Supporting Information

Reprogramming the Mechanism of Action of Chlorambucil by coupling to a G-quadruplex Ligand.

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Contents

1. General Experimental Procedures

All solvents and reagents were purified by standard techniques reported in Armarego, W. L. F., Chai, C. L. L., Purification of Laboratory Chemicals, 5th edition, Elsevier, 2003; or used as supplied from commercial sources (Sigma-Aldrich Corporation® unless stated otherwise). NMR spectra were acquired on Bruker® DRX-400, Bruker® DPX-400 and DRX-500 instruments using deuterated solvents as detailed and at ambient probe temperature (300 K). Notation for the ^{1}H NMR spectral splitting patterns includes: singlet (*s*), doublet (*d*), triplet (*t*), broad (*br*) and multiplet/overlapping peaks (*m*). Signals are quoted as δ values in ppm, coupling constants (*J*), are quoted in Hertz and approximated to the nearest 0.5. Data analysis for the nuclear magnetic resonance (NMR) spectra was performed using TopSpin® software. Mass spectra were recorded on a Micromass® Q-Tof (ESI) spectrometer. Thin layer chromatography (TLC) was performed on Merck Kieselgel 60 F254 plates, and spots were visualized under UV light. Flash chromatography (FC) was performed using Merck Kieselgel 60 at room temperature under a positive pressure of nitrogen using previously distilled solvents. High performance liquid chromatography (HPLC) purification was carried out on all final compounds by using a Varian Pursuit C18, 5 μ column (250 × 21.2 mm) and a gradient elution with H_2O / acetonitrile (MeCN) containing 0.1% TFA at a flow rate of 12.0 ml/ min. All cellular assays have been performed with final compounds which had a purity of ≥98% according LC-MS analysis.

1.1 PAGE. 100 μM stock solutions of oligonucleotides were prepared in molecular biology grade DNase-free water. Further dilutions were carried out in 100 mM KCl,

Tris·HCl buffer, pH 7.4. PAGE experiments were carried out with a 200 nM oligonucleotide concentration. All labelled DNA oligonucleotides were supplied by IBA® GmbH. Dual fluorescently labeled DNA oligonucleotides used in these experiments: H-Telo (5′-FAM-GGG TTA GGG TTA GGG TTA GGG-TAMRA-3′), c-kit1 (5′-FAM-GGG AGG GCG CTG GGA GGA GGG-TAMRA-3′), c-myc (5′-FAM-TGA GGG TGG GTA GGG TGG GTA A-TAMRA-3′), c-myc MUT (5′-FAM-TGA GGG TCC CTA CCC TGG GTA A-TAMRA-3′) and ds-DNA (5′-FAM-TAT AGC TAT A and T ATA GCT ATA-TAMRA-3′). The two labelling fluorophores were 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA). The dual-labeled oligonucleotides were annealed at a concentration of 400 nM by heating at 95 ºC for 10 min followed by slow cooling to rt at a controlled rate of 0.1 ºC/min. The annealed oligonucleotides were diluted 1:1 by adding an equal volume of buffer containing different doses of PDS-Chlorambucil (**3**) (0 to 10 μM). The solution was incubated at 37 ºC for 1 h, added with Novex® TBE-Urea Sample Buffer (2X) and heated up at 75 ºC for 5 min prior gel loading. Pre-cast Novex® TBE-Urea Gels were loaded with 10 μl of the sample and electrophoresis run performed following the manufacturer's specifications. Gels were imaged using a GE Healthcare "Typhoon 9400″ at the appropriate wavelengths.

1.2 HPLC Analysis. All DNA oligonucleotides used in these experiments: H-Telo (GGG TTA GGG TTA GGG TTA GGG), c-myc (TGA GGG TGG GTA GGG TGG GTA A), and ds-DNA (TAT AGC TAT A and T ATA GCT ATA) were supplied from IBA® GmbH. The dual-labeled oligonucleotides were annealed at a concentration of 400 nM, in 100 mM KCl, Tris·HCl buffer, pH 7.4, by heating at 94 ºC for 10 min followed by slow cooling to rt at a controlled rate of 0.1 ºC/min.

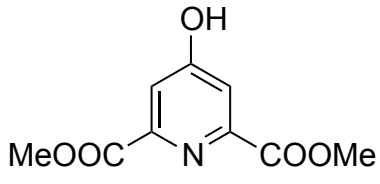
Annealed oligonucleotides were incubated with different doses of (**3**) in the same buffer at 37 ºC for 2 h. Incubated DNA was nuclease digested as by a literature protocol (S1), purified with Amicon Ultra 0.5 mL 10 kDa columns and analysed by LC-MS Ultimate 3000 Dionex MS Ion Trap AmaZon X Bruker (HyStar software). The system was equipped with an Eclipse Agilent column 3.5 mm XDB-C18 3.0 x 75 mm. The column temperature was maintained at 45°C. Eluting buffers were buffer A (500 mM Ammonium Acetate (Fisher) pH 5), Buffer B (Acetonitrile) and Buffer C (H_2O). Buffer A was held at 1% throughout the whole run and the gradient for the remaining buffers was 0 min – 0.5% B, 2 min – 1% B, 8 min – 4% B, 20 min – 95% B.

1.3 Cell Culture. Human apparently normal fibroblast cells and human Xeroderma pigmentosum, complementation group A fibroblast cells (GM00637, GM04312D and GM15876 derived from GM04312 supplemented with XPA cDNA Coriel Institute) were grown in Eagle Minimum Essential Media (Sigma Aldrich, M5650; supplemented with 10% foetal calf serum and 2 mM L-glutamine) at 37°C in 5% CO_2 in air. HCT116 colorectal carcinoma cells (ATCC# CCL-247) were grown in McCoy's 5a Medium (supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2 in air. HCT116 WT and BRCA2$^{-/-}$ cells were provided by Professor Carlos Caldas (CRUK Cambridge Institute, Cambridge, UK).[8c]

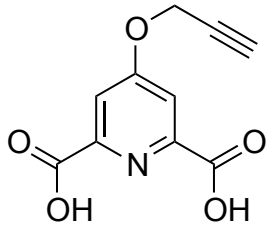
1.4 Measurement of Growth Inhibition. Cell growth was recorded continuously during treatment with small molecules using the xCELLigence RTCA-SP real-time cell monitoring system (ACEA) as previously reported.[8c] In a typical experiment cells are seeded in 96-well E-plates (5,000 cells/well) and allowed to adhere and begin log

phase growth (typically 18-20 h, 37°C in 5% CO_2 in air). The E-plate is removed at 20 h and cells treated with small molecule (generally 100, 20, 4 and 0.8 μM). The plate was re-installed into the RTCA-SP device and cell growth monitored for 72 h. All growth, measured as impedance and plotted as cell index, is monitored without disturbing the E-plate for the remainder of the experiment. Growth data were analyzed and GI_{50} curves plotted using proprietary xCELLigence software.

2. Synthesis of PDS-Chlorambucil

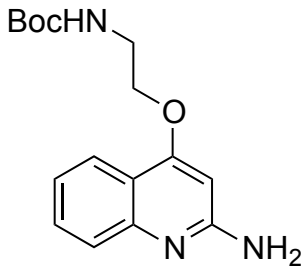


Chelidamic acid dimethyl ester. Chelidamic acid hydrate (2.0 g, 10.16 mmol) was suspended in 20 ml MeOH and $SOCl_2$ 500 µl was added at -10 °C under stirring. The solution was slowly allowed to warm up at room temperature and kept under stirring overnight. After that time the green solution was refluxed 2 h and the solvent removed *in vacuo*. The green oil obtained is re-crystallized from EtOH to afford a withe solid (1.5 g, 7.62 mmol, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.39 (2H, *br s*), 3.92 (6H, *s*).



4-(Prop-2-yn-1-yloxy)pyridine-2,6-dicarboxylic acid. Chelidamic acid dimethylester (0.8 g, 3.5 mmol), propargyl alcohol (0.3 ml, 4.6 mmol) and 1.6 g triphenylphosphine polymer bound (3.0 mmol loading/ g) were added to 50 ml freshly distilled THF and cooled to 0 °C. DIAD (0.9 ml, 4.9 mmol) was added dropwise under argon. The mixture was allowed to warm to rt and stirred for 3 d. The solution was filtered and the solvent was removed *in vacuo* and the product purified by column chromatography (50% EtOAc, 50% petroleum ether) to obtain the dimethyl ester of the title compound as a white powder. This compound was dissolved in 50 ml MeOH and deprotected by slowly adding a solution of NaOH (0.3 g, 7.7 mmol) in 50 ml H_2O. The methanol was evaporated *in vacuo* and the remaining suspension re-dissolved by adding H_2O. The solution was acidified with 5% HCOOH (aq.) and extracted with EtOAc. The organic layer was dried over $MgSO_4$, filtered, and the solvent removed *in vacuo* to obtain the title compound as a white powder (0.6

g, 2.7 mmol, 77%). ^{1}H NMR (400 MHz, CD_3OD) δ_H 7.95 (2H, *s*), 5.04 (2H, *d*, *J* 2.5), 3.16 (1H, *t*, *J* 2.5); ^{13}C NMR (100 MHz, CD_3OD) δ_C 167.1, 165.9, 149.5, 114.4, 78.8, 76.8, 56.5; HRMS (ES) calculated for $C_{10}H_8NO_5$ ([M + H]$^+$) m/z: 222.0402, found 222.0398.

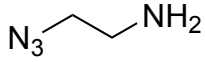


4-(2-*tert*-butoxycarbonylamino-ethoxy)-quinolin-2-ylamine. 2-amino-quinolinone (1.0 g, 6.2 mmol), N-Boc-ethanolamine (1.5 g, 9.3 mmol) and triphenylphosphine (3.3 g, 12.6 mmol) were dissolved in 10 ml freshly distilled THF and cooled to 0 °C. DIAD (1.8 ml, 9.4 mmol) was added dropwise under argon. The mixture was allowed to warm to rt and stirred for 3 d. The solvent was removed *in vacuo* and the product purified by column chromatography (90% EtOAc, 10% MeOH) to obtain the title compound as a white powder (1.2 g, 4.0 mmol, 65%). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.97 (1H, *dd*, *J* 8.0, 1.0 Hz), 7.59 (1H, *dd*, *J* 8.5, 1.0 Hz), 7.54 (1H, *ddd*, *J* 8.5, 7.0, 1.0 Hz), 7.23 (1H, *ddd*, *J* 8.0, 7.0, 1.0 Hz), 6.02 (1H, *s*), 5.00 (1H, *br s*), 4.69 (2H, *br s*), 4.16 (2H, *t*, *J* 5.0 Hz), 3.67 (2H, *dd*, *J* 5.0, 5.5), 1.47 (9H, *s*); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 162.3, 158.0, 155.9, 148.5, 130.3, 125.7, 122.0, 121.6, 117.5, 90.1, 79.8, 67.5, 39.8, 28.4; HRMS (ES) calculated for $C_{16}H_{22}N_3O_3$ ([M + H]$^+$) m/z: 304.1650, found 304.1668.

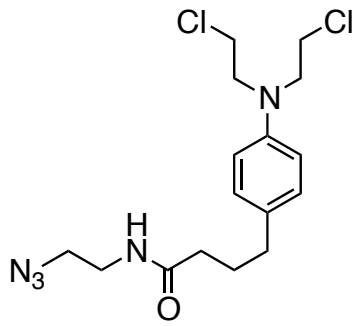
Pyridostatin precursor 1. One mole equivalent of **4-(prop-2-yn-1-yloxy)pyridine-2,6-dicarboxylate** was dissolved in DCM (≈ 0.5 M) and 2.2 mole equivalents of 1-chloro-*N*,*N*,2-trimethylpropenyl-amine were added slowly at 0 ºC. The reaction was allowed to stir at r.t, for 2 h. After total conversion was reached, as followed by LC-MS, the solution was cooled to 0 ºC and 2.2 mole equivalents of triethylamine were added dropwise. The solution was allowed to warm to rt and stirred for another hour.

2 mole equivalents of 4-(2-*tert*-butoxycarbonylamino-ethoxy)-quinolin-2-ylamine were added to the mixture as DCM suspensions (≈ 1.0 M) and stirred under argon at r.t. overnight. Product was precipitated from hot MeCN. Boc-protected compound **1** was dissolved in DCM and deprotected using TFA/DCM (1:2). The final compound was purified by HPLC (gradient: 10% MeCN/90% H_2O, 0.1% TFA to 100% MeCN, 0.1% TFA over 30 min, R_t=15.0-16.5 min).

(1) ^{1}H NMR (400 MHz, CD_3OD) δ_H 8.57 (2H, *d*, *J* 8.0 Hz), 8.27-8.24 (4H, *m*), 8.16 (2H, *d*, *J* 8.0 Hz), 8.08-8.02 (2H, *m*), 7.82-7.76 (2H, *m*), 5.20-5.17 (2H, *m*), 4.89-4.85 (4H, *m*), 3.76-3.70 (4H, *m*), 3.26-3.25 (1H, *m*); ^{13}C NMR (100 MHz, CD_3OD) δ_C 167.7, 167.2, 163.9, 151.3, 149.5, 139.1, 134.1, 127.5, 123.6, 121.4, 118.8, 114.2, 94.6, 78.6, 76.4, 67.4, 56.8, 38.6; HRMS (ES) calculated for $C_{32}H_{30}N_7O_5$ ($[M + H]^+$) m/z: 592.2284, found 592.2297.



2-Azidoethylamine. 2-chloroethylamine hydrocloride (1g, 8.6 mmol) and NaN_3 (650 mg 9 mmol) were dissolved in water and allowed to stir at 80 °C overnight. After this time the solution was cooled down and basified by addition of a KOH 2 M solution. The product was co-distilled with water and quenched in a HCl solution. The final azide was recovered as a hydrochloride salt after evaporating the solvent under reduced pressure (72% yield). Spectroscopic data were in agreement with previous reports.[12b]



***N*-(2-azidoethyl)-4-(4-(bis(2-chloroethyl)amino) phenyl)butanamide (2).** Chlorambucil (500 mg; 1.55 mmol) was dissolved in 4 ml of CH_2Cl_2 and cooled at 0 °C under nitrogen atmosphere in a ice bath. 1.25 ml of $SOCl_2$ were added drop wise under stir, as the solution turns to deep yellow to limpid again. Stirring in the ice bath was kept overnight. The solvent was then removed under vacuum and the reaction mixture was suspended in 5 ml of CH_2Cl_2 and cooled again at 0 °C in an ice bath. TEA (156.8 mg; 1.55 mmol) was added drop wise to the stirring solution followed by 1.33 mg (1.55 mmol) of 2-azidoethylamine dissolved in 1 ml of CH_2Cl_2. The reaction was allowed to warm up at room temperature and the kept under stirring for 1 h. Solvent was removed by vacuum and the crude product was purified by flash chromatography (EtOAc/Et_2O 3:7) to afford the product as yellow oil (78% yield).

(2) ^{1}H NMR (400 MHz, CD_3OD) δ_H 7.10 (2H, *d*, *J* 8.5 Hz), 6.55 (2H, *d*, *J* 8.5 Hz), 3.70 (4H, *t*, *J* 4.5 Hz), 3.60 (4 H, *t*, *J* 4.5 Hz), 3.45-3.30 (4H, *m*), 2.5 (2H, *t*, *J* 4.0 Hz), 2.1 (2H, *t*, *J* 4.0 Hz), 1.85-1.75 (2H, *m*); ^{13}C NMR (100 MHz, CD_3OD) δ_C 144.2, 130.6, 129.6, 121.4, 112.2, 53.5, 50.8 40.3, 38.7, 35.6, 33.9 27.0; HRMS (ES) calculated for $C_{16}H_{23}Cl_2N_5O_0$ ($[M + H]^+$) m/z: 372.2927, found 372.2971.

Chl-PDS. 23 mg (38 mmol) of precursor (**1**) were suspended in 1.2 ml of water and 1.2 ml of t-BuOH under nitrogen atmosphere. 100 µl of a solution 0.1 M of $CuSO_4{\cdot}5H_2O$ were added to the stirred solution at 0 °C, followed by 300 µl of a Sodium Ascorbate solution 1 M. The solution turns yellow and allowed to stir overnight at room temperature. The reaction is monitored by LC-MS and after solvent removal under vacuum compound **3** can be purified by preparative HPLC.

(Chl-PDS) ^{1}H NMR (400 MHz, CD_3OD) δ_H 8.55 (2H, *d*, *J* 8.5 Hz), 8.20-8.15 (4H, *m*), 8.10 (2H, *d*, *J* 8.5 Hz), 8.00-7.90 (2H, *m*), 7.70-7.65 (2H, *m*), 6.95 (2H, *d*, *J* 8.5 Hz), 6.57 (2H, *d*, *J* 8.5 Hz), 5.50 (2H, *s*), 4.75 (4H, *t*, *J* 4.5 Hz), 4.56 (2H, *t*, *J* 5.5 Hz), 3.69-3.63 (6H, *m*) 3.59-3.48 (8H, *m*), 2.37 (2H, *t*, *J* 7 Hz), 2.1 (2H, *t*, *J* 7 Hz), 1.75 (2H, *t*, *J* 7 Hz),; ^{13}C NMR (125 MHz, CD_3OD) δ_C 176.4, 169.3, 167.1, 164.3, 152.3, 150.8, 145.9, 143.2, 142.7, 134.5, 131.6, 130.6, 127.8, 126.5, 124.2, 123.8, 120.0, 114.7, 113.4, 95.2, 67.8, 63.5, 54.5, 49.5, 41.7, 40.2, 39.8, 36.3, 35.0, 28.8 ; HRMS (ES) calculated for $C_{48}H_{53}O_6N_{12}{}^{35}Cl_2([M + H]^+)$ m/z: 963.3583, found 963.3551.

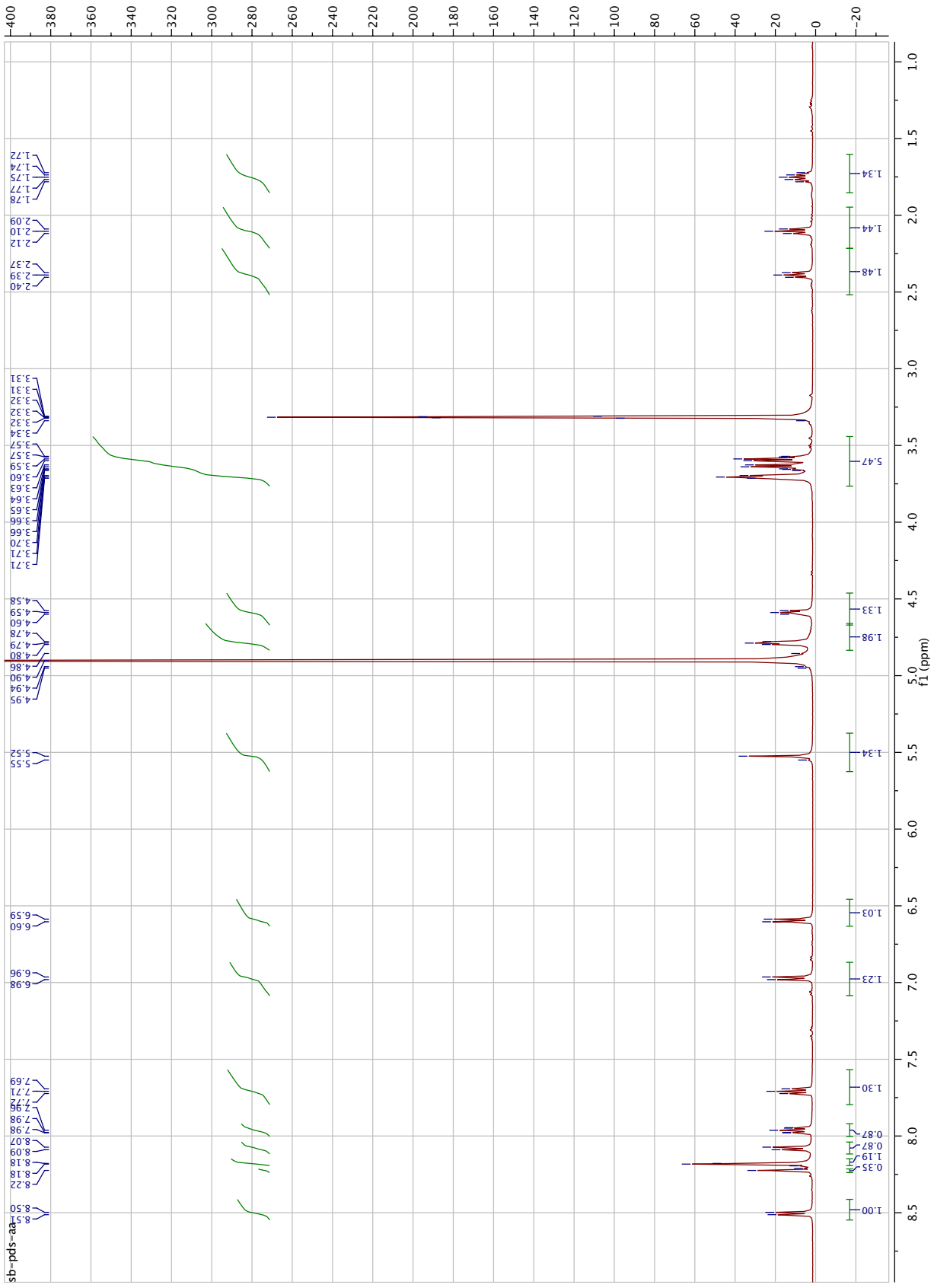


Figure S1. ^{1}H-NMR spectra of PDS-Chl in CD_3OD

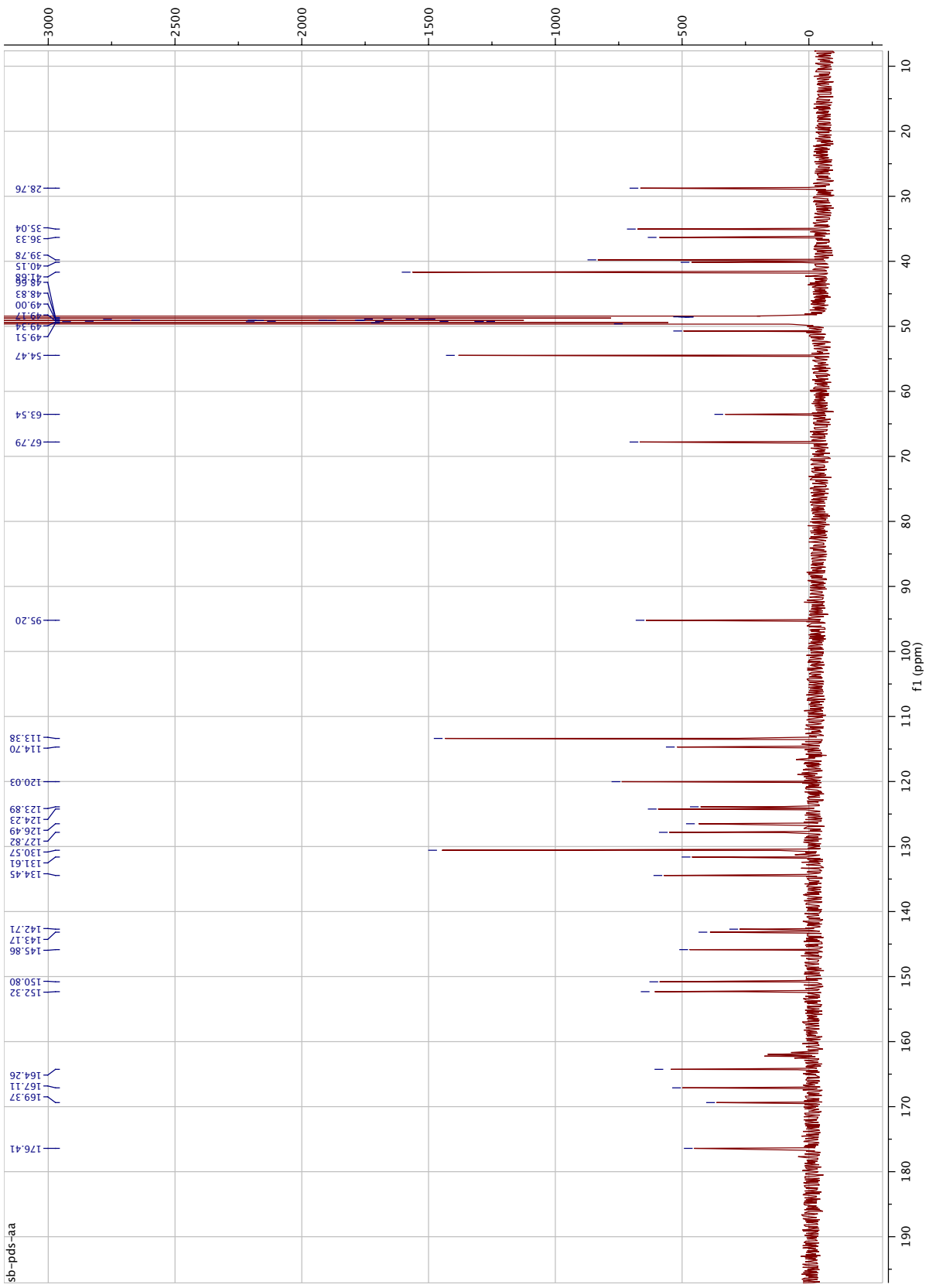


Figure S2. ^{13}C-NMR spectra of PDS-Chl in CD_3OD

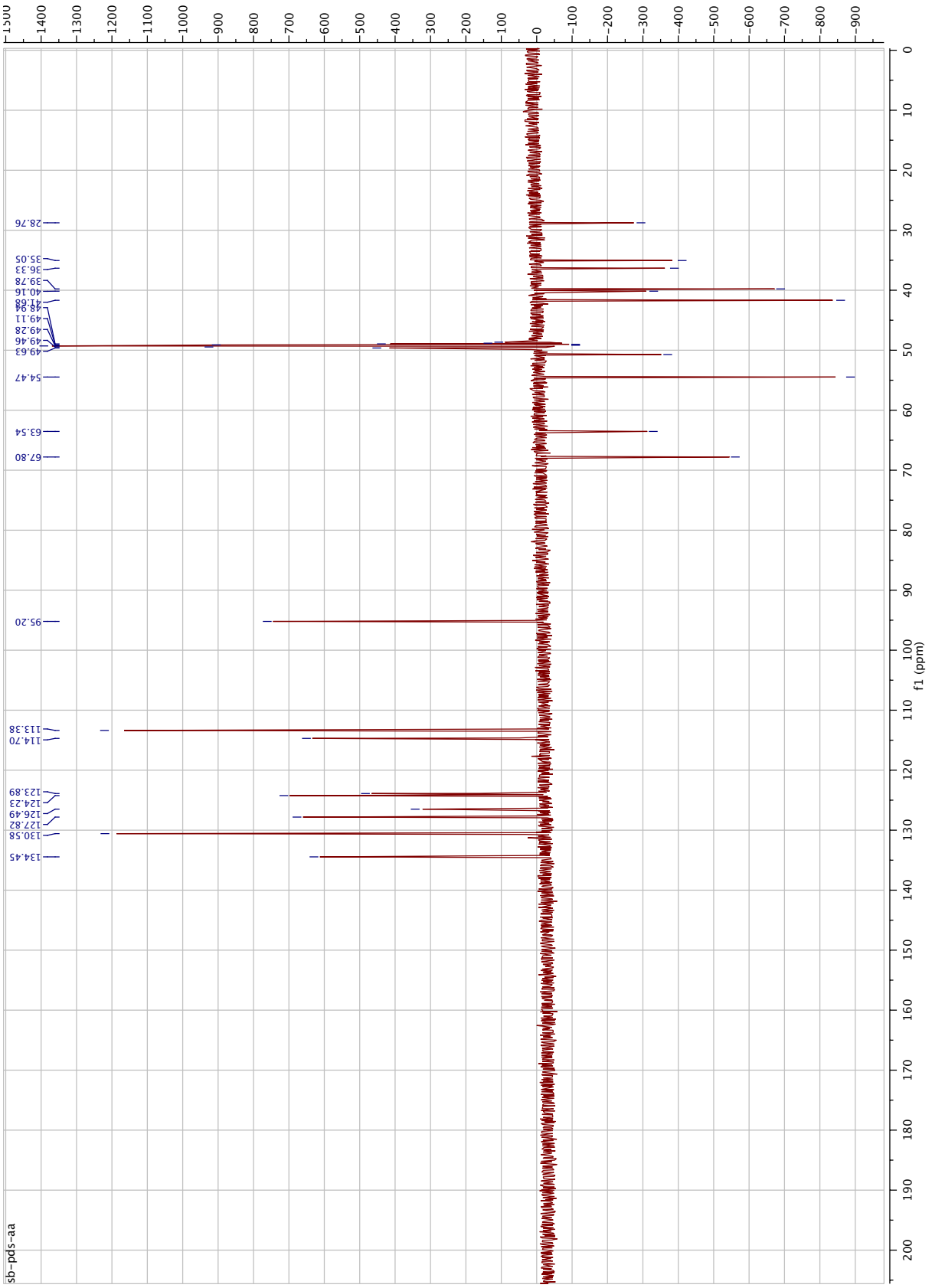


Figure S3. DEPT spectra of PDS-Chl in CD_3OD

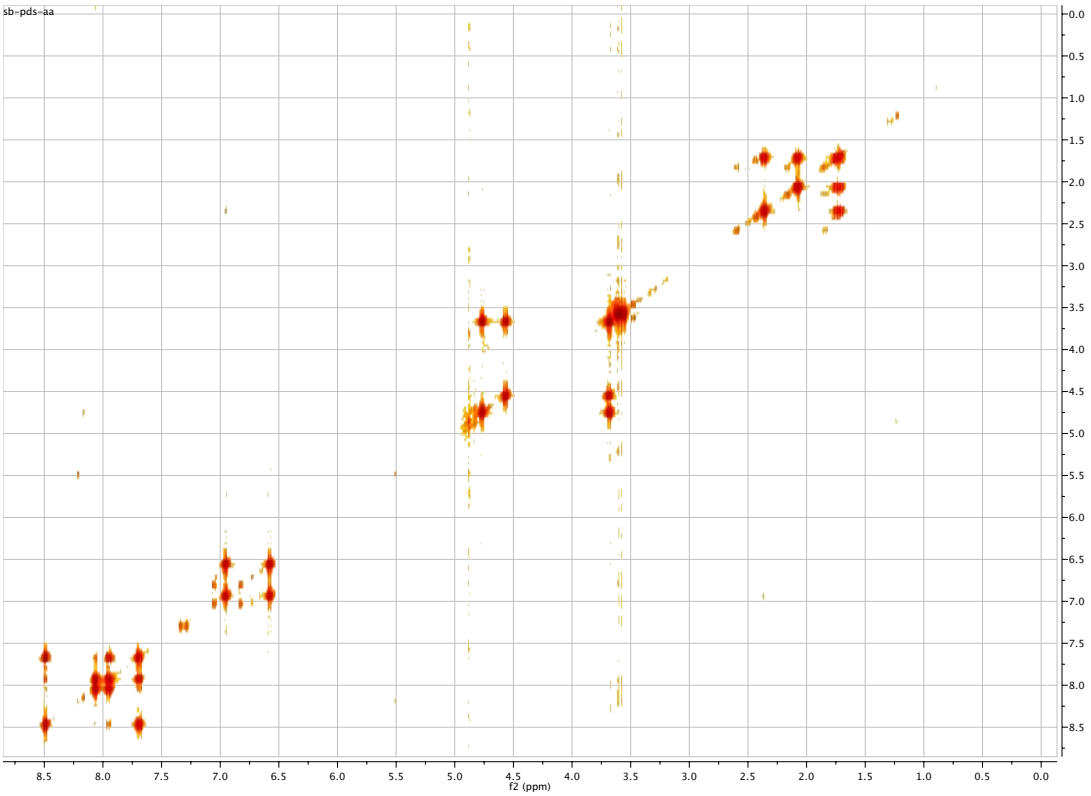


Figure S4. COSY analysis of PDS-Chl in CD_3OD

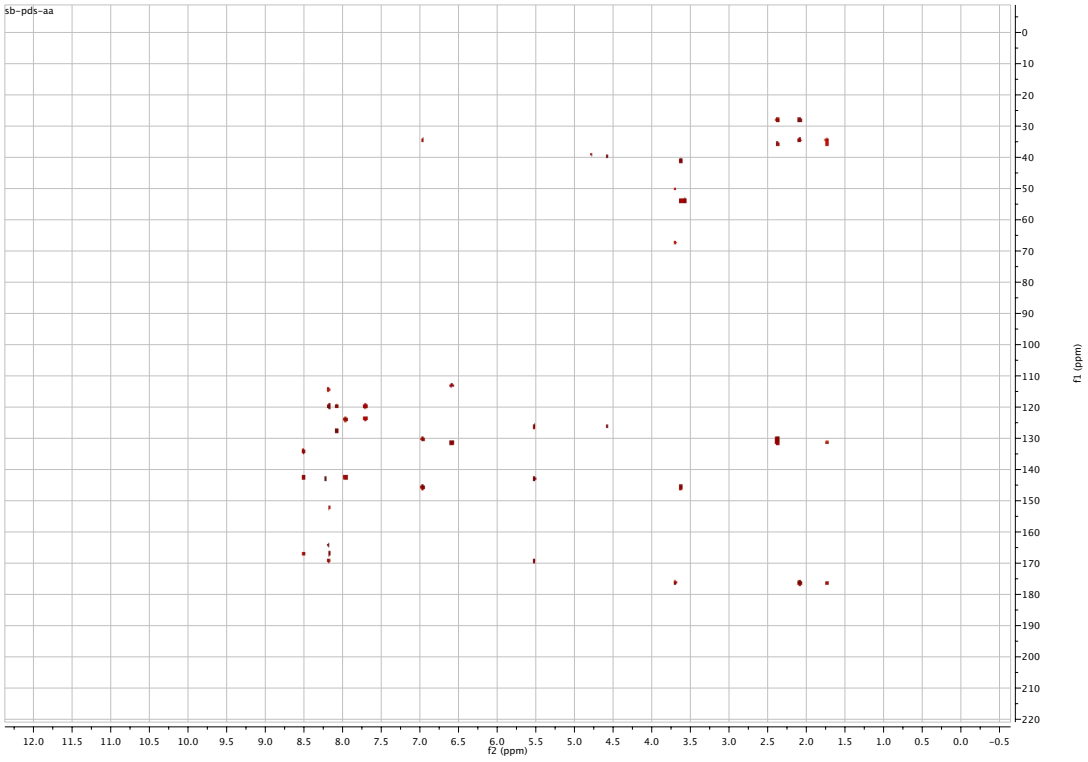


Figure S5. HETCOR analysis of PDS-Chl in CD_3OD

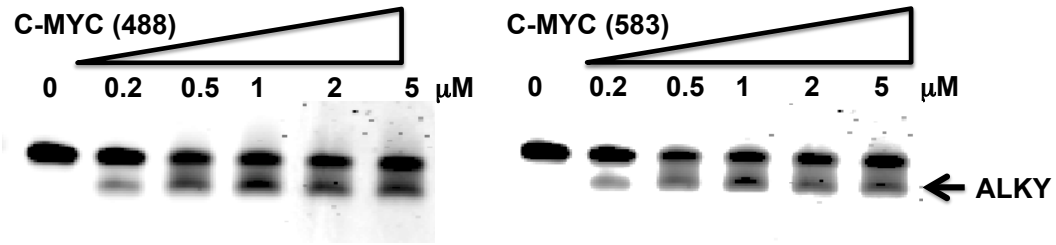


Figure S6. Urea denaturing gel shown in Figure 2 obtained by fluorophore excitation at 488 nm (left) and 583 (right).

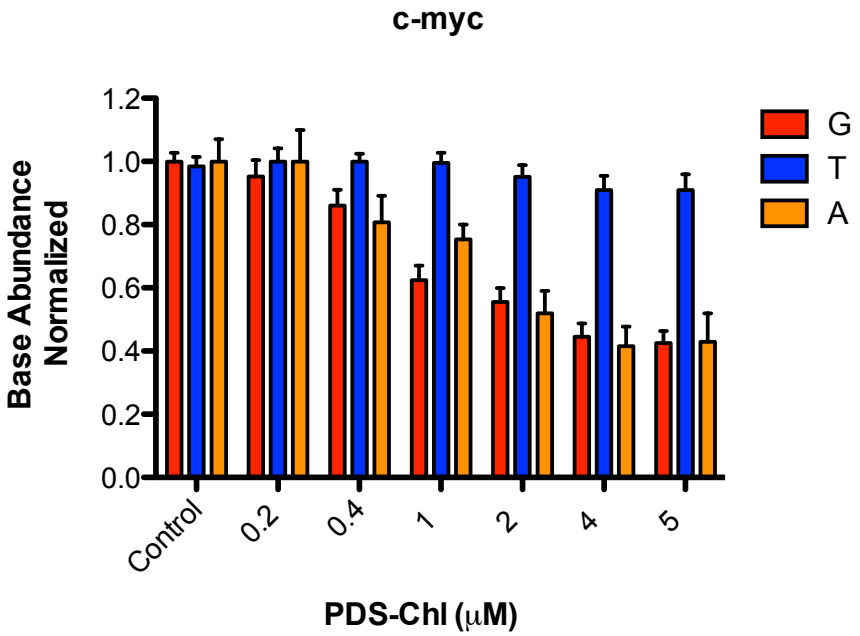


Figure S7. Relative base abundance measured by analytical HPCL after incubation of c-myc with different doses of PDS-Chl and nuclease treatment

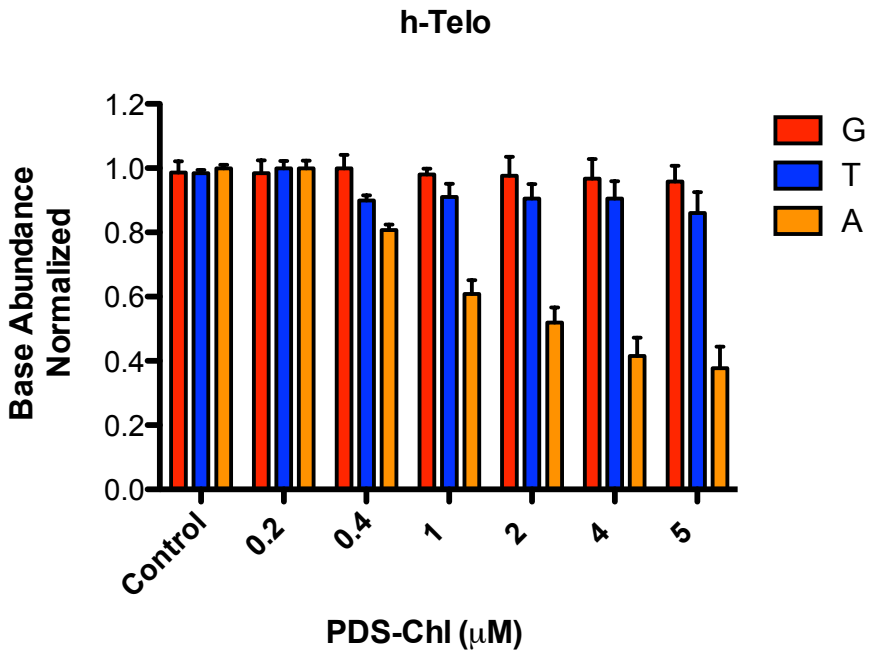


Figure S8. Relative base abundance measured by analytical HPCL after incubation of h-Telo with different doses of PDS-Chl and nuclease treatment

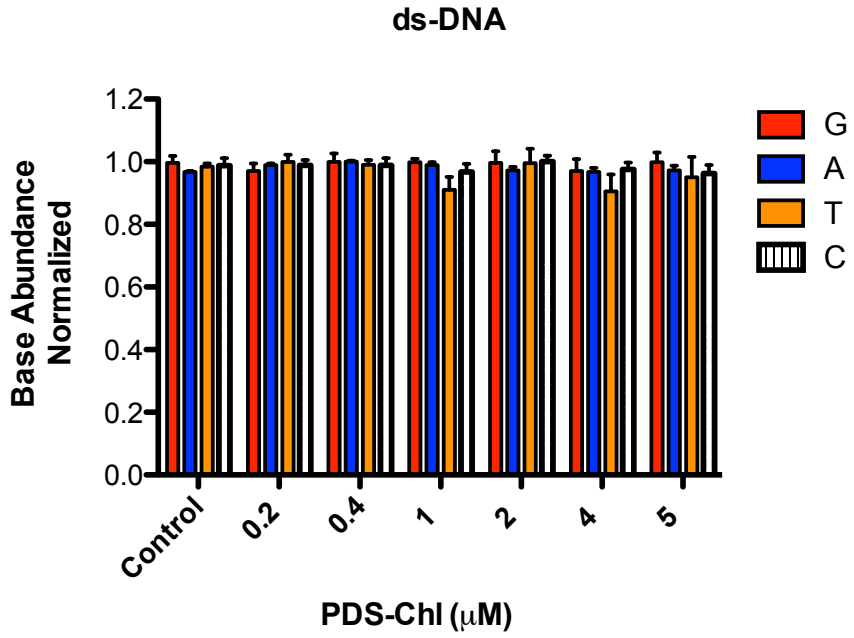


Figure S9. Relative base abundance measured by analytical HPCL after incubation of ds-DNA with different doses of PDS-Chl and nuclease treatment

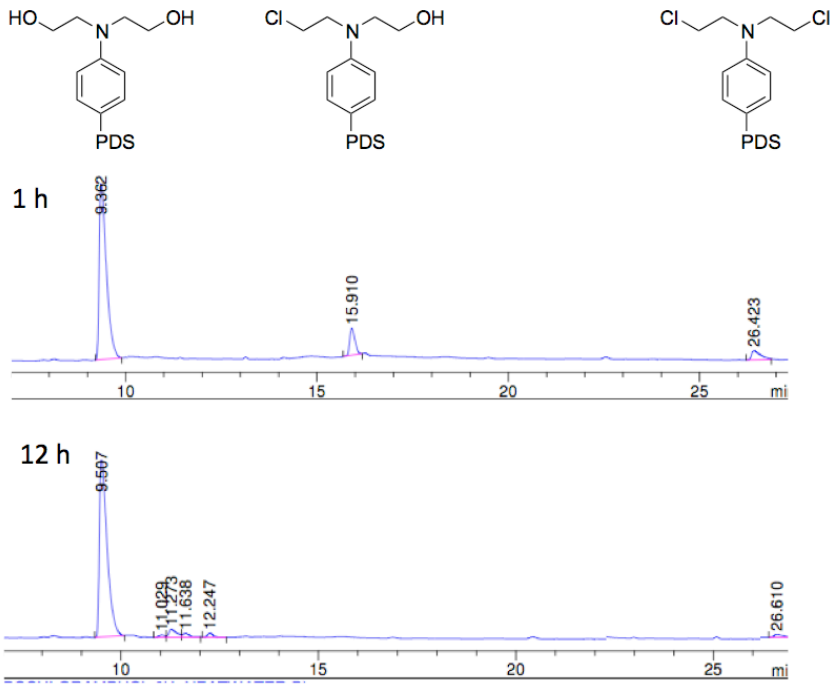


Figure S10. HPLC chromatographic traces of PDS-Chl hydrolysis at 37 °C in water after 1 h (top) and 12 h (bottom) incubation.

(S1) Gu, H.; Smith, Z. D.; Bock, C.; Boyle, P.; Gnirke, A.; Meissner, A. *Nat. Protoc.* **2011**, *6*, 468.