.09 (dd, J = 12.5, 2.6 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.0, 155.8, 106.0, 87.9, 86.2, 71.8, 62.8, 42.1, 26.0, 25.8, 18.4, 18.0, -4.5, -4.9, -5.3, -5.4. **ESI-HRMS** (M+Na) Calculated: 508.2639, found: 508.2629.

4-amino-1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-5-(hydroxymethyl)pyrimidin-2(1H)-one (5-^{HOMe}dC)

A solution of 7 (140 mg, 0.288 mmol) in THF (1 mL) was added to 50% TFA aqueous solution (0.8 mL) at 20 °C. The reaction was stirred for 1 h and concentrated in vacuo to afford 5-^{HOMe}dC (72 mg, 56%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.45 (s, 1H), 6.24 (t, J = 6.1 Hz, 1H), 4.47 (s, 2H), 4.45 – 4.40 (m, 1H), 4.00 (d, J = 3.4 Hz, 1H), 3.90 – 3.72 (m, 2H), 2.42 (dt, J = 13.4, 5.1 Hz, 1H), 2.28 (dt, J = 13.2, 6.2 Hz, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 142.9, 105.8, 88.2, 86.6, 70.3, 70.2, 61.0, 56.9, 40.5. **ESI-HRMS** (M+H) Calculated: 258.1090, found: 258.1088.

HPLC Chromatogram of Fe^{III}bTAML complex

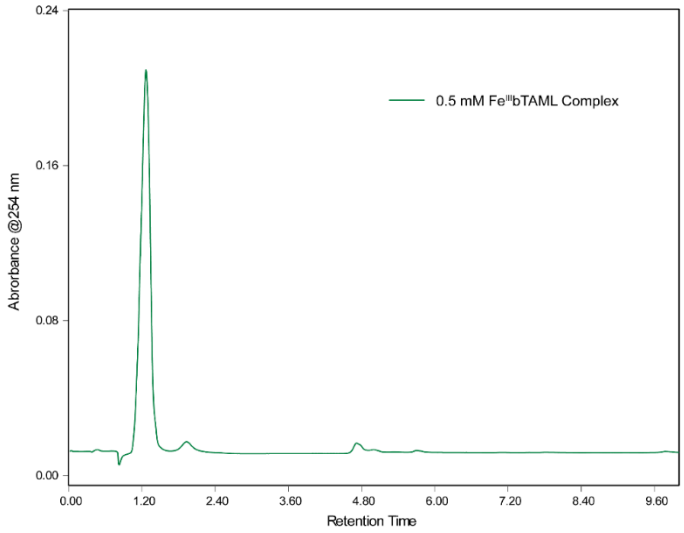
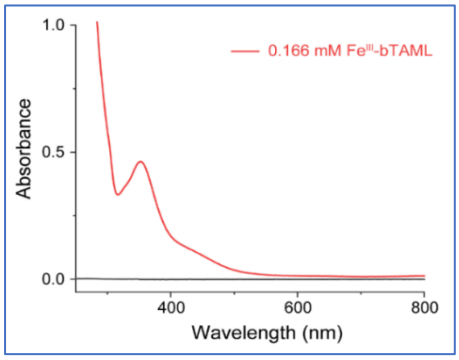


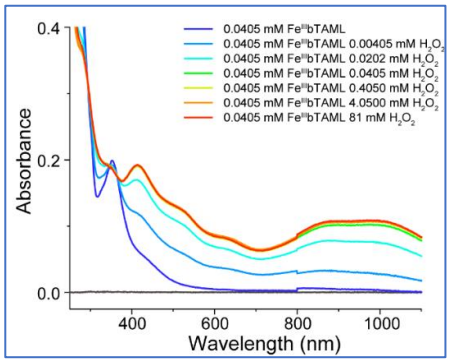
Figure S1: HPLC chromatogram of the Fe^{III}bTAML complex. Mobile phase: A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) was used with an binary gradient mobile phase having 2% A, and 98% D, at 0 minutes to 98% A and 2% D at 10 minutes. where A: Acetonitrile, D: Water with 0.1% TFA.

Absorbance spectra of Fe^{III}bTAML and Fe-oxo species in different types of solvent

A)



B)



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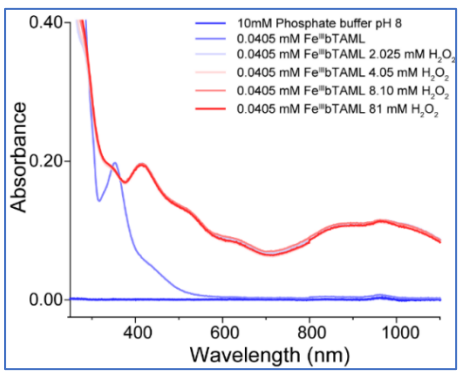

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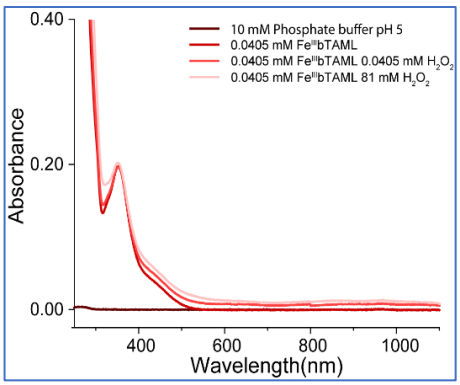


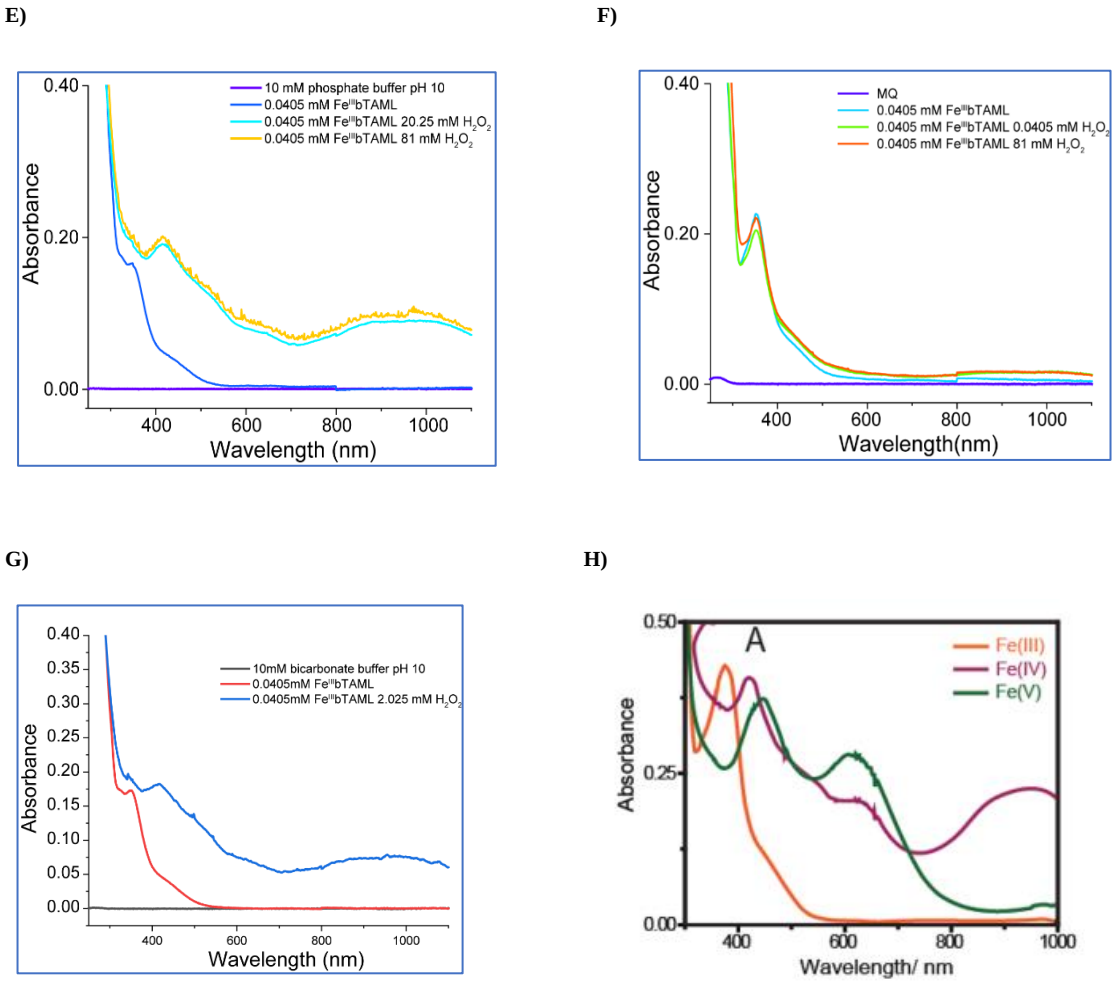


Figure S2. (A) UV-Vis spectra of Fe^{III}bTAML complex. The formation of µ-Fe^{IV}-oxo dimer species in 10 mM Phosphate buffer of different pH of (B) 7.5; (C) pH 8; (D) pH 5; (E) pH 10; (F) in aqueous solution and (G) in 10 mM Bicarbonate Buffer of pH 10 at room temperature in the presence of different equivalents of H_2O_2. (H) Reported UV−vis spectral changes of Fe^{III}bTAML (10^{-4} M) (orange) upon the addition of 0.5 equiv of NaOCl (5×10^{-5} M) in CH_3CN, forming the µ-Fe^{IV}-oxo dimer species (violet).[5] At 354 nm, we measure the presence of Fe^{III}bTAML. In a pH 7.5 and pH 8 phosphate buffer, we observed the formation of µ-Fe^{IV}-oxo dimer species.

The general procedure of HPLC studies

HPLC was performed in Waters Alliance e2695 equipped with Waters 2998 PDA detector and the data was analyzed with EMPOWER software. A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) was used with an isocratic binary mobile phase having 2 % C, 98 % D (C: MeOH, D: water with 0.1 % TFA) for HPLC analysis.

HPLC trace of a mixture of 5-mC, 5-hmC, 5-fC, 5-caC in Phosphate buffer

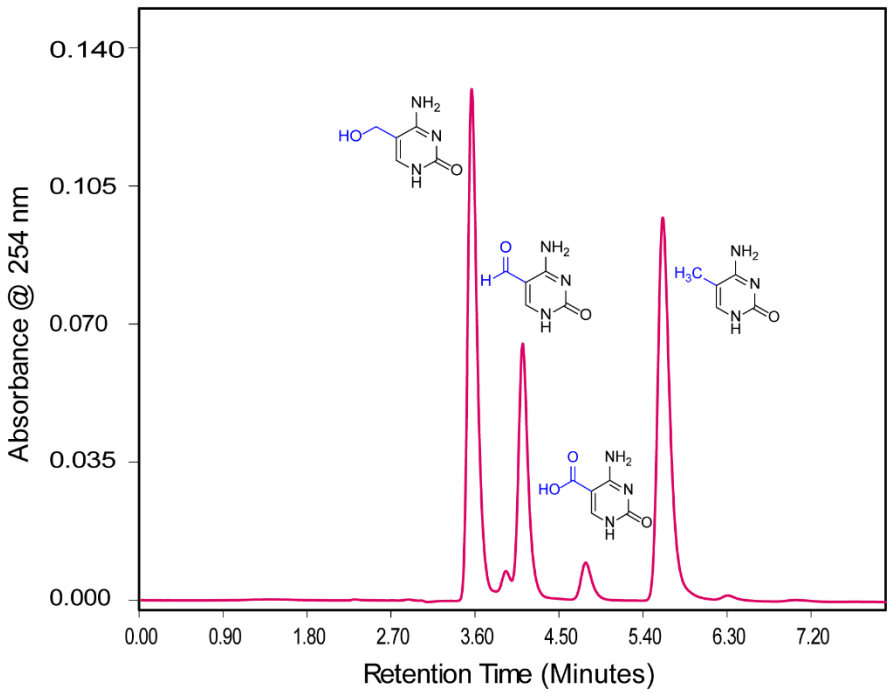


Figure S3. HPLC chromatogram of a mixture of 5-mC, 5-hmC, 5-fC, and 5-caC. A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) was used with an isocratic binary mobile phase having 2 % C, 98 % D (C: MeOH, D: water with 0.1 % TFA) for HPLC analysis.

Calibration of 5-hmC, 5-fC, 5-caC for quantitative experiment

From the calibration curves, we can quantify the amount of reactant remaining and the products formed in a reaction mixture by measuring the peak areas. A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) was used with an isocratic binary mobile phase having 2 % C, 98 % D (C: MeOH, D: water with 0.1 % TFA) for HPLC analysis.

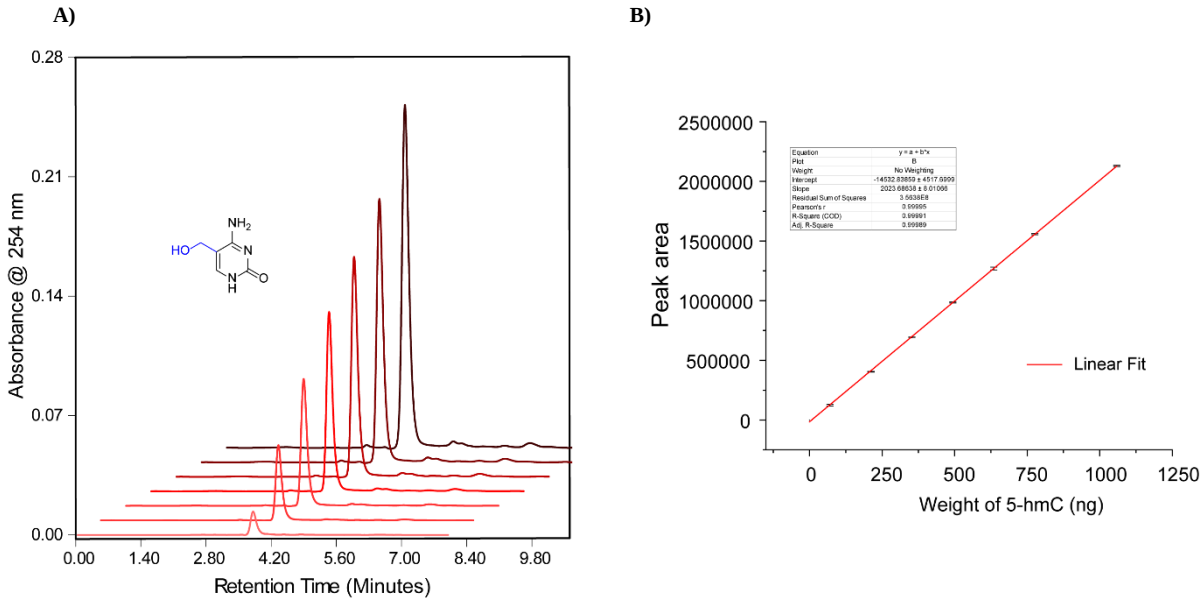


Figure S4. A) HPLC chromatogram of 5-hmC with increasing concentration of 5-hmC. B) Linear fit plots of HPLC peak area vs the amount of 5-hmC (ng). From this calibration curve, the amount of 5-hmC can be quantified in a reaction mixture.

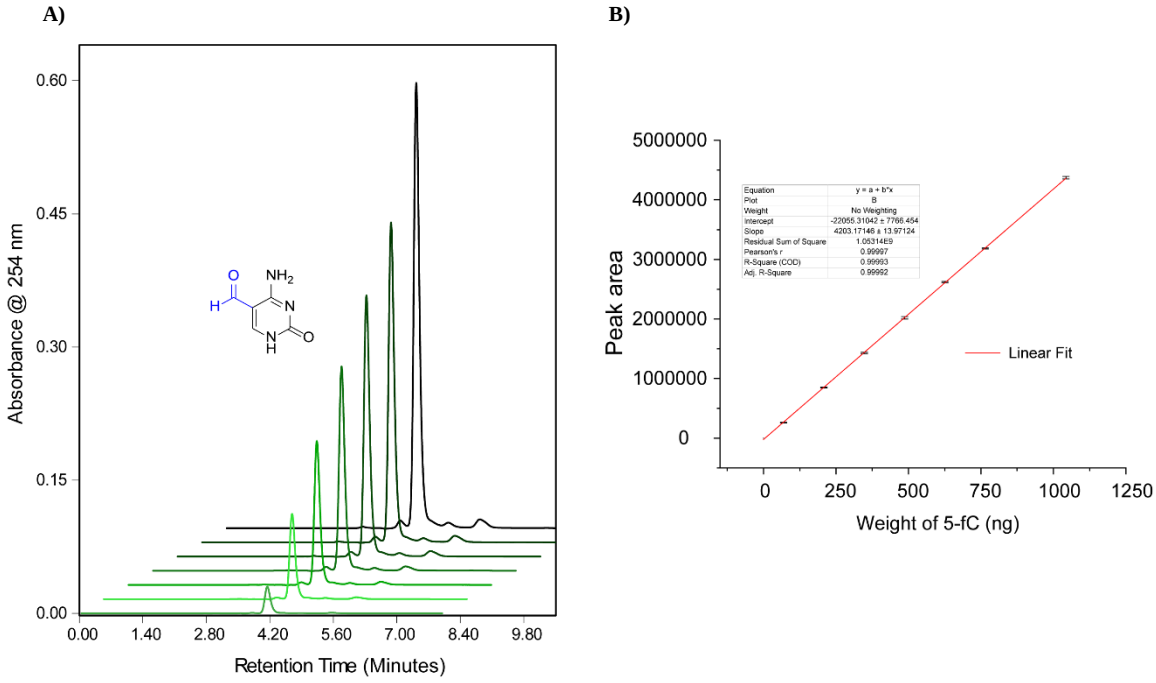


Figure S5. A) HPLC chromatogram of 5-fC with increasing concentration of 5-fC. B) Linear fit plots of HPLC peak area *vs* the amount of 5-fC (ng). From this calibration curve, the amount of 5-fC can be quantified in a reaction mixture.

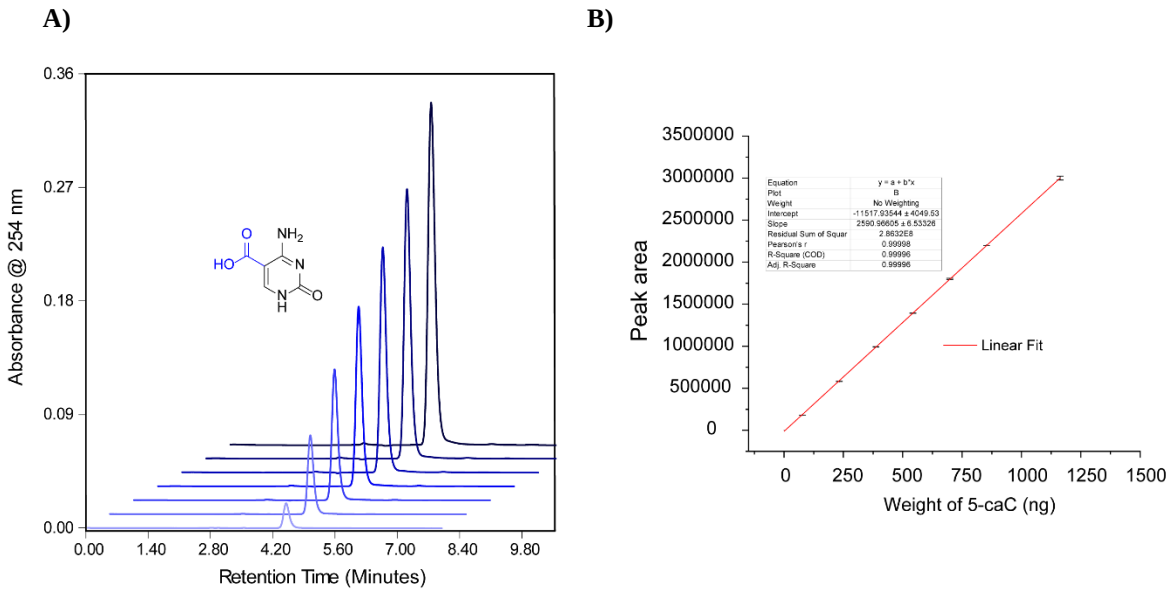


Figure S6. A) HPLC chromatogram of 5-caC with increasing concentration of 5-caC. B) Linear fit plots of HPLC peak area *vs* the amount of 5-caC (ng). From this calibration curve, the amount of 5-caC can be quantified in a reaction mixture.

HPLC chromatograms showing the selectivity of Fe^{III}bTAML towards 5-hmC to 5-fC oxidation.

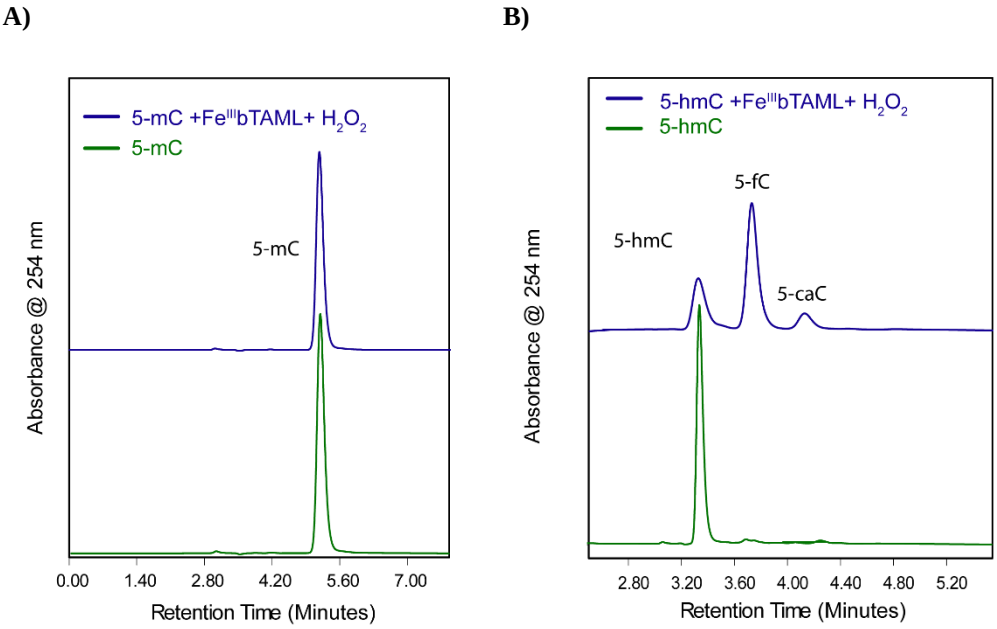


Figure S7. (A) HPLC Chromatogram of pure 5-mC (5 mM) and reaction mixture containing 5-mC (5 mM), Fe^{III}bTAML (0.05 mM), and H_2O_2 (500 mM) in 10 mM Phosphate Buffer of pH 7.5. B) HPLC Chromatogram of pure 5-hmC (5 mM) and reaction mixture containing 5-hmC (5 mM), Fe^{III}bTAML (0.05 mM) and H_2O_2 (50 mM) in 10 mM Phosphate Buffer of pH 7.5. A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) was used with an isocratic binary mobile phase having 2 % C, 98 % D (C: MeOH, D: water with 0.1 % TFA) for HPLC analysis.

HPLC chromatograms showing the selectivity of Fe^{III}bTAML towards 5-hmC over 5-mC for oxidation in presence of both the substrates.

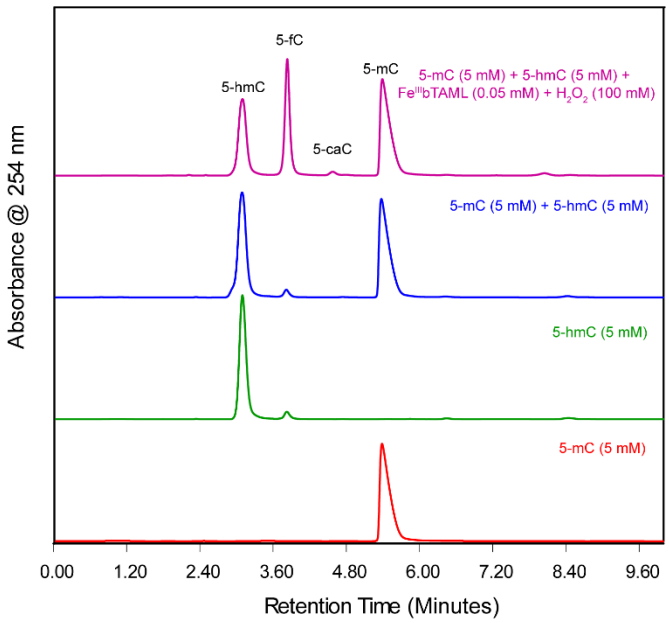


Figure S8. HPLC chromatograms showing the selectivity of Fe^{III}bTAML towards 5-hmC over 5-mC for oxidation in presence of both the substrates. Conditions: [5-mC] = 5 mM; [5-hmC] = 5 mM; [Fe^{III}bTAML] = 0.05 mM; [H_2O_2] = 100 mM; Solvent: 10 mM Phosphate Buffer of pH 8; T = 25 ^oC. Mobile phase: 100 % D, Flow rate = 0.6 ml/min [Where D = 0.1 % TFA in MQ]. We used a Cosmosil Packed HPLC column (4.6 ID x 100 mm; 3C18-MS-II) here.

HPLC chromatograms showing the conversion of 5-hmC to 5-fC, 5-caC with varying H_2O_2 concentrations.

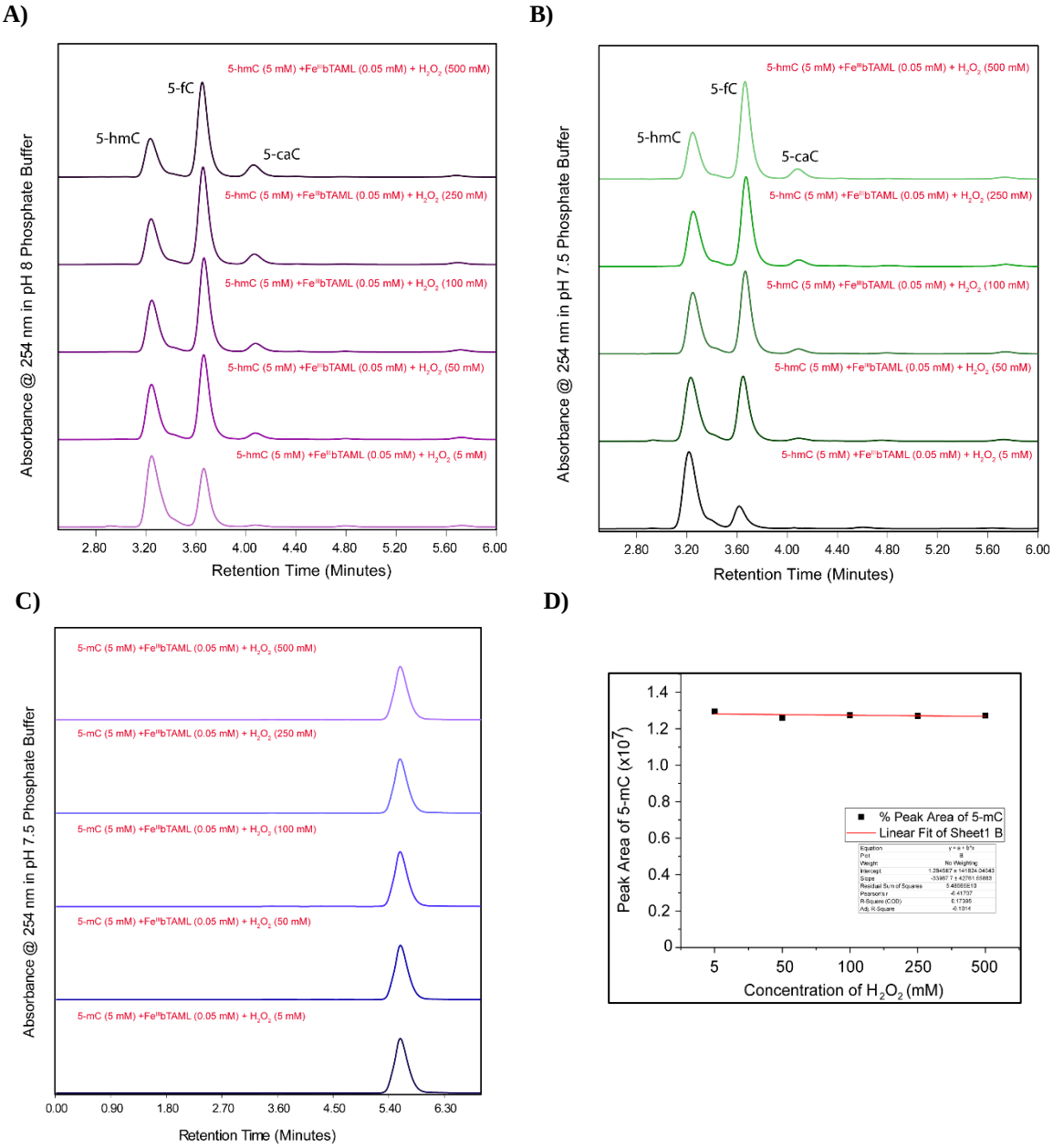


Figure S9. A, B) HPLC chromatogram showing the conversion of 5-hmC to 5-fC and 5-caC with varying concentrations of H_2O_2 (100, 1000, 2000, 5000, 10000 equivalents of H_2O_2 with respect to the Fe^{III}bTAML). Conditions: [5-hmC] = 5 mM; [Fe^{III}TAML] = 0.05 mM; [H_2O_2] = 5 mM, 50 mM, 100 mM, 250 mM, 500 mM; Solvent: 10 mM Phosphate Buffer of pH 8 (A) and pH 7.5 (B); T = 25 ^{o}C. (C) HPLC chromatogram of reaction mixture containing 5-mC, Fe^{III}bTAML with varying H_2O_2 concentration. Conditions: [5-mC] = 5 mM; [Fe^{III}TAML] = 0.05 mM; [H_2O_2] = 5 mM, 50 mM, 100 mM, 250 mM, 500 mM; Solvent: 10 mM Phosphate Buffer of pH 7.5; T = 25 ^{o}C. (D) The plot of the peak area of 5-mC (obtained from Figure S9.C) vs. H_2O_2 concentration shows no change in the 5-mC peak area. A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) was used with an isocratic binary mobile phase having 2 % C, 98 % D (C: MeOH, D: water with 0.1 % TFA) for HPLC analysis.

Effect of H_2O_2 and pH on the reactivity of Fe^{III}bTAML

A) **B)**

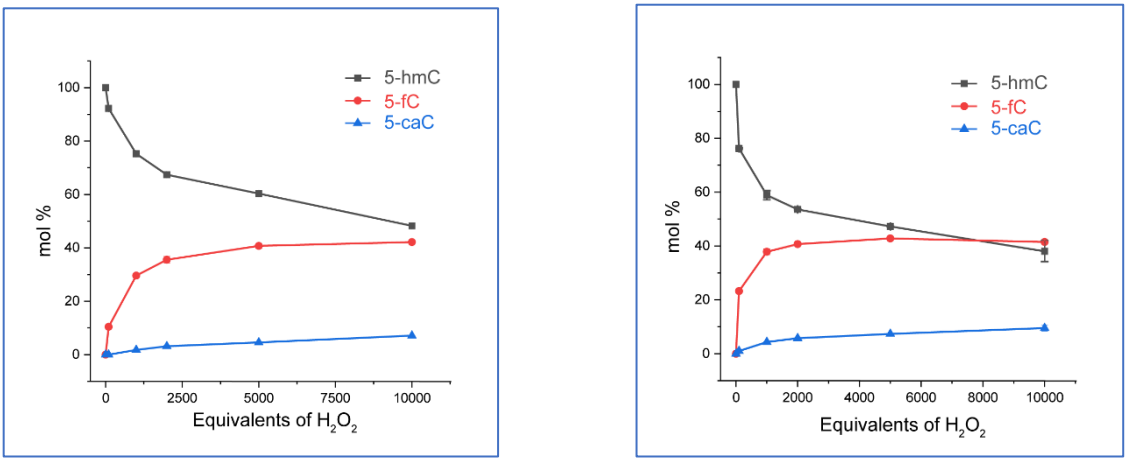


Figure S10. Quantification of 5-hmC, 5-fC, or 5-caC with varying concentrations of H_2O_2 (100, 1000, 2000, 5000, 10000 equivalents of H_2O_2 with respect to the Fe^{III}bTAML). Conditions: [5-hmC] = 5 mM; [Fe^{III}TAML] = 0.05 mM; [H_2O_2] = 5 mM, 50 mM, 100 mM, 250 mM, 500 mM; Solvent: 10 mM Phosphate Buffer of pH 7.5 (A) and pH 8 (B); T = 25 °C. A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) was used with an isocratic binary mobile phase having 2 % C, 98 % D (C: MeOH, D: water with 0.1 % TFA) for HPLC analysis.

Reactivity of Fe^{III}bTAML on different concentrations of reactant

A) **B)**

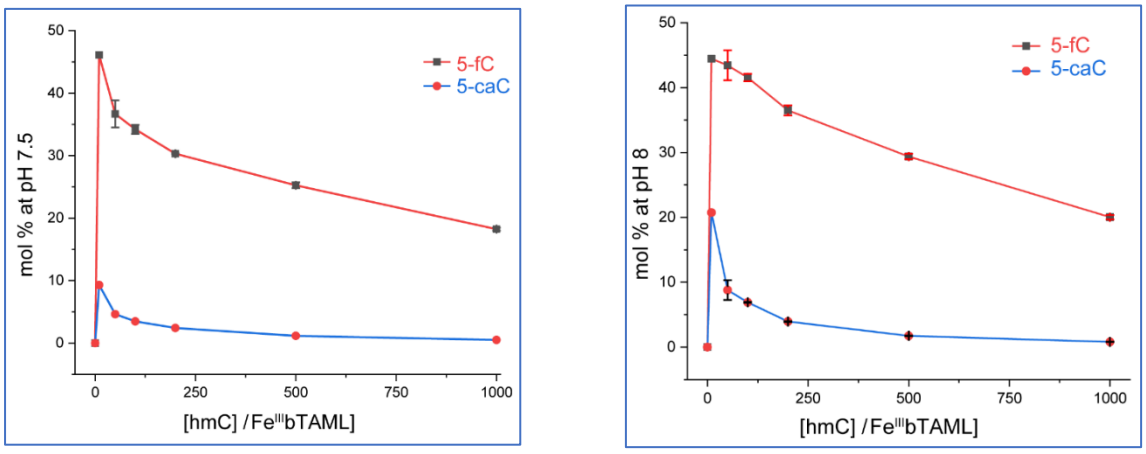


Figure S11. The effect of substrate (5-hmC) concentration variation keeping the catalyst concentration fixed. The formation of 5-fC and 5-caC were quantified from the calibration curve and after incubating the reaction mixture for 100 minutes at different equivalents of 5- hmC (10, 50, 100, 200, 500, 1000 equivalents of 5-hmC with respect to the Fe^{III}bTAML). Conditions: [H_2O_2] = 100 mM; [Fe^{III}bTAML] = 0.05 mM; [5-hmC] = 0.5 mM, 2.5 mM, 5 mM, 10 mM, 25 mM, and 50 mM; Solvent: 10 mM Phosphate Buffer of pH 7.5 (A) and pH 8 (B); T = 25 °C. A Chromachemie HPLC column (4.6 mm x 100 mm; C18, 3 µm, 100 Å) was used with an isocratic binary mobile phase having 2 % C, 98 % D (C: MeOH, D: water with 0.1 % TFA) for HPLC analysis.

Stability of μ-Fe^{IV}-oxo dimer in the absence and presence of 5-hmC

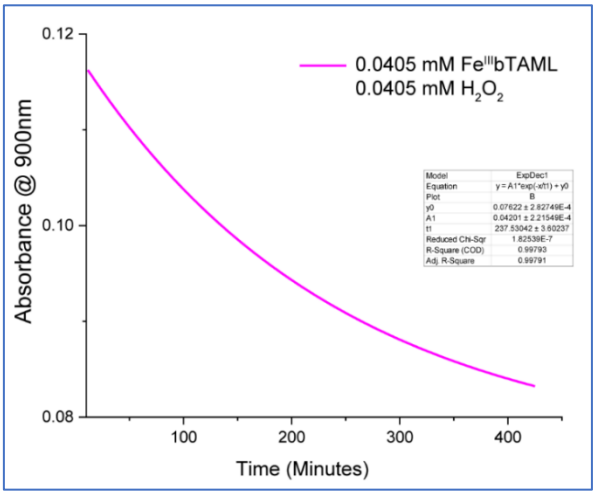


Figure S12. Monitoring the change in the absorbance (@ 900 nm) of μ-Fe^{IV}-oxo dimer species. Conditions: [H_2O_2] = 0.0405 mM; [Fe^{III}bTAML] = 0.0405 mM; Solvent: water; T = 25 ^oC. The rate of disappearance of Fe-oxo species in the reaction mixture is **4.3427 x 10^{-9} Ms^{-1}.**

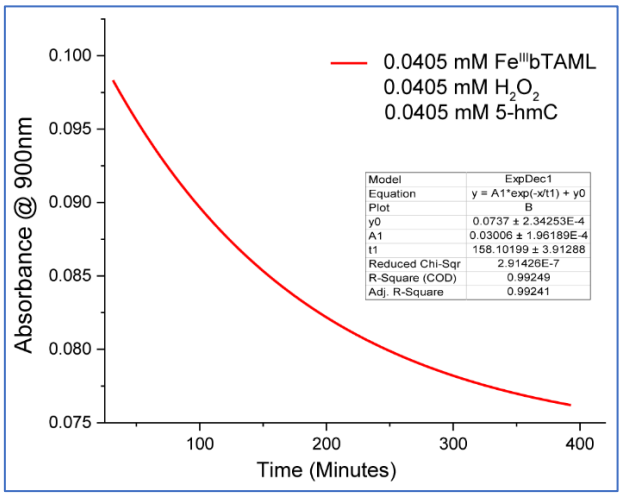


Figure S13. Monitoring the change in the absorbance (@ 900 nm) of μ-Fe^{IV}-oxo dimer species in the reaction mixture. Conditions: [H_2O_2] = 0.0405 mM; [5-hmC] = 0.0405 mM; [Fe^{III}bTAML] = 0.0405 mM; Solvent: 10 mM Phosphate Buffer of pH 7.5; T = 25 ^oC. We have calculated the initial rate based on this. The rate of disappearance of Fe-oxo species in the presence of 1 equivalent of reactant in the reaction mixture is **8.772 x 10^{-9} Ms^{-1}.**

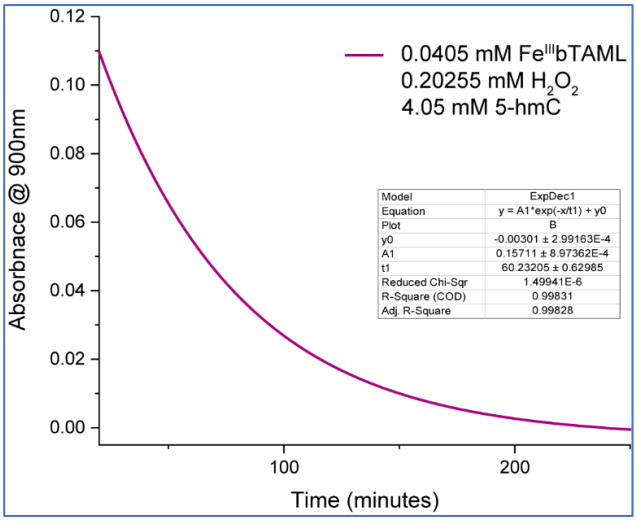


Figure S14. Monitoring the change in the absorbance (@ 900 nm) of µ-Fe^{IV}-oxo dimer species in the reaction mixture. Conditions: [H_2O_2] = 0.2025 mM; [5-hmC] = 4.05 mM; [Fe^{III}bTAML] = 0.0405 mM; Solvent: 10 mM Phosphate Buffer of pH 7.5; T = 25 ºC. We have calculated the initial rate based on this. The rate of disappearance of Fe-oxo species in the presence of 100 equivalent of reactant in the reaction mixture is **68.529 x 10^{-9} Ms^{-1}.**

Effect of H_2O_2 on the initial rate of oxidation of 5-hmC.

A) **B)**

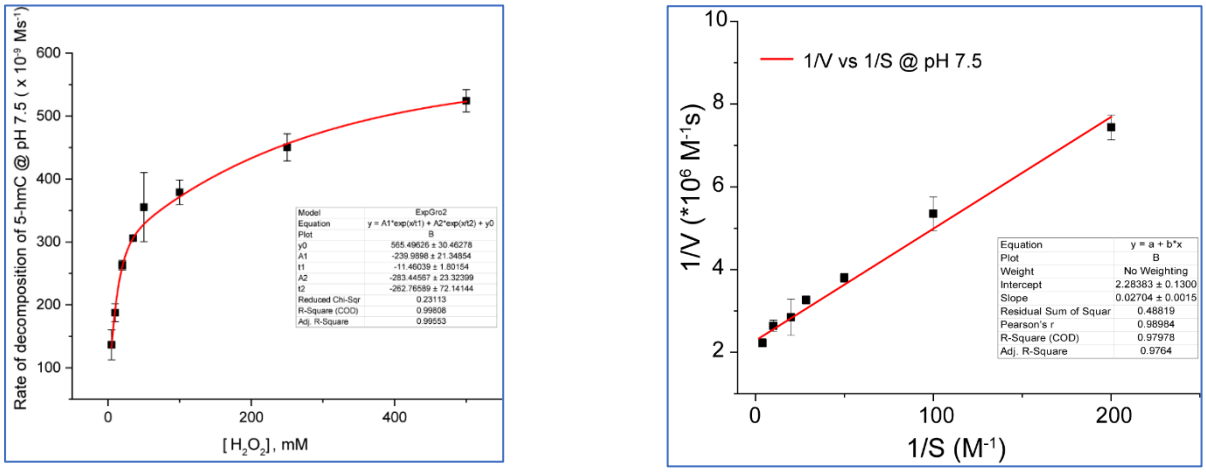


Figure S15. A) Rate of decomposition of 5-hmC with an increase in the concentration of H_2O_2. Conditions: [5-hmC] = 5 mM; [Fe^{III}bTAML] = 0.05 mM; [H_2O_2] = 5 mM, 50 mM, 100 mM, 250 mM, 500 mM; Solvent: 10 mM Phosphate Buffer of pH 7.5. B) Double inverse plot of (1/V) vs (1/S) [Reciprocal of H_2O_2 concentration].

V_{max} = 0.437860 x 10^{-6} Ms^{-1}, K_M = 0.0118 M.

A) **B)**

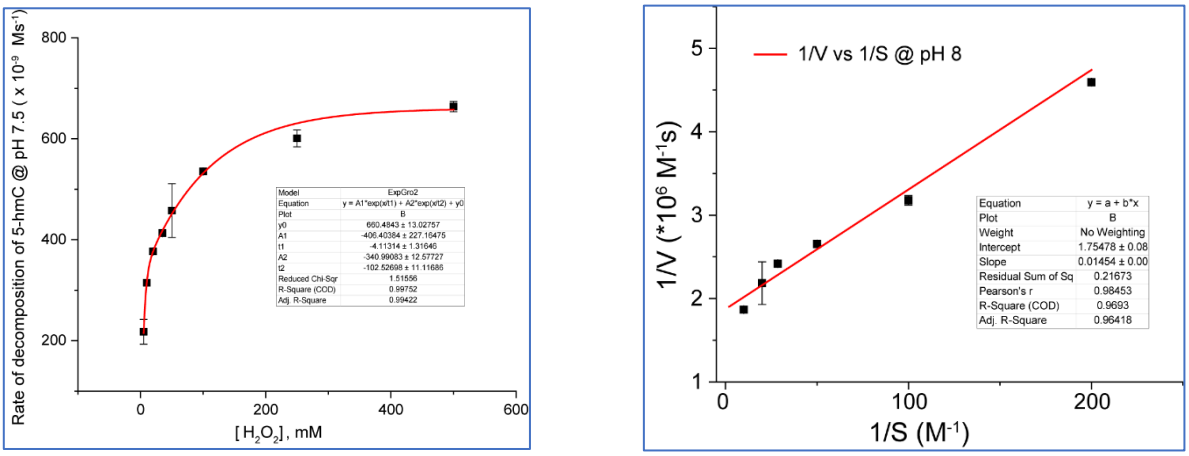


Figure S16. A) Rate of decomposition of 5-hmC with an increase in the concentration of H_2O_2. Conditions: [5-hmC] = 5 mM; [Fe^{III}bTAML] = 0.05 mM; [H_2O_2] = 5 mM, 50 mM, 100 mM, 250 mM, 500 mM; Solvent: 10 mM Phosphate Buffer of pH 8. B) Double inverse plot of (1/V) vs (1/S) [Reciprocal of H_2O_2 concentration].

V_{max} = 0.5698 x 10^{-6} Ms^{-1}, K_M = 0.00828 M.

HPLC chromatograms showing the oxidation of 5-mC by Fe^{III}bTAML.

A) **B)**

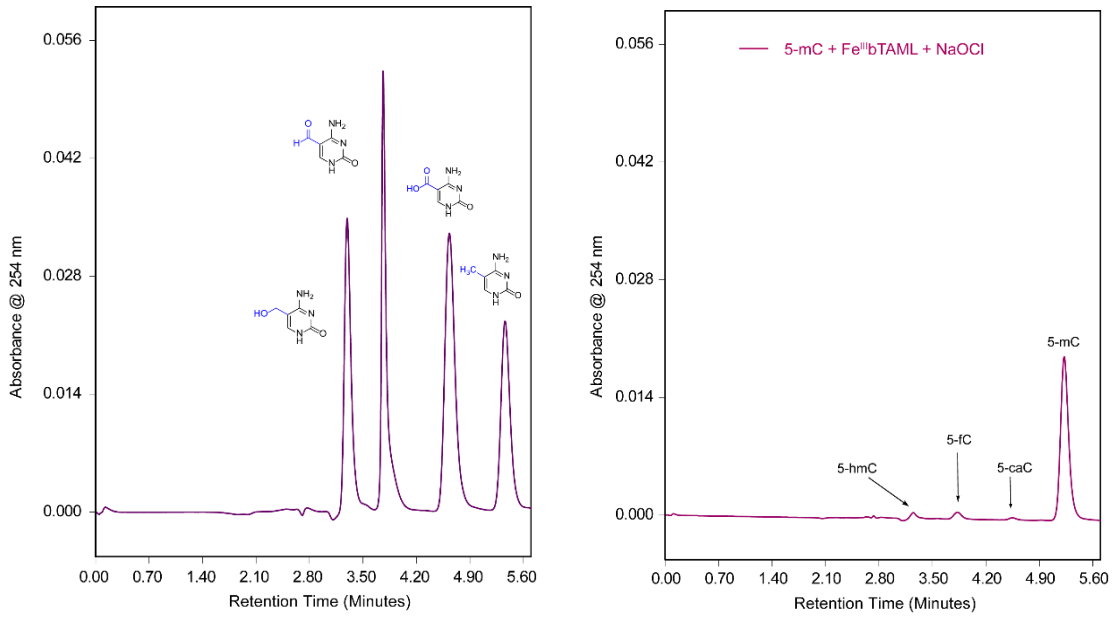


Figure S17. A) HPLC chromatogram of a mixture of 5-mC, 5-hmC, 5-fC, and 5-caC. B) HPLC chromatogram of 5-mC in the presence of NaOCl and Fe^{III}bTAML. Conditions: [5-mC] = 0.5 mM; [Fe^{III}bTAML] = 0.5 mM; [NaOCl] = 0.5 mM; Solvent: 50 % ACN-H_2O mixture. Here we used a Cosmosil Packed HPLC column (4.6 ID x 100 mm; $3C_{18}$-MS-II).

HPLC Chromatogram showing the selectivity towards 5-^{HOMe}dC over 5-^{Me}dC.

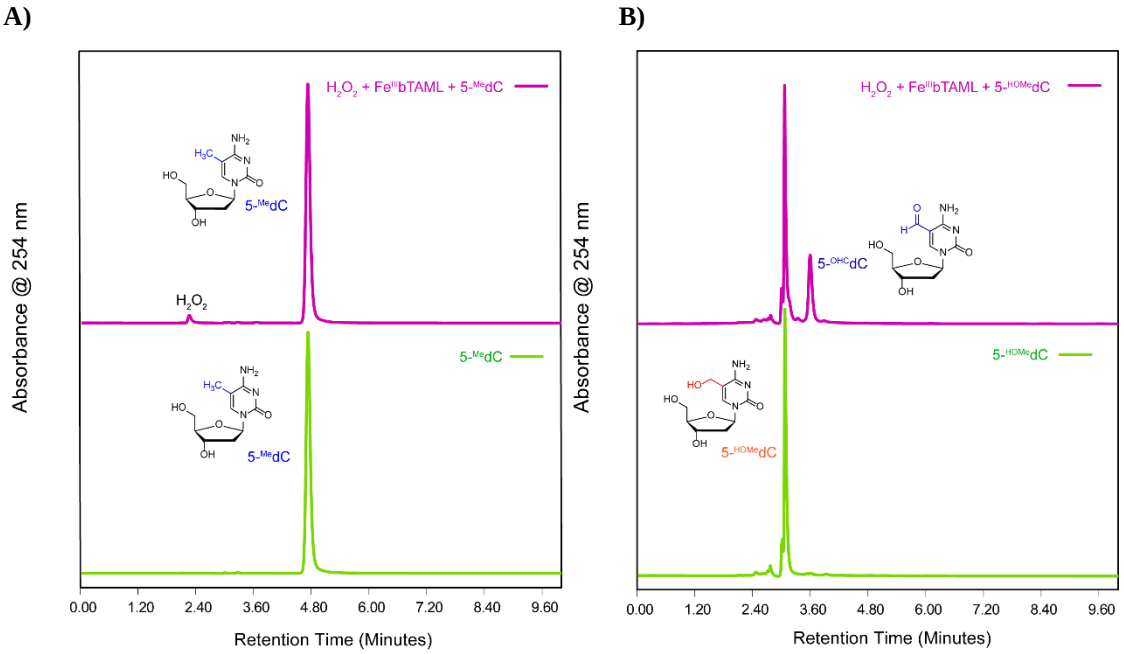


Figure S18. HPLC chromatogram showing the oxidation of 5-^{HOMe}dC over 5-^{Me}dC. A) HPLC chromatogram of 5-^{Me}dC in presence of H_2O_2, and Fe^{III}bTAML and B) HPLC chromatogram of 5-^{HOMe}dC in presence of H_2O_2, and Fe^{III}bTAML. Condition: [5-^{HOMe}dC] = 1 mM, [5-^{Me}dC] = 1 mM, [H_2O_2] = 20 mM, and [Fe^{III}bTAML] = 20 µM; 10 mM Phosphate buffer of pH 8. Temperature: 25 ^{o}C. Solvent: 8% A, 92% D, Flow rate = 0.5 ml/min [Where A = Acetonitrile, D = 0.1 % TFA in MQ]. Cosmosil Packed HPLC column (4.6 ID x 100 mm; 3C18-MS-II) was used here.

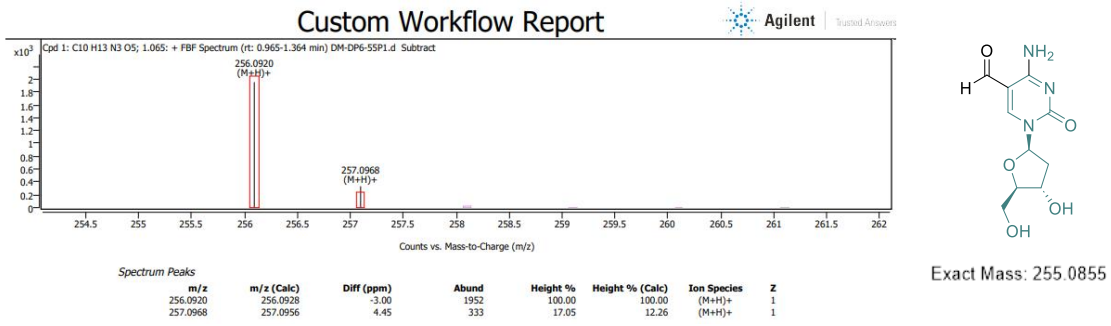
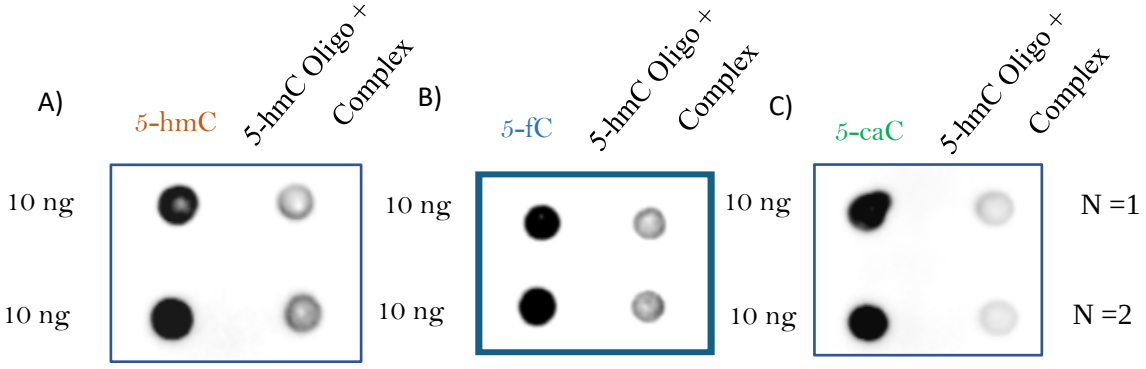


Figure S19. High resolution mass spectra of the new peak formed (Figure S18B) in the reaction mixture of 5-^{HOMe}dC + Fe^{III}bTAML + H_2O_2. **ESI-HRMS** (M+H) Calculated: 256.0933, found: 256.0920.

Oxidation of 5-hmC by Fe^{III}bTAML in dsDNA and Dot Blot Assay.

For checking the oxidation property of the Fe^{III}bTAML in the ds DNA we have purchased the Methylated cytosine DNA standard kit (GTX400004), which contains a set of five dsDNA, 426 bp long, and has the same sequence of 5'CGGGGTACCTTCACTTCAGAATCAACCAAACAGCCAAAACTGTTACATCAGGTTGTGGAGCAGTT ACAAAAGGTTCATTTTATCACAGATACCCTGTCAAAGGGTGAGACAAAGTTCATGGGTGTTTGCCA GCTTCCCAGTAAAAATGATGAAAAAGAATATCCACACAGAAGAATTGATATCAGGTTGATACCCA AAGATCAGTATTACTGTGGTGTTCTCTATTTCACTGGGAGTGATATTTTCAATAAGAATATGAGGG CTCATGCCCTAGAAAAGGGTTTCACAATCAATGAGTACACCATCCGTCCCTTGGGAGTCACTGGAG TTGCAGGAGAACCCCTGCCAGTGGATAGTGAAAAAGACATCTTTGATTACATCCAGTGGAAATAC CGGGAACCCAAGGACCGGAGCGAAGAATTC CCG 3'.

The only difference is that each contains either normal cytosines (C) or any specific modification among 5-mC, 5-hmC, 5-fC, or 5-caC. Before blotting on the positively charged nylon membrane the DNA sample was denatured at 99 ^{o}C for 5 min followed by cooling down the sample on ice. For the experiment, the 5-hmC containing DNA sample was incubated with the reaction mixture of Fe^{III}bTAML (20 μM) and H_2O_2 (1 mM) for 2.5 hours at 37 ^{o}C. 2 μL sample was spotted each time in such a way that 10 ng dsDNA sample was spotted at each place. The membrane was then baked at 80 ^{o}C in the oven for half an hour. The membrane was then blocked with 5% BSA in TBST for 1.5 hours at room temperature. After three times washing in TBST buffer, the membrane was incubated with primary antibody against the 5-hmC (HMC31, Mouse mAb #51660), 5-fC (D5D4K, Rabbit mAb #74178), 5-caC (D7S8U, Rabbit mAb #36836) overnight at 4 ^{o}C. Followed by three times washing with TBST the membrane was incubated with the peroxidase (HRP)-conjugated secondary antibody (Anti-mouse IgG, HRP-linked Antibody #7076, 1:1,500) and (Anti-rabbit IgG, HRP-linked Antibody #7074, 1:3,000) for 1 h at room temperature. Membranes were washed three times in TBST before visualizing with the help of Clarity ECL Western Substrates (Bio-Rad, 1705060) and the ChemiDoc MP system (Bio-Rad).



α 5hmC

100	25.82709
100	39.23278

α 5fC

100	35.45134
100	39.21536

α 5caC

100	13.12517
100	10.72079

Figure S20. Dot Blot Assay showing the oxidation of 5-hmC in the dsDNA to 5-fC and 5-caC. A) 10 ng pure 5-hmC containing dsDNA (Left) and 10 ng 5-hmC containing dsDNA with the reaction mixture of Fe^{III}bTAML and H_2O_2, where the intensity of the blot decreased by ~60% (Right), treated with primary antibody against the 5-hmC. B) 10 ng pure 5-fC containing dsDNA (Left) and 10 ng 5-hmC containing dsDNA with the reaction mixture of Fe^{III}bTAML and H_2O_2, showing significant amounts of formation of 5-fC (Intensity appeared by ~37% wrt standard) (Right), treated with primary antibody against 5-fC. C) 10 ng pure 5-caC containing dsDNA (Left) and 10 ng 5-hmC containing dsDNA with the reaction mixture of Fe^{III}bTAML and H_2O_2, showing minor amounts of formation of 5-caC (Intensity appeared by ~10% wrt standard) (Right), treated with primary antibody against 5-caC.

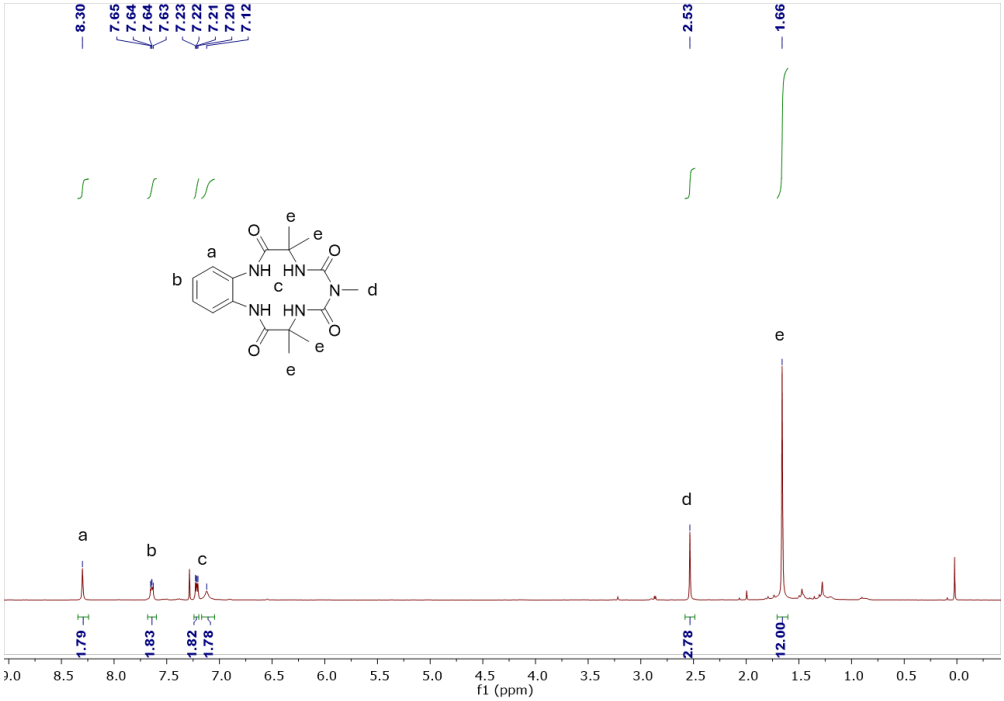


Figure S21: ^{1}H NMR spectra of bTAML.

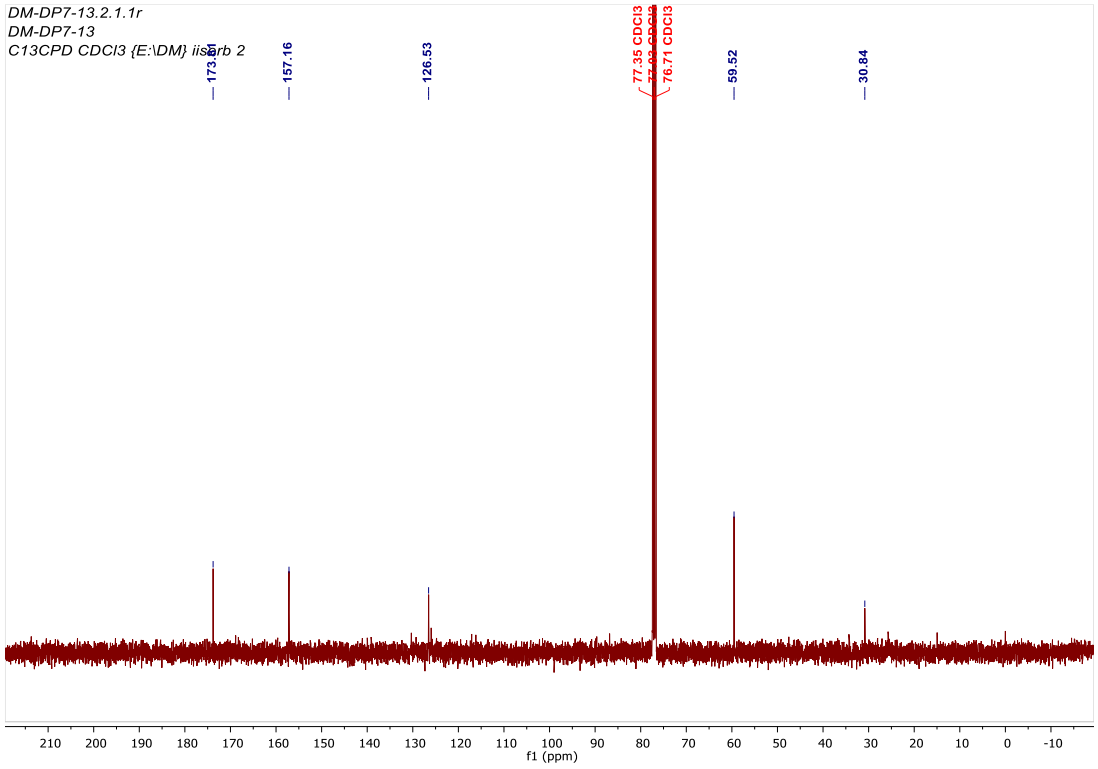


Figure S22: ^{13}C NMR spectra of bTAML.

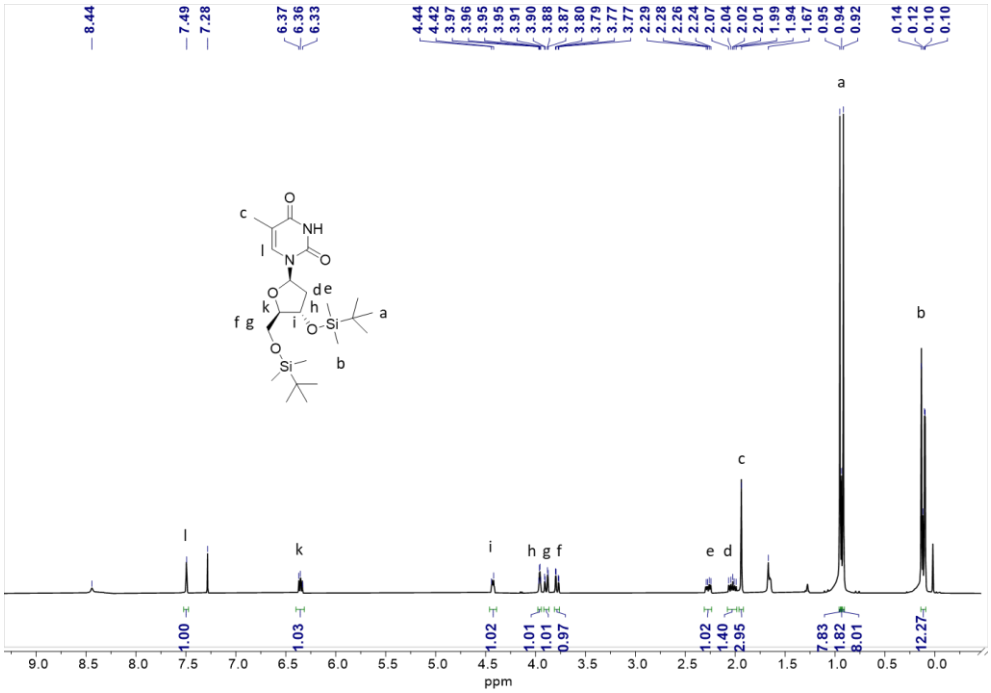


Figure S23: ^{1}H NMR spectra of 1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy) methyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H) dione (2).

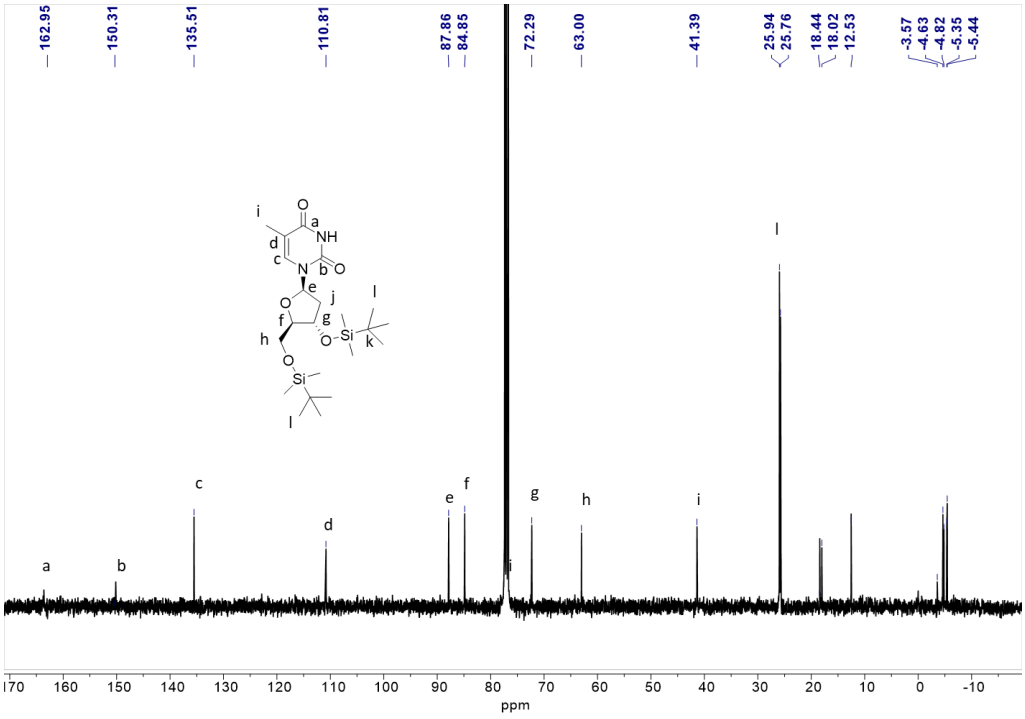


Figure S24: ^{13}C NMR spectra of 1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H) dione (2).

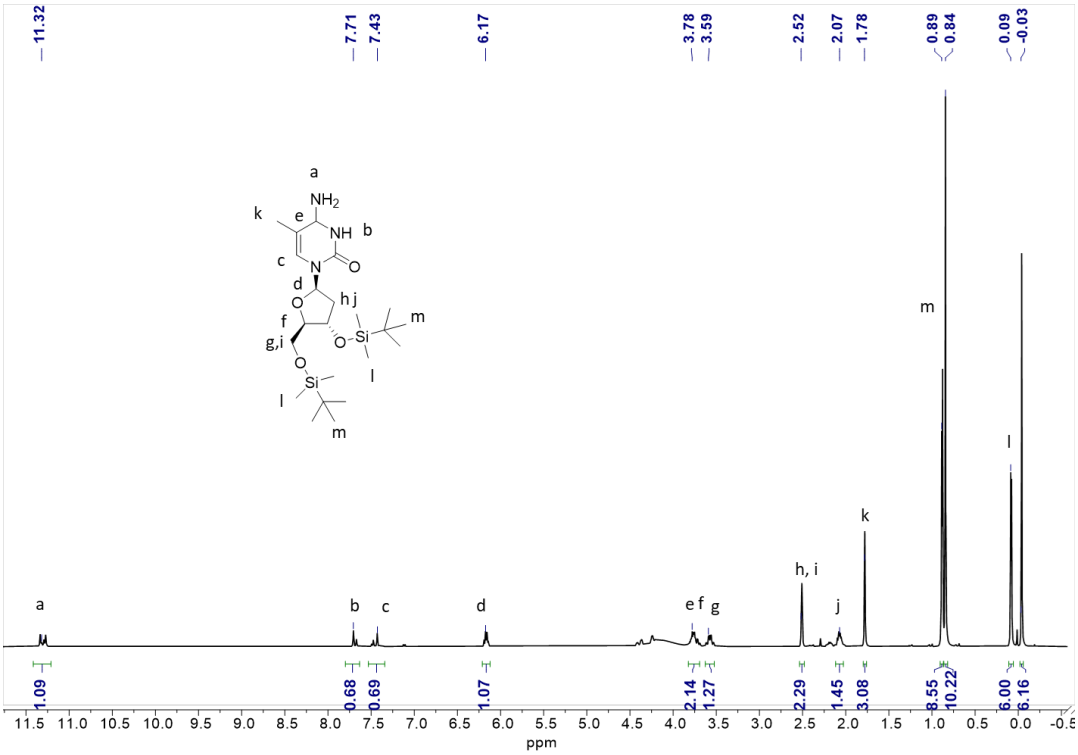


Figure S25: [1]H NMR spectra of 4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-methylpyrimidin-2(1H)-one (3)

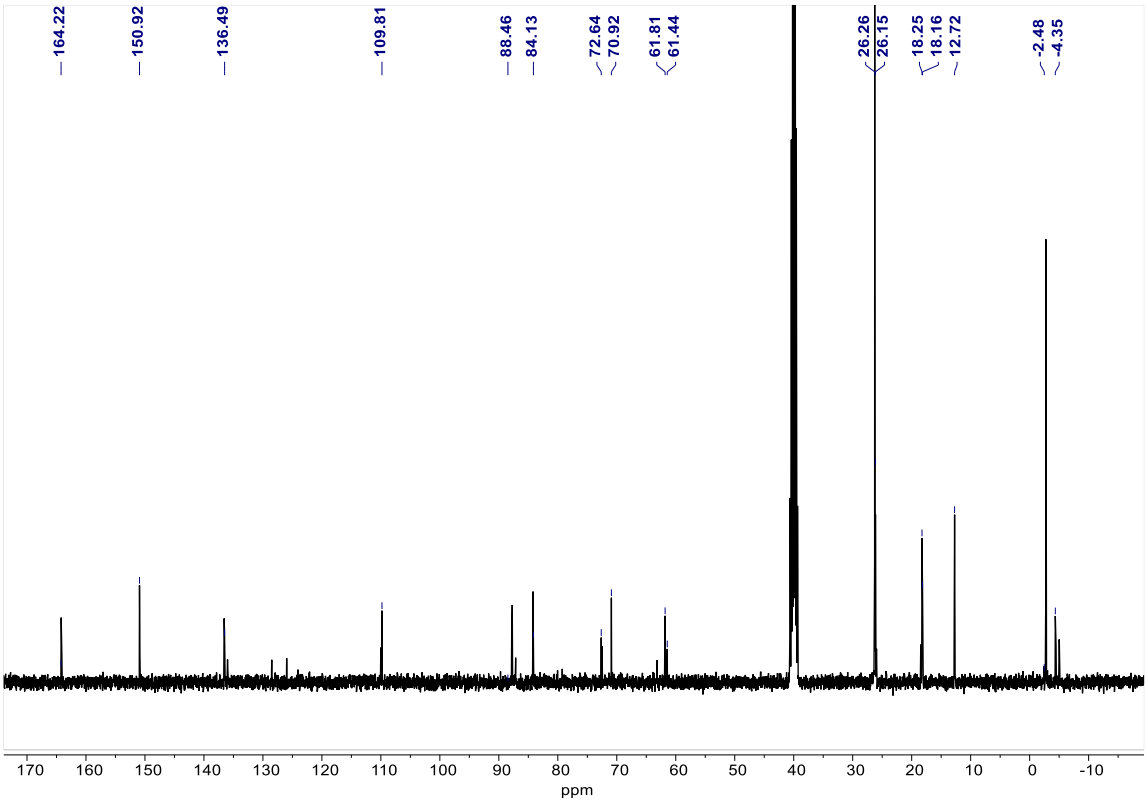


Figure S26: [13]C NMR spectra of 4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-methylpyrimidin-2(1H)-one (3)

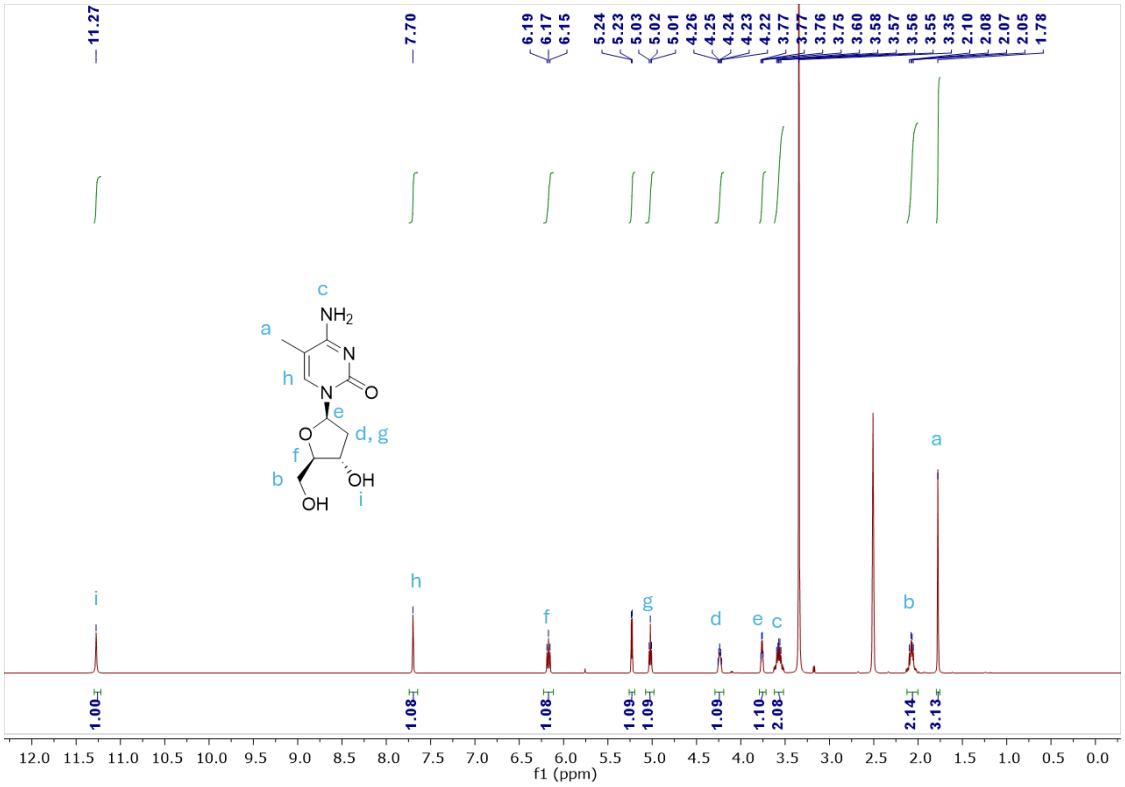


Figure S27: ^{1}H NMR spectra of 5-^{Me}dC.

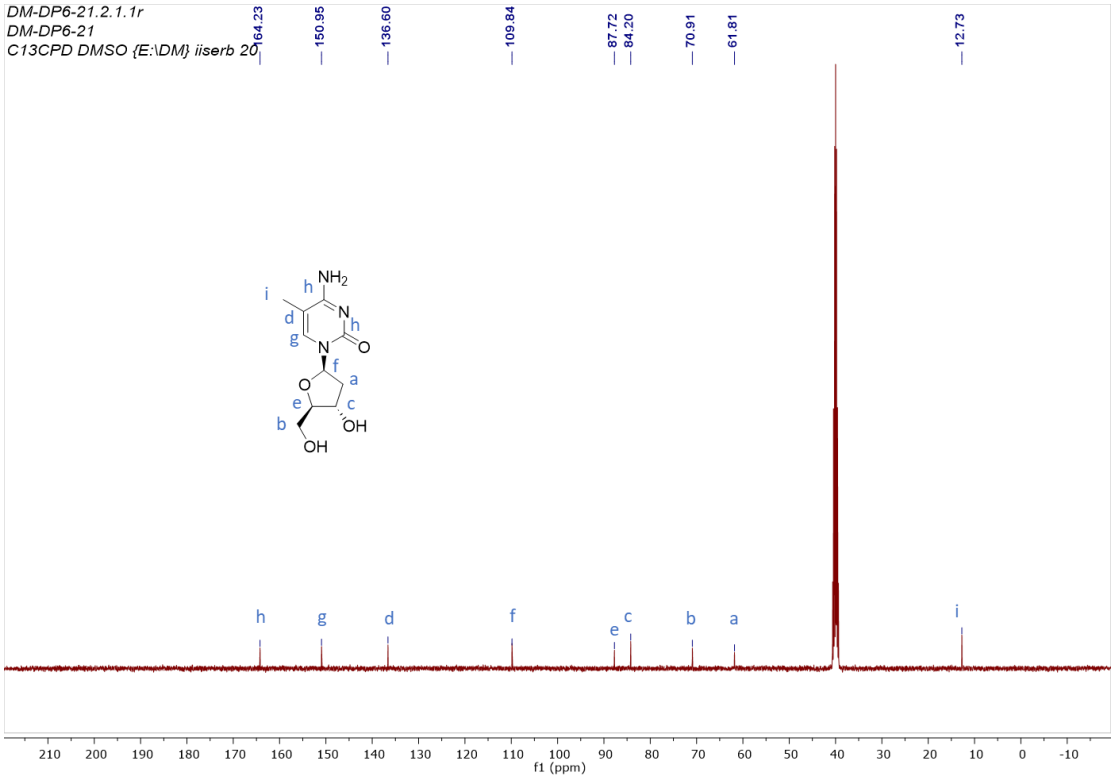


Figure S28: ^{13}C NMR spectra of 5-^{Me}dC.

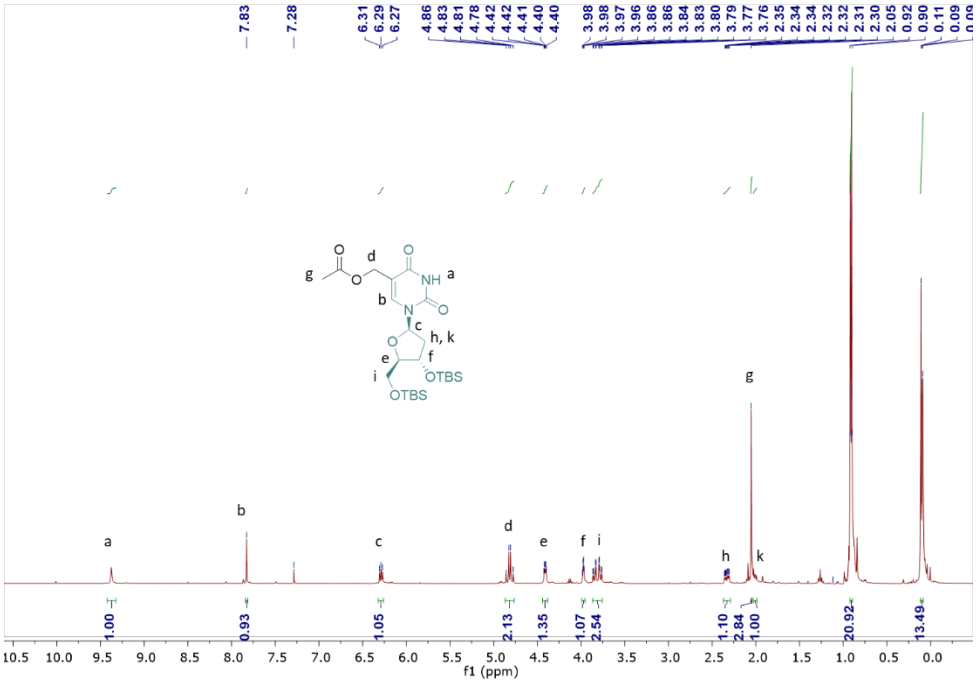


Figure S29: [1]H NMR spectra of (1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tertbutyldimethyl-silyl)oxy)methyl)tetra-hydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)methyl acetate (5)

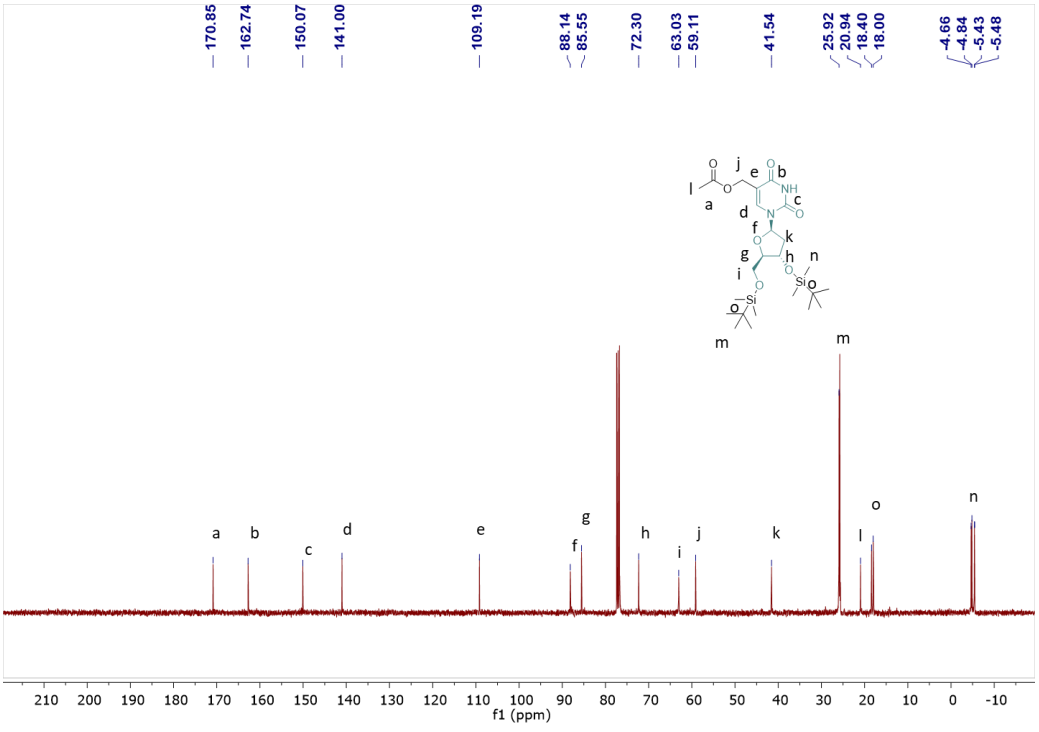


Figure S30: [13]C NMR spectra of (1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tertbutyldimethylsilyl)-oxy)methyl)tetra-hydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)methyl acetate (5)

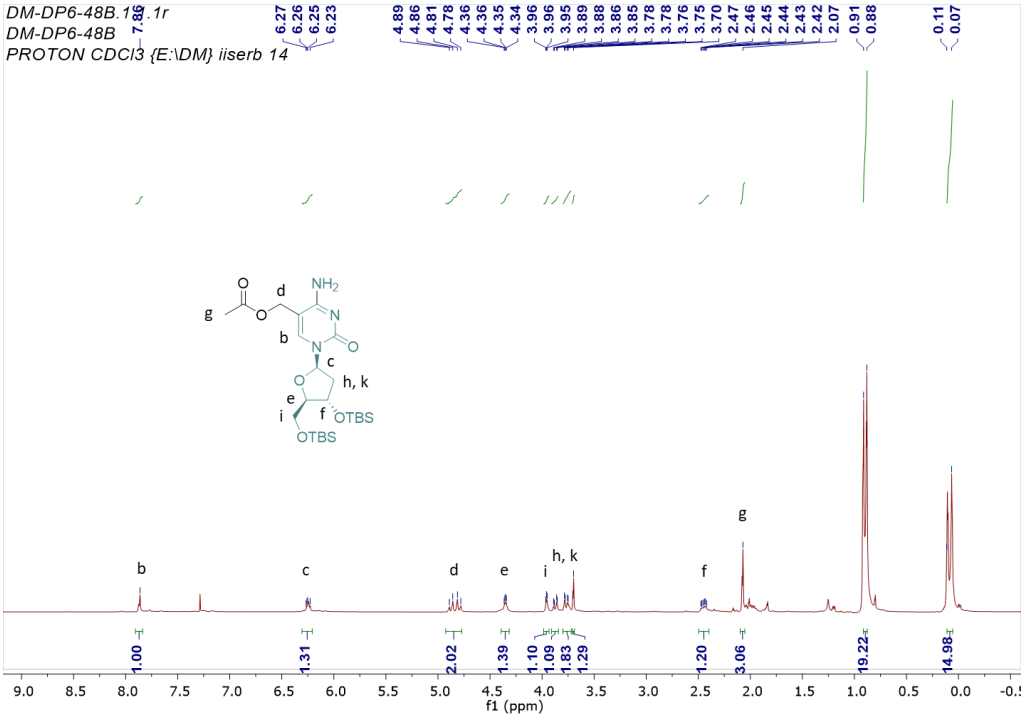


Figure S31: [1]H NMR spectra of (4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-5-yl)methyl acetate (6)

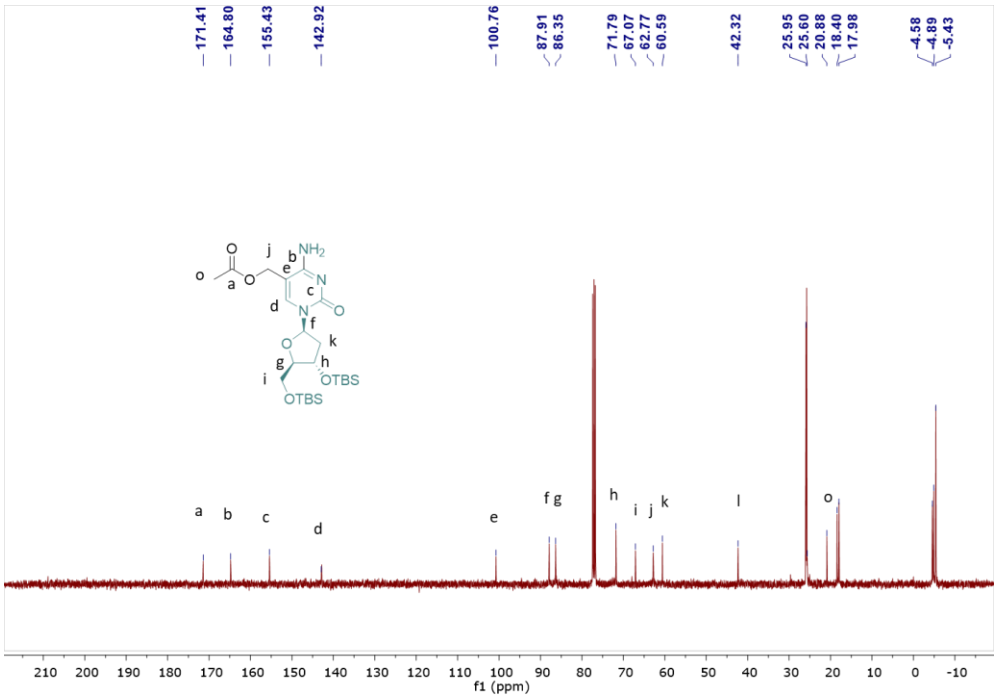


Figure S32: [13]C NMR spectra of (4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-5-yl)methyl acetate (6)

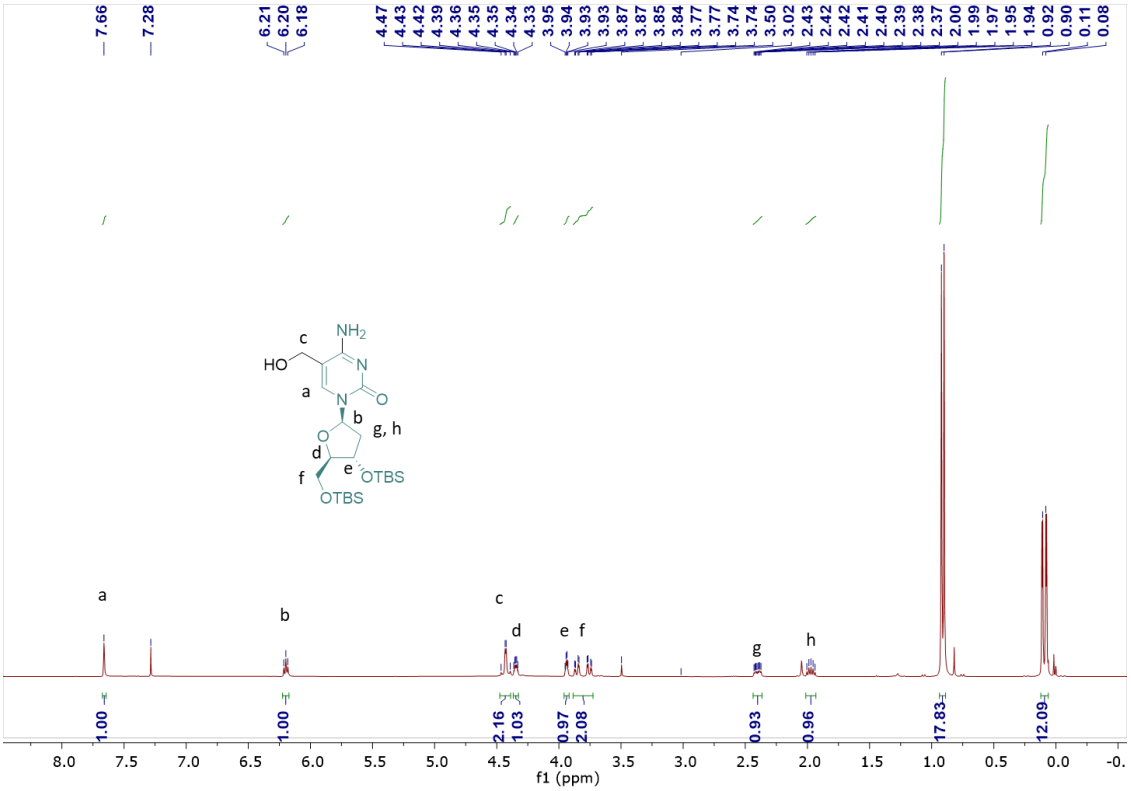


Figure S33: ^{1}H NMR spectra of 4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethyl-silyl)oxy)methyl)tetrahydrofuran-2-yl)-5-(hydroxymethyl)pyrimidin-2(1H)-one (7)

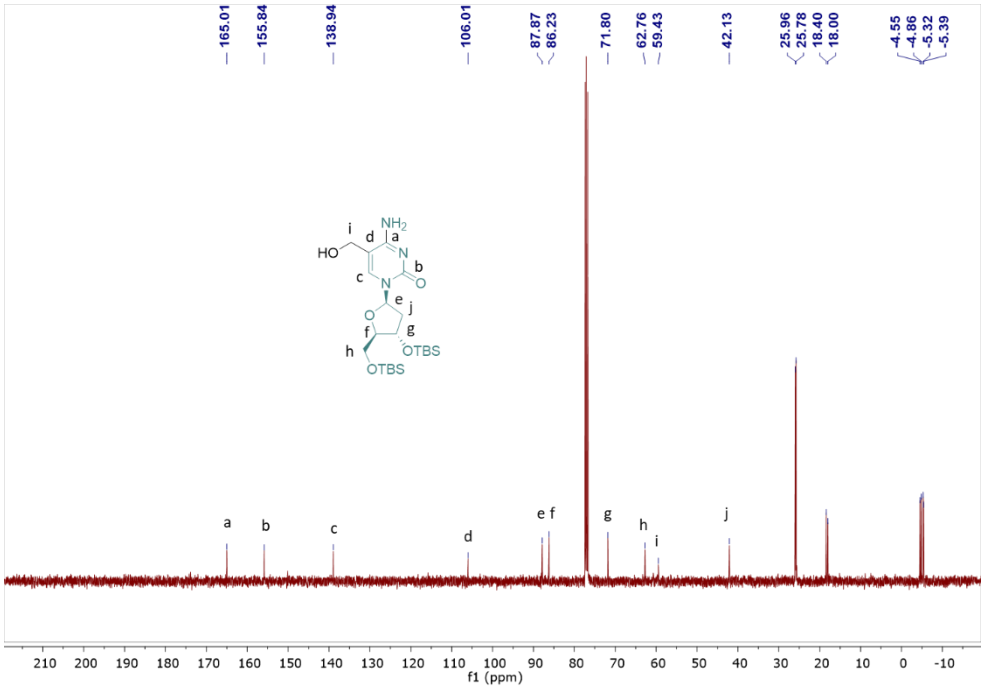


Figure S34: ^{13}C NMR spectra of 4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-(hydroxymethyl)pyrimidin-2(1H)-one (7)

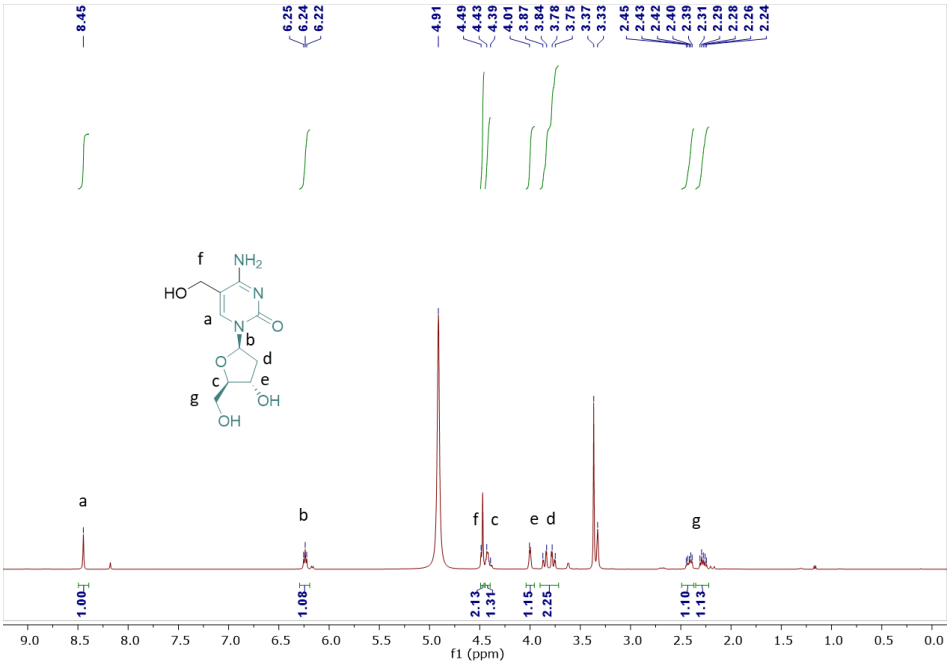


Figure S35: ^{1}H NMR spectra of 5-^{HOMe}dC.

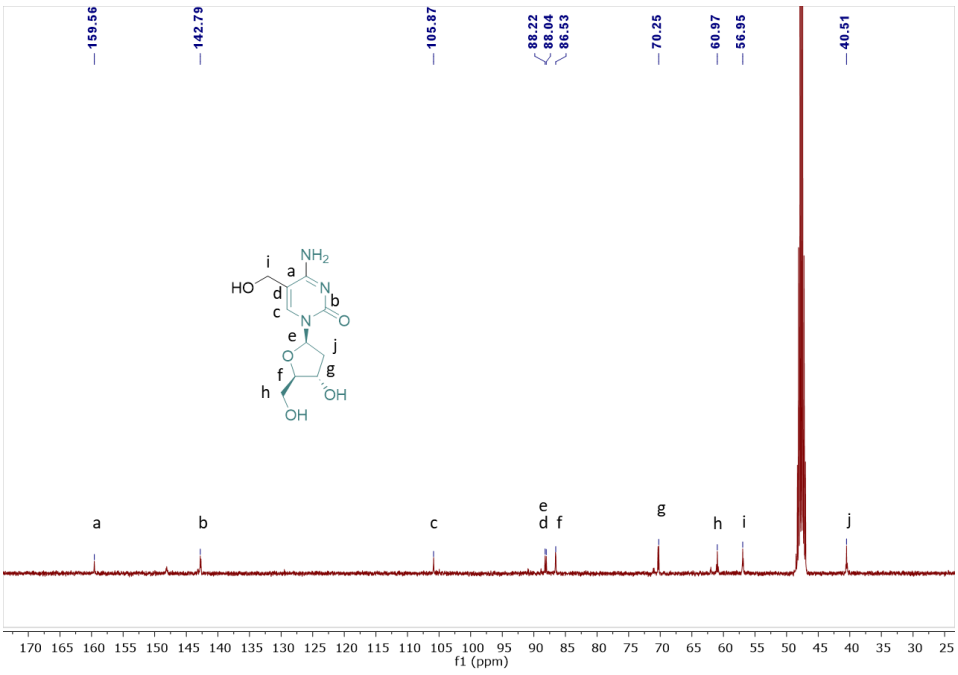


Figure S36: ^{13}C NMR spectra of 5-^{HOMe}dC.

Mass Spectrometry

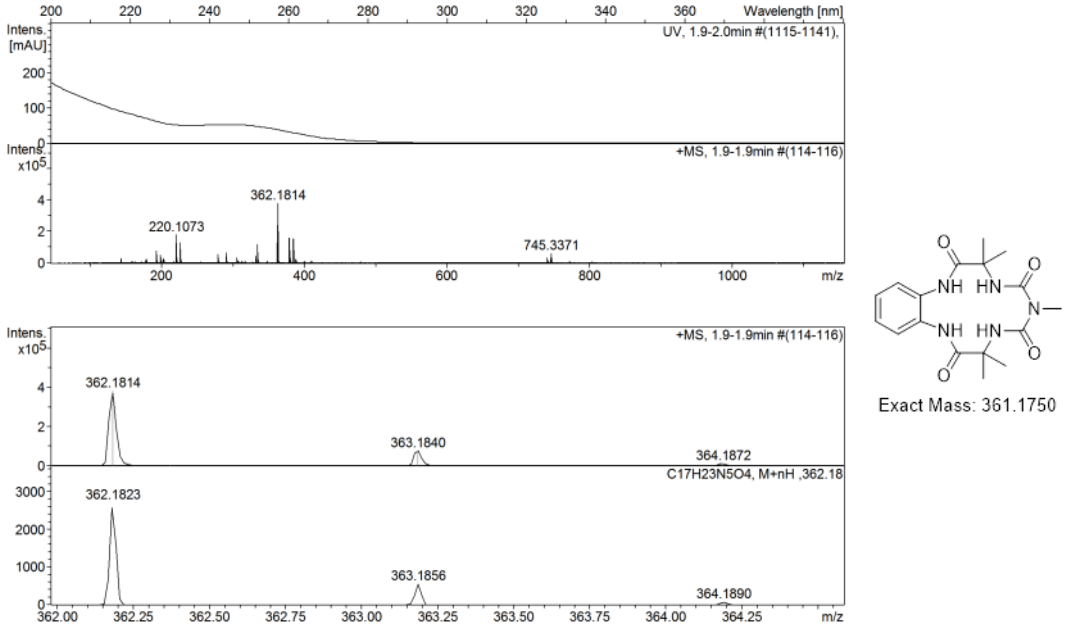


Figure S37. High resolution mass spectra of bTAML ligand. **ESI-HRMS** (M+H) Calculated: 362.1828, found: 362.1814.

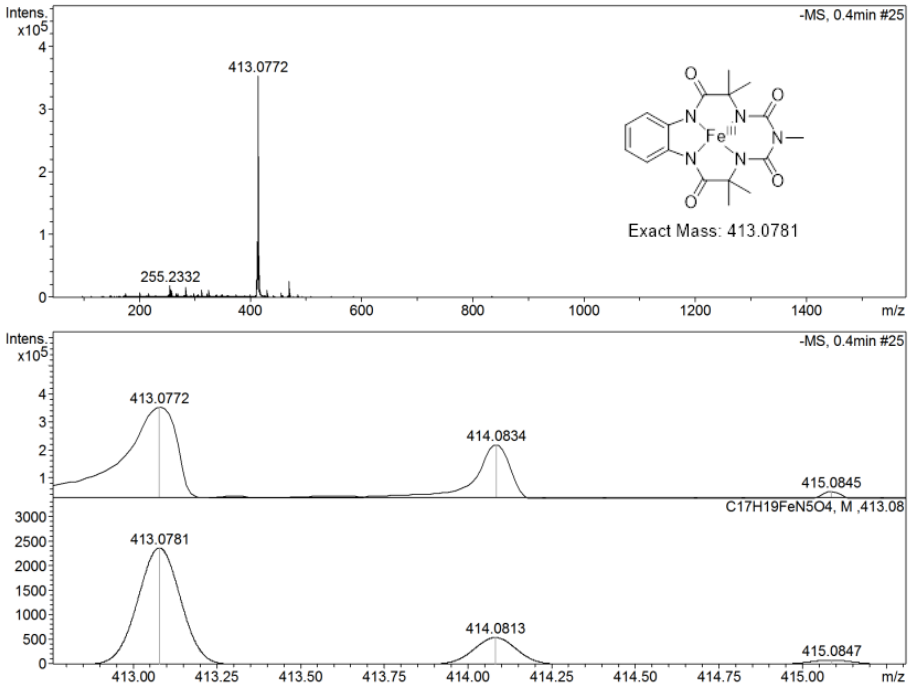
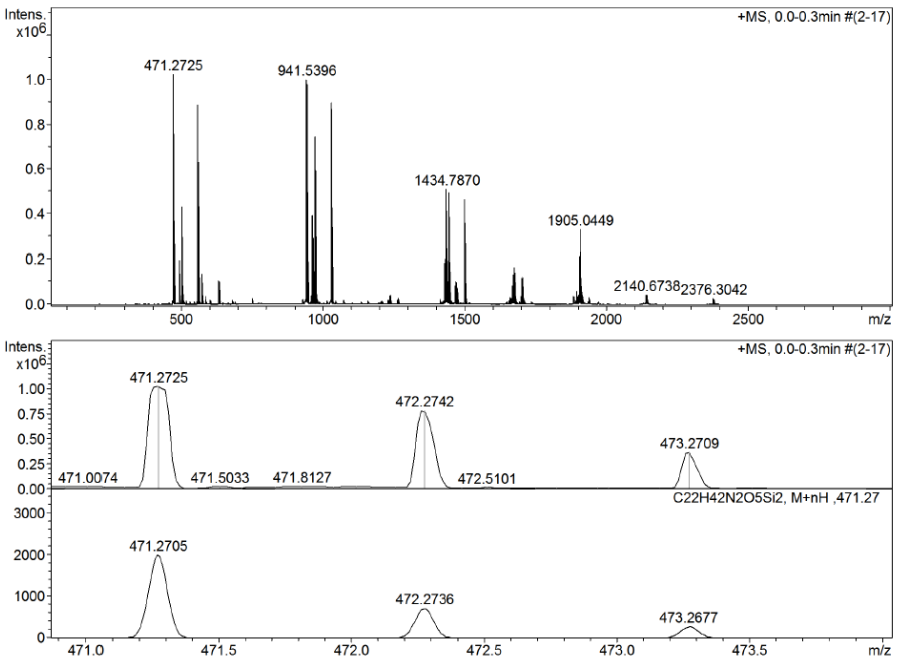


Figure S38. High resolution mass spectra of Fe^{III}bTAML complex. **ESI-HRMS** (negative ion mode) Calculated $[C_{17}H_{19}FeN_5O_4]^-$: 413.0781, found: 413.0772.



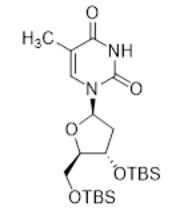
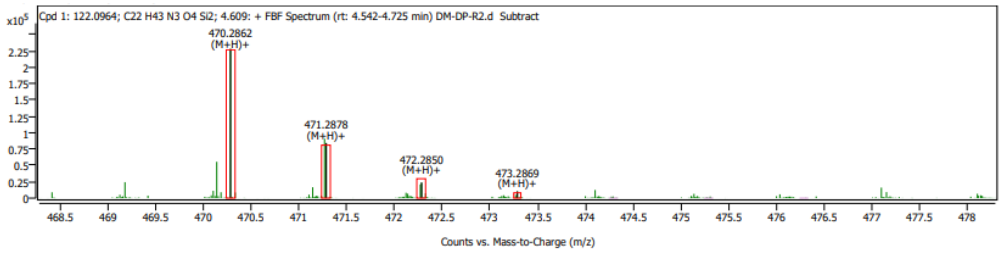


Figure S39. High resolution mass spectra 1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H) dione (2). **ESI-HRMS** (M+H) Calculated: 471.2711, found: 471.2725.



Spectrum Peaks

m/z	Z	Abund	Diff (ppm)	Height %	Height % (Calc)	Ion Species	Formula
470.2862	1	227999	-0.54	100.00	100.00	(M+H)+	C22H43N3O4Si2
471.2878	1	84136	-1.73	36.90	35.71	(M+H)+	C22H43N3O4Si2
472.2850	1	23741	-4.64	10.41	13.51	(M+H)+	C22H43N3O4Si2
473.2869	1	10349	-2.60	4.54	2.98	(M+H)+	C22H43N3O4Si2

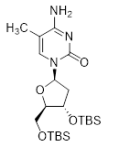


Figure S40. High resolution mass spectra of 4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-methylpyrimidin-2(1H)-one (3). **ESI-HRMS** (M+H) Calculated: 470.2870, found: 470.2862.

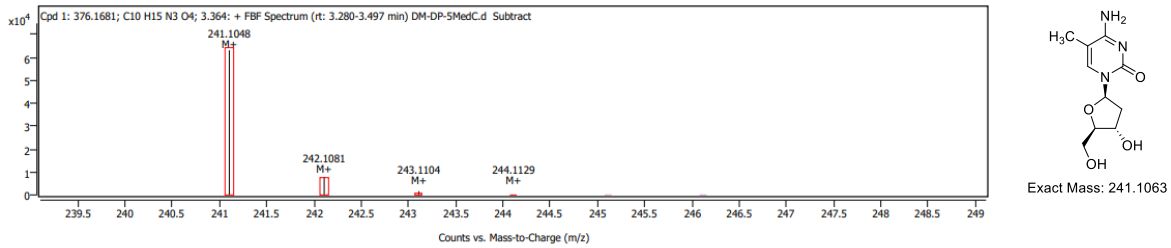


Figure S41: High resolution mass spectra of 5-^{Me}dC. **ESI-HRMS** (M+) Calculated: 241.1063, found: 241.1048.

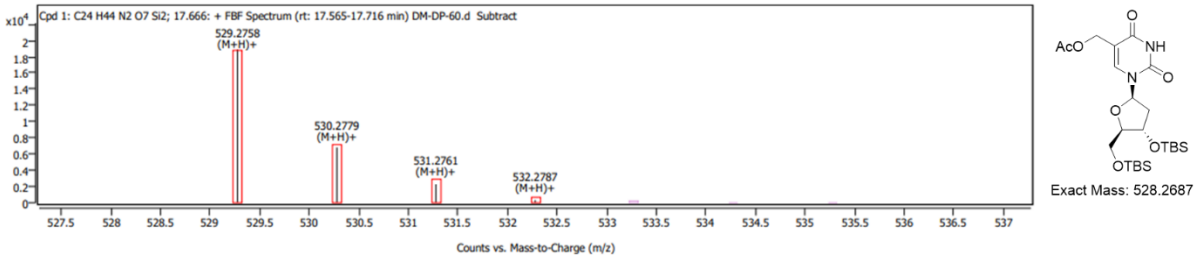


Figure S42: High resolution mass spectra of (1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tertbutyldimethylsilyl)oxy)methyl)tetra-hydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)methyl acetate (5). **ESI-HRMS** (M+H) Calculated: 529.2765, found: 529.2758.

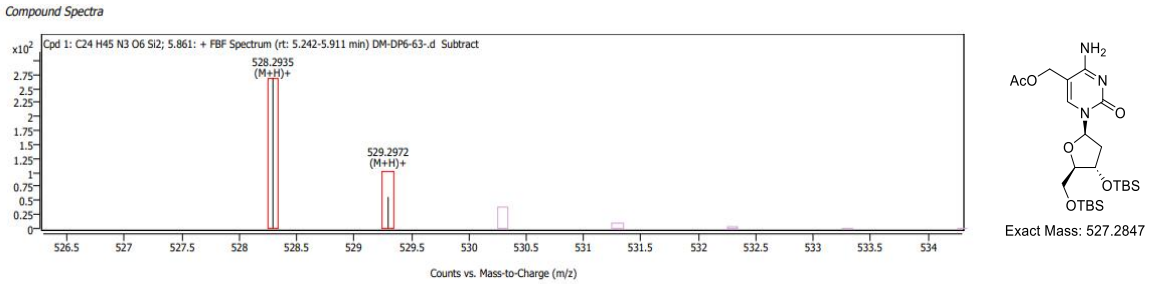


Figure S43: High resolution mass spectra of (4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)me-thyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-5-yl)methyl acetate (6). **ESI-HRMS** (M+H) Calculated: 528.2925, found: 528.2935.

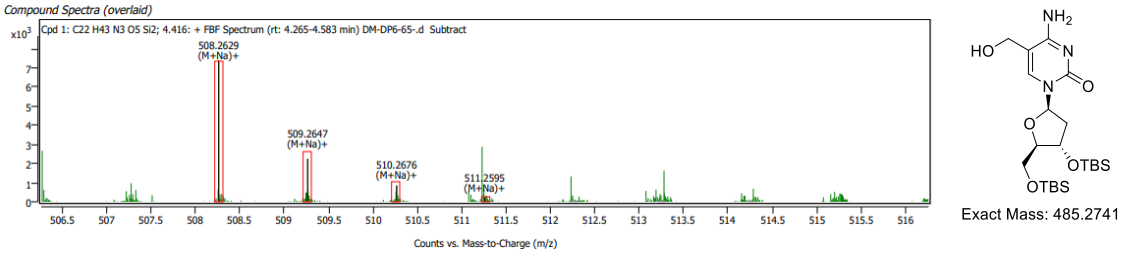


Figure S44: High resolution mass spectra of 4-amino-1-((2R,4S,5R)-4-((tert-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-5-(hydroxymethyl)pyrimidin-2(1H)-one (7). **ESI-HRMS** (M+Na) Calculated: 508.2639, found: 508.2629.

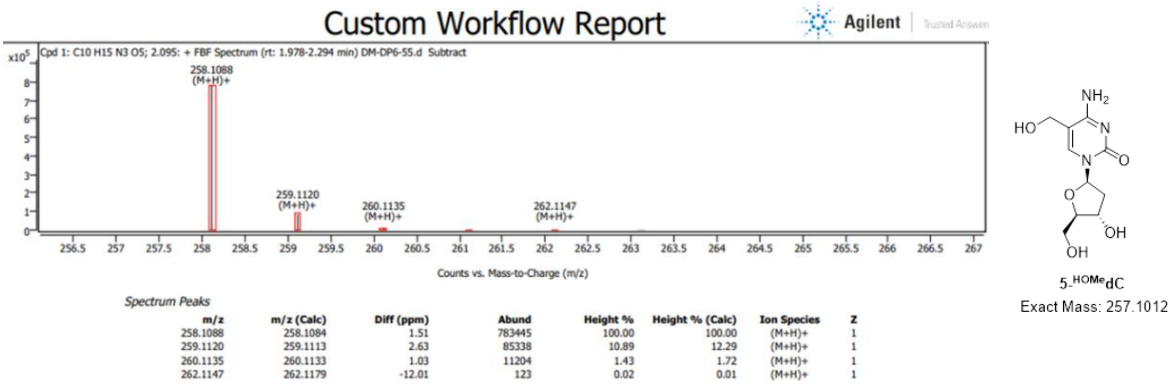


Figure S45: High resolution mass spectra of 5-^{MeOH}dC. **ESI-HRMS** (M+H) Calculated: 258.1090, found: 258.1088.

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