Published in final edited form as:

Chemistry. 2020 October 01; 26(55): 12570–12578. doi:10.1002/chem.202002452.

Synthesis of Oligonucleotides containing the *cis*-Interstrand Crosslink Produced by Mitomycins in their Reaction with DNA.

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Abstract

Mitomycin C, (MC), an antitumor drug and decarbamoylmitomycin C, (DMC), a derivative of MC lacking the carbamoyl moiety, are DNA alkylating agents which can form DNA interstrand crosslinks (ICLs) between deoxyguanosine residues located on opposing DNA strands. MC forms primarily deoxyguanosine adducts with a 1''-*R* stereochemistry at the guanine-mitosene bond (1''- α , *trans*) whereas DMC forms mainly adducts with a 1''-*S* stereochemistry (1''- β , *cis*). The crosslinking reaction is diastereospecific: *trans*-crosslinks are formed exclusively at CpG sequences, while *cis*-crosslinks are formed only at GpC sequences. Until now, oligonucleotides containing 1''- β -deoxyguanosine adducts or ICL at a specific site could not be synthesized, thus limiting the investigation of the role played by the stereochemical configuration at C1'' in the toxicity of these compounds. Here, we present a novel biomimetic synthesis to access these substrates. Structural proof of the adducted oligonucleotides and ICL were provided by enzymatic digestion to nucleosides, high resolution mass spectral analysis, CD spectroscopy and UV melting temperature studies. Finally, a virtual model of the 25 mer 1''- β ICL synthesized was created to explore the conformational space and structural features of the crosslinked duplex.

Graphical Abstract

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Supporting information for this article is given via a link at the end of the document.

A novel biomimetic synthesis to access mitomycins-DNA adducts with a *S* stereochemical configuration at C1'' is presented. Structural proof of the adducted oligonucleotides and ICL were provided by enzymatic digestion to nucleosides, MS/MS fragmentation, CD spectroscopy and UV melting temperature studies. A virtual model of the crosslinked DNA duplex was created to explore its conformational space and structural features.

Keywords

Interstrand crosslink; Mitomycin; Biomimetic synthesis; Conformational studies; Site-specific alkylation

Introduction

Mitomycin C^[1] (MC, Fig. 1) is a potent anticancer drug currently used to treat stomach, anal and lung cancers.^[2–4] Although MC is inert in its native state, it is reduced *in vivo* to a reactive intermediate which can form DNA interstrand crosslinks (ICLs) between the exocyclic amino groups of deoxyguanosine residues located on opposing DNA strands (Fig. 1).^[5–7] These lesions impede DNA replication and, thereby, block cancer cells proliferation. A mere 40 unrepaired ICLs can kill mammalian cells.^[8] MC is also able to generate monoadducts in its reaction with DNA but the current consensus, supported by strong experimental evidence, is that ICLs constitute the molecular basis for the cytotoxic effects of mitomycins.^[9]

The stereochemical configuration at C1'' of MC major monoadducts and ICL is *R* (**α** or *trans*, Fig.1). In contrast, decarbamoylmitomycin C (DMC, Fig. 1), a synthetic derivative of MC lacking the O10 carbamoyl group, generates preferentially the *S* stereoisomeric monoadducts and ICL (**β** or *cis*, Fig. 1).^[9] The crosslinking reaction between mitomycins and DNA is also diastereospecific: *trans*-crosslinks are formed exclusively at CpG sequences, while *cis*-crosslinks are formed only at GpC sequences.^[10]

Importantly, the *S* stereochemical configuration (*cis*) at C1'' of the ICL produced by DMC correlates with the higher toxicity of DMC in human cancer cells compared to MC.^[11–17] In this manuscript, from now on, we will refer to dG adducts and ICLs with a *R* stereochemistry at C1'' as **α** -adducts (*trans*), while adducts with a C1''-*S* configuration will be denoted as **β** -adducts (*cis*).

Recent studies have focused on the distinct cellular responses triggered by the two drugs and have shown, in particular, that DMC induces cell cycle arrest at lower concentrations when compared to MC.^[16] Moreover, DNA-adducts generated by DMC treatment (1''- **β** adducts) rapidly activate a p53-independent cell death signaling pathway.^[12–15] This may explain why DMC is so toxic against cells lacking a functioning p53. It also has clinical relevance because more than 50% of cancers have a mutated p53 gene.

Further research on the cellular processing of the α and β -ICLs has revealed that the two stereoisomeric ICLs are removed at different rates in EMT6 cells, with the β -ICL (**3b**) being eliminated faster than the α -ICL (**3a**).^[11] Another study on cellular signaling has shown that a duplex oligonucleotide crosslinked by the α -ICL and transfected to MCF-7 cells was able to activate p21^{WAF1/CIP1} to a similar extent than the drugs MC and DMC themselves.^[16]

These results point to the importance of ICL structure in the mode of action of both MC and DMC, however, the role of the ICLs' opposite stereochemical configuration at C1' (**3a** versus **3b**, Fig. 1) in the cytotoxicity of the two drugs is not fully understood. Our hypothesis is that the opposite stereochemical configuration of the ICL at C1' plays a major part in the differences between the mode of action of MC and DMC.

In support of our hypothesis, evidence of the importance of ICL structures in repair and recognition processes during replicative and non-replicative events has recently emerged.^[18–24] Solid data also indicate that *cis*- and *trans*-diastereomeric DNA adducts, such as those formed by benzo[*a*]pyrene diol epoxides, trigger very different biological effects.^[25] Our long-term goal is to establish relationships between the mode of action of mitomycin drugs and the stereochemistry of the ICLs they form. This will provide invaluable insight to design more efficient chemotherapeutics. Such studies require the use of modified oligonucleotides containing a specific adduct at a single position of their base sequences.

Syntheses of oligonucleotides containing α -monoadducts and ICL (**1a**, **2a** and **3a**, Fig. 1) have been performed in the past according to a protocol developed in the Tomasz Laboratory.^[26–29] In this methodology, the mitomycins are reduced under autocatalytic conditions using sub-stoichiometric reductant. The electrophilic mitosene produced through this autocatalytic pathway undergoes nucleophilic attack by the 2-amino group of guanine on the opposing strand only on its α face to yield 1''- α adducts (Scheme 1A).^[27] Further addition of reducing agent converts oligonucleotides monoadducted at CpG sequences into the 1''- α cfiledrosslinks.^[29] Until now, there was no method to access isomeric 1''- β monoadducts or β -ICLs produced by mitomycins (**2b**, **3b**, Fig. 1). We recently showed that the formation of 1''- β adducts requires an alternative “bifunctional” reductive activation whereby mitomycins are fully reduced preceding DNA alkylation (Scheme 1B).^[10,30] Under these conditions, both α and β nucleophilic additions are possible. Furthermore, we found that the formation of ICLs by mitomycins is sequence dependent: Only the α -diastereomer of the initially formed monoadduct can form crosslinks at GpC sequences, and only the β -diastereomer of the monoadduct can form crosslinks at CpG sequences.^[10]

These findings paved the way for the biomimetic synthesis of the β -crosslink presented here. We describe, for the first time, the synthesis of oligonucleotides containing 1''- β deoxyguanosine adducts or ICL at a specific site on a scale which allowed their characterization. Structural proof of the crosslinked oligonucleotide was obtained by enzymatic digestion to nucleosides, high resolution mass spectra analyses, CD spectroscopy and UV melting temperature studies. Finally, virtual models of the 25 mer native and of the β -ICL were created to explore the conformational space and the structural features of the two duplexes.

Results and Discussion

Mitomycin C and decarbamoylmitomycin C form two diastereomeric crosslinks in cancer cells, but the synthesis of oligonucleotides containing the β -diastereomer has proved elusive.^[9] Our research group has attempted this synthesis during the past years, starting with a post-oligomerization approach that succeeded at the nucleoside level but ultimately failed at the oligonucleotide level.^[31] The successful strategy presented here to access the β -ICL follows three steps: (a) synthesis of 1''- β monoadducted oligonucleotides (b) purification of the monoalkylated strand, annealing with a complementary strand and (c) treating the annealed duplex with sodium dithionite to trigger the second arm alkylation (Scheme 2).

Determination of the Optimal Sequence for the Formation of the 1''- β -monoadduct

In our previous work, we found that the structure of the nucleobases flanking the target sequence influences both alkylation yields and the ratio of diastereomeric monoadducts.^[30] In order to fine tune conditions that favor the formation of 1''- β adducts, we further investigated this effect by comparing the frequency of **2b** and **2a** in 5'-AGCT; 5'-TGCA and 5'-AGCA sequence contexts. Duplex oligonucleotides were reacted with fully reduced DMC according to a protocol described previously.^[10,30] Alkylated oligonucleotides were digested by nucleases and phosphatases to the individual nucleosides and the adducted nucleosides (**2a** and **2b**) were identified by their UV spectra, their retention time and co-elution with synthetically prepared standards to confirm their identity.^[10,30] A method to calculate the percent yields (frequency) for adducts **2a** and **2b** is described in the supporting information and in previous work.^[10,30] Results (Table 1) showed that the yield of β -monoalkylation (**2b**) in duplex DNA is enhanced when adenine is located at the 3' position of the GpC step.

Next, we tested if the presence of 5-methylcytosine (m^5C) influences the alkylation of GpC sequences by DMC under bifunctional reductive conditions. For mitomycin C (MC), the presence of a 5-methylcytosine (m^5C) at CpG steps increases the nucleophilic reactivity of the 2 amino group of guanine during the first alkylation step; which, as a result, enhances more than two-fold the yield of 1''- α -adducts (**1a**, **2a**, **3a**).^[32–37]

Our experiments (Fig. 2 and Table 2) showed that the presence of a m^5C at GpC steps strongly increased the frequency of adduct **2a** (α) but had little effect on the frequency of adduct **2b** (β). The frequency of **2a** increases 2–3-fold when a m^5C at GpC step is present but this modification only yields a slight increase in **2b** frequency (compare the frequency of **2a** and **2b** in methylated duplexes **e** and **f**, table 2, versus the frequency of **2a** and **2b** in non-methylated duplexes **c** and **d**, table 1). Reasons for this phenomenon are unclear. We suggest that, in the case of adduct **2b**, the enhanced nucleophilic reactivity of the 2-amino group of guanine is mitigated by steric or electronic interactions between the methyl group on cytosine and activated DMC which may hinder nucleophilic attack on the β face of the drug's tetrahydropyrrole ring.

In summary, optimization experiments have shown that a GpCpA sequence context enhances β -monoalkylation in duplex DNA and that methylation of the opposing cytosine at GpCpA steps increases the frequency of adduct **2a** but has little effect on the frequency of adduct **2b**.

Design of Oligonucleotide Substrates for the Synthesis of β -monoadducts and Crosslinks

Our synthetic strategy is similar to the one used previously to synthesize non-self-complementary oligonucleotides containing the α -crosslink induced by MC.^[38] Oligonucleotides containing the α -monoadduct induced by MC (**1a** or **2a**, Scheme 1A) were obtained by using monofunctional activation conditions (autocatalytic activation of MC). In a second step, crosslinking was achieved by annealing the monoadducted oligonucleotide with the complementary strand and further treatment with reducing agent.

For DMC, the use of bifunctional conditions and cold temperatures are pre-requisites to obtain β -monoadducts (step a, scheme 2), since (1) monofunctional conditions result in the formation of the α -monoadducts (Scheme 1A) and (2) low temperatures preclude the formation of unwanted crosslinked oligonucleotides during this first step.^[10,30] For the design of the oligonucleotide substrate, we had to consider that the subsequent crosslinking step (step c, scheme 2) requires a high temperature (40°C minimum for significant crosslinking)^[10,30], therefore we selected a 25-mer oligonucleotide as starting substrate to prevent DNA melting during the crosslinking step. To facilitate the purification of the monoadducted 25-mer oligonucleotide from the monoalkylation reaction (step a, scheme 2) we decided to use a duplex composed of a 12 mer and a 25 mer. With these considerations in mind, the following sequences were chosen: 25-mer: 5'-TCCTCCTAATAGCAATAATCCTCCT and 12-mer: 5'-TTATTGCTATTA-3' (**4** and **5** scheme 2). This design meets the criteria to maximize the yield of β -monoadduct **2b** that we determined in a previous work: (1) the GpC step is located in an A/T rich region^[10,30] and (2), there is no 5' or 3' terminal deoxyadenosine residues in the overhang part of the duplex, that are known targets for alkylation by bifunctionally activated DMC.^[39,40] Finally, it contains a GpCpA central sequence which favors the formation of **2b**, as we found in the optimization experiments described above.

Biomimetic Synthesis of 1''- β -Monoadducted Oligonucleotides

The (25 mer).(12 mer) duplex (**4-5**, Scheme 2) was reacted with fully reduced DMC at 0° C under our recently developed bifunctional reductive alkylation conditions that result in the exclusive formation of monoadducts (Scheme 2, (a)).^[10,30] DNA material was isolated by size-exclusion chromatography and the alkylated oligonucleotides were purified by HPLC (Fig. S1). The monoalkylation efficiency was 18 % ($\pm 2\%$) for the 25 mer and 9.5 % ($\pm 1\%$) for the 12 mer (Fig. S2). This confirms that the yield of β -monoalkylation in duplex DNA is enhanced when adenine is located at the 3' position of the GpC step. This biomimetic synthesis is highly reproducible and gave consistent yields in repeated experiments. The UV spectra of the modified oligonucleotides (Fig. 3) showed a shoulder peak at 310 nm, characteristic of the mitosene chromophore. The HPLC analysis of enzymatic digestions (Fig. 4) demonstrated the formation of adduct **2b**, which coeluted with an authentic standard synthesized in our laboratory.^[31] The mass spectra of the modified oligonucleotides (Fig. S3 and S4) show peaks 259 mu higher than those of the non-alkylated parent strands, corresponding to the mass of the alkylating mitosene. This data confirmed the successful synthesis of the monoadducted oligonucleotides.

Biomimetic Synthesis of an Oligonucleotide Containing the the β -Interstrand Crosslink

The β -ICL was generated by hybridizing the 25-mer 1''- β monoadduct with its complementary strand (**6**, Scheme 2) and further reducing the quinone moiety with sodium dithionite to trigger the second arm alkylation (Scheme 2, (b)). Although crosslinking does not occur at low temperature, at 42°C the β -ICL is formed in a 30 % yield (Fig 5A).^[30]

The crosslinked oligonucleotide was purified by HPLC and characterized by UV spectroscopy, CD spectroscopy (Fig. S5 Fig. 6, Fig. 8), enzymatic digestion (Fig. 5B), MS and MS/MS experiments. The crosslinked oligonucleotide can also be purified by denaturing PAGE (data not shown). Results and interpretation from the UV data, CD data, thermal melting curves and MS experiments are detailed below.

Mass Spectrometry Characterization

Nano-ESI-MS spectrum of the crosslinked duplex showed a mass of 15,562 Da corresponding to the mass of the duplex (15,321 Da) containing a mitosene crosslinking fragment (241 Da) (Fig. S6–S8). In source fragmentation yielded two major fragments: one with a mass of 7,824 $\pm$ 0.5 Da corresponding to oligonucleotide **6** and one with a mass of 7,755 $\pm$ 0.5 Da corresponding to the oligonucleotide **5** (7496 Da) with the adducted mitosene fragment (259 Da) (Fig. S9, S10).

The structure of the crosslinked duplex was further confirmed by MS/MS fragmentation in collision activated mode (Fig. 6, 7 and Fig. S11). Identification of the observed fragments were based on accurate mass-measurements (4 ppm or better). Notably, a high-resolution spectrum for the crosslinking fragment **7** (Fig. 6 and Fig. S12) was obtained.

Oligonucleotide fragments containing a mitosene moiety linked to a guanine base located either on the top strand (**8**, **9**, **10**) or on the bottom strand (**11** and **12**) of the β -ICL were also detected (Fig. 6 and Fig. S13–S17). These fragments unequivocally confirmed the position and the nature of the crosslinking moiety.

The fragmentation pattern of the β -ICL also showed the formation of expected w_n and $[a_n-B]$ ions which did not contain any mitosene moiety (Fig. 7 and Tables S1 and S2). The nomenclature for fragment ions follows that reported by McLuckey et al.^[41] In addition, ions with a mass corresponding to oligonucleotides **4**, **5** and **13** (Fig. 7 and Fig. S11) were detected.

UV-vis and Circular Dichroism (CD) Spectroscopy

UV-Vis spectra of the crosslinked (Fig. 8) and monoalkylated duplexes (Fig. 3) show a peak at around 260 nm (present also in the native duplex at the same wavelength) and a shoulder absorbance at higher wavelength (320 nm) due to the presence of the mitosene chromophore.

Circular dichroism (CD) spectroscopy of the crosslinked duplex versus the native sequence show very similar profiles at lower wavelengths with a negative band at around 245 nm and a positive one at around 220 nm (Fig. 8). A positive band in the region 270–280 nm is present for both the native and the crosslinked sequences, however the band for the

crosslinked species is slightly red shifted. This is the opposite behavior compared to the previously described α -ICL.^[42] The crosslinked DNA presents an additional weak negative band in the 320–380 nm region. This band which is characteristic of the crosslinked chromophore is also red-shifted compared to the α -ICL.^[42,43]

Thermal Denaturation Studies

Thermal denaturation studies of the native, β -monoalkylated and β -crosslinked duplexes were performed. The calculated melting temperatures for the native and monoalkylated oligonucleotide duplexes are respectively 58.3°C and 60.5°C suggesting that the mitosene moiety exerts a weak stabilizing effect. This stabilizing effect is likely due to extensive non-covalent contacts between the mitosene moiety and the DNA duplex and favorable stacking with the neighboring base pairs as was previously observed for the stereoisomeric α -monoadduct.^[44]

In the case of the β -crosslinked duplex, the shoulder in the melting curve (Fig. 9) between 60°C and 85°C suggests the presence of an intermediate state where the DNA is in both double-stranded and single stranded configurations resulting in two melting phases. A possible denaturation mechanism would involve the melting of terminal base-pairs first (calculated $T_m(1)$ = 58.6°C) followed by separation of bases in the central region. This region is likely stabilized by the crosslink and probably maintains its dsDNA configuration until the temperature is sufficiently high to cause the base pairs to melt.^[45,46] The second melting temperature was calculated at $T_m(2)$ = 79°C.

Conformational Survey of the Native and Crosslinked 25-mer DNA duplexes

Molecular dynamics (MD), in duration of 500 ns, was carried out on the native 25-mer and the β -ICL in an explicit TIP3P water box with stabilizing sodium cations at the backbone. The MD algorithm was implemented based on OPLS3 force field. MD trajectories of the native 25 mer duplex and the β -ICL disclose a vast conformational diversity of geometries. In order to have a manageable set of geometries for comparative analysis, we have extracted three representative conformations from each trajectory: the first geometry within the equilibrated MD trajectory (conf-1), the geometry with the lowest root mean square deviation in atom dispositions (*RMSD*) relative to all the other structures within the entire MD trajectory (conf-2), and the last geometry within the MD trajectory (conf-3).

The three conformations of the crosslinked duplex are represented in Fig. 10 (the conformations of the native duplex are available in Fig. S20). Figure 11 shows the position of the mitosene aromatic chromophore in the DNA duplex of the β -ICL.

Basic structural parameters of the crosslinked versus the native DNA duplex were quantified and subtle differences were observed. Namely, the width between opposing DNA strands around the crosslinking site has increased by one tenth of an Å on-average. The change in width is the most discernable for conf-3 associated with the two model systems. Specifically, for the native duplex, the width between both strands is ~ 14.15 Å, while in the case of the β -ICL it is extended to ~ 14.28 Å (Compare Fig. S21, table S3 with Fig. S22, table S4, supporting information).

Our conformational and CD studies show that the crosslinking chromophore only slightly affects the DNA structural parameters and that the overall structure of the β -ICL duplex remains a DNA-B conformation. In our model, the Watson-Crick base pairing is maintained at the (GC).(GC) crosslink site and base stacking is preserved. There is a slight widening (1.4 Å) of the minor groove at the crosslinking site which was measured for conf. 2 (Fig. S 23). Other conformational parameters of the crosslinked DNA helix, such as rise and tilt, barely deviate from the native structure. The helix pitch around the crosslinking site varies slightly and is 0.3 Å smaller (average measure from the three conformations) in the case of the β -ICL compared to the native duplex. Finally, the structural similarity between the β -ICL and the native 25 mer is visually evident when both structures are superimposed (Fig S. 25).

Conclusion

In conclusion, we have developed a novel biomimetic method to access oligonucleotides containing β -monoadducts at a specific site and β -crosslink at a specific site *as a single orientational isomer*. These β adducts (*cis*) are produced by mitomycins in cells alongside their α isomers (*trans*). Since α -monoadducts and α -crosslinks are already available through known protocols, access to the β isomers will allow us to compare the biological processing of the two types of DNA lesions (α and β) by the cell's machinery. In the future, we will use the α and β ICLs to investigate the role of the ICL stereochemical configuration in the signaling and ICL repair mechanisms induced by mitomycins.

Experimental Section

Materials.

MC was generously gifted by Professor Maria Tomasz. DMC was synthesized from MC by a published procedure.^[47] 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) was 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).

Methods. Quantitative Analysis.

Quantitation of nucleosides, DMC or MC-DNA adducts was based on UV spectrophotometry using the following molar extinction coefficients: dT, 6600 (254 nm); dA, 14200 (254 nm); DMC monoadducts (**2a**, **2b**), 24000 (254 nm); MC/DMC ICL **3b**, 30000 (254 nm). In enzymatic digests, the percent yields of DMC DNA-adducts designate the mole percent adducted guanine (**2a**, **2b**, **3b**) of total guanine nucleotide residues present in the oligonucleotides. A full description of the method is available in previous publications^[10,29] and in the supporting information. Quantitation of single stranded oligonucleotides was also based on UV spectrophotometry using extension coefficient derived from the "Base composition Method".^[48,49] For calculating the E_{260} value of crosslinked oligonucleotide duplexes and monoadducts, the E_{260} bound mitosene was assumed to be 12000 and added to E_{260} of the non-crosslinked duplex or single strand. This formula assumes negligible

hypochromicity associated with the crosslinked duplex; this is justified by the low hyperchromicity on melting observed in the present work. Extensions coefficient values used were the following: $113.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (ss 5'-ATTATTGCTATT); $188.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (duplex 5'-ATTATTGCTATT); $230 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (single strand 5'-TCCTCCTAATAGCAATAATCCTCCT); $398.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (duplex 5'-TCCTCCTAATAGCAATAATCCTCCT); $125.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (monoadduct 5'-ATTATTGCTATT); $242 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (monoadduct 5'-CCTCCTAATAGCAATAATCCTCCT); $410.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (for crosslinked duplex 5'-CCTCCTAATAGCAATAATCCTCCT).

Reproducibility.

Syntheses were repeated at least three times independently and average yield is reported with deviation from the mean value is represented in brackets.

Identification of Adducts 2a, 2b, 3b in Enzymatic Digests.

Adducts **2a**, **2b**, **3b** were identified by their UV spectra, retention times and co-elution with authentic standards synthesized previously in our laboratory.^[10,31,50]

Optimization of DMC Alkylation Frequency at GpC Steps.

12 mer oligonucleotides (10 A_{260} unit scale; corresponding to about 0.072 μmol) were mixed with an equal amount of their complementary strand containing, or not, a methylated dC (10 A_{260} unit scale, about 0.072 μmol). The mixture was lyophilized. Oligonucleotides were then annealed by heating (55°C, 10 min) after the addition of 10 mM potassium phosphate buffer, pH 5.8 (355 μL) followed by slow cooling. The reaction mixture was put under ice and deaerated *via* argon bubbling (30 min, 0°C). Excess $\text{Na}_2\text{S}_2\text{O}_4$ (5 eq; 6.48 μmol in 20 μL of potassium phosphate buffer, pH 5.8) from a freshly prepared anaerobic solution in water was added to the mixture quickly followed by a dropwise addition of DMC (1.3 μmol in 200 μL of potassium phosphate buffer, pH 5.8). The reaction was stirred for 10 min before another addition of $\text{Na}_2\text{S}_2\text{O}_4$ (2.5 eq, 3.24 μmol) was immediately followed by a dropwise addition of an anaerobic DMC solution (1.3 μmol in 200 μL). This process was repeated 3 more times until the reaction was treated with a total of 6.48 μmol of DMC. The reaction mixture was stirred in ice with argon bubbling for 1 h and then was removed from ice and opened to air, followed by gentle stirring until a consistent purple colour was obtained. The mixture was then stirred for 20 min under air and then chromatographed on a $2.5 \times 56 \text{ cm}$ Sephadex G-25 column using 20 mM NH_4HCO_3 as eluent. Oligonucleotide containing fractions were lyophilized.

Synthesis of Oligonucleotides Containing 1"- β -deoxyguanosine Monoadducts.

12 MER Oligonucleotide 5'-ATTATTGCTATT-3' (50 A_{260} unit scale; 0.405 μmol) was mixed with 25 MER oligonucleotide 5'-TCCTCCTAATAGCAATAATCCTCCT-3', (100 A_{260} unit scale, 0.401 μmol) in 5.32 mL of 10 mM potassium phosphate buffer, pH 5.8. Oligonucleotides were then annealed by heating (90°C, 10 min) followed by slow cooling to RT. The reaction mixture was then put under ice and deaerated *via* argon bubbling (30 min, 0°C). A 0.3 M solution of sodium dithionite and a 12.2 mM solution of

decarbomoylmitomycin C were freshly prepared in deaerated potassium phosphate buffer (10 mM, pH 5.8). 160 μ L (48 μ mol) of sodium dithionite solution was added to the reaction mixture, quickly followed by the addition of 800 μ L (9.76 μ mol) of DMC solution. The reaction was stirred for 10 min before another addition of sodium dithionite (80 μ L, 24 μ mol) was immediately followed by DMC (800 μ L, 9.76 μ mol). This process was repeated three more times until the reaction was treated with a total of 48.8 μ mol of DMC. The reaction mixture was stirred in ice with argon bubbling for 1 h after the last addition and then was removed from ice and opened to air, followed by gentle stirring until a consistent purple colour was obtained. The mixture was then stirred for 30 min under air and then chromatographed on a 2.5 $\times$ 56 cm Sephadex G-25 column using 20 mM NH_4HCO_3 as eluent. Oligonucleotide containing fractions were lyophilized. This synthesis was repeated twice more on the same starting materials.

Alkylation Efficiency of the First Alkylation Step.

The monoalkylation efficiency on the 12 and 25 mer (**4** and **5**) was determined by (1) separating the 12 mer oligonucleotides from the 25 mer oligonucleotides by PAGE from the crude reaction mixture and (2) measuring peak areas from the HPLC chromatogram of the mixtures containing non-alkylated and alkylated 12-mer or 25-mer oligonucleotides at λ 254 nm. The conversion efficiency was 18% ($\pm 2\%$) for the 25-mer alkylated oligonucleotide and 9.5% ($\pm 1\%$) for 12-mer alkylated oligonucleotide.

Purification of Oligonucleotides Containing 1''- β -deoxyguanosine Monoadducts.

Alkylated oligonucleotides were purified by semi-prep HPLC using an Agilent 1200 HPLC system and a Phenomenex Clarity Oligo-RP C-18 reverse phase Column (5 μ m, 10 mm $\times$ 250 mm). Buffer A: 0.1 M triethylammonium acetate pH 7.0; Buffer B: Acetonitrile. Flow Rate: 4 mL/min. The temperature was set to 45 $^\circ$ C. The elution gradient was: t=0 to t=3 min: 6 to 10 % acetonitrile; t=3min to t=12 min: 10% acetonitrile; t=12 min to t=27 min: 10 to 13% acetonitrile; t=27 min to t=30 min: 13 to 50 % acetonitrile. Oligonucleotide containing fractions were collected manually. The elution times of the 4 oligonucleotides were as follow: non-alkylated 25-mer (19 min); non-alkylated 12-mer (21min); monoalkylated 25 mer (22 min); monoalkylated 12-mer (23 min). After lyophilisation, oligonucleotides were redissolved in water (3 mL) and further desalted by centrifugation using 3K Amicon centrifuge filters. In some cases, the monoalkylated oligonucleotides were further purified by page as described below for the purification of the crosslinked duplex. The yield of monoalkylated oligonucleotide was calculated by: %yield of monoalkylated oligonucleotide=100*(moles of monoalkylated oligonucleotide)/(mole of starting parent oligonucleotide). The yield of the 25-mer alkylated oligonucleotide was 0.044 μ mol (11%; $\pm 2\%$) and the yield of the 12-mer alkylated oligonucleotide was 0.024 μ mol (6 %; $\pm 1\%$).

Synthesis of 1''- β -Interstrandcrosslinked Duplex (β -ICL).

Lyophilized monoalkylated 25-mer oligonucleotide (10 A_{260} unit, 0.040 μ mol) and a slight excess of complementary strand (13 A_{260} unit, 0.050 μ mol) were dissolved in 0.01 M potassium phosphate buffer, pH 5.8 (355 μ L). Oligonucleotides were annealed by heating (90 $^\circ$ C, 10 min) followed by slow cooling to 42 $^\circ$ C. A freshly made and deaerated solution of

sodium dithionite (0.161 M in potassium phosphate buffer, pH 5.8) was quickly added to the mixture (10 μ L, 1.61 μ mol). The reaction turned colourless and the mixture was stirred at 42°C with argon slowly bubbling. The mixture was opened to air after 1 h followed by gentle mixing until a consistent purple colour 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. Enzymatic digestion of the crude mixture consistently showed a conversion from monoadduct to crosslink of 30% ($\pm$ 2%).

Small Scale Analysis of Oligonucleotides Containing 1''- β -deoxyguanosine Adducts and of the β -ICL.

The purity of all synthesized oligonucleotides was verified by using an Agilent 1200 HPLC system and Phenomenex Clarity Oligo-RP C-18 reverse phase Column (5 μ m, 4.6 mm*250 mm). The elution system was the same as the one described above for the isolation of alkylated oligonucleotides. The temperature was set at 45°C. The elution times were as follow: non-alkylated 25-mer (30 min); non-alkylated 12-mer (33min); monoalkylated 25 mer (31 min); monoalkylated 12-mer (35 min); ICL (44 min).

Purification of the β -ICL.

The β -ICL was purified by 1) the system described above or 2) denaturing PAGE on a 20% acrylamide-bis (19:1) gel containing 7 M urea. In this case, an average of 10 A_{260} unit of crude material was purified for each run using 1.6 mm preparative comb (gel dimension: 20 cm*20 cm*1.6 mm). The gel was run for 3.5 h at 290 V. Bands were visualized by UV shadowing and excised. The DNA was eluted into TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) by the crush and soak method to yield 3.5 A_{260} unit; 0.008 μ mol of pure crosslinked oligonucleotide (20 % yield ($\pm$ 3%)).

Enzymatic Digestion of Alkylated Oligonucleotides and Analysis of DNA Adducts.

Nuclease P_1 /SVD/AP protocol: 1 A_{260} unit of oligonucleotide and 1 unit of nuclease P_1 were incubated at 37°C for 2 h in 0.8 mL of 20 mM ammonium acetate, pH 5.5; 100 mM MgCl_2 (20 μ L) was added, and the pH was adjusted to 8.2 by addition of 20 μ L of 200 mM NaOH. SVD (2 units) and AP (2 units) were added and incubation was continued at 37°C for 2.5 h. 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 elution system was 6–18% acetonitrile in 30 mM potassium phosphate, pH 5.4, in 60 min, 1 mL/min flow rate.

UV melting studies.

UV melting study were performed using a Shimadzu UV 2600 UV-vis spectrometer at 260 nm (slit width 1 nm). The melting studies were carried out in the temperature range 25–95°C using a scan rate of 1°C min⁻¹. Samples were recorded in the following buffer: 100 mM sodium chloride, 10 mM sodium phosphate monobasic, 0.1mM EDTA pH 7.4. Melting temperatures were determined using the first derivative of the melting curve.

Circular dichroism.

Circular Dichroism (CD) spectra were recorded on a Jasco J-1500 (serial number A0045PM539) spectrophotometer. The experiment parameters are the following: band width, 1 nm; data pitch, 0.1 nm; scanning speed, 100 nm/min. CD Spectra were recorded in the interval 650 nm-200 nm. The CD/UV-Vis spectrum of 10 mM potassium phosphate buffer pH 7 (blank) was subtracted from each of the recorded spectra also in the same buffer. Kaleidagraph software (version 4.1.3) was used to generate the graphs presented in the manuscript.

Mass spectrometry.

All mass spectrometry experiments were performed on a maXis-II-ETD ESI-Qq-TOF instrument (Bruker Daltonics Inc.) equipped with an off-line nano-ESI source. Off-line nano-electrospray was carried out by using borosilicate emitters (Thermo Scientific Inc.). Mass-spectra of all oligonucleotides (at 3 μ M concentration) were acquired in positive ion mode. Agilent's ESI-MS tuning mix was used to calibrate the instrument. To reduce sodium adduction in ESI-MS, we prepared DNA samples in 500 mM ammonium acetate. The instruments parameters were as follows: ion-source voltage 900V; dry N₂ gas flow 4L/min at 150°C; nebulizer gas flow kept at 0.1 bar. Bruker's software "Data Analysis 5.1" was used for de-convolution of all the acquired intact and collision activated dissociation (MS/MS) spectra. Additionally, we used online tool (developed at the RNA Institute of University of Albany) for analysis of DNA fragmentation spectra.^[51]

Conformational Survey of the Unmodified 25-mer DNA Duplex and of the β -ICL.

Virtual models of the native 25-mer DNA duplex and of the β -ICL were created via Maestro visualizer within the Schrodinger suite of programs (*Schrodinger, LLC, New York, NY, 2019*). In order to explore the conformational space of the two model structures, each duplex was subjected to 500 ns MD trajectory via Desmond software. Each duplex structure was imbedded into a 10 Å orthorhombic solvent box, with TIP3P model explicitly simulating H₂O media. The negative charge on phosphates was stabilized with sodium cations (Na⁺). The MD simulation was run based on OPLS-3 molecular mechanics force field. The energies for representative structures have been obtained at the same level of theory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank the program for initiatives in math and sciences (PRISM) at John Jay College for the purchase of some research equipment. This work was supported by NIH grant 2SC3GM105460-06 to E.C.

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Figure 1.
Structure of the major mitomycin C (MC) and decarbamoylmitomycin C (DMC) deoxyguanosine adducts detected in cells.

Figure 2. Effect of the presence of a methylated cytosine at GpC steps. HPLC chromatogram (A₂₅₄) of the enzymatic digest of duplexes: (A) (AATAGCAATAAT).(TTATCGTTATTA); (B) (AATAGCAATAAT).(TTAT(m5C)GTTATTA).

Figure 3.
UV spectra of alkylated 12 mer (left) and 25 mer (right).

Figure 4.
HPLC chromatogram (A_{254}): (1) standard **2b**; (2) enzymatic digest of monoadduct 5'-TCCTCCTAATA(**2b**)CAATAATCCTCCT; (3) co-injection of (1) and (2); (4) enzymatic digest of monoadduct 5'-TTATT(**2b**)CTATTA; (5) co-injection of (1) and (4).

Figure 5. HPLC chromatogram from enzymatic digests of (A): Crude reaction mixture after crosslinking between the monoalkylated 25 mer oligonucleotide and its complementary strand. (UV-vis spectra of **2b** and **3b** are inserted); (B) Pure crosslinked 25 mer.

Figure 6.

Mitosene containing fragments from the β-ICL detected by MS/MS fragmentation in collision activated mode.

Figure 7.

Fragmentation pattern observed for the β -ICL by MS/MS fragmentation in collision activated mode. Top strand : $[a_n-B]$ with $n=3,5,6,8,9,11,12$ and w_n with $n=7,8,10,11,12,13$. Bottom strand : $[a_n-B]$ with $n=2,3,4,5,6,7,13$ and w_n with $n=1,3,4,5,6,9,11,12$. **13**: top 25 mer strand containing an abasic site where the adducted guanine was located.

Figure 8.

Normalized UV-vis and CD spectra of the β -ICL (solid lines) and native 25 mer (bracketed line). The crosslinked sequence was measured at two different concentrations respectively $1.48 \cdot 10^{-6}$ M and $0.67 \cdot 10^{-6}$ M while the native sequence was measured at $1.13 \cdot 10^{-6}$ M.

Figure 9.
Melting curves for the native 25 mer duplex (blue), the monoalkylated 25 mer duplex (purple) and the crosslink 25 mer duplex (red).

Figure 10. Conformations of the crosslinked 25-mer DNA duplex with three representative conformations isolated from the MD trajectory. Conf. 1 (left); conf. 2 (middle) and conf. 3 (right).

Figure 11.
Position of the mitosene aromatic chromophore in the DNA duplex for conf. 2 of the β -ICL.

Scheme 1.

(A) Auto-catalytic activation of MC and DMC, MC: $\text{R}=\text{CONH}_2$, DMC: $\text{R}=\text{H}$, alpha attack only (B) Bifunctional activation of DMC. Alpha and beta attacks occur.

4: 5'-TCCTCCTAATAGCAATAATCCTCCT-3'

5: 5'-TTATTGCTATTA-3'

6: 5'-AGGAGGATTATGCTATTAGGAGGA-3'

Scheme 2.

Synthesis of 1''-β-monoadducted oligonucleotides and ICL. (a) DMC, excess Na₂S₂O₄, 0°C, then O₂. This step is repeated three times (b) Isolation of the monoalkylated 25 mer. Annealing with the complementary strand (c) Na₂S₂O₄, 42°C followed by O₂.

Table 1.

Frequencies (%) of Guanine Adducts in Duplex DNA under Bifunctional Conditions. Role of Pre-Covalent Interactions with Deoxyadenosine.

Duplex DNA	2a	2b	2a+2b	2b/2a
a: 5'-ATTATAGCTATT-3' 3'-TAATATCGATAA-5'	0.16 (± 0.01)	1.9 (± 0.08)	1.2	12
b: 5'-ATATAGCTATAT-3' 3'-TATATCGATATA-5'	0.38 (± 0.1)	2.5 (± 0.9)	2.9	6.6
c: 5'-TTATTGCAATTA-3' 3'-AATAACGTTAAT-5'	0.50 (± 0.03)	4.3 (± 0.1)	4.8	8.6
d: 5'-AATAGCAATAAT-3' 3'-TTATCGTTATTA-5'	0.37(± 0.03)	4.1 (± 0.3)	4.5	11

Table 2.

Frequencies (%) of Guanine Adducts in Duplex DNA under Bifunctional Conditions. Effect of the Presence of a Methylated Cytosine (C*=m⁵C) at GpCpA Steps.

Duplex DNA	2a	2b	2a+2b	2b/2a
e: 5'-AATAGCAATAAT-3' 3'-TTATC*GTTATTA-5'	1.1 (±0.08)	4.9 (±0.06)	6.0	4.4
f: 5'-TTATTGCAATTA-3' 3'-AATAAC*GTTAAT-3'	1.3 (±0.1)	4.6 (±0.2)	5.9	3.6