Allele-Specific Inhibition of Histone Demethylases

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([#] Equal contribution)

Contents

1. General materials, methods and equipment

Chemicals: All chemicals were purchased from established vendors (e.g. Sigma-Aldrich, Acros Organics) and used without purification unless otherwise noted. Optima grade acetonitrile was obtained from Fisher Scientific and degassed under vacuum prior to HPLC purification. All reactions to prepare N-oxalylglycine (NOG) analogues were carried out in round bottom flasks and stirred with Teflon®-coated magnetic stir bars under inert atmosphere when needed. Analytical thin layer chromatography (TLC) was performed using EMD 250 micron flexible aluminum backed, UV F254 pre-coated silica gel plates and visualized under UV light (254 nm) or by staining with phosphomolybdic acid, ninhydrin or anisaldehyde. Reaction solvents were removed by a Büchi rotary evaporator equipped with a dry ice-acetone condenser. Analytic and preparative HPLC was carried out on an Agilent 1220 Infinity HPLC with diode array detector. Concentration and lyophilization of aqueous samples were performed using Savant Sc210A SpeedVac Concentrator (Thermo), followed by Labconco Freeze-Dryer system.

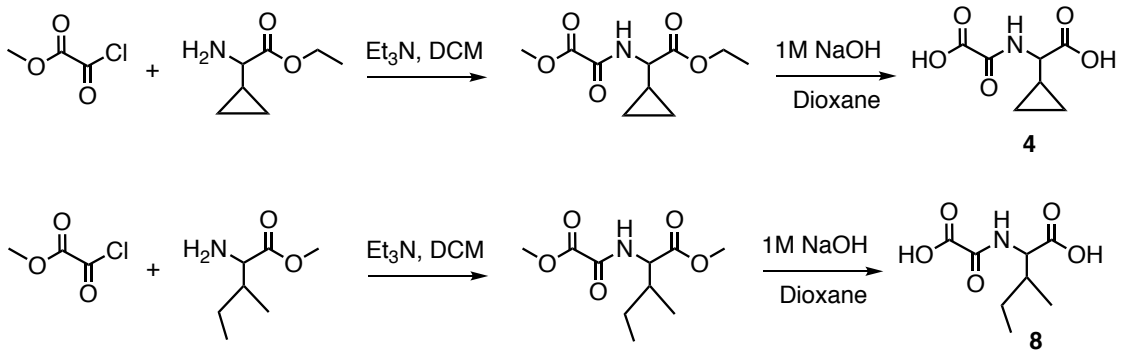
Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on Bruker Ultrashield^{TM} Plus 600/500/400/300 MHz instruments at 24 ^{o}C. Chemical shifts of ^{1}H and ^{13}C NMR spectra are reported as δ in units of parts per million (ppm) relative to tetramethylsilane (δ 0.0) or residual solvent signals: chloroform-d (δ 7.26, singlet), methanol-d4 (δ 3.30, quintet), and deuterium oxide-d2 (δ 4.80, singlet). Coupling constants are expressed in Hz. Mass spectra were collected at the UPITT MASSSPEC lab on a Q-ExactiveTM Thermo Scientific LC-MS with electron spray ionization (ESI) probe.

Plasmids, mutagenic primers, cell lines and antibodies: All the plasmids are for bacterial expression and obtained as gifts from individual laboratories or purchased from Addgene. Mutagenic primers are obtained from Integrated DNA Technologies (Table S1). Commercially purchased competent bacterial cells are used for protein expression and mutagenesis. All the antibodies used in the current study are purchased from established vendors and used following manufacturer's protocol.

2. Synthesis of NOG analogues

Scheme S1 depicts a general approach to the synthesis of NOG analogues. Details of the

synthetic method and characterization data for compounds **3**, **5**, **6**, **7**, **9** and **10** can be found in ref.[1],[2],[3] For NOG analogue **4** and **8**, commercially available ethyl ester of cyclopropyl-alanine and methyl esters of isoleucine were reacted with methoxyoxalyl chloride to obtain the ester derivative of the NOG analogues that were subsequently hydrolyzed to furnish the final compounds.



Scheme S1: Synthetic scheme for NOG analogues **4** and **8**

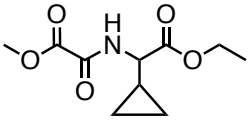
General method for coupling step:

To a solution of amino acid methyl ester hydrochloride (2 mmol) in 10 ml anhydrous DCM was added Et_3N (2.2 mmol) followed by methoxyoxalyl chloride (4.4 mmol) on ice bath. Then the reaction was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate, washed with 1N HCl, saturated Na_2CO_3 and brine. The organic layer was dried over Na_2SO_4, filtered and concentrated to give dimethyl ester derivative of NOG analogues as viscous liquid (67-80% yield).

General method for ester hydrolysis step:

Dimethyl ester derivative of NOG analogues (1.2 mmol) was dissolved in 3 ml dioxane and 6 ml 1M NaOH. The reaction was stirred overnight at room temperature. The reaction was acidified to pH 2 using 2N HCl and extract with iPrOH/DCM (2:1) six times. The organic layer was dried with Na_2SO_4, filtered and concentrated. The crude material was subsequently purified by HPLC to give NOG analogues 3 - 5 and 7 as white solids. The crude compound was dissolved in deionized H2O and filtered through 0.2µM syringe filter. NOG analogues were purified with preparative reversed-phase HPLC (XBridge™ Prep C18 5µm OBD™ 10×250mm) eluting at a flow rate of 4 mL/min. The purification was monitored at 220 nm eluting with acetonitrile (linear

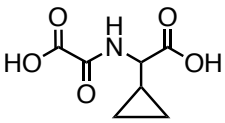
gradient to 80% in first 10 mins) in aqueous trifluoroacetic acid (0.1%). The peaks were concentrated by SpeedVac for 2h followed by lyophilization overnight. The dried product was re-dissolved in 0.1% TFA (v/v). The concentration of NOG analogues was measured using the standard curve generated for NOG **2**, or the extinction coefficients for aromatic NOGs and stored in -80 °C for further use.



^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58 (br, 1H), 4.16 (q, J = 7.2 Hz, 2H), 3.91 (s, 3H), 1.66-1.63 (m, 2H), 1.26-1.22 (m, 5H);

^{13}C NMR (100 MHz, $CDCl_3$): δ 171.0, 160.9, 156.9, 61.7, 53.6, 33.7, 17.2, 14.2, 14.1.

ESI-HRMS: m/z calcd. for $C_9H_{14}NO_5$ $[M+H]^+$ 216.08665, found 216.08719.



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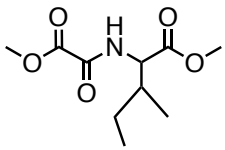
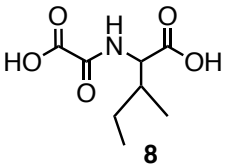
^{1}H NMR (400 MHz, DMSO-d_6): δ 12.67 (br, 1H), 9.45 (br, 1H), 1.37-1.36 (m, 2H), 1.08-1.08 (m, 2H);

^{13}C NMR (100 MHz, DMSO-d_6): δ 173.3, 161.4, 158.3, 33.2, 16.8.

ESI-HRMS: m/z calcd for $C_6H_6NO_5$ $[M-H]^-$ 172.02405, found 172.02496.



^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53 (d, J = 7.6 Hz, 1H), 4.62-4.59 (m, 1H), 3.92 (s, 3H), 3.77 (s, 3H), 2.00-1.96 (m, 1H), 1.47 (m, 1H), 1.24-1.21 (m, 1H), 0.96-0.92 (m, 6H).



8

^{1}H NMR (300 MHz, DMSO-d_6): δ 13.38 (br, 2H), 8.56 (d, J = 8.4 Hz, 1H), 4.19-4.14 (m, 1H), 1.92-1.90 (m, 1H), 1.44 (m, 1H), 1.22-1.16 (m, 1H), 0.89-0.86 (m, 6H).

ESI-HRMS: m/z calcd for $C_8H_{14}NO_5$ $[M+H]^+$ 204.08665, found 204.08768.

3. Synthesis and purification of peptides

The H3K9Me$_3$ peptide was synthesized by the University of Pittsburgh Peptide Synthesis Facility (Table S2). Analytical-scale separation was performed using ZORBAX reversed-phase C18 (5μm, 4.6 × 250 mm) column with UV detection at 280 nm. The column was equilibrated with 0.1% aqueous trifluoroacetic acid solution prior to each injection. Analytical separation was performed with a linear gradient of acetonitrile to 10% in 15 min and then to 70% in 5 min in

0.1% aqueous trifluoroacetic acid with a flow rate of 1 mL/min. The crude peptides were purified using preparative reversed-phase HPLC (XBridge C18, 5 μm, 10 x 250 mm column) eluting with a flow rate of 5 mL/min and a gradient of acetonitrile starting from 0% to 90% in 15 min and then to 100% in 18 min in aqueous trifluoroacetic acid (0.01%). The purified peptides were first concentrated by SpeedVac concentrator followed by lyophilization. The dried peptides were resuspended in water containing 0.01% TFA and stored at -80°C before use. Concentrations of the peptides were determined based on the observation that 1 mg/ml peptide generates an absorbance value (A_{205}) of 30 at 205 nm. The integrity of the purified peptide was confirmed by MALDI mass spectrometry.

4. Mutagenesis, expression and purification of KDM4A

The N-terminal 6xHis-tagged human KDM4A-jmjC domain (catalytic domain of KDM4A) bacterial expression construct pNIC28-Bsa4 (Addgene ID: 38846) was obtained from Addgene. The wild type KDM4A plasmid was transformed into E. Coli BL21 (DE3) Star competent cells (Invitrogen) using pNIC28-Bsa4 kanamycin-resistant vector. A single colony was picked up and grown overnight at 37 °C in 10 mL of Luria-Bertani (LB) broth in the presence of 50 μg/mL kanamycin.[4] The culture was diluted 100-fold and allowed to grow at 37 °C to an optical density (OD600) of 0.8, and protein expression was induced overnight at 17 °C with 0.6 mM IPTG in an Innova 44® Incubator shaker (New Brunswick Scientific). Proteins were purified as follows: harvested cells were resuspended in 15 mL lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 25 mM imidazole, Lysozyme, DNase, and Roche protease inhibitor cocktail). Resuspended cells were lysed by pulsed sonication (Qsonica-Q700) then centrifuged at 13000 rpm for 40 min at 4 °C. The soluble extracts were subject to Ni-NTA agarose resin (Thermo) according to manufacturer's instructions. After passing 20 volumes of washing buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 25 mM imidazole), proteins were eluted with a buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 400 mM imidazole. Proteins were further purified by size exclusion chromatography (Superdex-200) using AKTA pure FPLC system (GE healthcare) with buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, and 10% glycerol. Purified proteins were concentrated using Amicon Ultra-10k centrifugal filter device (Merck Millipore Ltd.). The protein concentration was determined using

Bradford assay kit (BioRad Laboratories) with BSA as a standard. The concentrated proteins were stored at -80°C before use. KDM4A variants (N198A/G and S288A/G) were generated using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). The resulting mutant plasmids were confirmed by DNA sequencing. The mutants were expressed and purified as stated for KDM4A-WT.

5. Expression and purification of Formaldehyde Dehydrogenase (FDH)

The N-terminal 6xHis-tagged P. putida FDH bacterial expression construct in pET28 vector was obtained from the Bhagwat laboratory at the Wayne State University.[5] The plasmid was transformed into E. coli Rosetta [DE3] competent cells. A single colony was picked up and grown overnight at 37 °C in 10 mL of Luria-Bertani (LB) broth in the presence of 50 µg/mL kanamycin and 35 µg/mL chloramphenicol. The culture was diluted 100-fold and allowed to grow at 37 °C to an optical density (OD600) of 0.8, and protein expression was induced overnight at 18 °C with 1 mM IPTG in an Innova 44® Incubator shaker (New Brunswick Scientific). Proteins were purified as follows: harvested cells were resuspended in 15 mL lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 25 mM imidazole, Lysozyme, DNase, and Roche protease inhibitor cocktail). The cells were lysed by pulsed sonication (Qsonica-Q700), and centrifuged at 13000 rpm for 40 min at 4 °C. The soluble extracts were subject to Ni-NTA agarose resin (Thermo) according to manufacturer's instructions. After passing 20 volumes of washing buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 25 mM imidazole), proteins were eluted with S16 a buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 400 mM imidazole. Proteins were further purified by size exclusion chromatography (Superdex-200) using AKTA pure FPLC system (GE healthcare) with buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, and 10% glycerol. Purified proteins were concentrated using Amicon Ultra-10k centrifugal filter device (Merck Millipore Ltd.). The protein concentration was determined using Bradford assay kit (BioRad Laboratories) with BSA as a standard. The concentrated proteins were stored at -80°C before use.

6. Expression and purification of histone H3 and its mutants

Gene sequence encoding wild type *Xenopus laevis* histone H3 was a kind gift from Prof. Minkui

Luo at the Memorial Sloan-Kettering Cancer Center. The plasmid containing histone H3-C110A was transformed into BL21 codon plus (DE3) RIPL competent cells. A single colony was picked up and grown overnight at 37 ^{o}C in 10 mL of LB broth with 100 µg/mL ampicillin and 35 µg/mL chloramphenicol. The inoculation culture was diluted 1:100 fold in fresh LB medium and cells were grown at 37 ^{o}C until OD_{600} reached to ~0.7. Protein expression was induced by the addition of 0.3 mM IPTG followed by growing for an additional 3 h at 37^{o}C. Cells were harvested by centrifugation at 5000 rpm for 30 min, and then resuspension of the pellet in 5 mL of lysis buffer (10 mM Tris-HCl pH 7.5, 2 M guanidinium hydrochloride (GdnHCl), 5 mM β-mercaptoethanol, 10% glycerol, DNase, Lysozyme and Roche protease inhibitor cocktail). The cells were lysed by pulsed sonication and centrifuged at 20,000g for 40 min at 4^{o}C. Insoluble histone was recovered from inclusion bodies by dissolving in 6 M GdnHCl and 10 mM Tris-HCl pH 7.5, and incubated for 10 min at room temperature followed by centrifugation at 20,000g for 40 min at 4 ^{o}C. The soluble histone supernatant was purified by size exclusion chromatography on a Superdex-200 using AKTA pure FPLC system.[6] Fractions were concentrated using Amicon Ultra-4 centrifugal 3K filter and further purified with preparative reverse-phase HPLC (XBridge C18, 5 µm, 10 x 250 mm column) eluting with a flow rate of 4 mL/min starting from 10% acetonitrile to 70 % in 15 min and then to 100 % over 5 min in aqueous trifluoroacetic acid (0.01%). The purified protein was concentrated by SpeedVac followed by lyophilization. The protein was stored at -80^{o}C before use.

Histone H3 mutant K9C ($H3K_C9$) was generated by the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies) following manufacturer's protocol. The resulting mutant plasmid was confirmed by DNA sequencing. $H3K_C9$ was expressed and purified as described above for the wild type Histone H3.

7. MALDI demethylase activity assay

To monitor enzymatic activity, a demethylase activity assay was optimized and then observed through MALDI-TOF MS.[4] Each histone demethylase assay sample included 10 µM enzyme, 10 µM peptide, 1 mM 2KG or 80 µm 2KG, 50 mM Tris pH 8, 50 µM $(NH_4)_2Fe(SO_4)_2$, and 2 mM L-ascorbic acid with a total assay volume of 20 µl. Fe(II) supplement and L-ascorbic acid were prepared freshly. The 2KG and peptide was added last to the assay sample and briefly centrifuged. The samples were incubated at 37°C for three hours. To observe demethylase

activity, 0.7 μL of assay sample was applied to the MALDI plate followed by 0.7 μL of CHCA matrix. The sample was analyzed using Voyager on the Reflector Positive mode. The negative control included all components of the assay except for the demethylating enzyme. For KDM4 inhibition screening studies, the demethylation protocol remains the same as for the activity, but 100 μM inhibitor was added to the samples which were then incubated on ice ten minutes prior to the addition of 2KG (50 μM) or peptide. Inhibition studies were incubated at 37°C for four minutes, based on a time-dependence assay and kinetic constants. For IC_{50}, a series of inhibitor concentrations were adjusted to cover 100% to <10% activity. Activity was calculated on a weighted basis such that:

$\%Activity = (Area_{monomethylated}*2+Area_{dimethylated})/(Area_{monomethylated}*2+ Area_{dimethylated} +Area_{trimethylated})$.

8. Coupled fluorescence assay

To determine the catalytic efficiency of the wild type and engineered KDM4A-2KG pairs, the demethylase activity was measure by fluorescence intensity of accumulated NADH by employing a coupled fluorescence intensity assay.[5],[7] The experiments were performed in 384-well white Corning plates. The assay was composed of an enzyme cocktail (50 mM HEPES pH 8.0, 50 μM $(NH_4)_2Fe(SO_4)_2$, 1 mM L-ascorbic acid, 1 mM NAD^+, 200 nM FDH, and 1 μM KDM4A, KDM4A-N198G, KDM4A-N198A, KDM4A-S288A, or KDM4A-S288G and a substrate cocktail (0-750 μM 2KG/analogue and 300 μM H3K9Me_3 peptide). The substrate cocktail was first applied to the microplate followed by the enzyme cocktail. The microplate was centrifuged at 3000 rpm for one minute prior to measurement to remove air bubbles and ensure mixing. The accumulation of NADH was measured by fluorescence intensity (excitation= 340 nm, emission= 490 nm) every 30 seconds over a period of 15 minutes on a TECAN Infinite M1000Pro. An optimized gain for the instrument was obtained within the minimum and maximum NADH produced by the assay. The data was analyzed using Graphpad Prism software and converted to μM of H3K9Me_3 demethylated using the NADH calibration curve. Only points within the linear range were used to calculate the slope for each 2KG concentration and the values were fitted to the Michaelis-Menten equation to get the K_M and k_{cat} values. Experiments were performed in duplicates.

9. NADH calibration curve

To convert the relative fluorescence unit (RFU) values collected in the coupled fluorescence intensity assay, an NADH calibration curve was generated by measuring fluorescence intensity[6]. NADH (ACROS, cat #124530050) was added to assay buffer (50 mM HEPES pH 8, 50 μM S18 (NH4)2Fe(SO4)2, 1 mM L-ascorbic acid, 1 mM NAD^+, 200 nM FDH, and 1 μM KDM4A) in varying concentrations (0-10 μM). The experiments were performed in 384-well white Corning plates. The fluorescence intensity was measured with excitation = 340 nm and emission = 490 nm on a TECAN Infinite M1000Pro. Triplicate values were taken for each concentration.

10. Chemical trimethylation of $H3K_C9$

For the generation of trimethylated $H3K_C9Me_3$, 1 mg of lyophilized histone was dissolved in 98 ml of alkylation buffer (4 M Guanidine-Hydrochloride, 1 M HEPES pH 7.8, 10 mM D/L-methionine).[8],[9] Once histone was fully dissolved, 2 ml of 1 M DTT (prepared fresh) was added to the histone solution followed by 1 h incubation at 37 ^oC. The solution was then added to 10 mg of (2-bromoethyl) trimethyl ammonium bromide (Sigma, cat# 117196) and protected from light. After incubating for 2.5 h at 50 ^oC (gently agitating every 30 min), 1 ml of 1 M DTT was added to quench the reaction. The solution incubated at 50 ^oC for an additional 2.5 h. The reaction was quenched by adding 5 ml β-mercaptoethanol (β-ME). A PD10 (GE, cat# 17-0851-01) column was used to desalt the solution and the histones were eluted with 2 mM β-ME. SDS-PAGE was performed to determine presence of the histone protein. The samples were lyophilized and then resuspended in 50 mM Tris pH 8.0. Protein concentration was determined by Bradford assay and the molecular weight was verified by LC-MS. Protein was stored in -80 ^oC until used in demethylase activity assay.

11. Demethylation assay with $H3K_C9Me_3$

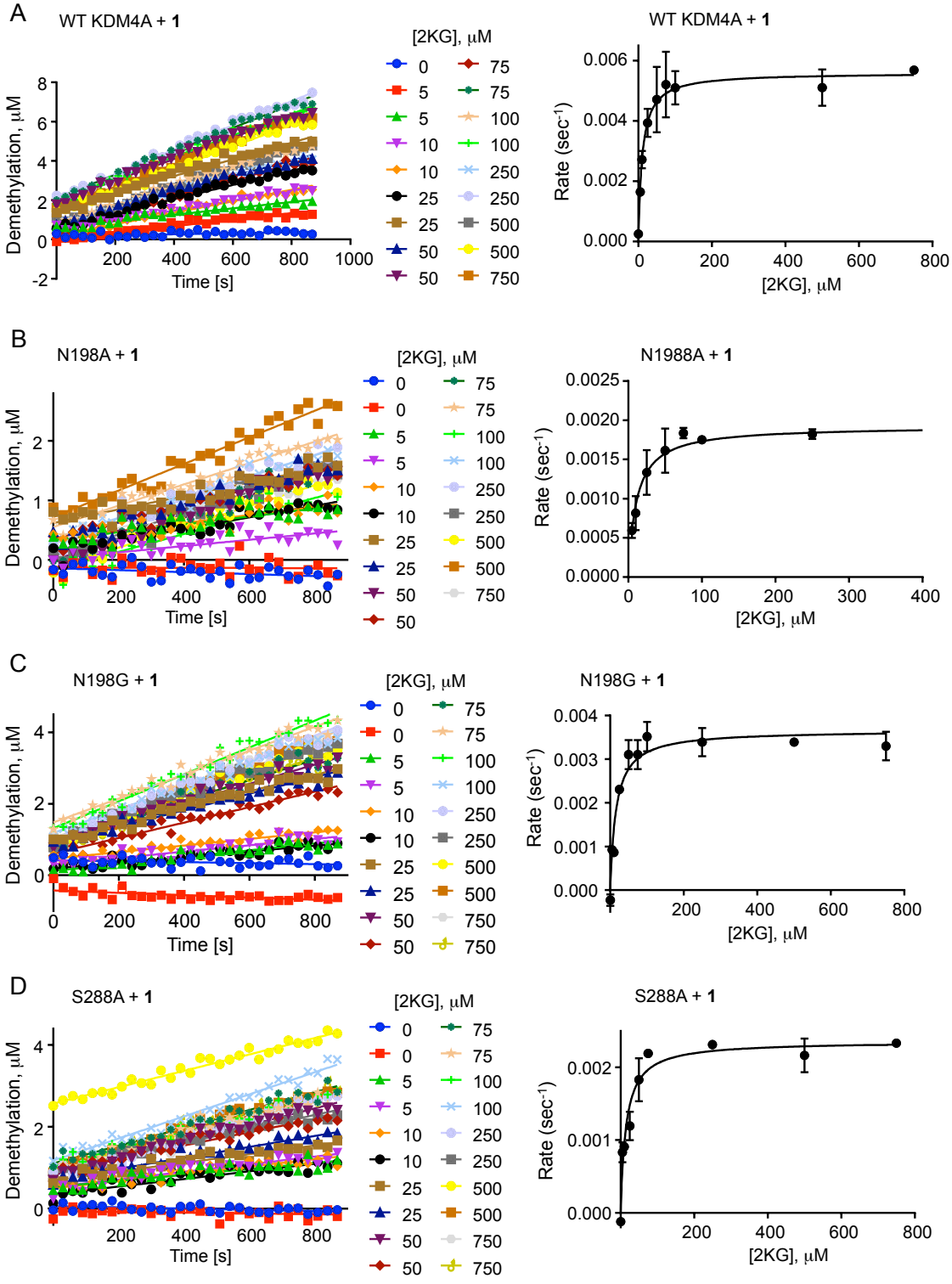
10 μM of each enzyme N198A and N198G was incubated with 200 μM of inhibitor (ethyl NOG, leucine NOG or propyl NOG) in 2 mM ascorbic acid and 50 μM ammonium ferrous (II) sulphate hexahydrate for 10 mins at 4^oC in 50 mM tris pH 8.0.[4] The assay was initiated by adding 100 μM 2KG and 20 μM $H3K_C9Me_3$ and incubated at 37^oC for 4 mins. Equal amounts of assay was loaded on 4-12% Bis-Tris SDS PAGE (Criterion XT Precast Gel, Bio-Rad) and transferred to 0.45 μm PVDF membrane (Immobilon-P). Immunoblotting was performed with 1: 1000 dilution

for H3 c-terminal (Invitrogen, 701517), H3K9me$_2$ (Active Motif, 39683) and H3K9Me$_3$ (Active Motif, 61013) primary antibodies over-night at 4^{o}C. Membranes were washed in TBST buffer (3x5 mins) and incubated with 1: 5000 dilution of HRP conjugated anti-mouse IgG (CST, 7076S) and 1:10000 of HRP-conjugated goat anti-rabbit IgG (Active Motif, 15015) for 1.5 hours at room temperature with gentle shaking followed by washing in TBST (3X5 mins) and visualized using chemiluminescence element (Perkin Elmer) following manufacturer's protocol on Bio-Rad Universal Hood III.

12. Acquisition and analysis of MALDI data

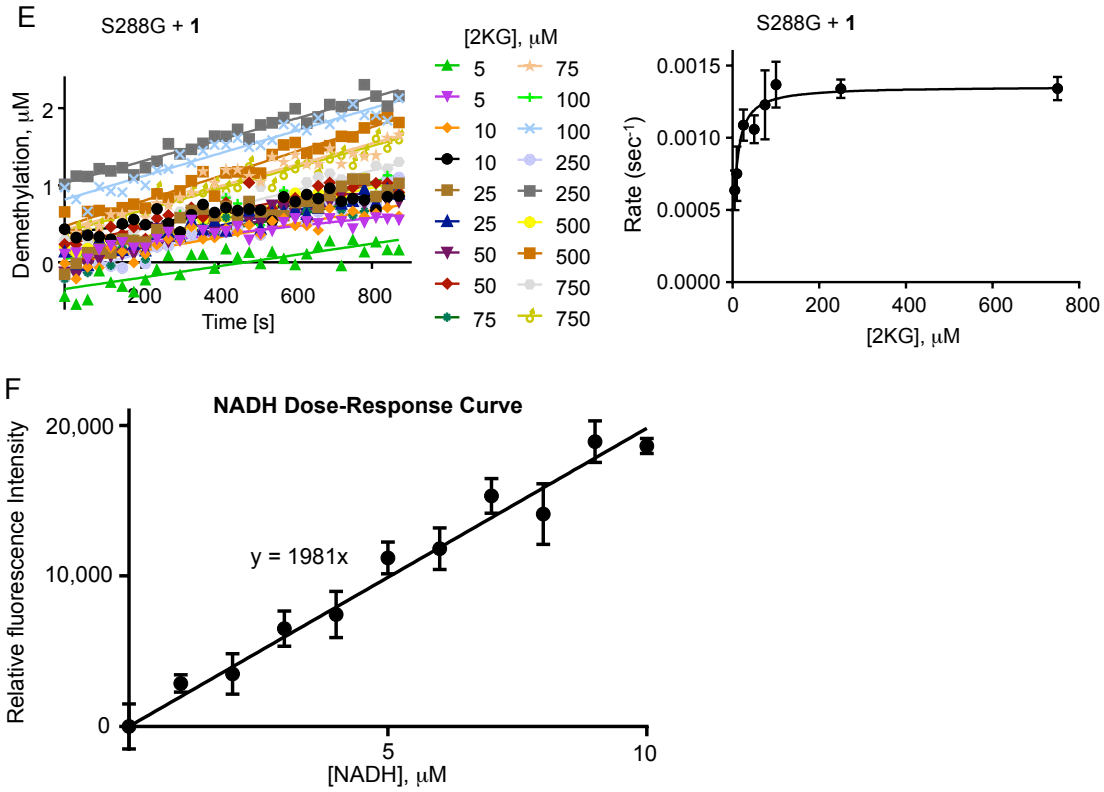
The MALDI data were collected on Bruker-ultrafleXtreme™ MALDI-TOF/TOF mass spectrometer. The experiments were conducted in reflectron positive mode and the ion intensities were normalized to the highest peak. The background was corrected for noise by smoothing and baselining prior to finding peak areas in flexAnalysis 3.4 program. Any signal at peaks 2281 or 2267 in the "no enzyme" control was subtracted from each additional trial.[3],[10]

13. Supplementary figures and table

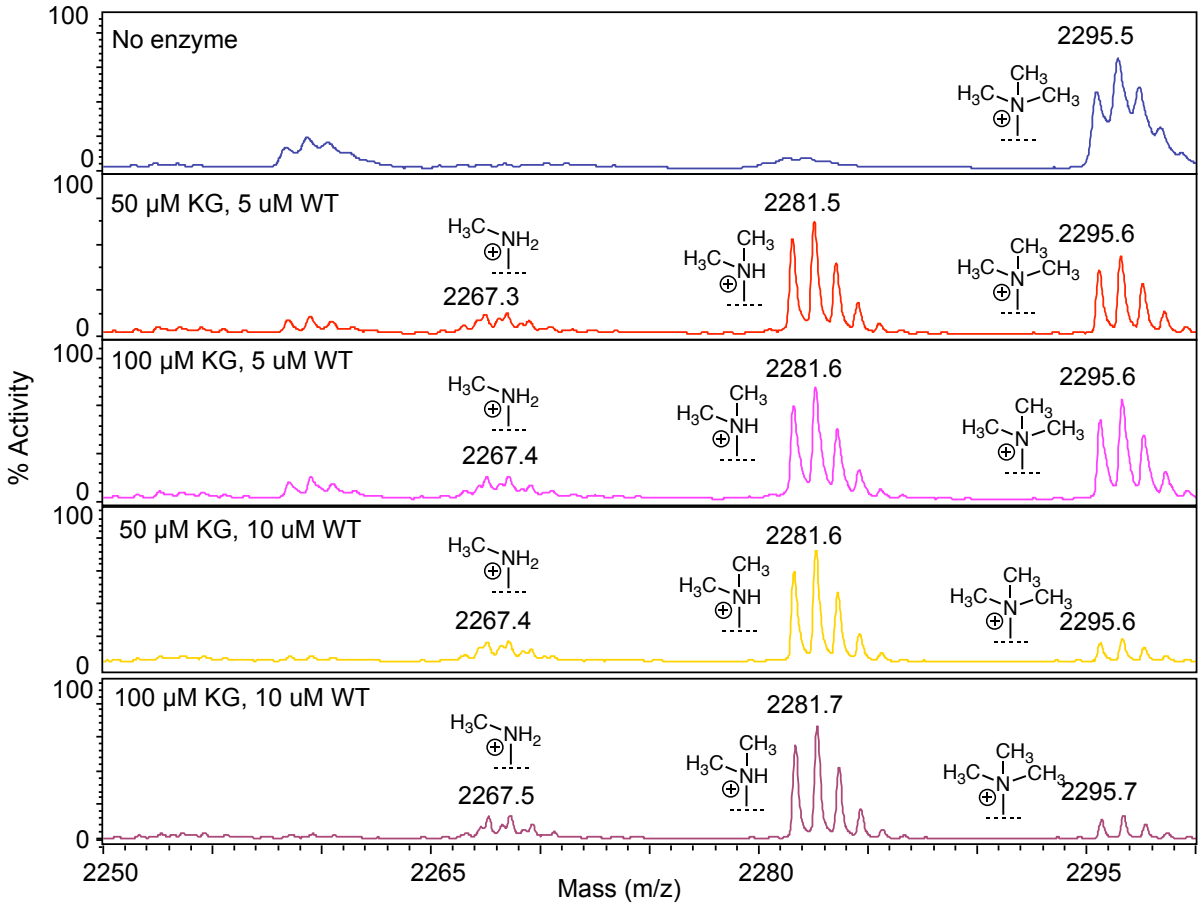


Supplementary Figure S1: Kinetic constants of the wild type and the KDM4A mutants. (A-E) Time- and 2KG concentration-dependent enzymatic activity of wild type KDM4A-**1** (A),

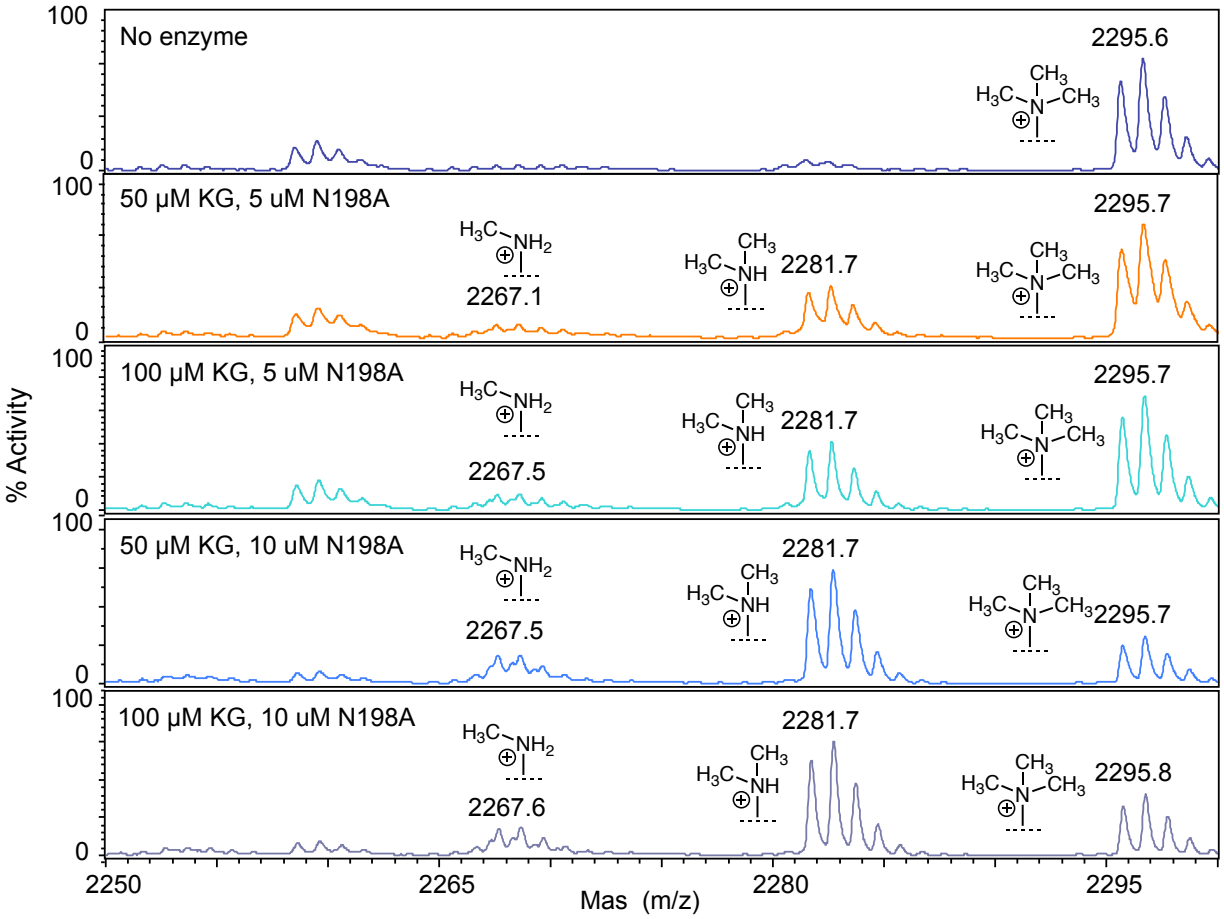
N198A-**1** (B), N198G-**1** (C), S288A-**1** (D) and S288G-**1** (E). The Michaelis-Menten curves are used to determine the kinetic parameters (k_{cat}, K_M and k_{cat}/K_M) of enzyme-cofactor pairs. (F) NADH dose response curve for correlation between RFU and μM of NADH produced.



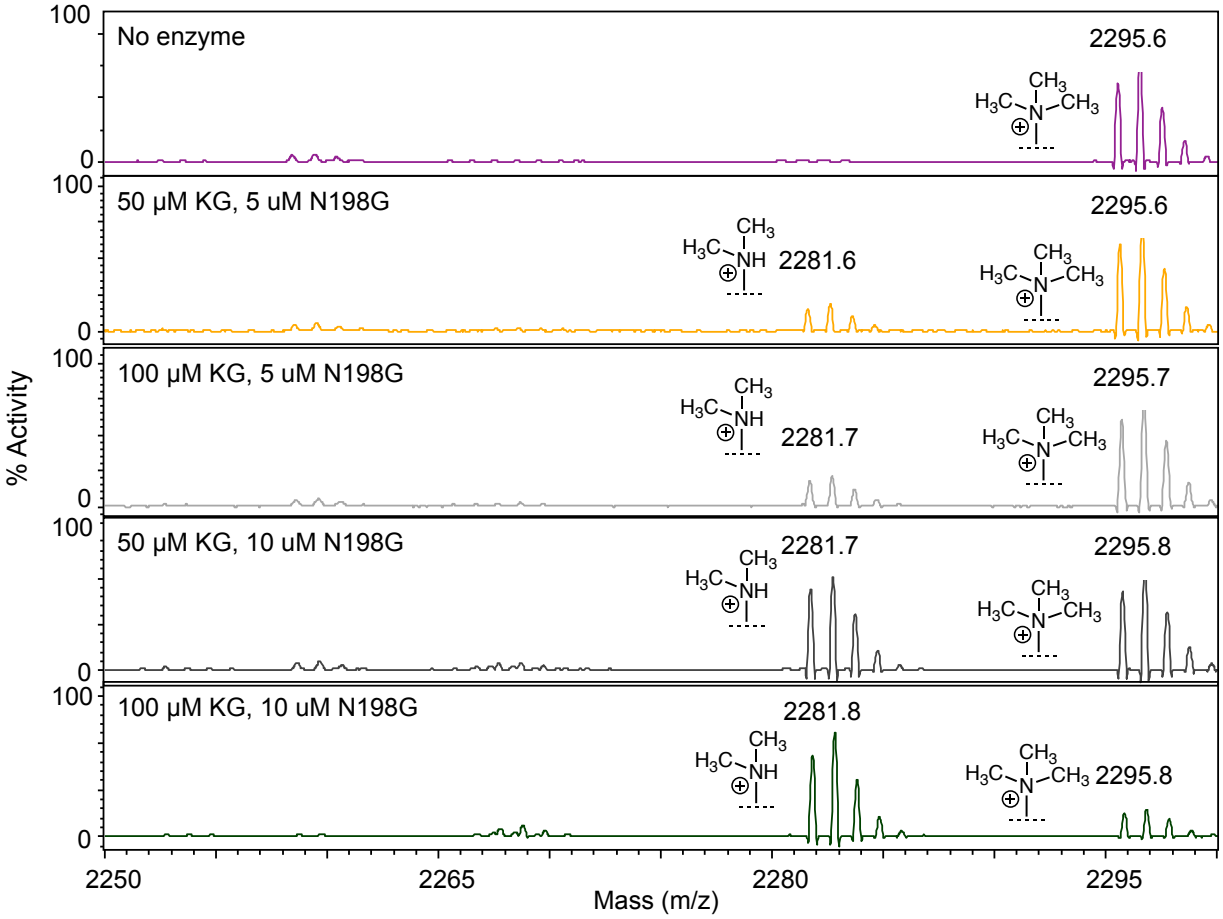
Supplementary Figure S1 Continued: Kinetic constants of the wild type and the KDM4A mutants. (A-E) Time- and 2KG concentration-dependent enzymatic activity of wild type KDM4A-**1** (A), N198A-**1** (B), N198G-**1** (C), S288A-**1** (D) and S288G-**1** (E). The Michaelis-Menten curves are used to determine the kinetic parameters (k_{cat}, K_M and k_{cat}/K_M) of the enzyme-cofactor pairs. (F) NADH dose response curve for correlation between RFU and μM of NADH produced.



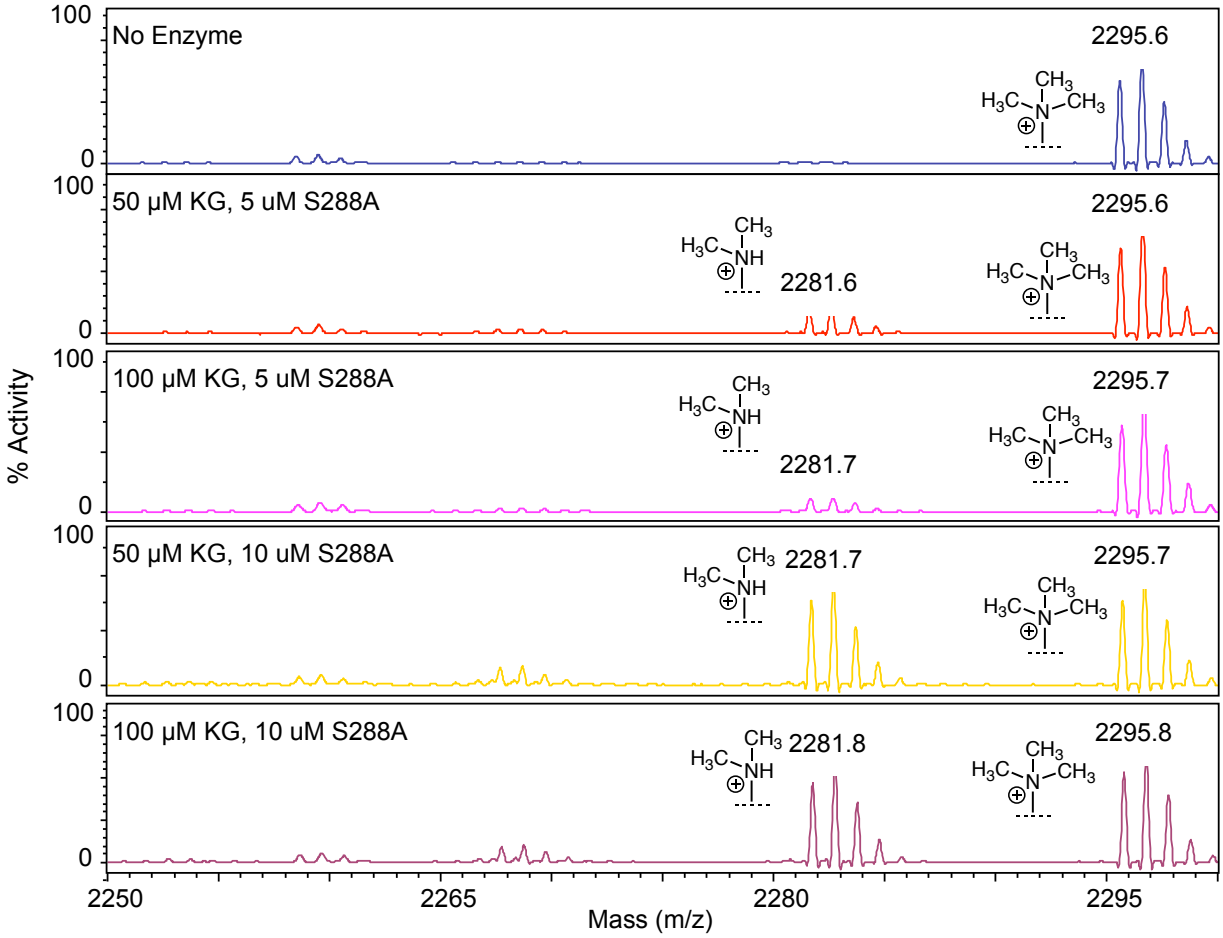
Supplemental Figure S2: MALDI demethylase assay for finding optimized screening condition for wild type KDM4A. The optimal condition was selected as 50 µM KG and 5 µM enzyme.



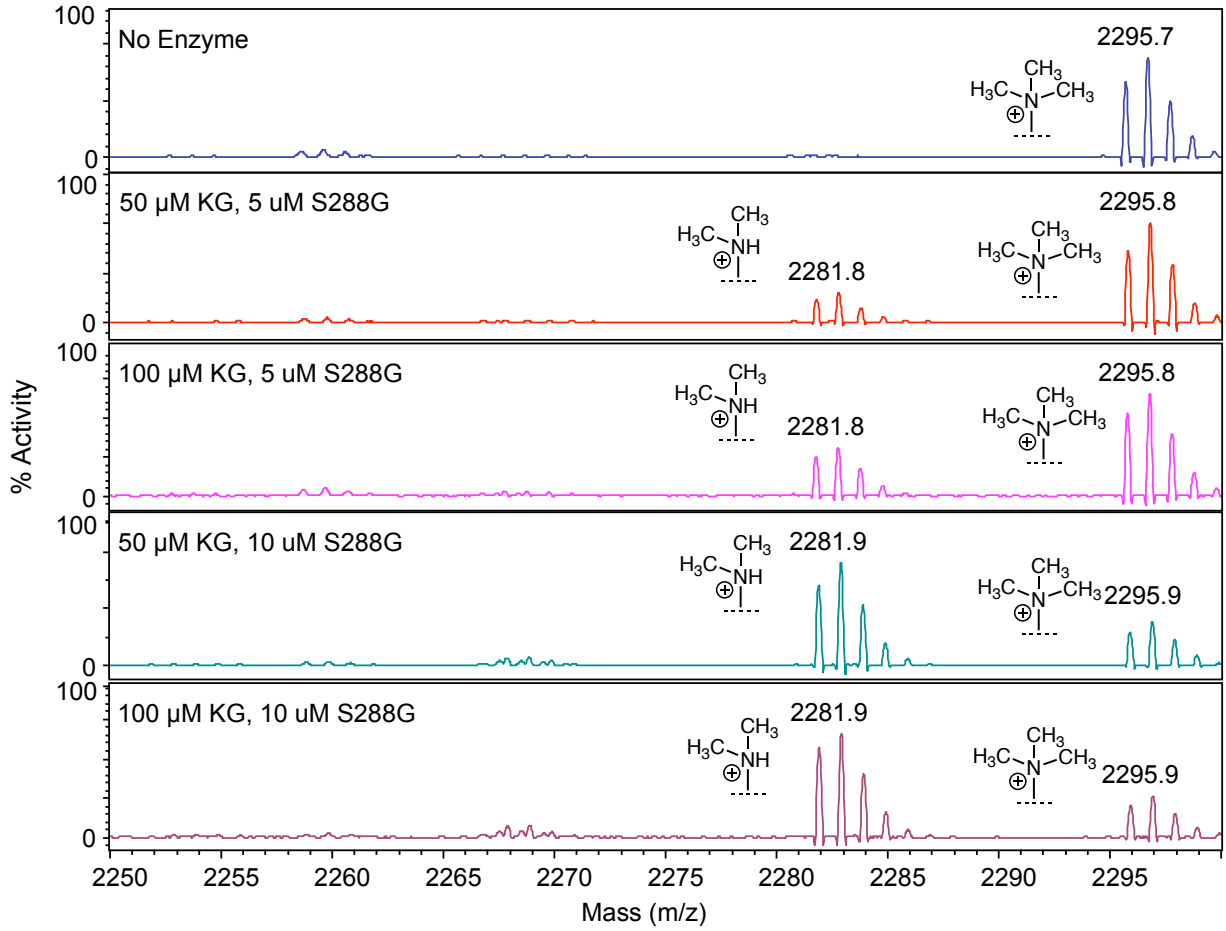
Supplemental Figure S3: MALDI demethylase assay for finding optimized screening condition for N198A mutant. The optimal condition was selected as 50 μM KG and 10 μM enzyme.



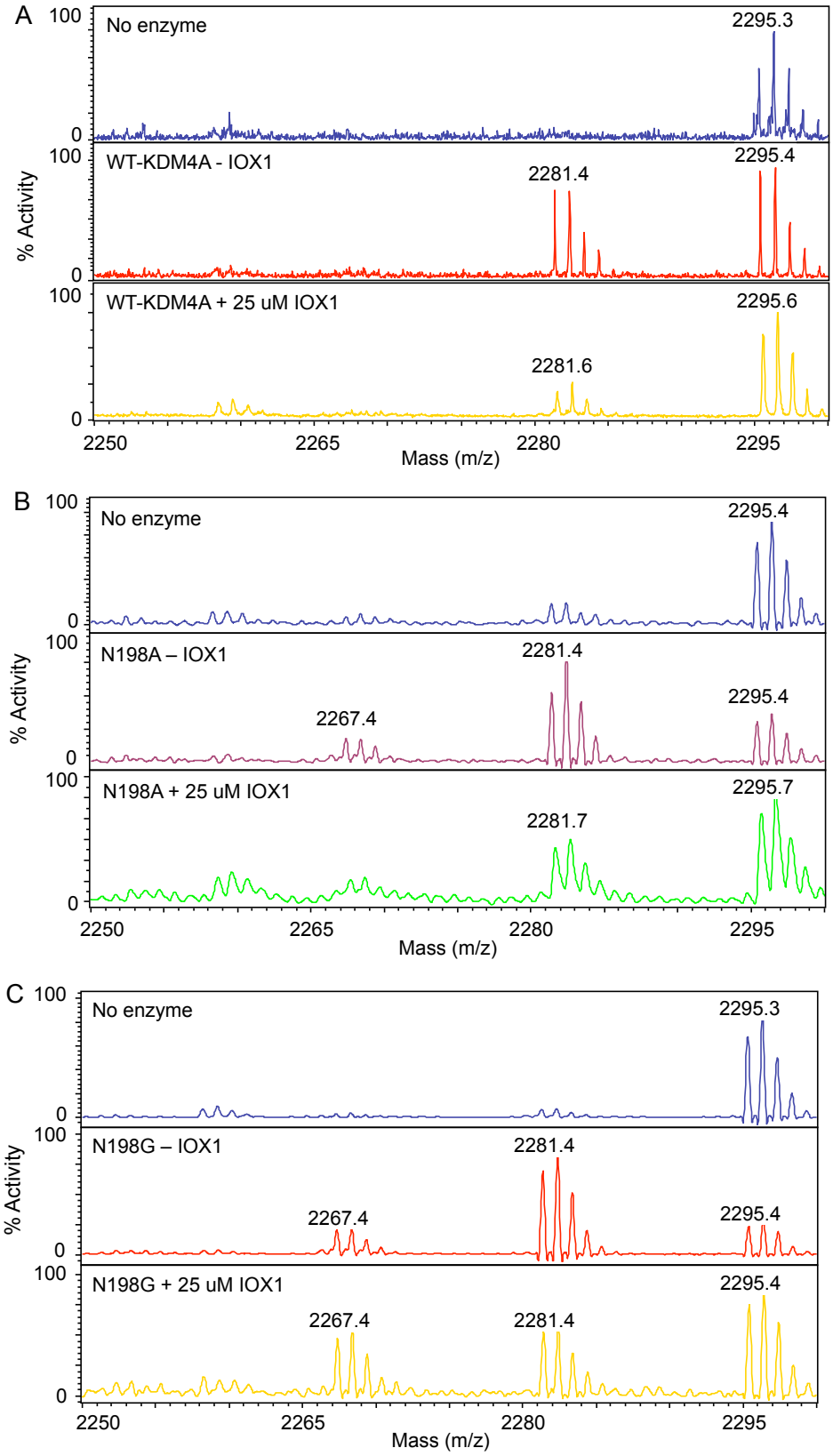
Supplemental Figure S4: MALDI demethylase assay for finding optimized screening condition for N198G mutant. The optimal condition was selected as 50 μM KG and 10 μM enzyme.



Supplemental Figure S5: MALDI demethylase assay for finding optimized screening condition for S288A mutant. The optimal condition was selected as 50 μM KG and 10 μM enzyme.

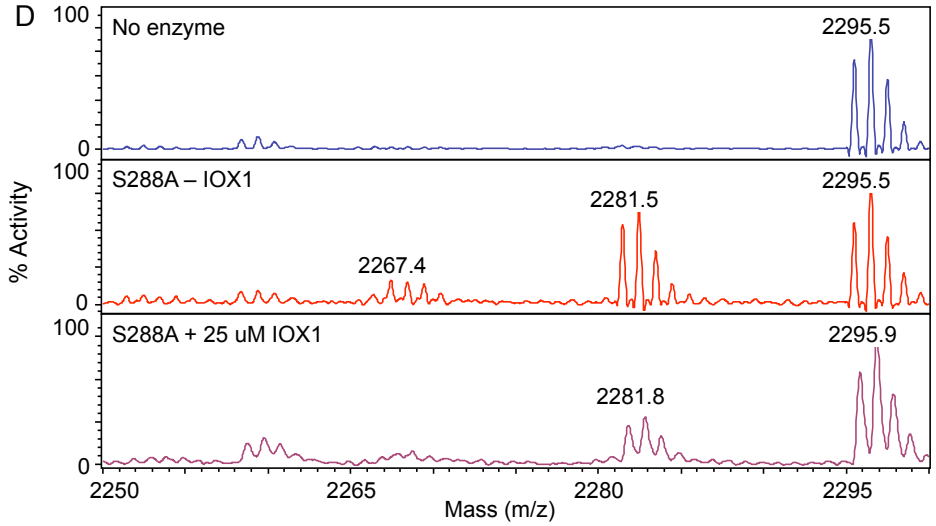


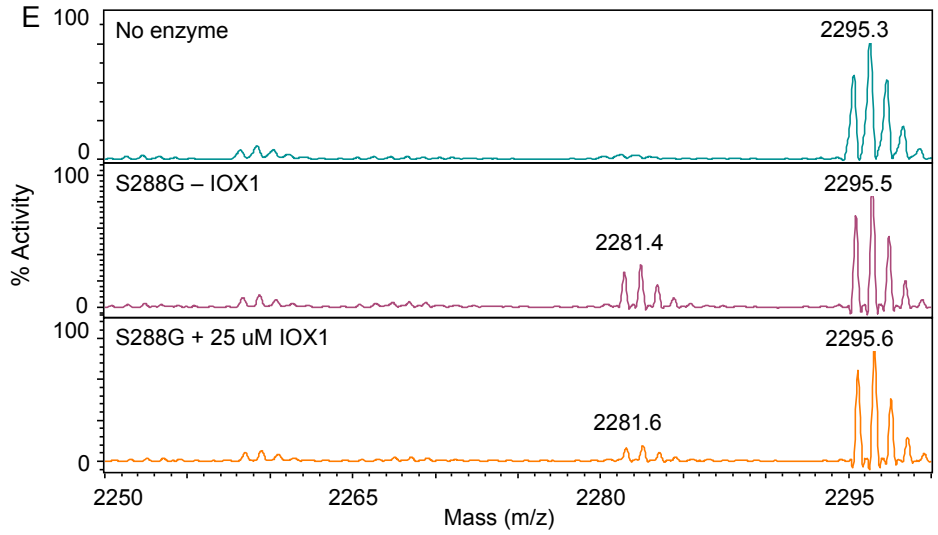
Supplemental Figure S6: MALDI demethylase assay for finding optimized screening condition for S288G mutant. The optimal condition was selected as 50 µM KG and 10 µM enzyme.

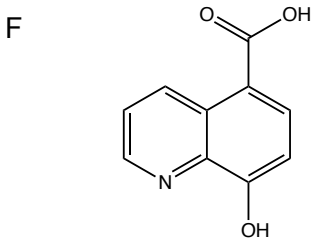


Supplemental Figure S7: MALDI demethylase assay to examine inhibitory activity of IOX1

towards wild type KDM4A (A), and N198A (B), N198G (C), S288A (D) and S288G (E) mutants. (F) Chemical structure of IOX1.

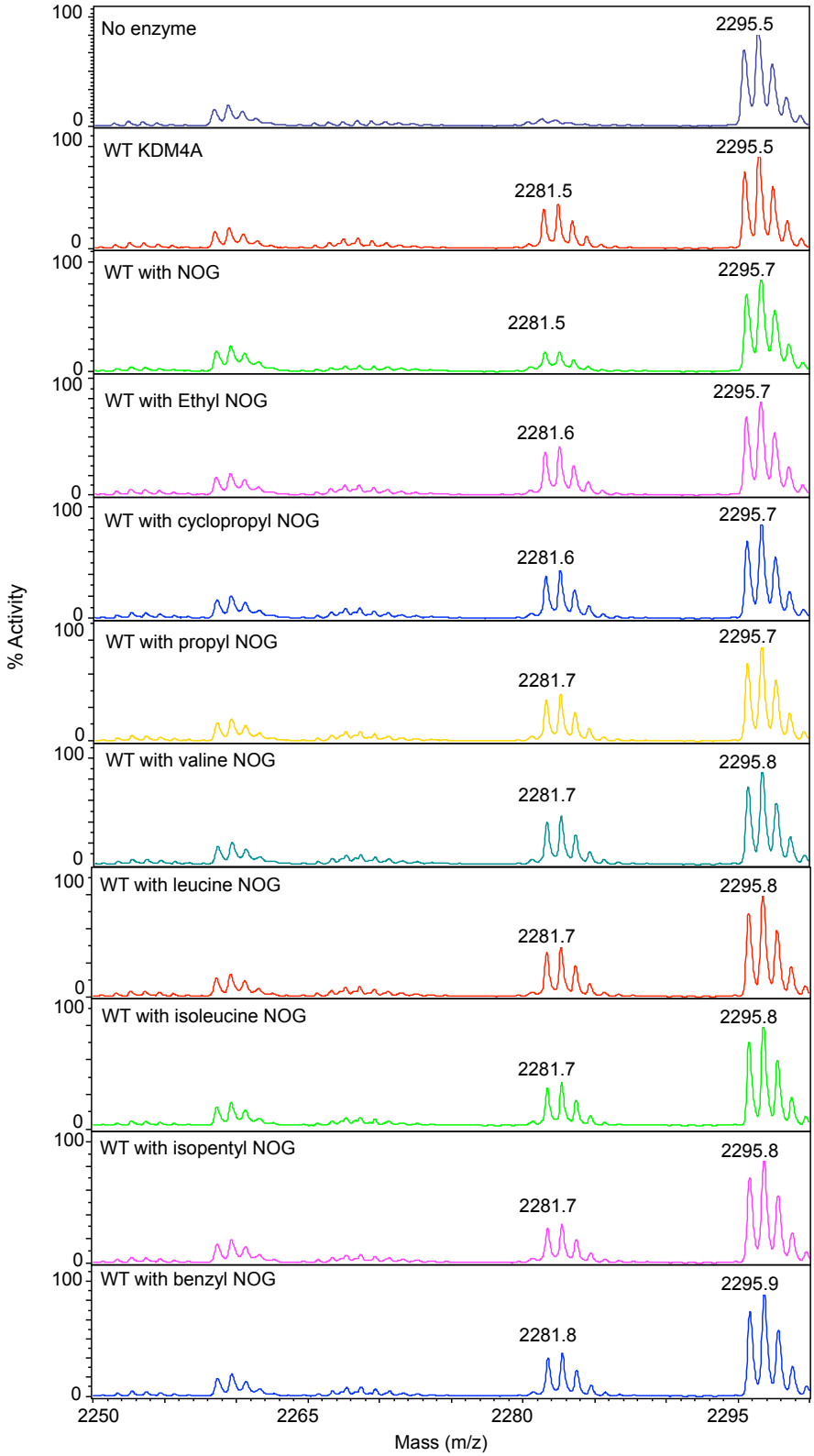




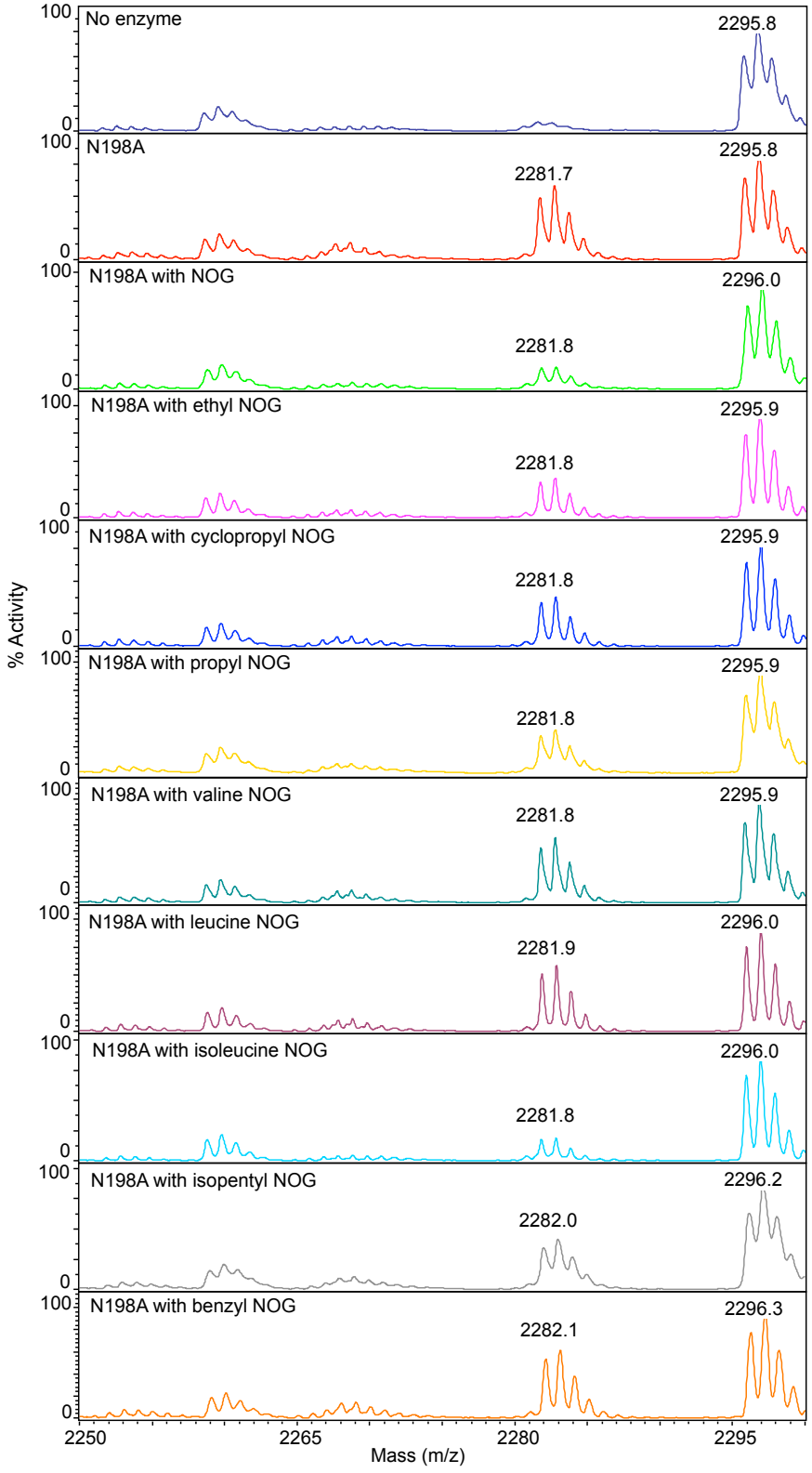


5-carboxy-8-hydroxyquinone (IOX1)

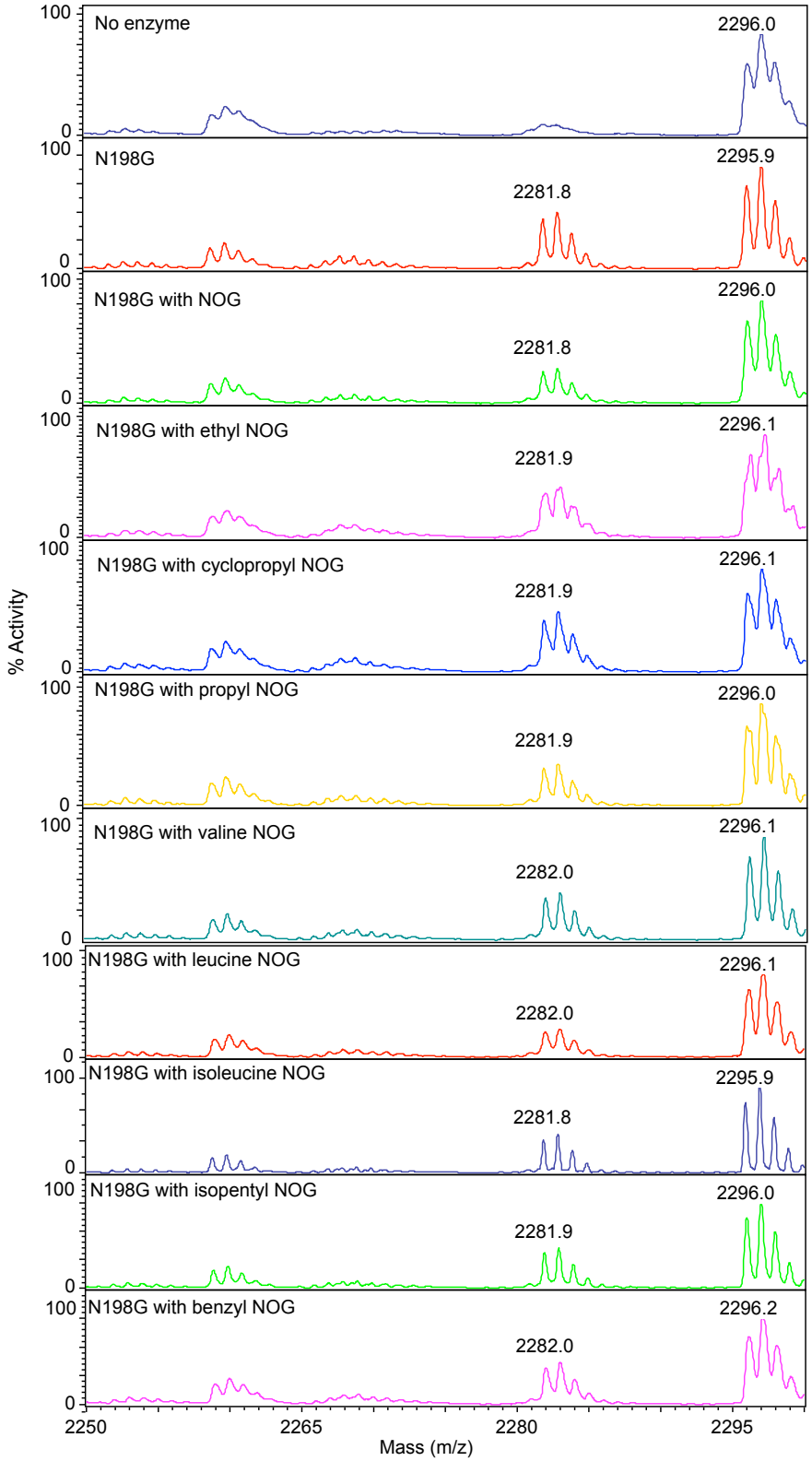
Supplemental Figure S7 Continued: MALDI demethylase assay to examine inhibitory activity of IOX1 towards wild type KDM4A (A), and N198A (B), N198G (C), S288A (D) and S288G (E) mutants. (F) Chemical structure of IOX1.



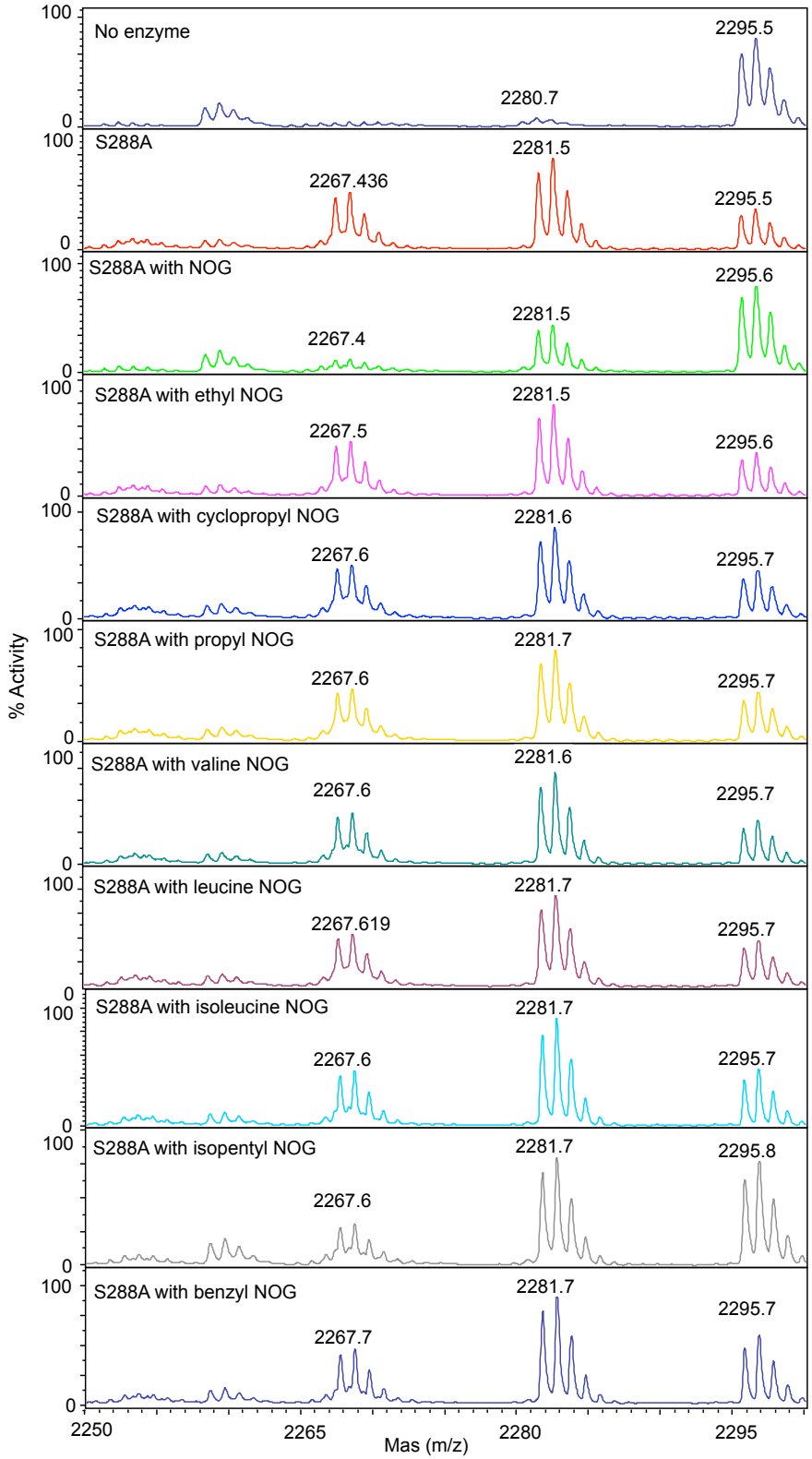
Supplemental Figure S8: MALDI demethylase assay to examine inhibitory activity of a panel of NOG analogues at 100 μM concentration towards wild type KDM4A.



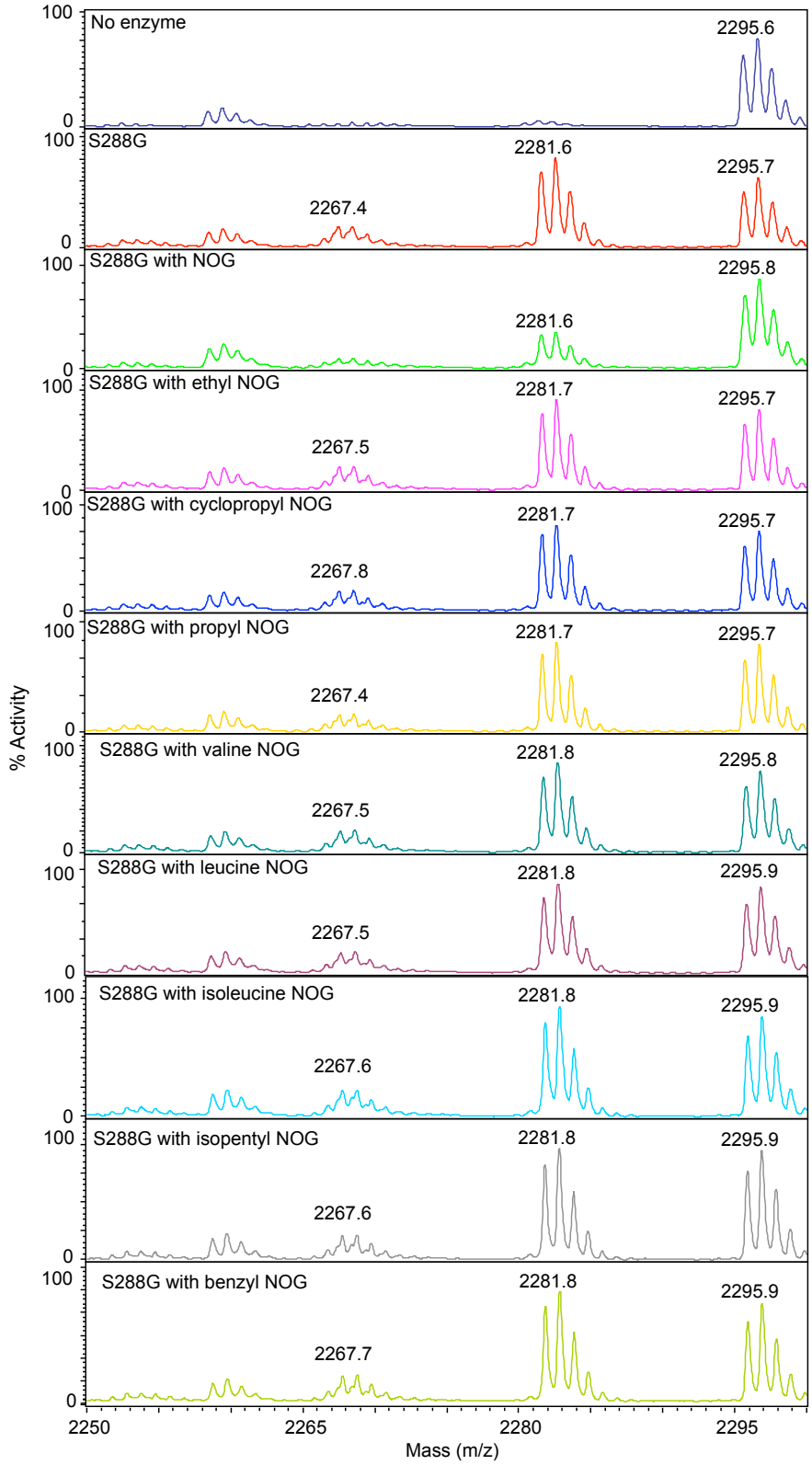
Supplemental Figure S9: MALDI demethylase assay to examine inhibitory activity of a panel of NOG analogues at 100 μM concentration towards the N198A mutant.



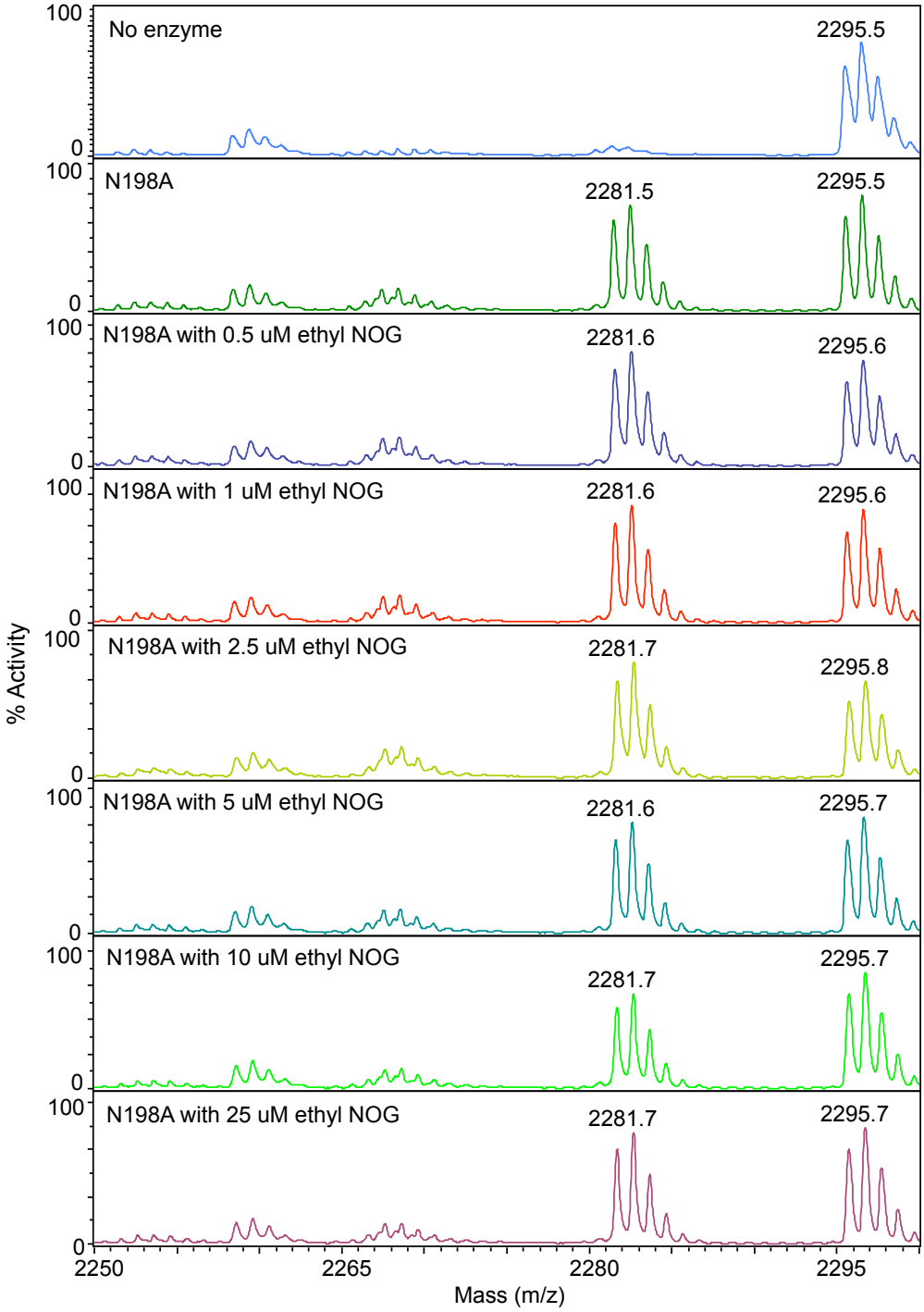
Supplemental Figure S10: MALDI demethylase assay to examine inhibitory activity of a panel of NOG analogues at 100 μM concentration towards the N198G mutant.



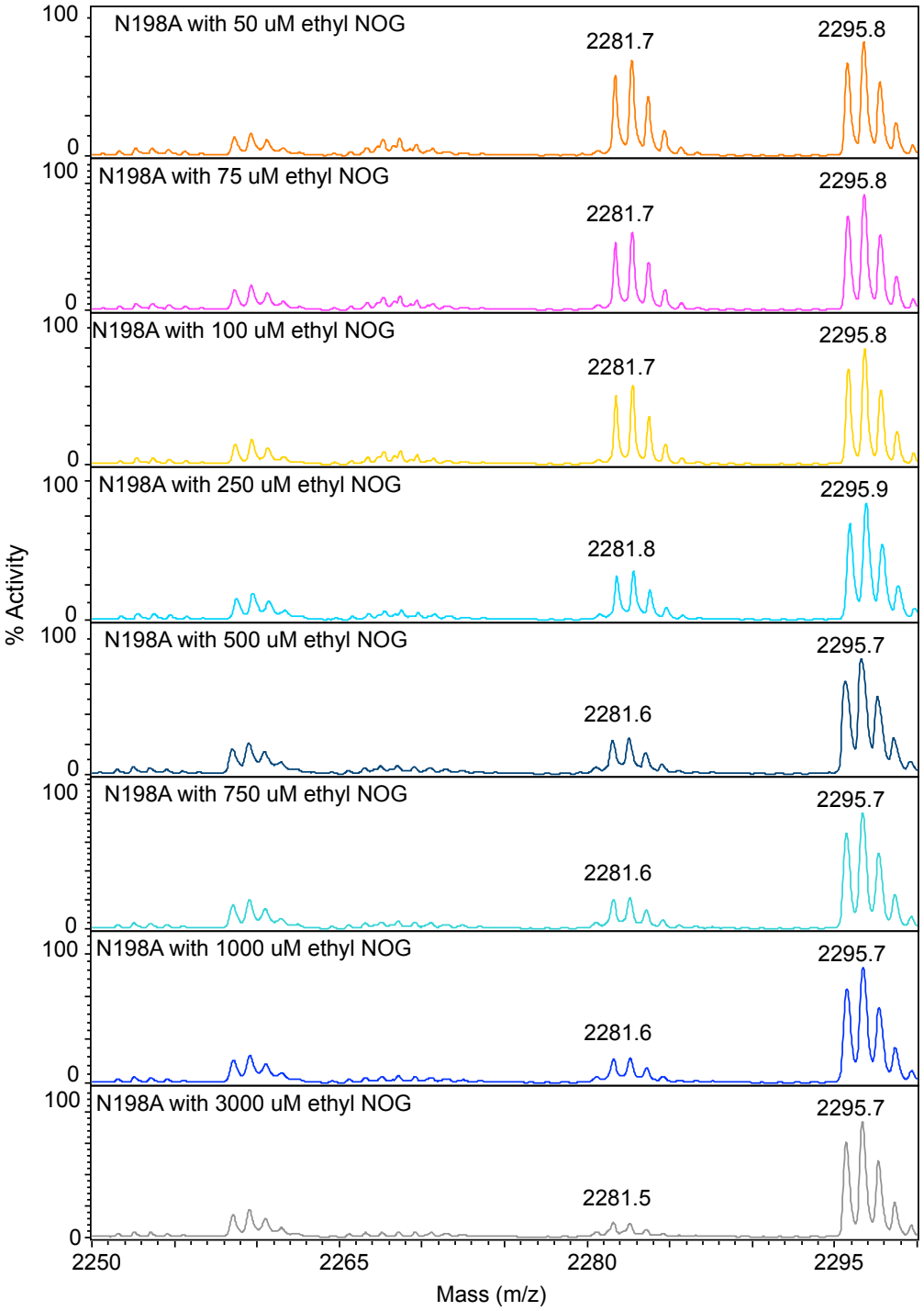
Supplemental Figure S11: MALDI demethylase assay to examine inhibitory activity of a panel of NOG analogues at 100 μM concentration towards the S288A mutant.



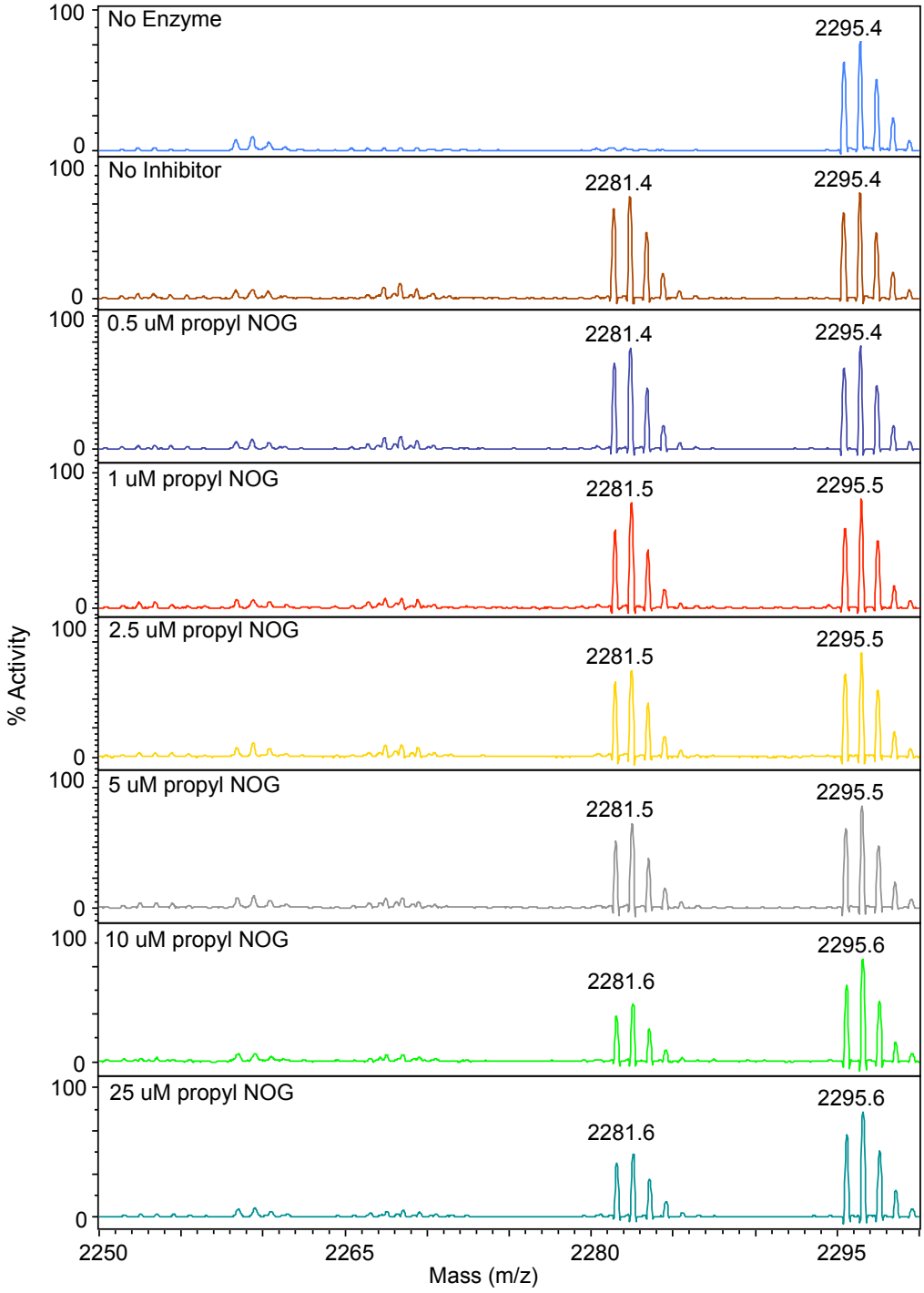
Supplemental Figure S12: MALDI demethylase assay to examine inhibitory activity of a panel of NOG analogues at 100 μM concentration towards the S288G mutant.



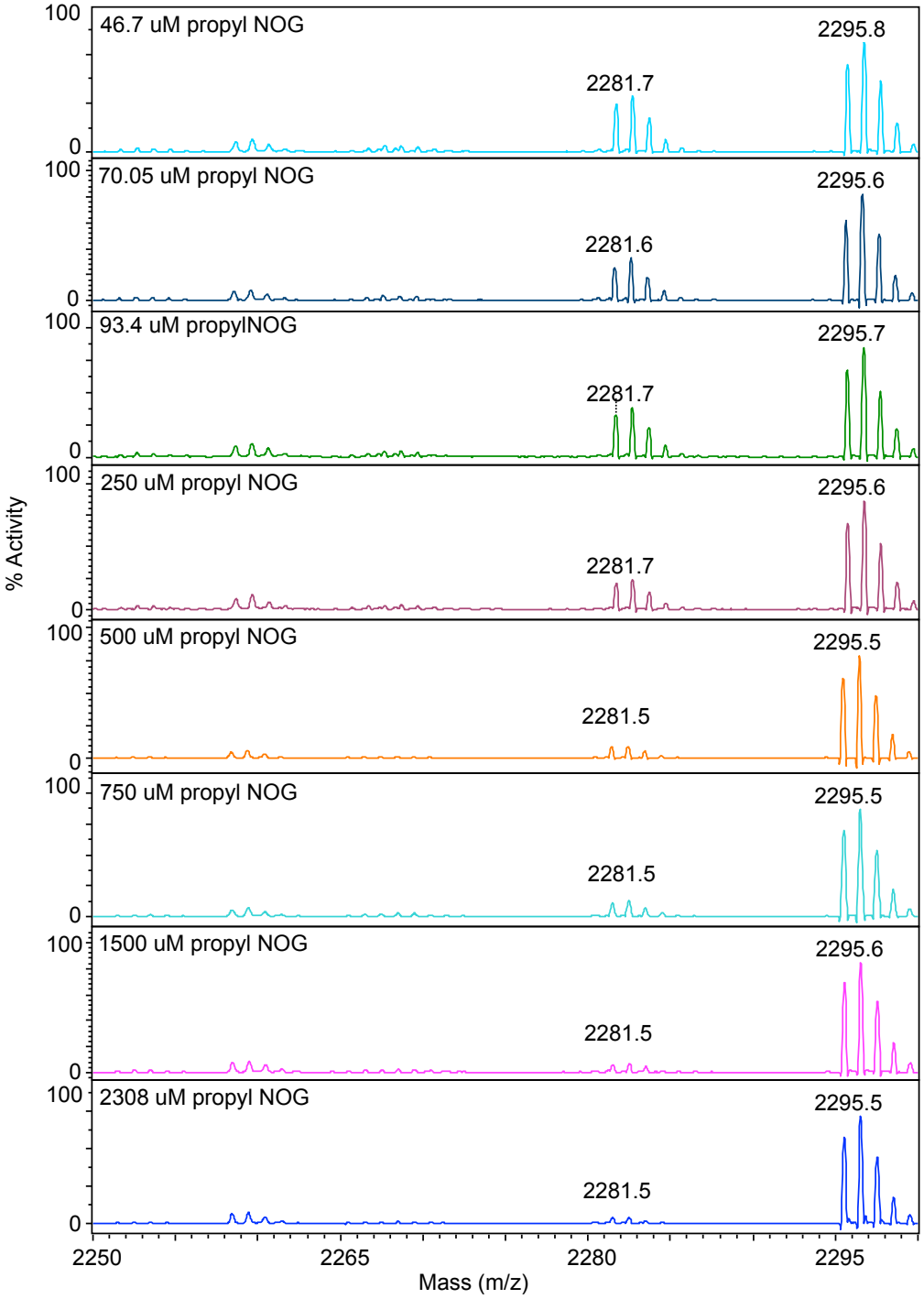
Supplemental Figure S13: MALDI demethylase assay to determine IC_{50} value of Ethyl-NOG **3** against the N198A mutant.



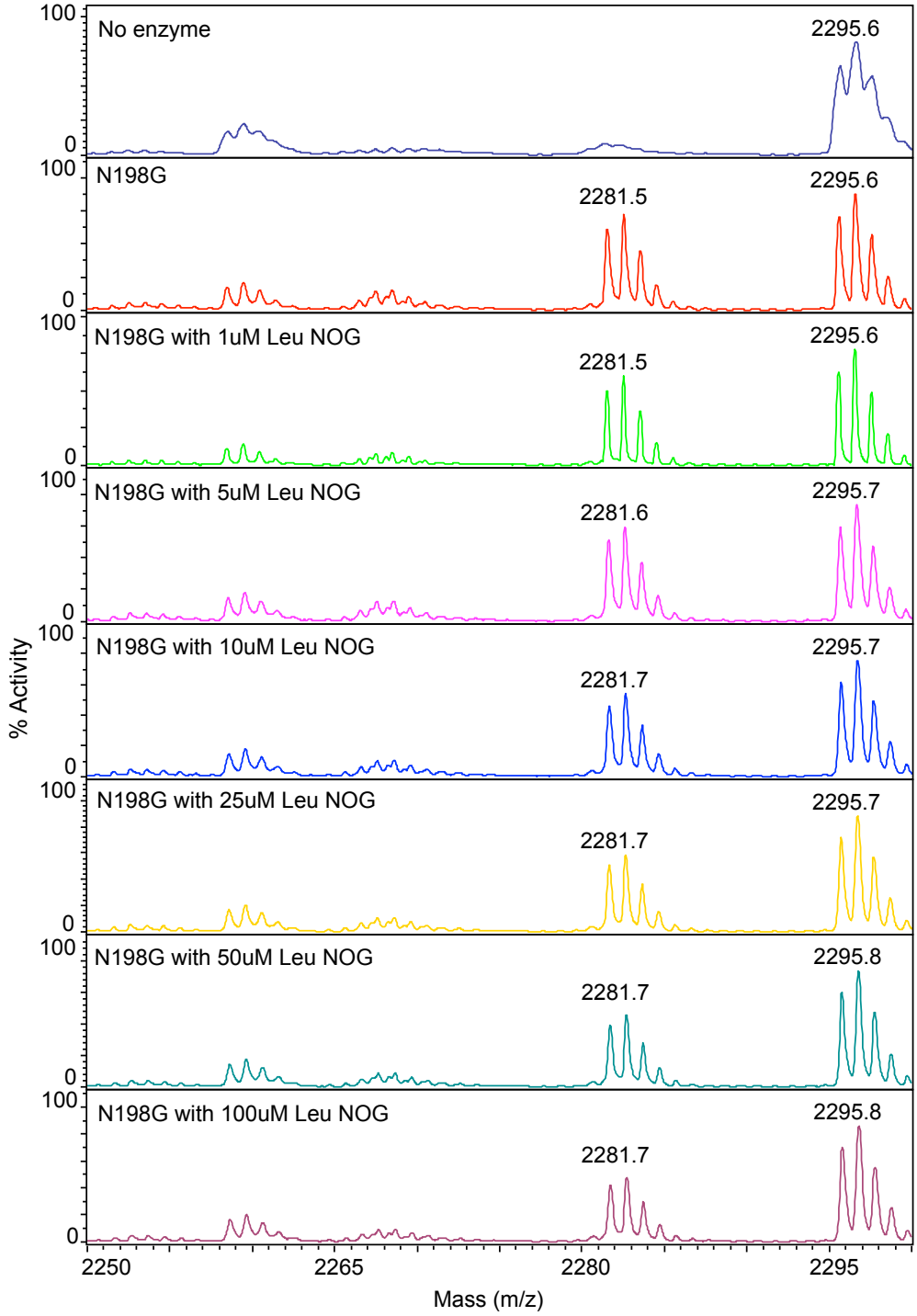
Supplemental Figure S13 Continued: MALDI demethylase assay to determine IC_{50} value of Ethyl-NOG **3** against the N198A mutant.



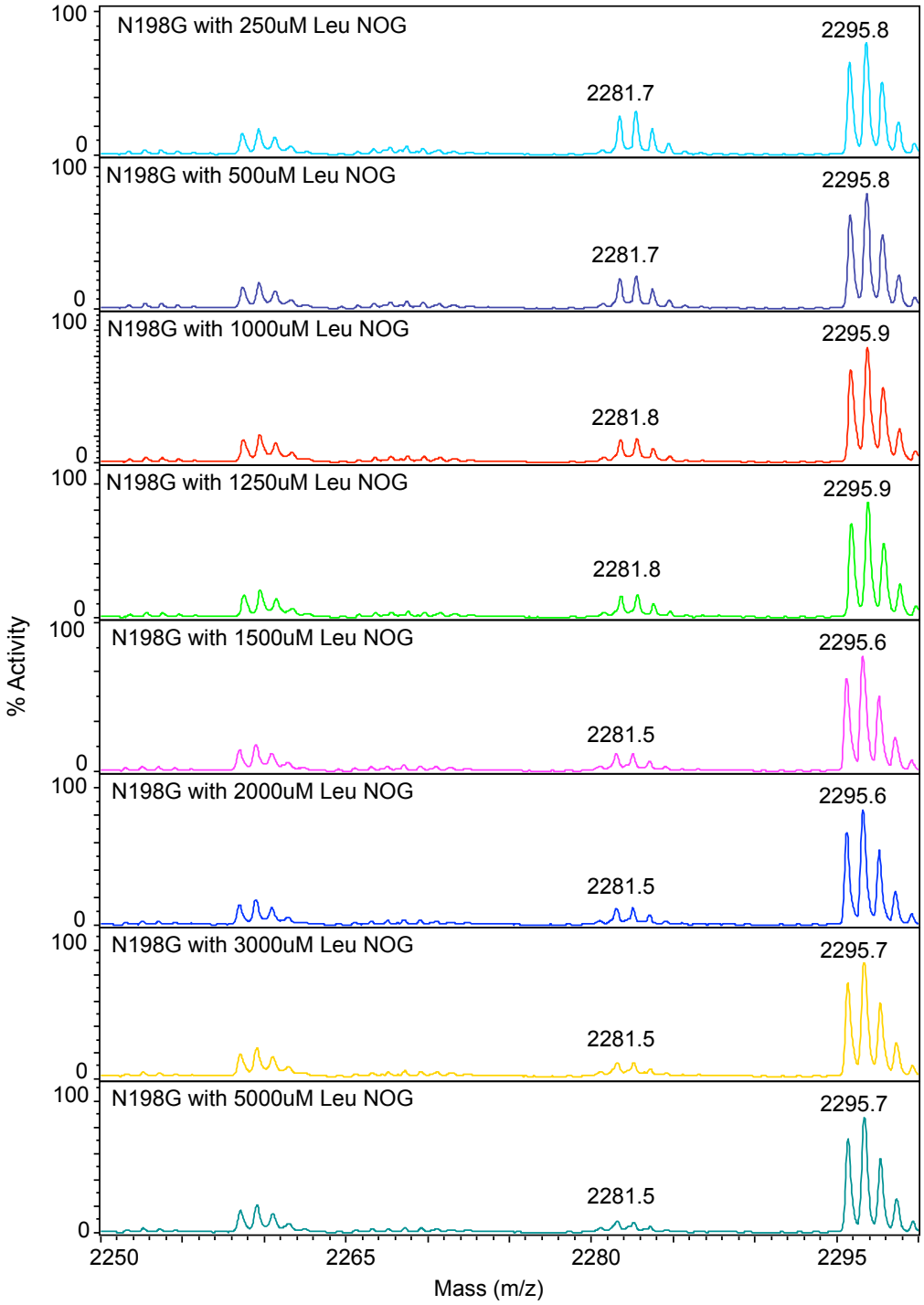
Supplemental Figure S14: MALDI demethylase assay to determine IC_{50} value of Propyl-NOG **5** against the N198A mutant.



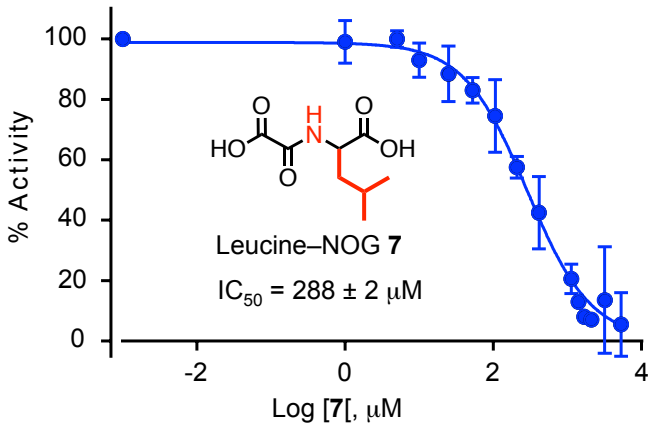
Supplemental Figure S14 Continued: MALDI demethylase assay to determine IC_{50} value of Propyl-NOG **5** against the N198A mutant.



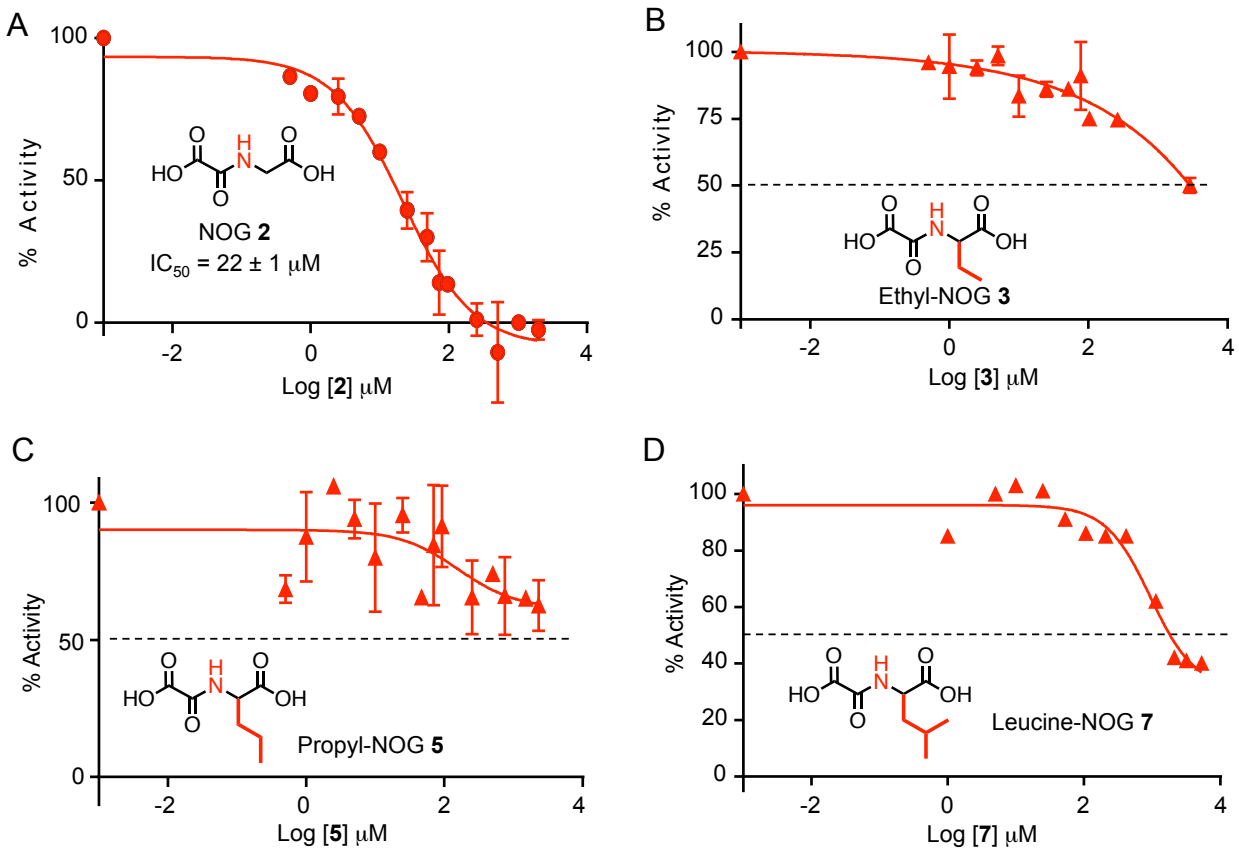
Supplemental Figure S15: MALDI demethylase assay to determine IC_{50} value of Leucine-NOG **7** against the N198G mutant.



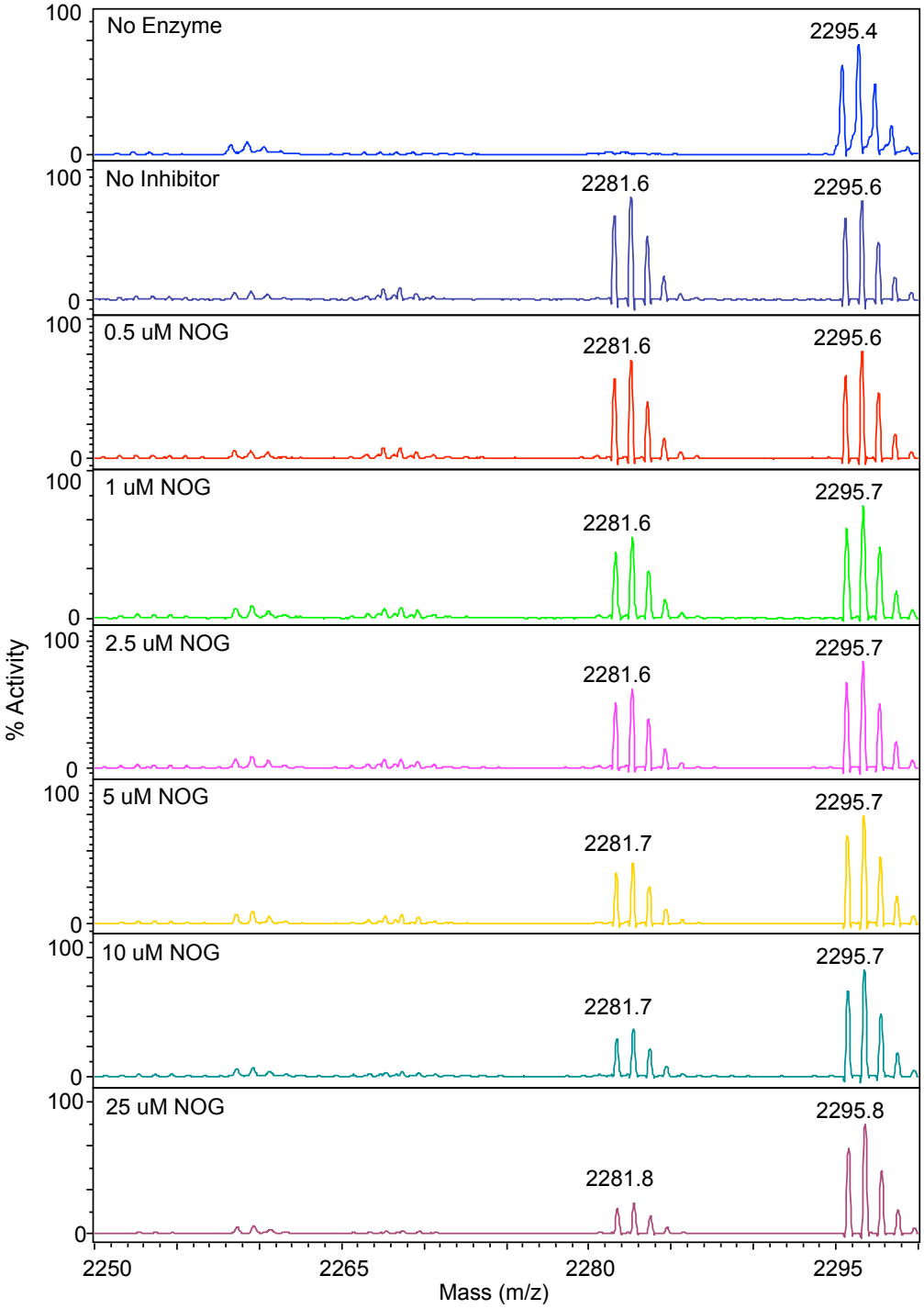
Supplemental Figure S15 Continued: MALDI demethylase assay to determine IC_{50} value of Leucine-NOG **7** against the N198G mutant.



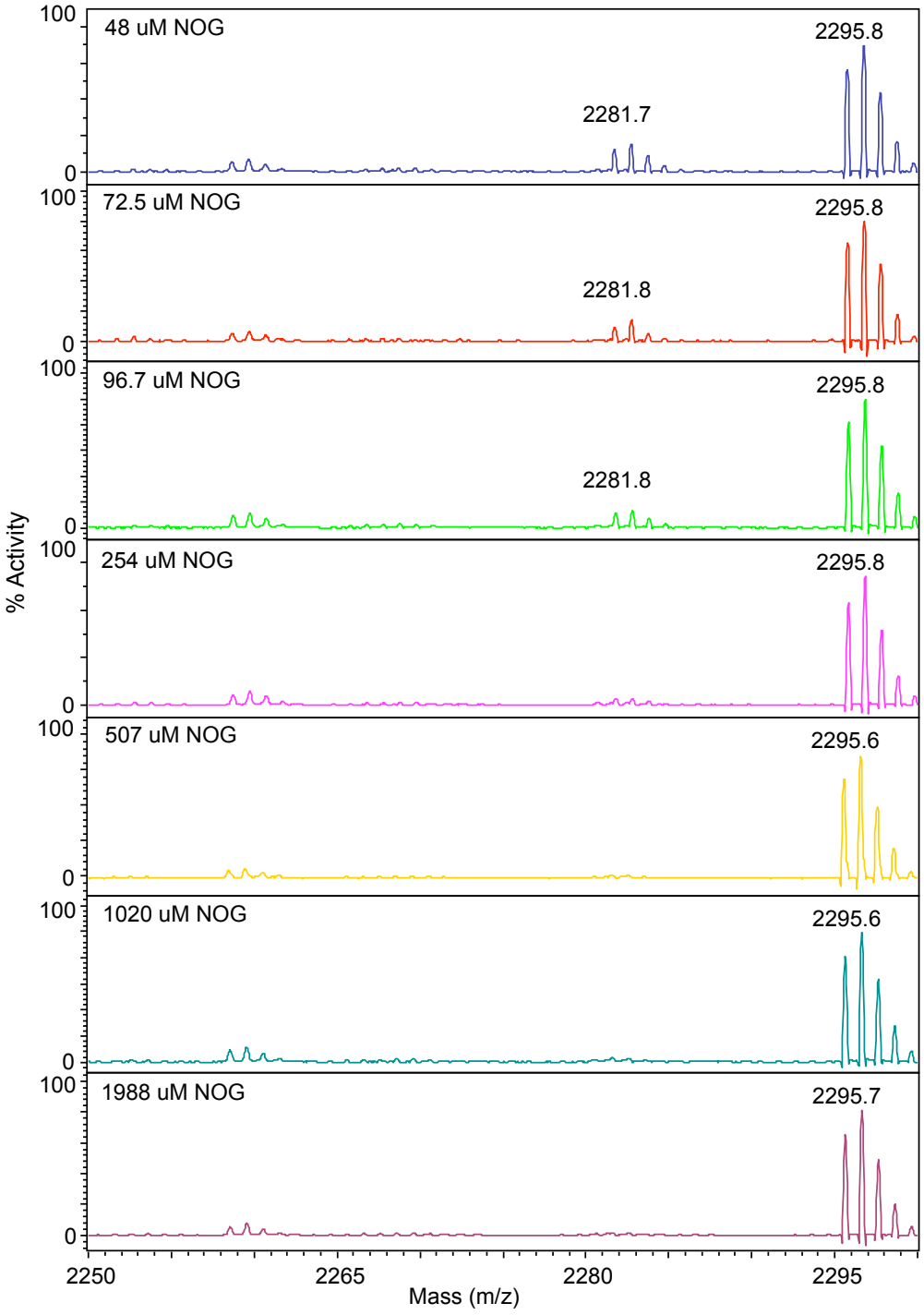
Supplemental Figure S16: IC_{50} value of Leucine-NOG **7** against the N198G mutant based on MALDI demethylase data provided in Supplementary Figure S15.



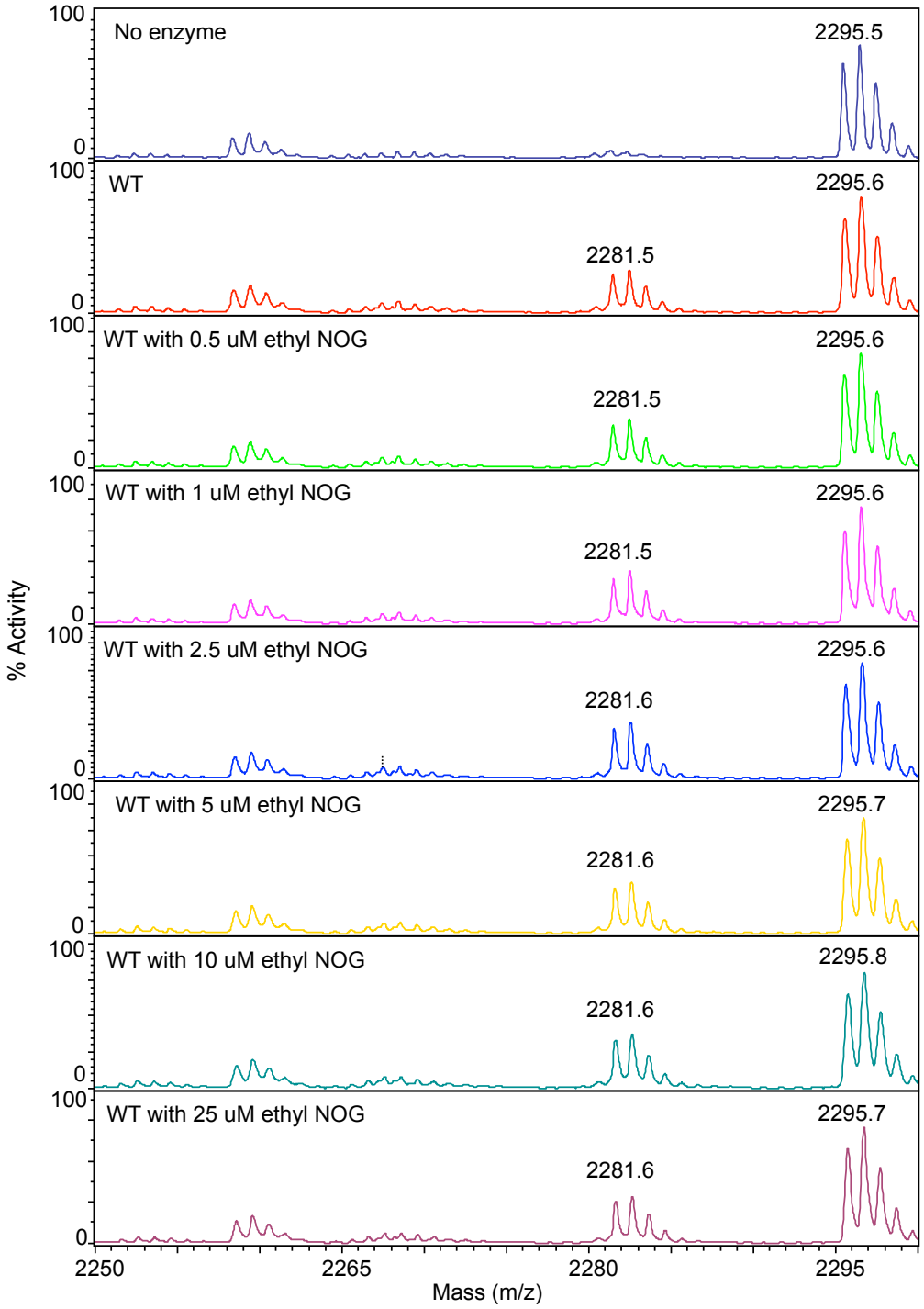
Supplemental Figure S17: Dose-dependent inhibition of demethylase activity of wild type KDM4A by NOG **2** (A), Ethyl-NOG **3** (B), Propyl-NOG **5** (C) and Leucine-NOG **7** (D). The corresponding MALDI MS spectra are provided in Supplementary figures S18, S19, S20 and S21.



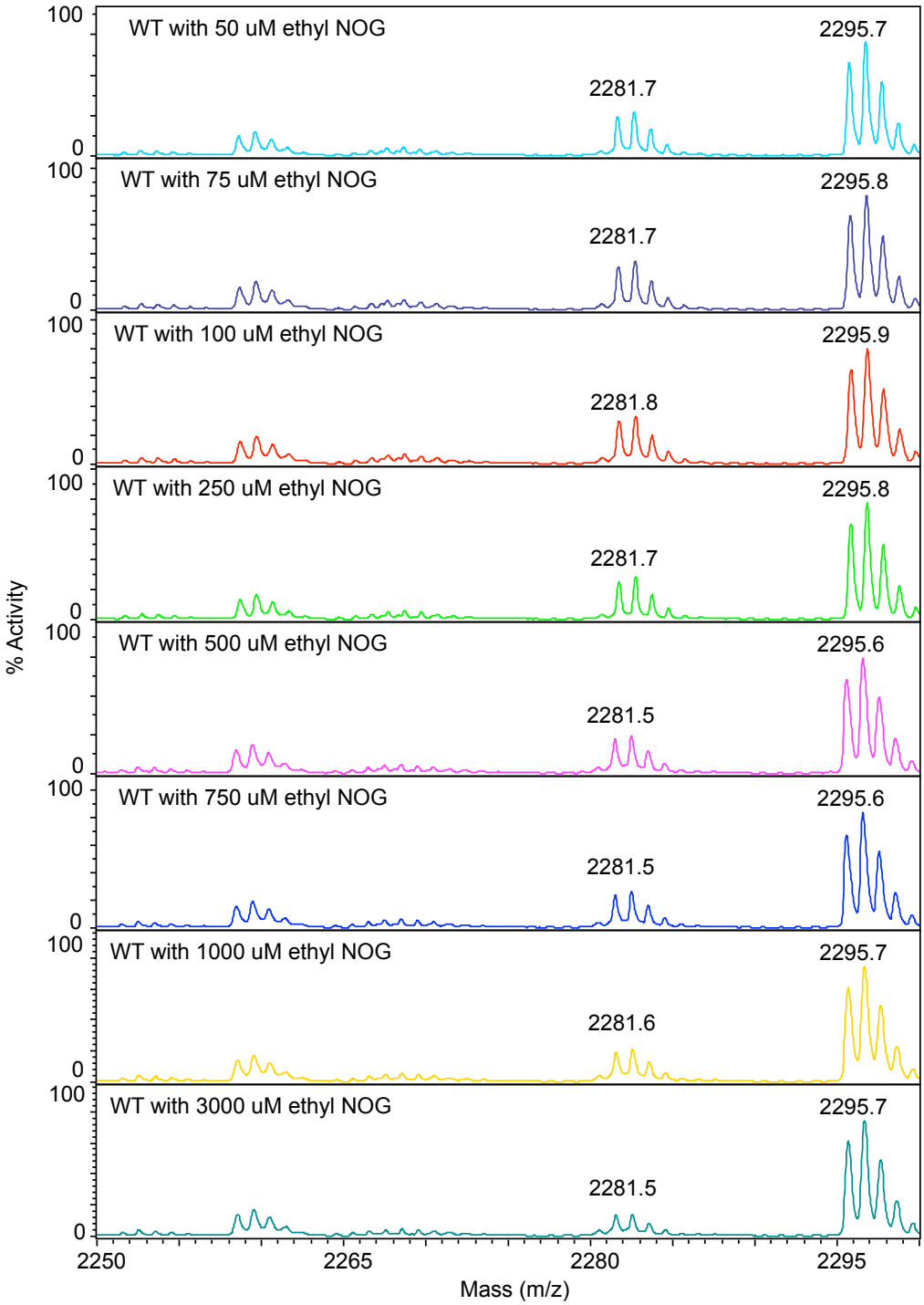
Supplemental Figure S18: MALDI demethylase assay to determine IC_{50} value of NOG **1** against the wild type KDM4A.



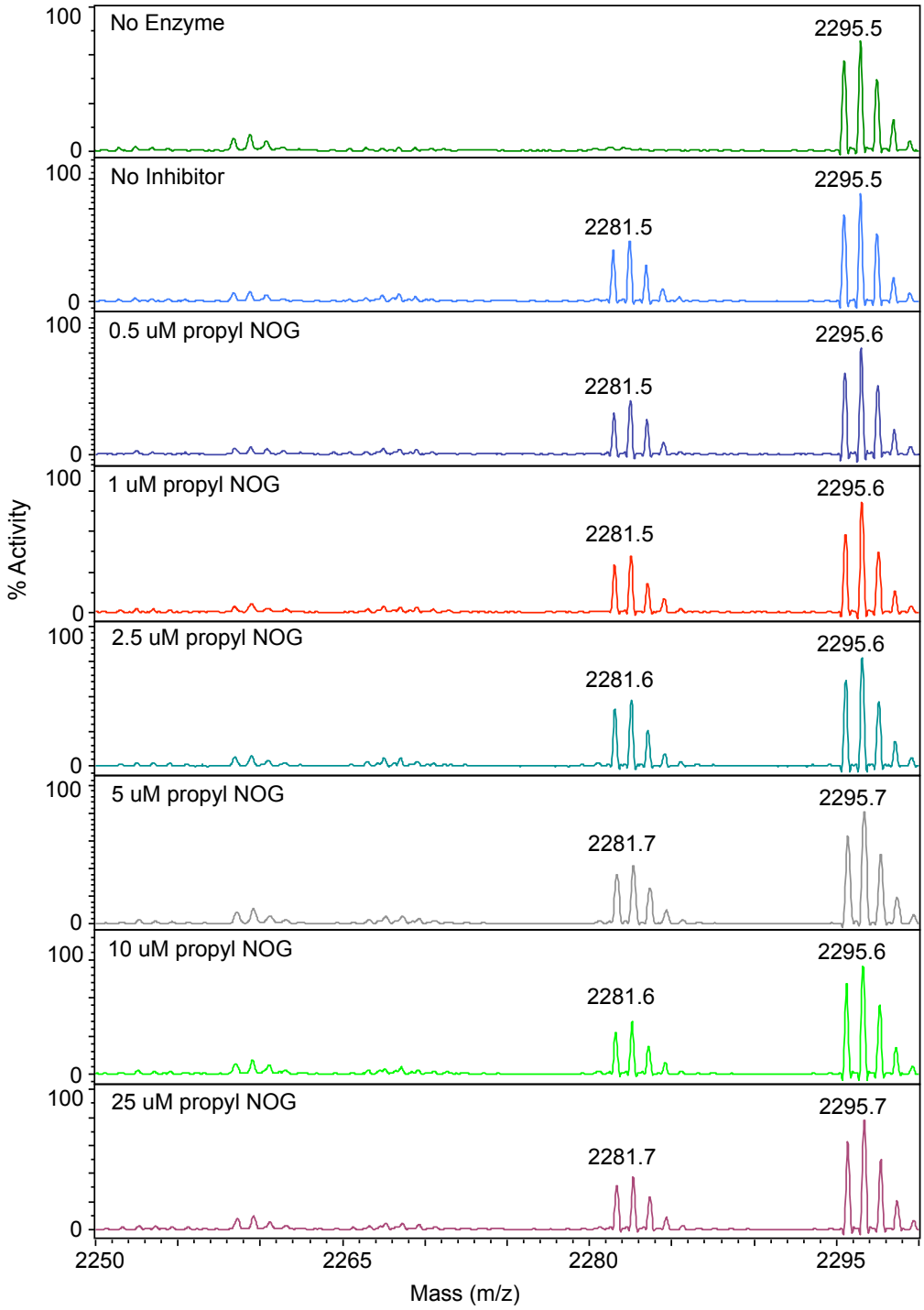
Supplemental Figure S18 Continued: MALDI demethylase assay to determine IC_{50} value of NOG **2** against the wild type KDM4A.



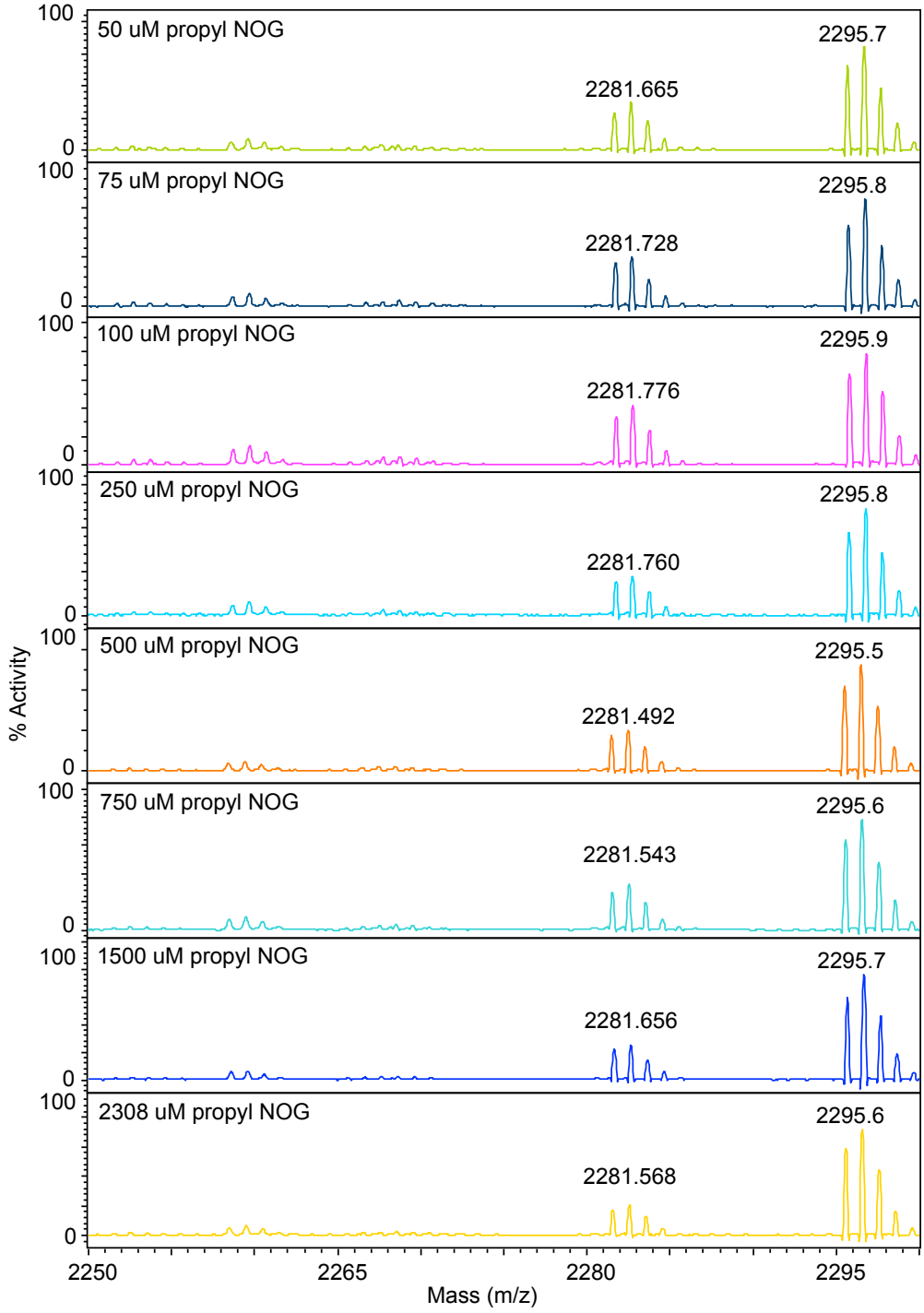
Supplemental Figure S19: MALDI demethylase assay to determine IC_{50} value of Ethyl-NOG **3** against the wild type KDM4A.



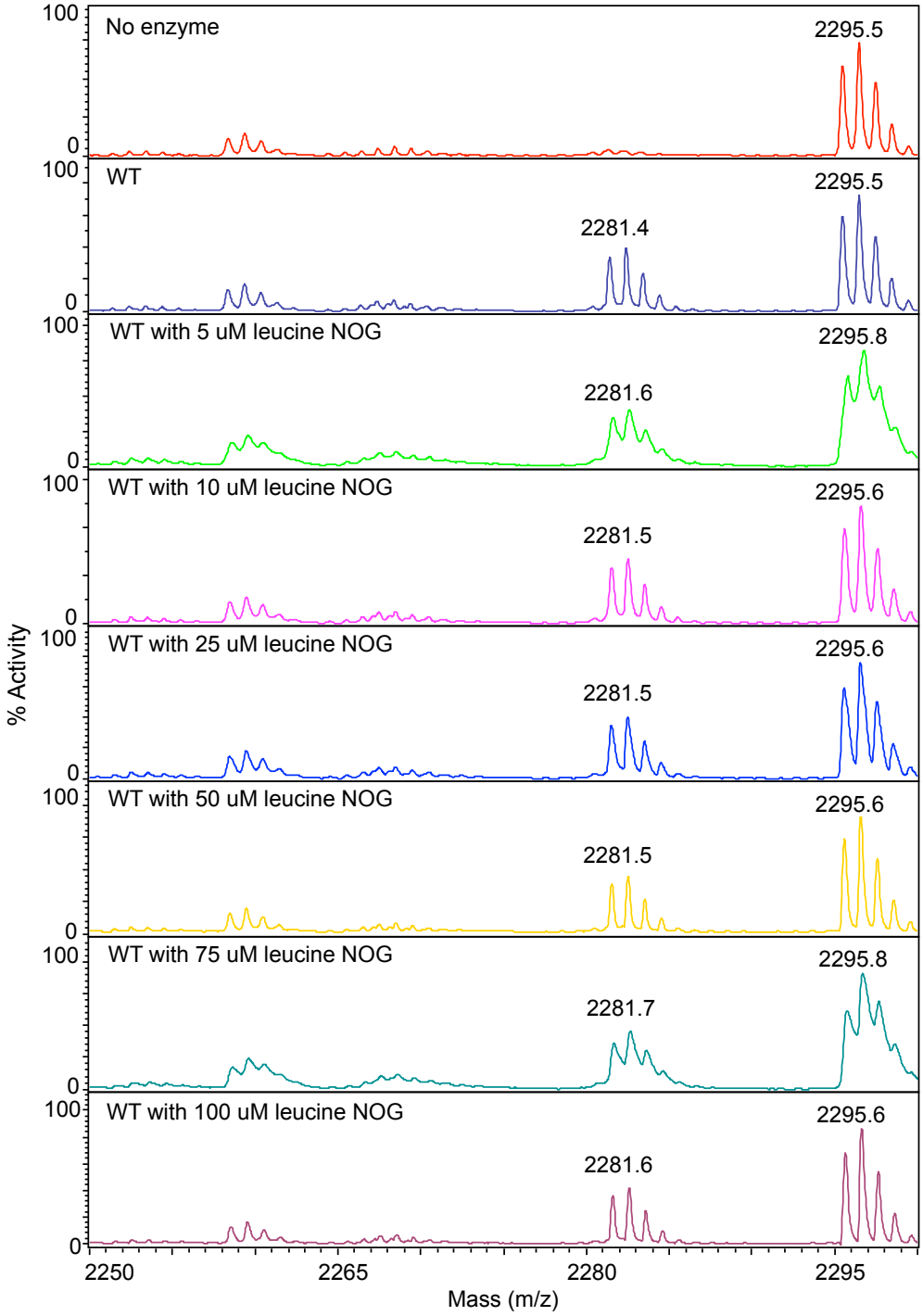
Supplemental Figure S19 Continued: MALDI demethylase assay to determine IC_{50} value of Ethyl-NOG **3** against the wild type KDM4A.



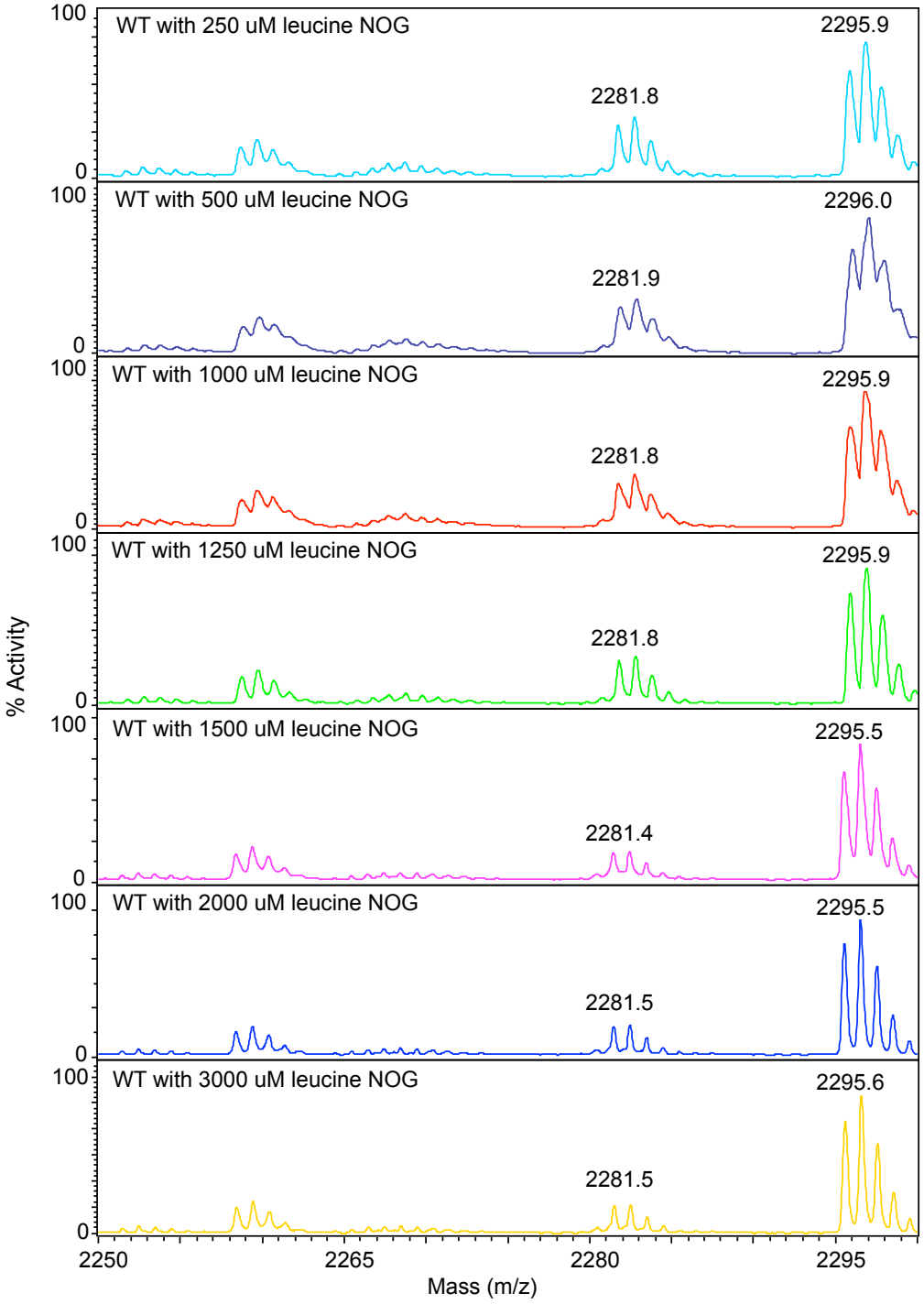
Supplemental Figure S20: MALDI demethylase assay to determine IC_{50} value of Propyl-NOG **5** against the wild type KDM4A.



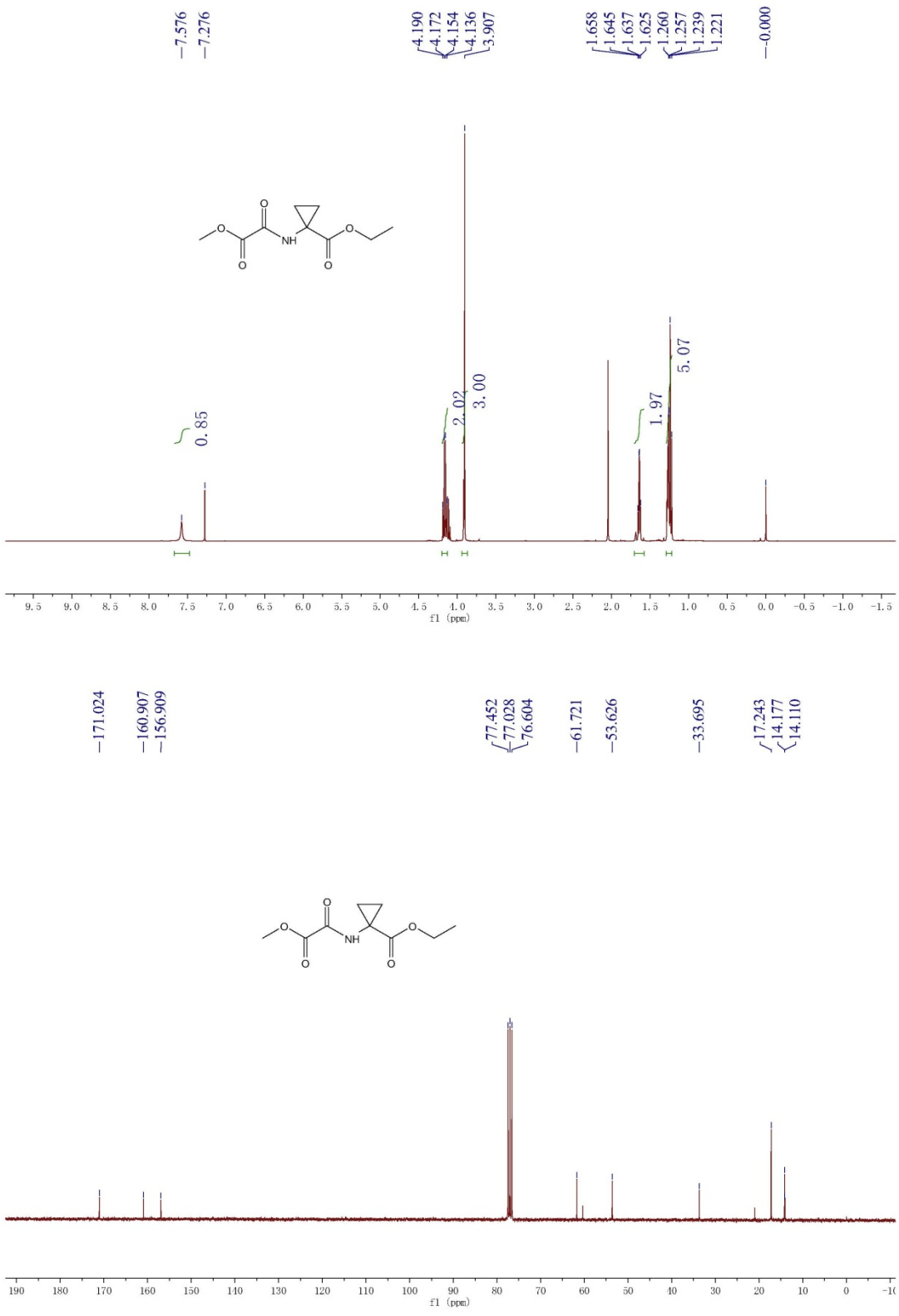
Supplemental Figure S20 Continued: MALDI demethylase assay to determine IC_{50} value of Propyl-NOG **5** against the wild type KDM4A.



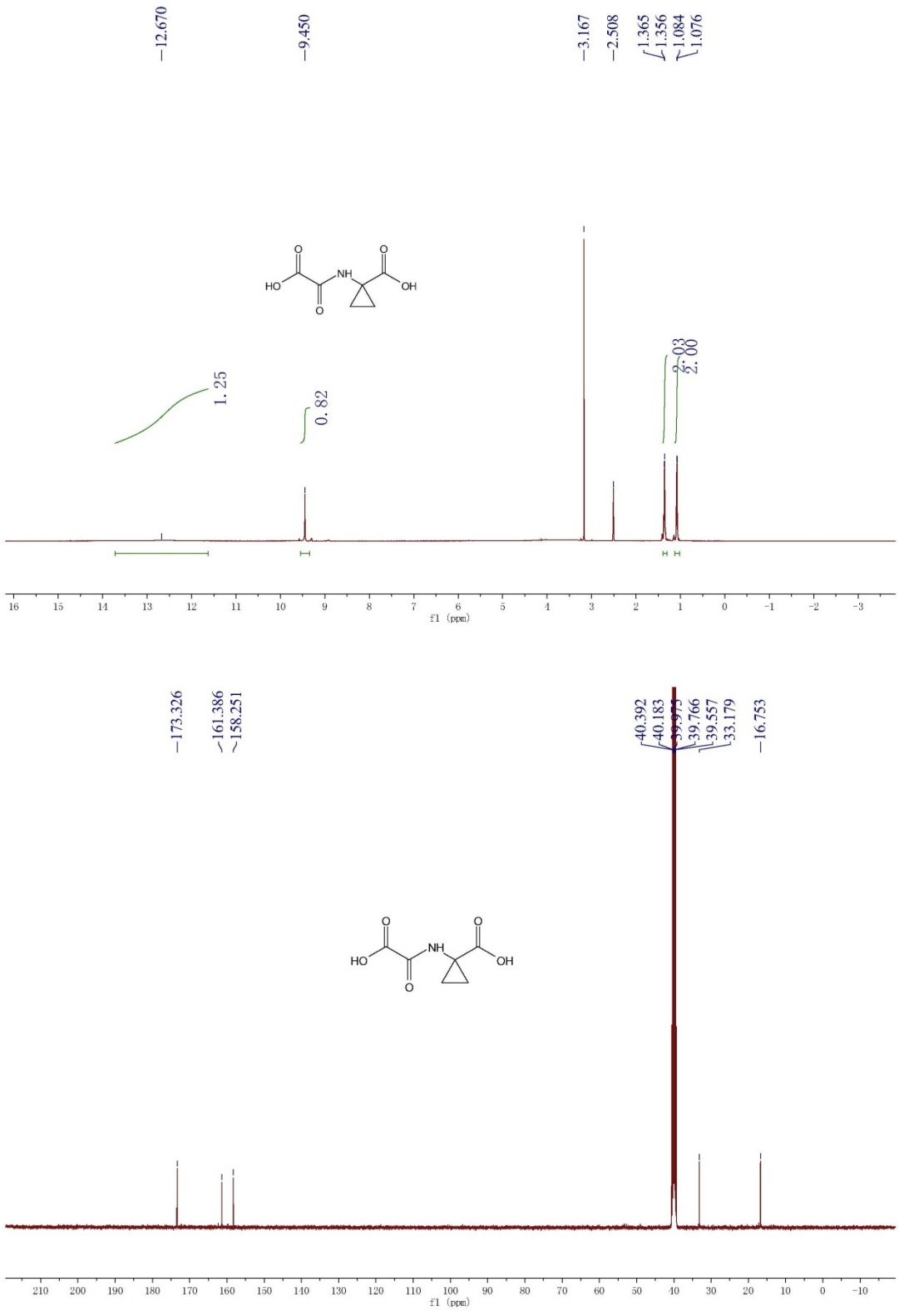
Supplemental Figure S21: MALDI demethylase assay to determine IC_{50} value of leucine-NOG **7** against the wild type KDM4A.



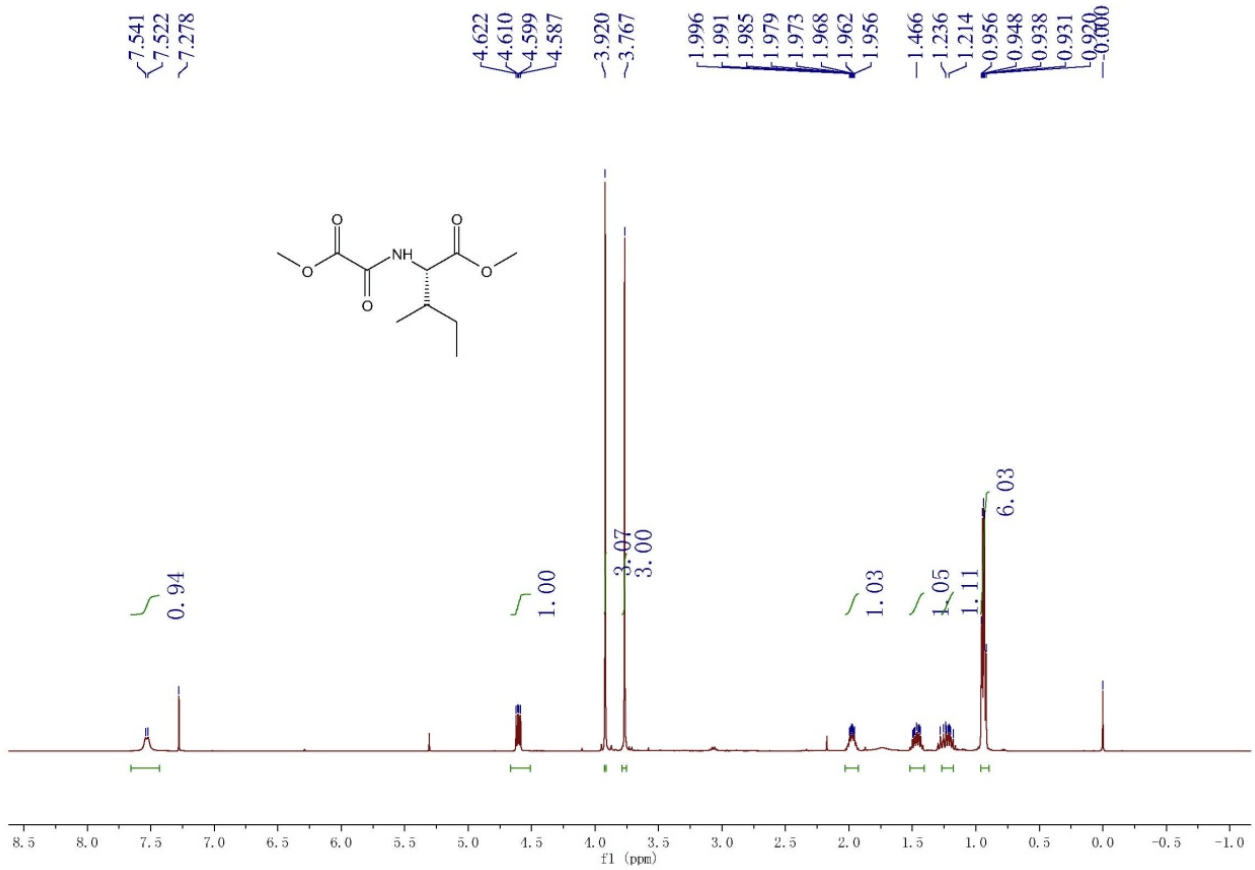
Supplemental Figure S21 Continued: MALDI demethylase assay to determine IC_{50} value of leucine-NOG **7** against the wild type KDM4A.



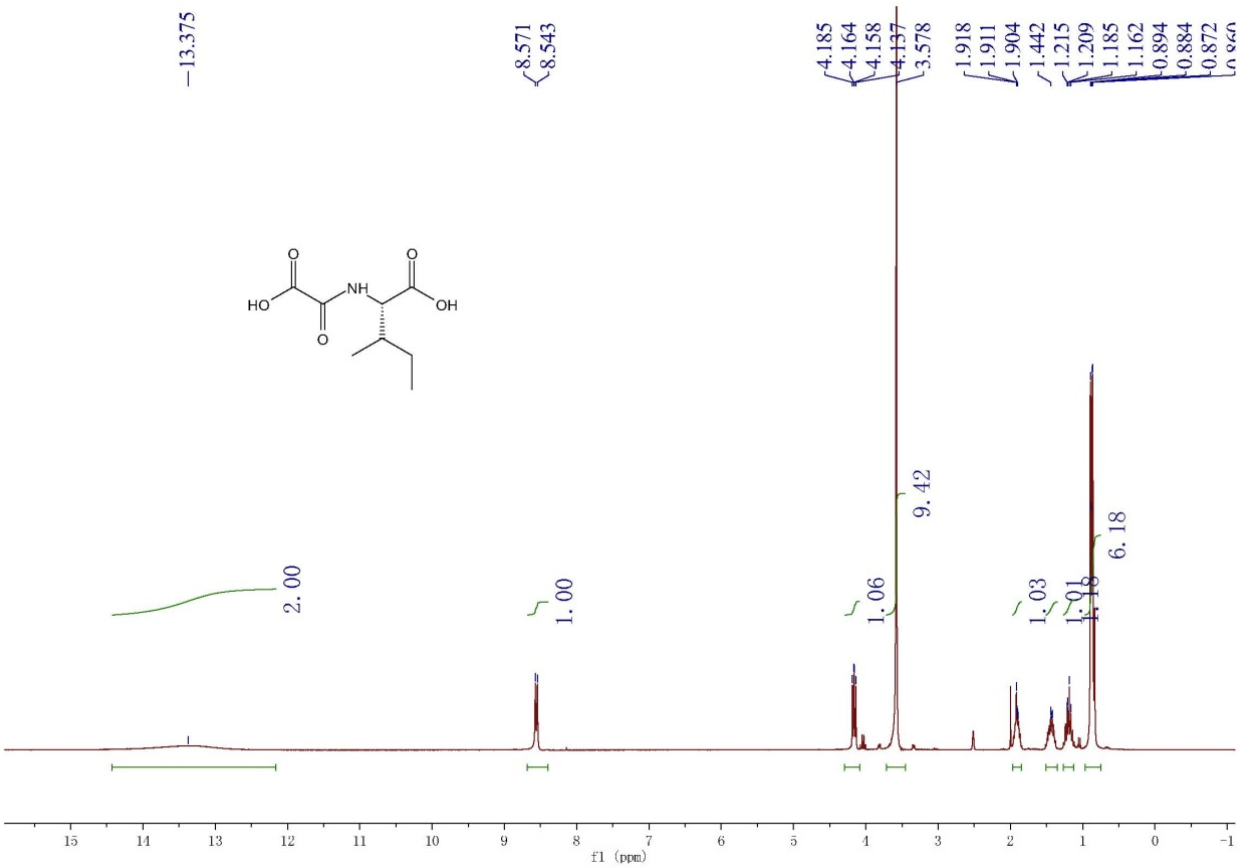
Supplementary Figure S22: ^{1}H and ^{13}C NMR spectra of the indicated compound in $CDCl_3$



Supplementary Figure S23: ^{1}H and ^{13}C NMR spectra of NOG analogue **4** in DMSO-d_6



Supplementary Figure S24: ^{1}H spectrum of the indicated compound in $CDCl_3$



Supplementary Figure S25: ^{1}H NMR spectrum of NOG analogue **8** in DMSO-d_6

Bump-Hole Mutant Primers	
Mutant	**Fwd. Primer**
KDM4A-N198A	5'-GACCTCTACAGCATC**GCC**TACCTGCACTTTGG-3'
KDM4A-N198G	5'-GGACCTCTACAGCATC**GGC**TACCTGCACTTTGGAG-3'
KDM4A-S288A	5'-GGTTTTAACTGTGCGGAG**GCC**ACCAATTTTGCTACCC-3'
KDM4A-S288G	5-GGTTTTAACTGTGCGGAG**GGC**ACCAATTTTGCTACCCG-3'

Supplementary Table S1. List of primers designed for site-directed mutagenesis. Reverse primers used are the reverse-complement to the given forward primers.

Peptide ID	Peptide Sequence	Molecular Weight (Daltons)
H3K9Me3 NO TAG	H_2N-ARTKQTARK$_{(Me3)}$STGGKAPRKQLA-$CONH_2$	2295

Supplementary Table S2. Peptide Sequence used for all experiments.

14. References

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