Sequence-Dependent Diastereospecific and Diastereodivergent Crosslinking of DNA by Decarbamoylmitomycin C

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Abstract: Mitomycin C (MC), a potent antitumor drug, and decarbamoylmitomycin C (DMC), a derivative lacking the carbamoyl group, form highly cytotoxic DNA interstrand crosslinks. The major interstrand crosslink formed by DMC is the C1" epimer of the major crosslink formed by MC. The molecular basis for the stereochemical configuration exhibited by DMC was investigated using biomimetic synthesis. The formation of DNA-DNA crosslinks by DMC is diastereospecific and diastereodivergent: Only the 1"S-diastereomer of the initially formed monoadduct can form crosslinks at GpC sequences, and only the 1"R-diastereomer of the monoadduct can form crosslinks at CpG sequences. We also show that CpG and GpC sequences react with divergent diastereoselectivity in the first alkylation step: 1"S stereochemistry is favored at GpC sequences and 1"R stereochemistry is favored at CpG sequences. Therefore, the first alkylation step results, at each sequence, in the selective formation of the diastereomer able to generate an interstrand DNA-DNA crosslink after the "second arm" alkylation. Examination of the known DNA adduct pattern obtained after treatment of cancer cell cultures with DMC indicates that the GpC sequence is the major target for the formation of DNA-DNA crosslinks *in vivo* by this drug.

Table of contents **Pages**

S-1 Materials.

Mitonycin C (MC) was a generous gift from Professor Maria Tomasz. Decarbamoylmitomycin C (DMC) was synthesized from MC by a published procedure.[1] Phosphodiesterase I (snake venom diesterase (SVD), *Crotalus adamanteus* venom, E.C. 3.1.4.1. and alkaline phosphatase (*Escherichia coli*, EC 3.1.3.1) were obtained from Worthington Biochemical Corp (Freehold, NJ). Nuclease P1 (penicillium citrinum, EC 3.1.30.1) and calf thymus DNA (type XV; 42% GC) were from Sigma Life Sciences (St. Louis, MO). Sep-Pak C-18 cartridges were purchased from Waters Corp (Milford, MA). Oligonucleotides were purchased from Midland Certified Reagent (Midland, TX).

S-2 Methods and Instrumentation.

S-2-1 Quantitative Analysis. Quantitation of DNA and DMC-DNA adducts was based on UV spectrophotometry using the following molar extinction coefficients: Calf Thymus DNA, 6500 (260 nm, mononucleotide); dT, 6600 (254 nm); dA, 14200 (254 nm); DMC monadducts (**9a**, **9b**), 24000 (254 nm); DMC Interstand Crosslinks (**10a**, **10b**), 30000 (254 nm).

The percent yields of DMC-DNA-adducts (**9a**, **9b**, **10a**, **10b**) designate the mole percent adducted guanine of total guanine nucleotide residues present in the oligonucleotides or DNA substrates. A full description of the method is available in references 2 and 3.[2,3]

S-2-2 High Resolution Mass Spectroscopy. HRMS spectra were recorded by direct infusion using Bruker's micrOTOF-II ESI instrument at Notre Dame University Mass Spectrometry Facility.

S-2-3 UV Spectra. UV spectra were obtained using a Shimadzu UV 2600 UV-vis spectrometer.

S-2-4 Reproducibility. Reactions were repeated at least twice independently and average deviation from the mean percentage yield of each adduct is represented in brackets in tables 2 and 3.

S-3 Reaction Protocols.

S-3-1 Oligonucleotide Alkylation by DMC under Bifunctional Conditions, Incremental Additions of $Na_2S_2O_4$ at 37°C (protocol 1). Oligonucleotides 5'-T**GC**TT**GC**TT**GC**TT**GC**TT**GC**TT**GC**T-3', T**GC**AT**GC**AT**GC**AA**GC**TA**GC**TA**GC**T-3', 5'-T**CG**TT**CG**TT**CG**TT**CG**TT**CG**TT**CG**T-3', T**CG**AT**CG**AT**CG**AA**CG**TA**CG**TA**CG**T-3' (10 A_{260}unit scale; Tm: 57°C) were each mixed with an equal amount of their complementary strand (10 A_{260}unit scale) in water. The mixture was lyophilized. Oligonucleotides were then annealed by heating (90°C, 10 min) after the addition of 0.01 M potassium phosphate buffer, pH 5.8 (355 µL) followed by slow cooling to 37°C. The reaction mixture was deaerated via argon bubbling (30 min) while kept at 37°C. Excess $Na_2S_2O_4$ (6.48 µmol in 20 µL of potassium phosphate buffer, pH 5.8) from a freshly prepared anaerobic solution was then added to the mixture quickly and immediately followed by addition of DMC (1.30 µmol). The reaction was allowed to stir for 10 min before another addition of $Na_2S_2O_4$ (3.24 µmol in 10 µL of potassium phosphate buffer, pH 5.8) was immediately followed by addition of DMC (1.30 µmol in 200µL). This process was repeated 3 more times until the reaction was treated with a total of 6.50 µmol of DMC. The reaction mixture was allowed to stir with argon bubbling for 1 hr and then opened to air, followed by gentle mixing until a consistent purple color was obtained. The mixture was then allowed to

stir for 20 min under air and chromatographed on a 2.5*56 cm Sephadex G-25 column using 0.02M NH_4HCO_3 as eluent. Oligonucleotide containing fractions were lyophilized.

S-3-2 Oligonucleotide Alkylation by DMC under Bifunctional Conditions, Incremental Additions of $Na_2S_2O_4$ at 0°C (protocol 2, first step of the 2-step crosslinking process). A similar process as protocol 1 was followed with oligonucleotides **11**, **12**, **13** and **14** except that the reaction was done at 0°C. The lyophilized products were used as such for the second step described below (2-step crosslinking).

S-3-3 Crosslinking of Monoalkylated Oligonucleotides at 37°C (protocol 2, second step of the 2-step crosslinking process). Lyophiliozed monoalkylated oligonucleotides **11, 12, 13, 14** (products from protocol 1, first step) were redissolved in 0.01 M potassium phosphate buffer, pH 5.8 (355 µL). Oligonucleotides were then annealed by heating (90°C, 10 min) followed by slow cooling to 37°C. Sodium dithionite (1.61 µmol in 10 µL of potassium phosphate buffer, pH 5.8) from a freshly prepared anaerobic solution was quickly added. The reaction mixture was stirred at 37°C with argon bubbling. The mixture was opened to air after 1 h followed by gentle mixing until a consistent purple color was obtained. The mixture was then stirred for 20 min under air and chromatographed on a 2.5*56 cm Sephadex G-25 column using 0.02M NH_4HCO_3 as eluent. Oligonucleotide containing fractions were lyophilized.

S-3-4 Calf thymus DNA Alkylation by DMC under $Na_2S_2O_4$ Activation. A solution of sonicated calf thymus DNA (12 mM) and DMC (1mM) was deaerated in 0.01 M potassium phosphate-0.001 M EDTA, pH 5.8 (1 mL). A deaerated solution of $Na_2S_2O_4$ (2mM) was added. $Na_2S_2O_4$ and DMC were added incrementally as described in protocol 1 until a total of 10 mM of $Na_2S_2O_4$ and 5 mM of DMC were added. The drug-DNA complexes were extracted by adding

0.01M potassium phosphate, pH 9.0 to adjust the pH to 8.0 and then adding 1 ml of phenol:$CHCl_3$:isoamyl alcohol extraction solution (25:24:1 v/v). The solution was vortexed vigorously and allowed to settle before it was transferred into vials and centrifuged (13,000 rpm, 10 min). DNA was isolated by applying only the top aqueous layer on a Sephadex G-25 column.

S-4 Enzymatic Digestions.

S-4-1 Enzymatic Digestion of the DMC-DNA Complexes to Nucleosides and DMC-Nucleoside Adducts. The lyophilized DMC-DNA complex (calf thymus) was dissolved in 0.02 M ammonium acetate, pH 5.5 (2.5 A_{260} units/mL). Nuclease P1 (1.0 unit/A_{260} unit of complex) was added to the mixture followed by incubation for 4 h at 37°C. The pH was adjusted to 8.2 by addition of 0.2 M NaOH, and $MgCl_2$ was added to a concentration of 0.9 mM. Addition of Snake Venom Diesterase (2.25 units/A_{260} unit of complex) was followed by incubation at 37°C (2 hr). Alkaline phosphatase (1.6 units/A_{260} unit of complex) was then added and the digest was incubated overnight at 37°C. Samples were lyophilized and redissolved for HPLC analysis.

S-4-2 Enzymatic Digestion of Alkylated Oligonucleotides. Each alkylated oligonucleotide (1 A_{260} unit) was incubated with 1 unit of nuclease P1 at 37°C for 2 hours in 0.8 mL of 0.02 M ammonium acetate, pH 5.5. The pH was then adjusted to 8.2 by addition of 0.2 M NaOH and 20μL of a 0.1 M $MgCl_2$ solution was added followed by SVD (2 units) and AP (2 units). Incubation continued at 37°C for 2.5 h.

S-5 Analysis and Identification of DMC DNA Adducts.

S-5-1 Analysis and Isolation of DNA Adducts after Enzymatic Digestion of Alkylated Oligonucleotides and Calf Thymus DNA. Digestion mixtures were analyzed by HPLC using an Agilent 1200 HPLC system and a Kromasil C-18 reverse phase column (0.46*25 cm). The elution system to analyze digested oligonucleotides was: 6-18% acetonitrile in 0.03 M potassium phosphate, pH 5.4, in 60 min, 1 mL/min flow rate. The temperature of the column was 45°C. Retention times: **9a**: 13 min; **10a**: 15 min; **9b**: 19 min; **10b**: 21 min. For the analysis of Calf Thymus DNA digests, see section S6: In that case, the column was kept at room temperature.

S-5-2 Identification of 9a, 9b, 10a. Adducts **9a**, **9b** and **10a** were identified by their UV spectra, retention times and co-elution with authentic standards synthesized previously in our laboratories.[4,5] Examples of co-elution chromatograms are shown in Figures S4, S6 and S7.

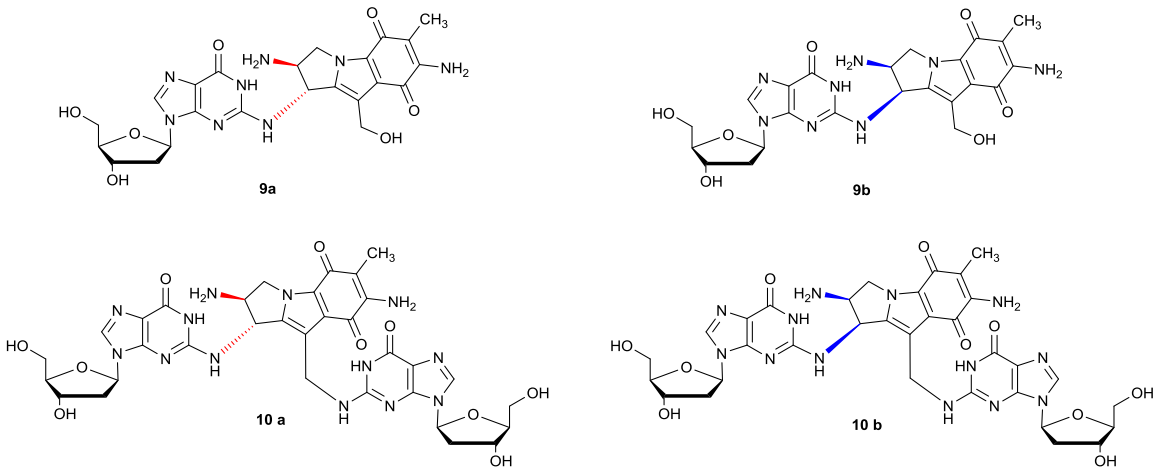

Figure S1: Adducts **9a**, **9b**, **10a, 10b**

S-5-3 Identification of adduct 10b. Adduct **10b** has previously been isolated from culture cells[6]. Its presence in HPLC digests of oligonucleotides and Calf Thymus DNA was assigned based on its UV spectrum (Figure S2) and retention time (HPLC, RT=21 min). In addition, we isolated an authentic standard of **10b** by HPLC from the reaction between DMC and Calf Thymus DNA and recorded a CD spectrum (Figure S2) as well as obtained HRMS data (*m*/*z* calcd for $C_{33}H_{38}N_{13}O_{10}$ $[M + H]^+$ 776.2859, found 776.2882). This is the first reported synthesis of an authentic standard of adduct **10b**. The weak positive cotton effect between 500 and 600 nm is diagnostic of the β configuration at C1".[7]

Co-injection between adduct **10b** isolated from calf thymus DNA (HPLC) and the digest of oligonucleotide **11** confirmed that the same adduct **10b** is present in DMC reactions between oligonucleotides and Calf Thymus DNA (Figure S4).

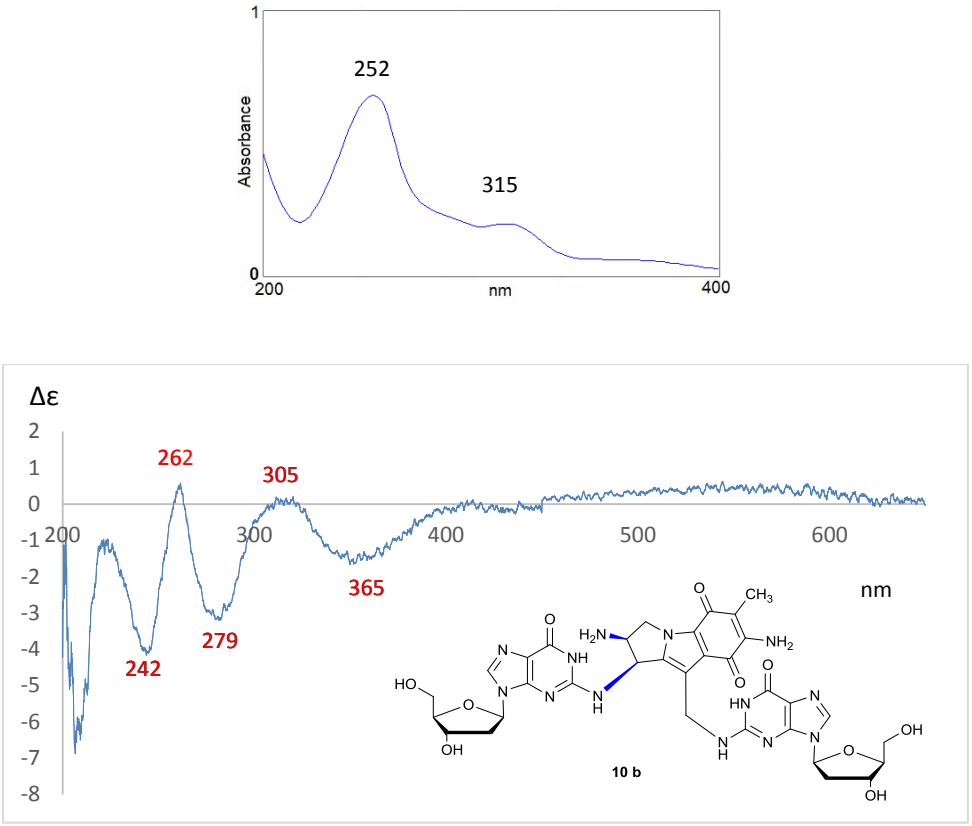

Figure S2. UV spectrum (top) and CD spectrum (bottom) of adduct **10b** (0.000029 M).

S-6 HPLC Chromatograms of Digests from the Reaction between DMC and Calf Thymus DNA at Various Temperatures.

Calft Thymus DNA was treated by DMC acccording to the protocol described above (S-3-4). Below are chromatograms of the digest from reactions performed at different temperatures.

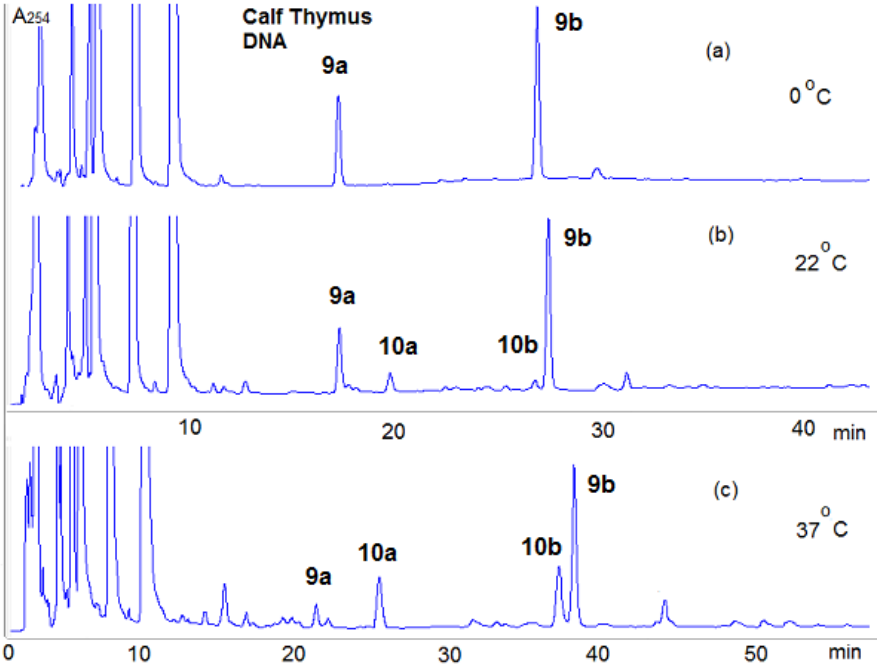

Figure S3. HPLC of digests from the reaction between DMC and Calf Thymus DNA at various temperatures. Digestion mixtures were directly analyzed by HPLC using an Agilent 1200 HPLC system and a Kromasil C-18 reverse phase column (0.46*25 cm). The column was kept at RT. (a) and (b), the elution system was: 6-18% acetonitrile in 0.03 M potassium phosphate, pH 5.4, in 60 min, 1 mL/min flow rate. (c), the elution gradient was modified: 6 to 18% acetonitrile in 105 minutes.

Formation of Interstrand Crosslinks **10a** and **10b** with DMC is temperature dependent: At 0°C, **10a** and **10b** did not form (Figure S3 (a)). At room temperature, crosslinking occurred and Interstrand Crosslinks **10a** and **10b** were detected (Figure S3 (b)). At 37°C, crosslinking increased and adducts **10a** and **10b** were formed in higher proportion (Figure S3 (c)). The

adduct pattern from calf thymus DNA DMC alkylation shows a remarkable resemblance to the adduct pattern from treatment of EMT6, FAA-A fibroblasts and MCF-7 cells with DMC.[6] The only difference is that the ratio **10b**/**10a** is much higher in cells than in our model system.

S7-Co-injection Chromatograms

S-7-1 Co-injections between oligonucleotide 11 digest and DMC β-adducts 9b and 10 b.

11: d(TGCTTGCTTGCTTGCTTGCTTGCT.
AGCAAGCAAGCAAGCAAGCAAGCA)

*S-7-1-1Co-injection between **9b** (authentic standard) and the digest of **11**.*

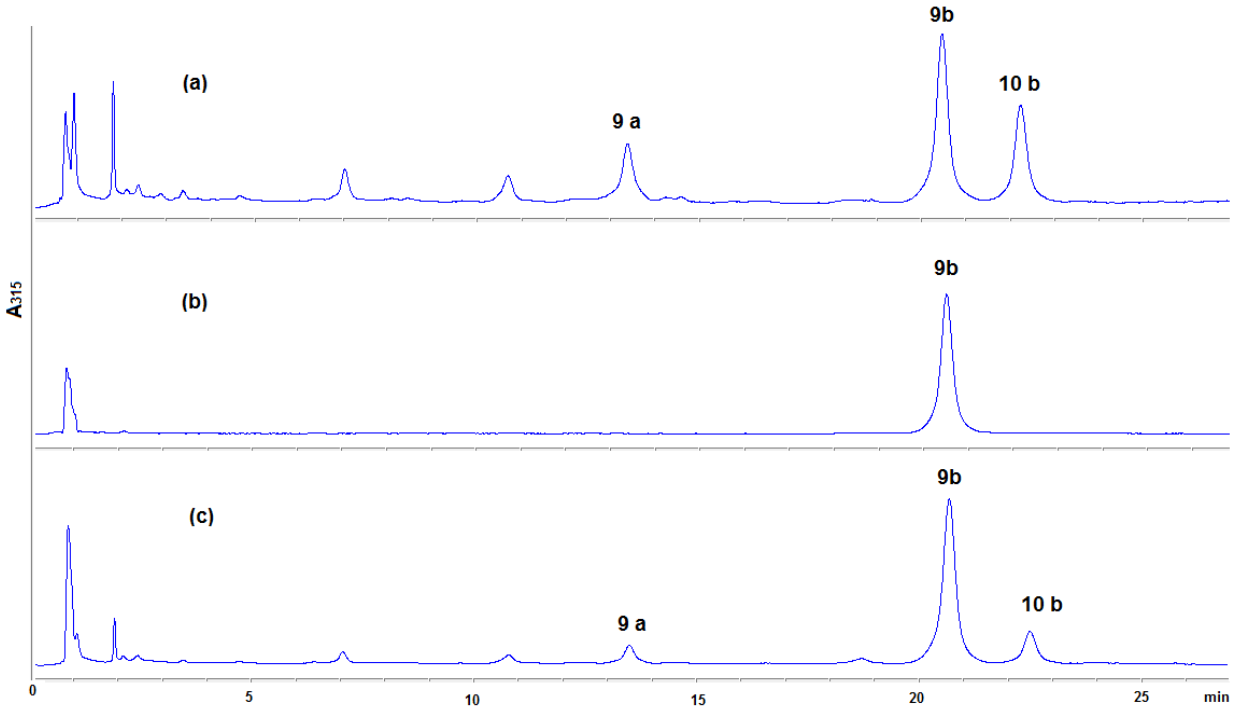

Figure S4. (a) Digest of oligonucleotide **11**. (b) Authentic standard adduct **9 b**. (c) Co-injection between oligonucleotide **11** digest and authentic standard adduct **9b**.

*S-7-1-2 Co-injection between **10b** (isolated from the reaction between calf thymus DNA and DMC) and the digest of **11**.*

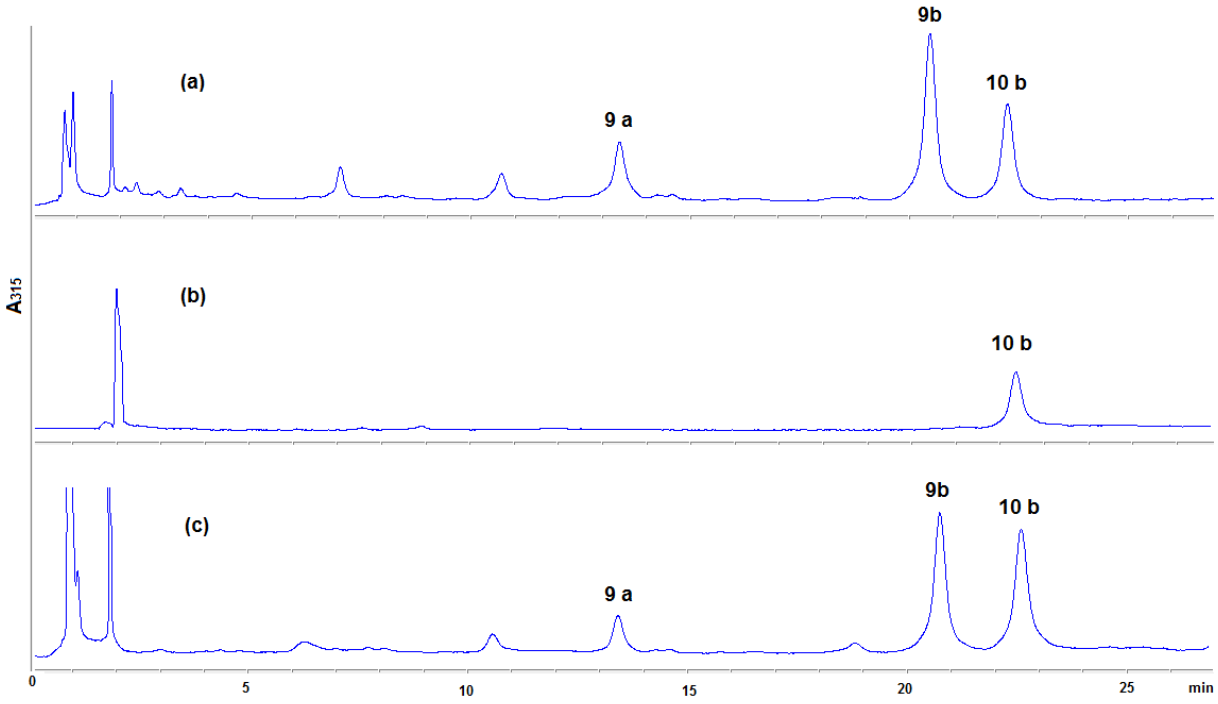

Figure S5. (a) Digest of oligonucleotide **11**. (b) Adduct **10 b** from Calf Thymus DNA. (c) Co-injection between oligonucleotide **11** digest and **10b** from Calf Thymus DNA.

S-7-2 Co-injections between oligonucleotide 13 digest and DMC α-adducts 9a and 10 a.

13: d(TCGTTCGTTCGTTCGTTCGTTCGT.
ACGAACGAACGAACGAACGAACGA)

*S-7-2-1 Co-injection between **9a** (authentic standard) and the digest of **13**.*

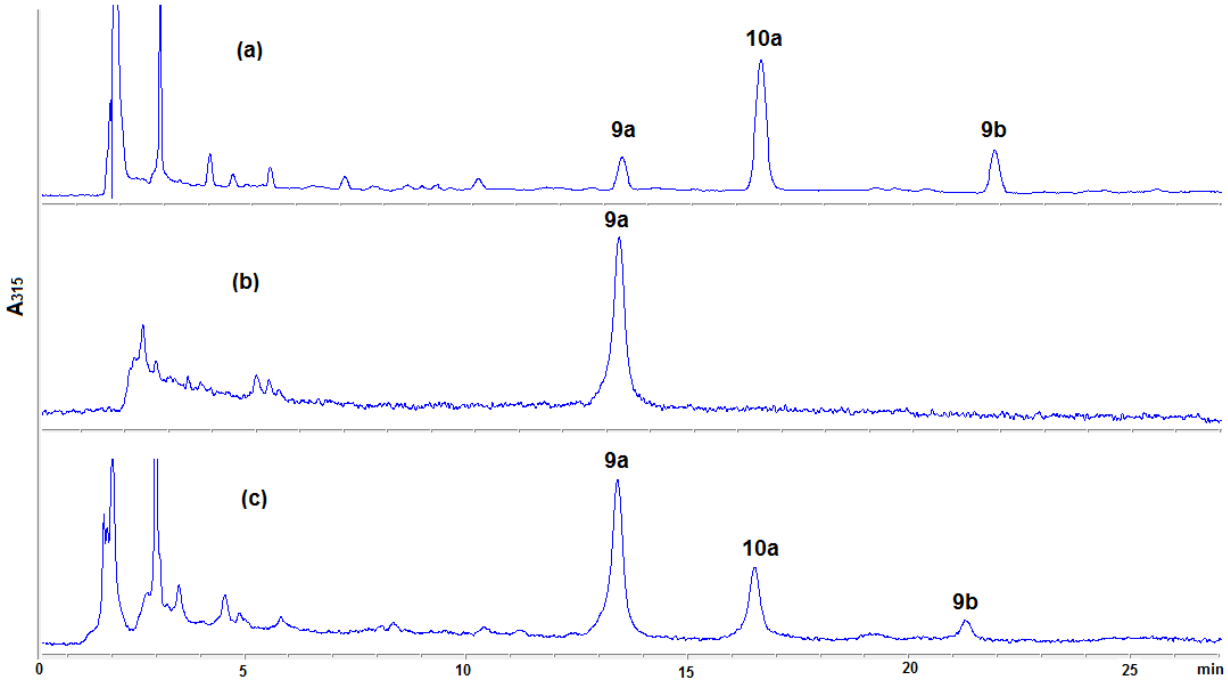

Figure S6. (a) Digest of oligonucleotide **13**. (b) Authentic standard adduct **9 a**. (c) Co-injection between oligonucleotide **12** digest and authentic standard **9a**.

*S-7-2-2 Co-injection between **10a** (authentic standard) and the digest of **13**.*

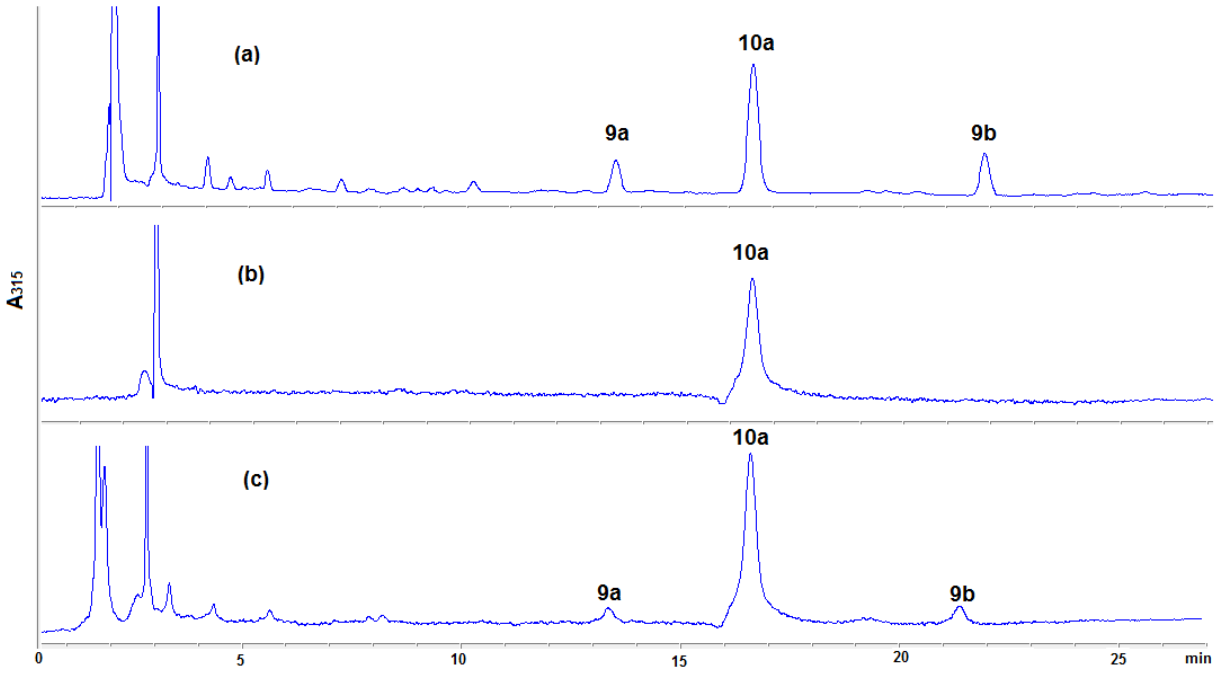

Figure S7. (a) Digest of oligonucleotide **13**. (b) Authentic standard adduct **10 a**. (c) Co-injection between oligonucleotide **12** digest and authentic standard **10a**.

S-8 Molecular modeling

Molecular modeling of alpha and beta DMC adducts.

The modeling of the two DMC monoadducts **9a** and **9b** was performed by using as template the original 199D.pdb which is an NMR solution structure of the monoalkylated MC-DNA 9-mer complex. In this template structure, the [MC]dG5 adduct is positioned opposite dC14 in the d(A3-C4-[MC]G5-T6).d(A13-C14-G15-T16) sequence context.[8] For the modeling of the cis adduct, we wanted to preserve the coordinates of this original structure and therefore we

selected one out the three reported NMR structures and modified the base sequence context by swapping the C4 with G5 in the 5'-3' strand and accordingly the C14 with G15 in the 3'-5' strand. We preserved the retention of the Watson-Crick alignment at the base-pair involving each monoalkylated guanine and at the flanking pairs in the complex, as was originally determined for the MC-monoadduct. The cis-DMC adduct **9b** was generated from the MC monoadduct by inverting the stereochemistry of the appropriate bond comprising the exo NH_2 group of the original G5. The trans-DMC adduct **9a** was generated from the trans MC adduct by preserving the trans stereochemistry of the appropriate bond. The MC adducts were further decarbamoylated to obtain the DMC cis- and trans-monoadducts **9b** and **9a**. The new adducts were assembled with the built-in modules from Maestro, version 11.3.016, release 2017-3 for Platform Windows-x64, a Schrödinger software. The finalized structures were further prepared using the "Ligprep" function in Maestro and exported as new pdb files for visualization with the PyMOL Molecular Graphics System, version 2.0.7 Schrödinger, LLC.

References

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