



Published in final edited form as:

Chemistry. 2018 September 06; 24(50): 13278–13289. doi:10.1002/chem.201802038.

Interdependent Sequence-Selectivity and Diastereoselectivity in the Alkylation of DNA by Decarbamoylmitomycin C.

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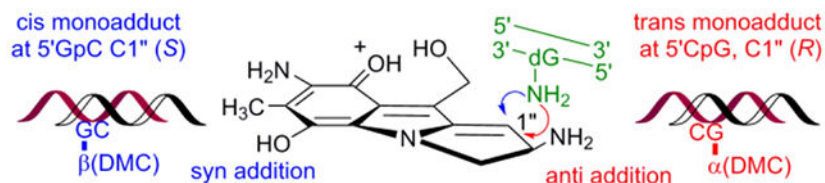
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Abstract

Mitomycin C (MC), an antitumor drug, and decarbamoylmitomycin C (DMC), a derivative of MC, alkylate DNA and form deoxyguanosine monoadducts and interstrand crosslinks (ICLs). Interestingly, in mammalian culture cells, MC forms primarily deoxyguanosine adducts with a 1''-*R* stereochemistry at the guanine-mitosene bond (1''- α) whereas DMC forms mainly adducts with a 1''-*S* stereochemistry (1''- β). The molecular basis for the stereochemical configuration exhibited by DMC has been investigated using biomimetic synthesis. Here, we present the results of our studies on the monoalkylation of DNA by DMC. We show that the formation of 1''- β -deoxyguanosine adducts requires bifunctional reductive activation of DMC, and that monofunctional activation only produces 1''- α -adducts. The stereochemistry of the deoxyguanosine adducts formed is also dependent on the regioselectivity of DNA alkylation and on the overall DNA CG content. Additionally, we found that temperature plays a determinant role in the regioselectivity of duplex DNA alkylation by mitomycins: At 0°C, both deoxyadenosine (dA) and deoxyguanosine (dG) alkylation occur whereas at 37 °C, mitomycins alkylate dG preferentially. The new reaction protocols developed in our laboratory to investigate DMC-DNA alkylation raise the possibility that oligonucleotides containing DMC 1''- β -deoxyguanosine adducts at a specific site may be synthesized by a biomimetic approach.

Graphical abstract



Decarbamoylmitomycin C (DMC) forms DNA deoxyguanosine monoadducts both at GpC and CpG sequences. The major deoxyguanosine adducts produced have the opposite stereochemical configuration at each sequence in mammalian DNA. The GpC sequence is the major target for mammalian DNA (42% CG) *in culture cells* by this drug and the major adduct has 1''- β (cis) stereochemistry at C1''. In CG rich DNA, formation of 1''- α (trans) adduct is favored.

Keywords

Mitomycins; Biomimetic synthesis; Antitumor agent; Oligonucleotides; Diastereoselectivity

Introduction

Mitomycin C (MC) is a DNA alkylating agent currently used as a cancer chemotherapeutic drug.^[1,2] Its cytotoxicity is attributed to the formation of DNA-adducts, in particular the formation of interstrand crosslinks (ICLs). MC monoadducts and ICLs are formed at the exocyclic amino group of 2'-deoxyguanosine (dG) in DNA.^[3,4] 10-decarbamoylmitomycin C (DMC) is a derivative of MC obtained from chemical removal of the 10-carbamoyl group. Despite lacking a carbamate group, DMC is able to form ICLs and generates 10 to 20 times more dG adducts than MC in mammalian culture cells.^[5-7] Furthermore, the major dG adducts generated by MC and DMC have opposite stereochemistry at C1 of the guanine-mitosene bond: C1''-*R* (trans or 1''- α) for MC and C1''-*S* (cis or 1''- β) for DMC (Figure 1: MC major adducts are **1a**, **2a** and the α -ICL **3a**,^[8,9] whereas DMC forms primarily the epimers **2b** and the β -ICL **3b**).^[6,7] Another common DNA adduct formed by MC and DMC at the 2-amino group of dG results from the alkylation of DNA by 2,7-diaminomitosenes **7a** and **7b** (Figure 1), the major metabolites of MC and DMC.^[10]

Both MC and DMC alkylation processes are dependent on the reductive activation of their quinone systems. These complex processes consist of 2 competing activation pathways (monofunctional and/or bifunctional) yielding 2 different DNA-reactive electrophiles (illustrated in Scheme 1A i.e., species **11** and **13**).^[11,12] The DNA adducts formed in cells by MC indicate that the reductive activation occurs through the bifunctional mechanism. In this case, both mitomycins' alkylating centers, the 1,2-aziridine and 10-carbamate for MC or 10-hydroxyl for DMC, can react with the exocyclic amino groups of deoxyguanosine residues located on opposing DNA strands to generate ICLs.^[12]

The role of the opposite stereochemistry of MC and DMC DNA adducts in the cytotoxicity of the two drugs is not fully understood. Strong evidence supports the hypothesis that the structure of ICLs generated by chemotherapeutics plays an important role in the mode of action of these drugs. The importance of the structure of ICLs in repair and recognition processes during replicative and non-replicative events has been previously evidenced.^[13,14] In line with this hypothesis, the structure of the two diastereomeric ICLs generated by MC and DMC (**3a** and **3b**, Figure 1) could be at the origin of the contrasting biochemical responses exhibited by the two drugs,^[15] exemplified as follows: (i) DMC induces cell cycle arrest at lower concentrations when compared with MC^[16] (ii) Removal of DMC 1'' β adducts **2b** and **3b** is faster than removal of MC 1'' α adducts **2a** and **3a** in EMT6 cells;^[7]

(iii) The DNA-adducts generated by DMC treatment (1''- β -ICL) rapidly activate a p53-independent signal transduction pathway.¹⁷

The comparison of the local DNA structure of MC and DMC adducts with the biological activity of both drugs should provide invaluable insight into the structure-activity relationship of the stereoisomeric DNA lesions. Such studies require the use of modified oligonucleotides containing a specific adduct at a single position of their base sequences. [18–22]

However, examination of the biochemical effects of DMC adducts has been severely limited by the inability to prepare sufficient quantities of oligonucleotides containing **2b** and **3b**. Our long-term objective is to synthesize duplex oligonucleotides bearing DMC 1''- β -monoadduct **2b** and ICL **3b** to elucidate the role of the adducts' β stereochemistry at C1'' in the drug biological activity. Consistent with this objective, the present work focuses on the molecular mechanisms responsible for the stereochemical outcome of DMC-DNA adducts formation. Previous DNA alkylation reactions between DMC and oligonucleotides or plasmids yielded only trans (1''- α) adducts (**2a** and **3a**, Figure 1) as major adducts.^[23–25] However, we have recently developed experimental conditions capable of generating DMC cis (1''- β) adducts **2b** and **3b** (Figure 1). We have established that DMC is able to generate crosslinks **3a** and **3b** both at CpG and GpC sequences and that the crosslinking reaction 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.^[26] We report here, that the diastereoselective formation of 1''- β adducts by DMC requires bifunctional activation of DMC. Furthermore, by using oligonucleotides with C-G base pairs flanked by different base sequences, we establish that: (i) DMC guanine monoadducts **2b** (1''- β) and **2a** (1''- α) are produced simultaneously in most sequence contexts; (ii) the regioselectivity and diastereoselectivity of DNA monoalkylation by DMC are interdependent; (iii) single-stranded DNA favors the α -monoadduct **2a**. We also report that, in DNA with a low CG content, formation of **2b** (1''- β adduct) is favored overall whereas formation of **2a** (1''- α adduct) is favored in DNA with a high CG content. Finally, we found that temperature plays a determinant role in the regioselectivity of duplex DNA alkylation by mitomycins: At 0 °C, both deoxyadenosine (dA) and deoxyguanosine (dG) alkylation occur whereas at 37 °C, DMC alkylates dG preferentially.

Results and Discussion

Results

DMC 1''- β Monoadduct (2b) is Produced only under Bifunctional Activation Conditions (Figure 2 and Table S1, supporting information).—The α -adducts **1a** (from MC) and **2a** (from DMC) were formed in the reaction of duplex oligonucleotides a: 5'-ATATACCGTATAT-3' and b: 5'-ATATAGCTTATAT-3' with MC or DMC under autocatalytic conditions at 0 °C (Figures 2A, 2B and Table S1, supporting information). In both cases, the frequency of adduct formation was higher at 5'-CpG steps. The 1''- β adducts **1b** (**1b** (C1'' *S*) is the stereoisomer of **1a** (C1'' *R*)) and **2b** (Figure 1) were not detected, and

the frequency of adduct formation was higher in the case of MC compared to DMC. No interstrand crosslink (**3a** or **3b**, Figure 1) was detected at that temperature. These results are in agreement with previous observations.^[23]

We then used a newly developed bio-mimetic protocol to reduce DMC fully and quickly so that DNA alkylation is achieved by **11** (i.e., under bifunctional conditions, scheme 1) rather than **13** (i.e., under auto-catalytic conditions, scheme 1). This protocol is described in the experimental section (Oligonucleotide Alkylation by DMC under Bifunctional Conditions, protocol 1). Bifunctional conditions require a complete and fast reduction of DMC to minimize the monofunctional pathway; path 1 (Scheme 1A). Reduction under bifunctional conditions was achieved by adding an excess of sodium dithionite and a low concentration of DMC under anaerobic conditions. This can be monitored visually: The reaction mixture becomes colorless as soon as sodium dithionite is added and remains so during the course of the reaction, indicating that compound **9**, which is colorless, is not reoxidized to aziridomitosene **12**, which is purple (Scheme 1A).

Under such bifunctional conditions, DMC monoadduct **2b** was formed both at 5'-CpG and at 5'-GpC steps (Figure 2C and 2D). Oligonucleotide **b** (GpC central sequence) yielded adduct **2b** as the major adduct, in contrast to oligonucleotide **a** (CpG central sequence). The formation of DMC 1''- β -monoadduct **2b** was favored at low concentrations of DMC (Figure S1, Supporting information). As the concentration of DMC decreases, the autocatalytic pathway is necessarily disfavored and this correlates with an increase in the ratio **2b/2a**. Taken together, these results indicate that the formation of **2b** (1''- β stereochemistry) is only observed when the dG alkylation step at C1'' is achieved by intermediate **11** (generated by bifunctional activation) and that the monoalkylation of duplex DNA by aziridinomitosene **13** (generated under autocatalytic conditions) produces mainly **2a** (1''- α stereochemistry) at 0 °C (Figure 2 and Scheme 1B). Additionally, deoxyadenosine (dA) monoadducts **4** and **5** (Chart 1) were also detected at 0 °C. A full description of the formation of these dA adducts is the subject of a separate manuscript.^[27]

Enzymatic activation (NADH-cytochrome *c* reductase) at pH 5.8 yielded adduct **2b** as a minor adduct and only at 5'-GpC steps (Supporting information, Table S2). This implies that adduct **2b** is produced less efficiently under enzymatic activation in our model system. This is probably due to the slower kinetic of enzymatic reduction and the presence of unreduced DMC in the reaction mixture. Consequently, the autocatalytic pathway is probably also activated (path 1, Scheme 1A). This supports the rationale that both **11** (from bifunctional activation, path 2, Scheme 1A) and **13** (from auto-catalytic activation, path 1, Scheme 1A) contribute to dG monoalkylation at pH 5.8 in this case.

Finally, alkylation of single stranded oligonucleotides under bifunctional conditions yielded both **2a** and **2b**. However, in single stranded oligonucleotides, the frequency of adducts **2a** and **2b** was similar both in a 5'-CpG and 5'-GpC sequence context and, in both cases, the major adduct detected was **2a** (Table 1).

In summary, when duplex DNA is alkylated at 0 °C by monofunctionally activated DMC, the 1'' α adduct **2a** is the only dG adduct observed, and it is formed more efficiently at 5'-

CpG steps than at 5'-GpC steps. When the same reactions are performed under bifunctional activation conditions both the 1''- β dG adduct **2b** and the 1''- α adduct **2a** are formed. The diastereoselectivity of the alkylation reaction is sequence-dependent: Isomer **2b** is strongly favored at 5'-GpC steps (10-fold higher frequency), while isomer **2a** is favored at CpG steps, but with lower diastereoselectivity (2-fold higher frequency). These results prove that the structure of the alkylating agent generated by reduction of DMC determines the stereochemical outcome of DNA alkylation by DMC. Aziridinomitosene **13** only produces α adducts whereas reduced DMC species **11** produces both α and β adducts (Scheme 1B). No crosslink was detected at 0 °C.

The Adduct Profile of DNA Alkylation is Temperature and pH Dependent. Development of Reaction Conditions for the Formation of DNA Crosslinks by DMC (Table S3, supporting information and Figures 3 and 4).

Treatment of Calf Thymus DNA and *M. Luteus* DNA with DMC at Various Temperatures (Figures 3 and 4).

Treatment of calf thymus DNA and *M. Luteus* DNA with DMC under bifunctional conditions resulted in the formation of dG adducts **2a**, **2b**, **3a**, **3b**, **6** (Figure 1) and dA adducts **4** and **5** (Chart 1). The adduct profile of the alkylation reaction depends on the DNA CG content, the reaction temperature, and the pH. The transmonoadduct **2a** was the major adduct formed in *M. Luteus* DNA (72% GC) but the cis-monoadduct **2b** was formed preferentially in calf thymus DNA (42% GC). This is a strong indication that the stereoselectivity exhibited by DMC is dependent and the CG content of the DNA substrate. The formation of the diastereomeric ICLs **3a** and **3b** with both types of DNA is temperature dependent. At 0 °C, ICLs **3a** and **3b** (Figures 3 and 4) did not form. In contrast, MC crosslinking is possible at 0-5 °C.^[24] Interestingly, adducts **4** and **5** (deoxyadenosine (dA) adducts, Chart 1) were observed at 0 °C in the reaction mixtures.^[27] At room temperature, crosslinking was observed and the trans crosslink **3a** was formed with both *M. luteus* DNA and calf thymus DNA (Figures 3 and 4). The cis crosslink **3b** was detected with calf thymus DNA, but not with *M. luteus* DNA. In addition, dG- N^2 -2,7 DAM adduct **6** (Figure 1) was detected with both types of DNA. The 2,7-DAM adduct **6** was also observed in cells treated with DMC as a minor adduct.^[10] Higher temperature (37 °C) promoted the formation of ICLs and adducts **3a** and **3b** were formed in higher proportion (Figures 3 and 4). The trans adduct **3a** was the major ICL adduct formed with *M. Luteus* DNA, while the cis-adduct **3b** was the major ICL adduct formed with calf thymus DNA. No deoxyadenosine adducts were detected at higher temperature in duplex DNA. The adduct pattern resulting from the alkylation of calf thymus DNA with DMC (Figure 4) shows a remarkable resemblance to the adduct pattern from treatment of EMT6, FAA-A fibroblasts, and MCF-7 cells with DMC.^[7] The major difference is that the ratio **2a/3a** is higher in cells than in our model systems.

Influence of pH on DMC Alkylation of Calf Thymus DNA (Table S3, supporting information).

A higher ratio crosslink/monoadduct (**3b/2b**) was observed when reactions were performed at a more acidic pH (pH 4.5). However, under those conditions, the adduct profile presented a high proportion of products derived from N^7 guanine monoalkylation, in addition to the

expected N^2 adducts (data not shown). This is in agreement with previous observations on the reaction of acid activated mitomycins with DNA.^[28] At higher pH (pH 7.5), as anticipated, the ratio crosslinks/monoadducts was slightly lower than at pH 5.8 and the 2,7 DAM adduct **6** could not be detected.^[12] At pH 5.8, the adduct profile from DMC treated calf thymus DNA was very similar to the adduct profile observed in cells (Figure 4). In addition, this pH also maximized the amount of crosslinks and it was chosen for further reactions (Table S3, supporting information). The study of the influence of temperature and pH on the alkylation of DNA by bifunctionally activated DMC discussed above resulted in the development of reaction conditions that produce significant yields of crosslink adducts.

These reaction conditions permitted us to perform a rigorous study on the regioselectivity and diastereospecificity of the formation of DNA interstrand crosslinks by DMC. The results of this work have been presented recently.^[26]

Sequence-Selectivity of Guanine Monoalkylation by Bifunctionally Activated DMC.

The results discussed above indicate that formation of **2b** is favored in DNA with a low CG content (as in calf thymus DNA) but that formation of **2a** is favored in DNA with a high proportion of CG base pairs (as in *M. Luteus* DNA). In order to gain insight into the regioselectivity of deoxyguanosine monoalkylation in mammalian DNA (42% CG), oligonucleotides in 17 different sequence contexts (Table 2) were hybridized with their complementary strand and reacted with reduced DMC.

Further Optimization of Reaction Conditions for the Alkylation of Oligonucleotides by DMC.

We performed an optimization of the bifunctional activation of DMC with the aim of increasing the frequency of adducts, thus facilitating their detection and improving the precision in their quantification. These reaction conditions ensured that DMC was kept fully reduced in solution to minimize the autocatalytic route. We hypothesized that repeated additions at low concentrations of fully reduced DMC would result in a significant increase of the yield of adducts, and we tested our hypothesis using the conditions described as protocol 2 in the experimental part. This new protocol gave consistently higher yields and was used in our subsequent investigations of DMC regioselectivity. Reactions were performed at least twice and the adduct pattern observed with oligonucleotides **14-31** was highly reproducible. Reactions were performed at 0 °C because carrying the reaction at this temperature prevented the formation of crosslinks, thus facilitating the study of the monoalkylation reaction. The 1''- β dG adduct **2b** was always produced alongside 1''- α dG adduct **2a** and the alkylation frequency of **2a** and **2b** was determined in each case (Figures 5 and 7).

Regioselectivity and Diastereoselectivity in the Alkylation of Oligonucleotides by DMC.

DMC generated adducts **2b** and **2a** in all oligonucleotides studied. Calculation of percent yields for the formation of **2a** and **2b** is described in the experimental section as well as in the supporting Information section (page S8) and in our previous publication.^[26] The

frequency of guanine alkylation was highest at **XpGpC** steps (X = C, G, A, T) and **XpGpY** (X, Y = A, T) (Figures 5 and 7). TpGpC steps produced the highest yield of dG alkylation and also the highest diastereoselectivity in the formation of **2b**: Oligonucleotide **17** (TpGpCpT) yielded 4.0% of **2b** (for a total of 4.4% of guanine adducts, Figure 5a and 6) and oligonucleotide **30** (TpGpCpA) 4.3% of **2b** (for a total of 4.8% of guanine adducts, data not shown). Both 5'-GpG and 5'-CpG sequences gave the lowest yield of dG adducts and the highest diastereoselectivity for **2a** (Figures 5, 6 and 7). This indicates that there is a competition between guanine- N^2 nucleophilic attack on the α and β face of **11** at 0 °C in all sequence contexts studied. In oligonucleotides **14-30**, DMC regioselectivity and stereoselectivity are interdependent, i.e., oligonucleotides with a high frequency of dG alkylation are also oligonucleotides yielding a high ratio **2b/2a** (Figures 5–7 and S2, supporting information). Formation of **2b** and therefore, guanine- N^2 nucleophilic attack on the β face of reduced DMC is favored at **XpGpC** steps (X=C, G, A, T; oligonucleotides **14**, **15**, **16**, **17**, **30**, Figures 6, 7 and Figure S2, supporting information) and **XpGpY** (X,Y=A,T; oligonucleotides **20**, **21**, **24**, **25**, Figures 6, 7 and S2, supporting information).

Formation of 1''- α adduct **2a** and, therefore, guanine- N^2 nucleophilic attack on the β face of reduced DMC is favored at **CpGpX** (with X = A, T, G; oligonucleotides **19**, **23** and **27**, Figures 6, 7 and S2, supporting information) and **TpGpGpY** (with Y = A, T; oligonucleotides **18** and **22**, Figures 6, 7 and S2, supporting information). In oligonucleotides **14-30** (low CG content), formation of 1''- β adduct **2b** is favored overall. Taken together, these results indicate that there is interdependence between regioselectivity and diastereoselectivity in the alkylation of guanine residues by DMC. The alkylation of dG is favored at NpGpN (where N = A or T) and at GpC steps, and this effect correlates with the stereoselective formation of the 1''- β monoadduct. Conversely, the stereoselective formation of the 1''- α monoadduct is favored at CpG and GpG steps.

The Ratio α/β Adducts is a Function of DNA G-C Content.

Results from CT DNA and *M. Luteus* DNA alkylation with DMC above, indicate that the overall DNA CG content has an influence on the stereoselectivity of DMC guanine alkylation. This phenomenon was further investigated by examining the ratio α/β adducts versus the CG content of all DNA substrates studied (Figure 8).

The data shown in Figure 8 highlights the interdependence between the CG content of DNA and the diastereoselectivity of dG alkylation by DMC: An increase of C-G content correlates with a decrease with the ratio β/α . In DNA with a low CG content, such as calf-thymus DNA, the formation of 1''- β adducts **2b** and **3b** is favored. However, in DNA with a high C-G content, such as *M. Luteus* DNA, the formation of 1''- α adducts **2a** and **3a** is favored. The only crosslink detected with oligonucleotide **31** (100% CG) at 37 °C was 1''- α crosslink **3a**. Although the regioselectivity of dG monoalkylation with DMC in DNA with a high C-G content has not been systematically determined in this work, the fact that α -crosslink **3a** is the major adduct formed with *M. Luteus* DNA suggests that, for DNA with a high CG content, the alkylation of DNA by DMC is selective for CpG steps since the crosslink adduct **3a** can only be formed at CpG sequences.^[26]

Influence of the Exocyclic Amino Group on the Opposite or Adjacent Guanine on DMC Stereoselectivity.

The results discussed above indicate that the 1''- α adduct is favored at most CpG sequences and the 1''- β adduct is favored at most GpC steps. These observations prompted an investigation into the role played by the 2-NH₂ group of guanine in the opposite strand (at CpG and GpC steps) in the regio- and stereospecificity of the alkylation of dG by DMC. To this end, one deoxyguanosine in duplexes **17** and **19** was replaced by deoxyinosine either at CpG (duplex **17**) or GpC steps (duplex **19**). The replacement of deoxyguanosine by deoxyinosine in the opposite strand at GpC in duplex **17** resulted in a significant decrease in the frequency of dG alkylation (-2%), and a significant decrease in the amount of **2b** versus **2a** (Table 3 and Figure S3, supporting information). In contrast, the replacement of deoxyguanosine by deoxyinosine in the opposite strand at the CpG step in duplex **19** resulted in a small increase in the global yield of dG adducts (+0.5%), and a significant increase in the amount of **2b** versus **2a** (Table 3 and Figure S4, supporting information). These results show that the 2-amino group on deoxyguanosine of the nonbonding strand contributes to the regio- and stereoselectivity exhibited by DMC at GpC and CpG steps and also suggest the presence of other contributing factors.

Similarly, the role played by the 2-NH₂ group on the adjacent deoxyguanosine at GpG steps was investigated (table 3 and Figure S5, supporting information). One deoxyguanosine in duplex **18** was replaced by deoxyinosine either at the 5' or at the 3' position. Replacement of deoxyguanosine by deoxyinosine at the 5' position at GpG resulted in an increase in the frequency of dG alkylation (+1%) by DMC, and in a reversal of stereoselectivity, as **2b** became the major adduct (Table 3 and Figure S5, supporting information). This suggests that the presence of the exocyclic 2-NH₂ group of the 5' guanine at GpG steps must be essential for the 1''- α stereoselectivity exhibited by DMC at GpG steps. On the other hand, replacement of deoxyguanosine by deoxyinosine at the 3' position at GpG steps resulted in smaller variations both in the frequency of guanine alkylation (+0.3%) and in the ratio **2b/2a** (**2a** was still the major adduct) (Table 3 and Figure S5, supporting information). Considered together, these results demonstrate that the exocyclic amino group on deoxyguanosine located at the 5' position at GpG steps and on the opposite strand at GpC and CpG steps plays an important but not exclusive role in the determination of DMC alkylation frequency and stereoselectivity.

Discussion

The identification of DNA adducts generated in mammalian culture cells upon treatment with MC and DMC showed that the two drugs generate stereoisomeric DNA adducts. The DMC major dG monoadduct and ICL, **2b** and **3b**, have a 1''- β (cis) stereochemistry at C1'' whereas the MC major DNA adducts, **1a**, **2a**, and **3a** have a 1''- α (trans) stereochemistry. DMC also generates 20 to 30 times more monoadducts than MC when administered at the same doses.^[7] The aim of this work was to provide a rationale for these observations in order to understand the molecular basis of DMC cytotoxicity. Previous reactions between short oligonucleotides, poly (dG-dC), or plasmids *in vitro* with reduced DMC produced the

1''- α (trans) adduct **2a** as the major monoadduct, while the diastereomeric adduct **2b** was not formed or formed in minimal proportions.^[25] This is in contrast to what happens in mammalian cultured cells where **2b** is the major DMC-DNA adduct formed. We hypothesized that the discrepancy between the adduct patterns observed with cultured cells and the ones obtained using *in vitro* chemical reactions was a direct consequence of the reductive conditions under which previous DNA alkylation reactions had been performed. Until now, research on the alkylation of DNA by MC or DMC has mostly used sequential additions of substoichiometric amounts of dithionite or slow enzymatic reductions of MC or DMC.^[25] Under such conditions, the auto-catalytic monofunctional pathway is promoted (path 1, Scheme 1A),^[30] and DNA alkylation is effected by aziridinomitosene **13**.^[12] To test our hypothesis, we reacted short oligonucleotides containing a CpG or GpC central sequence with DMC using autocatalytic or bifunctional reductive conditions and analyzed the adducts formed. The results showed that when autocatalytic conditions were used, the alkylation of short oligonucleotides by **13** was selective for CpG sequences and more importantly, produced mostly α -adducts (Figures 2A and 2B, Table S1). However, when DMC was reduced under conditions that minimize the formation of **13** and favor the formation of **11** (bifunctional pathway, Scheme 1A), the 1''- β dG adduct **2b** was observed, and its formation appeared to be selective for 5'-GpC sequences. These results clearly show that, in the case of DMC, the structure of the electrophilic species influences the stereochemical outcome of DMC DNA alkylation, meaning that **13** (generated under auto-catalytic conditions) produces only 1''- α dG adducts (Figure 2, left) but **11** (generated under bifunctional conditions) is able to generate 1''- β dG adducts (Figure 2, right). In contrast, in the case of MC, the major dG adducts produced under auto-catalytic,^[23-25] or bifunctional conditions,^[9] have the same α stereochemistry at C1''.

Although reduction of DMC under bifunctional conditions (to generate **11**) is a necessary condition for the formation of 1''- β dG adducts, it is not sufficient. The amount of CG base pairs in the DNA substrate also determines the stereochemical outcome of DNA alkylation by DMC (Figure 8). Substrates with a high proportion of CG base pairs favor the formation of 1''- α dG adducts and 1''- α ICLs, whereas substrates with a low proportion of CG base pairs favor the formation of 1''- β dG adducts and 1''- β ICLs. The reason for this phenomenon is not clear. AT rich segments tend to adopt very narrow minor grooves and this has implications for drug-DNA interactions.^[31] Consequently, the difference in minor groove structures between AT-rich and CG-rich segments may influence DMC stereoselectivity. In general, GC-rich DNA in the highest multicellular organisms constitutes gene-rich actively-transcribed genomic regions. It was recently established that cis-platin, another DNA alkylating agent, targets promoters and regions harboring transcription termination sites in genomic DNA.^[32] Our results suggest that, upon treatment with DMC, regions of mammalian DNA with a high CG content are likely to be rich in 1''- α dG adducts whereas regions with a low CG content are likely to be rich in 1''- β dG adducts. Further work is necessary to verify if this uneven ratio of 1''- α and 1''- β adducts is indeed present in DMC treated mammalian DNA and, if so, to thereby understand its biological relevance.

We have previously established that the formation of DNA-DNA crosslinks by DMC is diastereospecific and diastereodivergent.^[26] In this manuscript we further investigated the

regioselectivity and stereoselectivity of dG monoalkylation by DMC in oligonucleotides with a low CG content. To do so, we treated short oligonucleotides **14–30** with reduced DMC under optimized bifunctional conditions. The results show that the formation of either 1''- α -guanine **2a** or 1''- β -guanine **2b** is dependent on the local sequence context (Figures 5 and 6) and follows the general rule in our model systems (oligonucleotides **14–30**, Figures 5–7): Formation of **2b** and dG alkylation are favored at 5'-NpGpN (N = A or T) steps and at 5'-GpC steps (Figures 5–7, oligonucleotides **16, 17, 21, 24, 25**). In contrast, at 5'-CpG and 5'-GpG steps, formation of **2a** is favored (Figures 5–7, oligonucleotides **18, 19, 22, 23**). This demonstrates an interdependence between regioselectivity and stereoselectivity and implies that both the nature of the 3' and 5' side of the alkylated dG play a role in determining the regio- and diastereoselectivity for DMC alkylation in DNA with a low CG content (such as mammalian DNA; 42% CG). In contrast, MC alkylates mammalian DNA selectively at 5'-CpG steps and generates the 1''- α -guanine adduct **1a** as the major adduct.^[7] Our results also show that the frequency of alkylation is higher at GpC versus CpG in oligonucleotides **14–30** (Figure 5) and support the hypothesis that DMC targets GpC sequences over CpG sequences for dG monoalkylation in DNA. However, DMC is overall less regioselective than MC because it also targets NpGpN (N = A or T) sites with high frequency. Therefore, if DMC targets more sequence contexts than MC, the frequency of DMC DNA adducts should be higher than MC when equivalent drug treatment is performed, specifically since the rate of bioactivation of the 2 drugs is similar (assuming cellular uptake is similar for both drugs).^[6] This is in agreement with the fact that DMC forms 20–30-fold more DNA monoadducts than MC in mammalian cells.^[7] Results from our work also provide a rationale for the observed higher ratio monoadducts/crosslinks displayed by DMC versus MC in culture cells:^[6,7] On one hand, DMC targets dG in non-crosslinkable sequences (i.e., NpGpN; N = A, T) with high frequency; on the other hand, the formation of crosslinks is less efficient at GpC steps (the major target of DMC) than at CpG steps (the major target of MC), as we reported earlier.^[26] On average, the conversion of monoadduct to crosslink occurs with 31% efficiency at GpC steps and with 80% efficiency at CpG steps. As a result (and because DMC targets GpC sequences over CpG sequences in mammalian DNA), only a low proportion of monoadducts will be able to form interstrand crosslinks.

Finally, we experimentally verified the role of the 2-NH₂ group of the non-alkylated guanine in the formation of **2b** and **2a** at CpG, GpC and GpG steps when DNA is treated by **11**. It has been postulated that pre-covalent interactions between the hydroxyl oxygen of **11** (Figure 2) and the amino group of the non-alkylated guanine were responsible for the orientation of DMC in duplex DNA leading to the formation of 1''- β monoadduct **2b**.^[33] Our results show that the 2-amino group on the guanine of the non-reacting strand contributes to the stereoselectivity exhibited by DMC at GpC and CpG steps, but that it is not the only contributing factor (Tables 3 and 4 and Figures S3–S4, supporting information). We propose that pre-covalent interactions between the exocyclic 2-NH₂ group of guanine in the opposite strand and activated DMC largely contribute to placing DMC in an orientation which favors nucleophilic attack on the β face of the drug's tetrahydropyrrole ring at GpC steps and on the α face of the drug's tetrahydropyrrole ring at CpG steps. However, the diastereoselectivity is not fully abolished upon replacement of guanosine by inosine in either case, suggesting that other factors may contribute to DMC diastereoselectivity. In contrast,

the exocyclic 2-NH₂ group on the 5' guanine is essential for the 1''- α stereoselectivity exhibited by DMC at GpG steps (Table 4 and Figure S5, supporting information). We propose therefore, that pre-covalent interactions involving the 2-NH₂ group on the 5' guanine at GpG steps and activated DMC are the main contributors to the orientation of DMC in the duplex so that nucleophilic attack on the α face of the drug occurs preferentially (Table 3 and Figure S5, Supporting information). Therefore, the exocyclic amino groups described above play an important, but not exclusive, role in the diastereoselectivity of the alkylation of DNA by DMC. Two observations indicate that **11** may also form competing pre-covalent interactions with bases other than guanine, in particular, interactions involving deoxyadenosine are likely to occur because: i) DMC 1''- β adducts are always favored when at least one dA is in the 5' position of dG (as seen with oligonucleotides **14**, **15**, **16**, **17**, **20**, **24**, **28**, Figures 5–7) and ii) adenine can react with DMC under certain conditions in duplex DNA to generate stereoisomeric adducts.^[27]

What is the biological significance of the fact that MC and DMC target different sequence contexts in mammalian DNA and of the stereochemistry of the DNA adducts formed? We, and others have observed that the biochemical responses triggered by MC and DMC are widely different in cultured cells.^[15–17] Our results suggest that the recognition of damaged DNA is likely to occur differently when DNA is alkylated by MC or DMC. Proteins recognize damaged DNA by two mechanisms: The base read out and the shape read out.^[34] Since both drugs target different sequences, the recognition process of DNA adducts by base read out is likely to differ in the case of MC and DMC. The shape read out mechanism is also likely to vary if MC and DMC adducts induce different structural DNA distortion.^[13] In addition, *trans*-adducts **2a** and **3a** formed at CpG by MC and *cis*-adducts **2b** and **3b** formed at GpC by DMC may also confer different conformational flexibility near damaged DNA sites. The efficiency of DNA repair has been linked to DNA flexibility near a damaged site.^[35] If ICL **3a** (CpG) and ICL **3b** (GpC) do indeed confer different conformational flexibility to DNA, this may partly explain why the rate of removal of **3b** is enhanced compared to the rate of removal of **3a** in EMT6 cells treated with MC.^[7]

Previous research indicates that the MC and DMC major dG adducts are also likely to be processed differently because of the different stereochemistry of the DNA adducts formed in each case (1''- α versus 1''- β). Various steps in the processing of ICLs in cells and cell extracts are dependent on the structure of the ICL itself during replicative events.^[14,36] Furthermore, in the case of DNA monoadducts, recognition and incision by base excision repair are different for stereoisomeric lesions.^[37] The structure of oligonucleotides modified with adducts **2a** and **3a** are known (both monoadduct **2a** and crosslink **3a** lie in the slightly widened minor groove of a minimally distorted DNA helix)^[38,39] but there is no structural information on DMC adducts **2b** and **3b** at the oligonucleotide level. Therefore, further efforts to produce DMC 1''- β dG adducts **2b** and **3b** on a larger scale and examination of their local DNA structures are warranted, with the aim of linking the local DNA structure of 1''- α and 1''- β adducts with the DNA damage response triggered by the two drugs.

Lastly, the 2,7 DAM adduct formed by DMC (adduct **6**, figure 1) was only detected as a minor adduct and at a low frequency in this work. Previous work has also established that adduct **6** is formed at much lower frequency with DMC compared to MC in culture cells:^[7]

a likely consequence of the better leaving group ability of the carbamoyl at C10 in **7a** compared to the hydroxyl in **7b** (Figure 1). The relative low abundance of **6** in culture cells treated with DMC compared to MC correlates with DMC higher toxicity. This is in agreement with the hypothesis stating that formation of adduct **6**, a non-cytotoxic DNA adduct, is part of the reductive detoxification pathway of mitomycins.^[22,40]

Conclusion

The aim of this work was to determine the factors governing the formation of the 1''-β dG adducts produced by DMC. We have successfully developed conditions which reproduce the pattern of DNA alkylation by DMC observed in cells: In order to produce 1''-β (cis) DNA adducts as major adducts *in vitro* upon chemical reductive activation, DMC must be reduced in a bifunctional way, DNA must be in the duplex form and the C-G DNA content must be low so that CG base pairs are flanked by A, T residues. Our results also revealed the importance of temperature in the reaction outcome. At 0 °C, only monoadducts are formed and N²-adenine adducts are observed. However, high temperatures promote the formation of crosslinks and of 2,7-DAM adduct. The influence of the structure of DNA adducts generated by MC and DMC with respect to DNA repair and cytotoxicity is still not completely understood. The different sequence selectivity of DMC versus MC and the opposite stereochemistry of the major adducts produced by MC and DMC may play a role in the different biological activity of DMC and MC since it is likely to affect the processing of DNA adducts by cells. While the structure of 1''-β adducts has been elucidated at the nucleoside level, there are no available routes to prepare oligonucleotides bearing a single DMC monoadduct or crosslink with a 1''-β (cis) stereochemistry. The synthesis of such site-specifically modified oligonucleotides is necessary to understand the relationship between the structure of DMC and MC adducts and the activity of the drugs. Our results raise the possibility that such oligonucleotides containing DMC-dG 1''-β adducts may be synthesized by a biomimetic approach.

Experimental Section

Materials.

MC was generously gifted by Professor Maria Tomasz. DMC was synthesized from MC by a published procedure^[41]. 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), NADH-cytochrome *c* reductase, NADH, 2'-deoxyadenosine, *M. lysodeicticus* DNA (*M. luteus* DNA, type XI; 72% GC) 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).

Methods. Quantitative Analysis.

Quantitation of DNA, DMC or MC-DNA adducts was based on UV spectrophotometry using the following molar extinction coefficients: MC and DMC, 21800 (367 nm); *M.*

luteus-DNA, 6000 (260 nm, mononucleotide unit); calf thymus DNA, 6500 (260 nm, mononucleotide); dT, 6600 (254 nm); dA, 14200 (254 nm); MC and DMC monoadducts (**1a**, **2a**, **2b**, **4**, **5**, **6**), 24000 (254 nm); MC and DMC ICLs (**3a**, **3b**), 30000 (254 nm). The percent yields of DMC and MC DNA-adducts (**1a**, **2a**, **2b**, **3a**, **3b**, **4**, **5** and **6**) designate the mole percent adducted guanine (for **1a**, **2a**, **2b**, **3a**, **3b** and **6**) or adenine (for **4** and **5**) of total guanine or adenine nucleotide residues present in the oligonucleotides or DNA substrates. A full description of the method is available in previous publications and in the supporting information.^{[12],[13]}

Reproducibility.

Reactions under bifunctional conditions were repeated at least twice independently and average deviation from the mean value is represented with error bars on graphs or in brackets in tables. Autocatalytic activation reactions were only performed once as they were repeats of previous experiments.^[23]

Identification of Adducts **1a**, **2a**, **2b**, **3a**, **3b**, **4**, **5** and **6** in Enzymatic Digests.

Adducts **1a**, **2a**, **2b**, **3a**, **3b**, **4**, **5** and **6** were identified by their UV spectra, retention times and co-elution with authentic standards synthesized previously in our laboratory.
[10b, 7, 26, 27, 42, 43]

Oligonucleotide Alkylation by DMC and MC under Autocatalytic Conditions.

Oligonucleotide alkylation by DMC and MC under autocatalytic conditions has been previously described and is also available in the supporting information section.^[23]

Oligonucleotide Alkylation by DMC under Bifunctional Conditions, (protocol 1)

Self-complimentary duplex oligonucleotides (5'-ATATACCGTATAT-3' or 5'-ATATAGCTTATAT-3': 10 A_{260} units; 330 μg ; corresponding to 0.083 μmol) were annealed (55 °C heating for 10 min followed by slow cooling to RT) and then mixed with 0.648 μmol of DMC in 10 mM potassium phosphate buffer, pH 5.8 (355 μL). The mixture was cooled down to 0°C in an ice bath while deaerating *via* argon bubbling (30min). Freshly prepared $\text{Na}_2\text{S}_2\text{O}_4$ solution (1.62 μmol in 10 μL of 10 mM potassium phosphate buffer, pH 5.8) was added at once. After 1 hr at 0 °C, the reaction was then removed from ice and opened to air, followed by gentle stirring until a consistent purple color was obtained. The mixture was stirred for 20 min and chromatographed on a 2.5*56 cm Sephadex G-25 column using 20 mM NH_4HCO_3 as eluent. Oligonucleotide containing fractions were lyophilized. In the case of single stranded oligonucleotides, 5'-ATTATCGTTATT-3' and 5'-ATTATTGCTATT-3'. the reaction was performed without the annealing step.

Oligonucleotide Alkylation by DMC under Bifunctional Conditions, Repeated Addition of Fully Reduced DMC (Protocol 2)

Oligonucleotides **13-30** (5'-ATTATXGYTATT-3', 10 A_{260} unit scale; corresponding to about 0.072 μmol) were mixed with an equal amount of their complementary strand (5'-AATAXGYATAAT-3', 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 then 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 allowed to stir 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 hr and then was removed from ice and opened to air, followed by gentle stirring until a consistent purple color was obtained. The mixture was then stirred for 20 min under air and then chromatographed on a 2.5 x 56 cm Sephadex G-25 column using 20 mM NH_4HCO_3 as eluent. Oligonucleotide containing fractions were lyophilized. In the case of duplex oligonucleotide **31**, the reaction was performed both at 0 and 37°C .

Oligonucleotide Alkylation by Enzymatic Activation.

Self-complementary duplex oligonucleotides (5'-ATATACCGTATAT-3' or 5'-ATATAGCTTATAT-3': 10 A_{260} units; 330 μg ; corresponding to 0.083 μmol) were mixed with 4.0 μmol of DMC in 100 mM potassium phosphate buffer, pH 5.8 (500 μL) at 0°C in an ice bath while deaerating (30min). NADH (8 μmol in 515 μL of 10 mM potassium phosphate, pH 5.8) was added to the reaction followed by cytochrome c reductase (6 units in 12 μL of deaerated 100 mM potassium phosphate, pH 7.4). The reaction was left in an ice bath with argon bubbling for 1hr and then stirred open to air for 20min. The mixture was chromatographed on a 2.5 x 56 cm Sephadex G-25 column using 20 mM NH_4HCO_3 as eluent.

M. luteus and calf thymus DNA Alkylation by DMC under $\text{Na}_2\text{S}_2\text{O}_4$ Activation.

A solution of sonicated DNA (either *M. luteus* or calf thymus; 12 mM) and DMC (1mM) was deaerated in 10 mM potassium phosphate-0.001 M EDTA, pH 5.8, 4.5 or 7.5 (1 mL). A deaerated solution of $\text{Na}_2\text{S}_2\text{O}_4$ (2mM, from a freshly prepared anaerobic 120 mM solution in water) was added incrementally following protocol 2 until a total of 5 mM of DMC was added. The drug-DNA complexes were extracted by adding 10 mM 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 then vortexed vigorously and allowed to settle before transferring 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 as described above.

Enzymatic Digestion of the DMC-DNA Complexes to Nucleosides and DMC-Nucleoside Adducts.

The lyophilized DMC-DNA complex (*M. luteus* or calf thymus) was dissolved in 20 mM ammonium acetate, pH 5.5 (2.5 A_{260} units/mL). Nuclease P_1 (1.0 unit/ A_{260} unit of complex) was added to the mixture followed by incubation for 4 hr at 37°C . The pH was adjusted to 8.2 by addition of 200 mM NaOH (25 $\mu\text{L}/\text{mL}$ of ammonium acetate), 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) and 2 hr incubation at 37 °C were followed by the addition of alkaline phosphatase (1.6 units/ A_{260} unit of complex) and incubation overnight at 37 °C. Samples were lyophilized and redissolved for HPLC analysis.

Enzymatic Digestion of Alkylated Oligonucleotides.

Nuclease P₁/SVD/AP protocol: 1 A_{260} unit of oligonucleotide and 1 unit of nuclease P₁ were incubated at 37 °C for 2 hr in 0.8 mL of 20 mM ammonium acetate, pH 5.5; 100 mM MgCl₂ (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 hr.

Analysis of DNA Adducts after Enzymatic Digestion of Alkylated Oligonucleotides and Calf Thymus or *M. Luteus* DNA.

Digestion mixtures were directly analyzed by HPLC using an Agilent 1200 HPLC system and a Kromasil C-18 reverse phase column (0.46 x 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by a grant from the National Institute of Health (5SC3GM105460-04) to E.C. The authors would like to thank the PRISM (Program for Research Initiatives in Science and Math) program for support to A. Vargas and M. Zheng.

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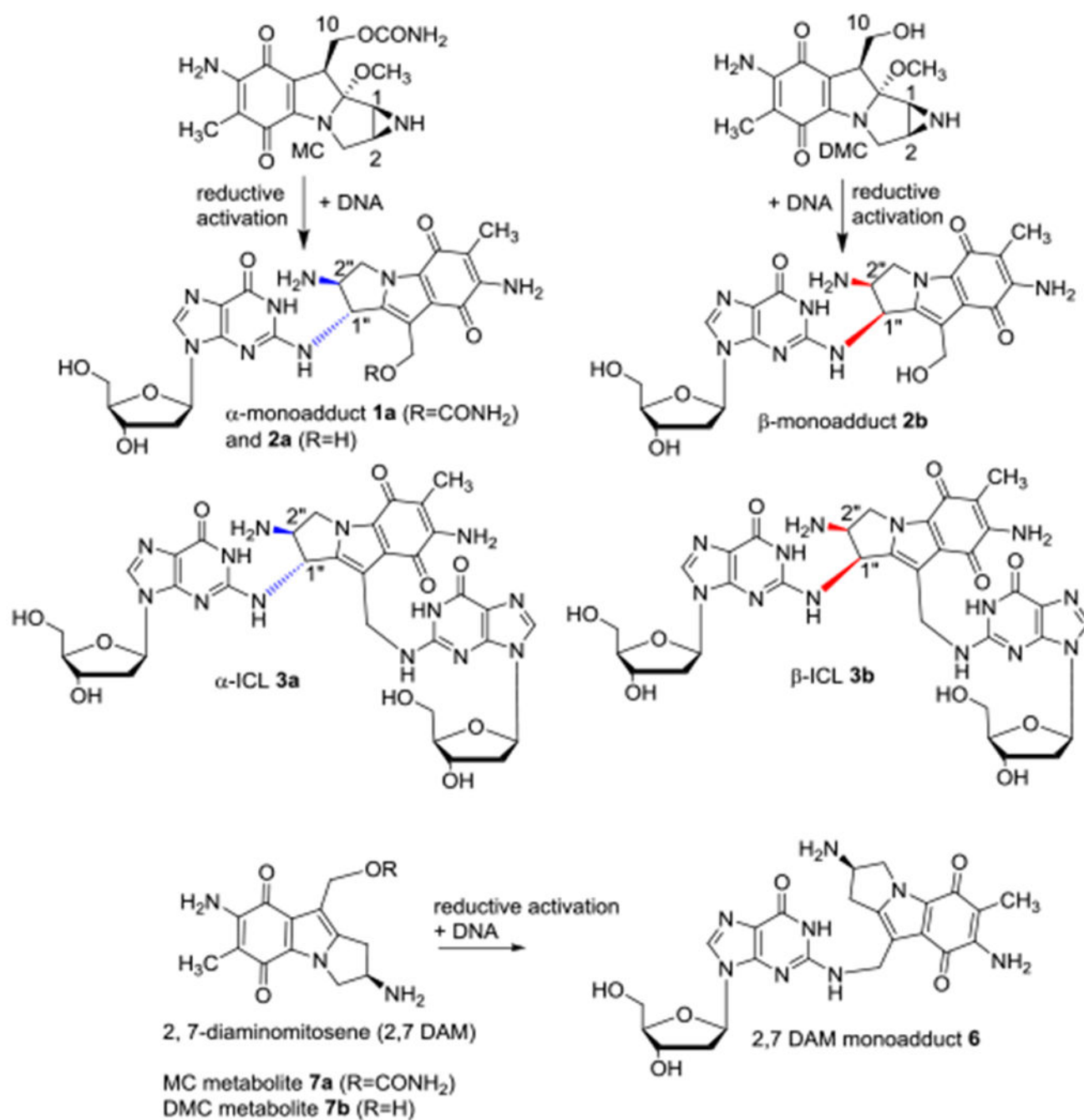


Figure 1. Structure of the major mitomycin C (MC), decarbamoylmitomycin C (DMC) and 2, 7-diaminomitosenes **7a** and **7b** deoxyguanosine adducts detected in cells.

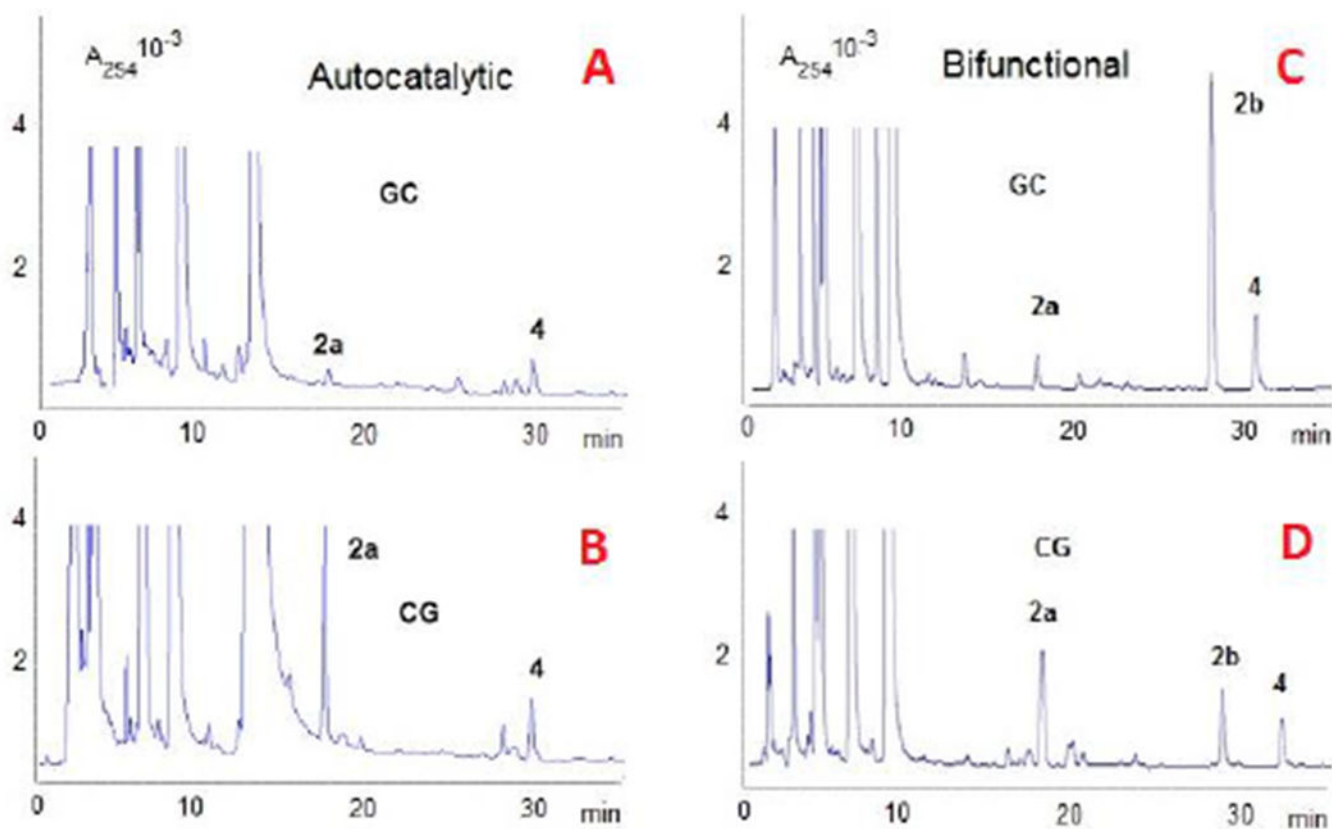


Figure 2. HPLC analysis of digests of DMC-oligonucleotide complexes formed under DMC autocatalytic activation (A and B) and bifunctional activation (C and D). Oligonucleotides used: duplex 5'-ATATAGCTATAT-3' (A and C) and duplex 5'-ATATACGTATAT-3' (B and D). 4: adenine adduct.²⁵

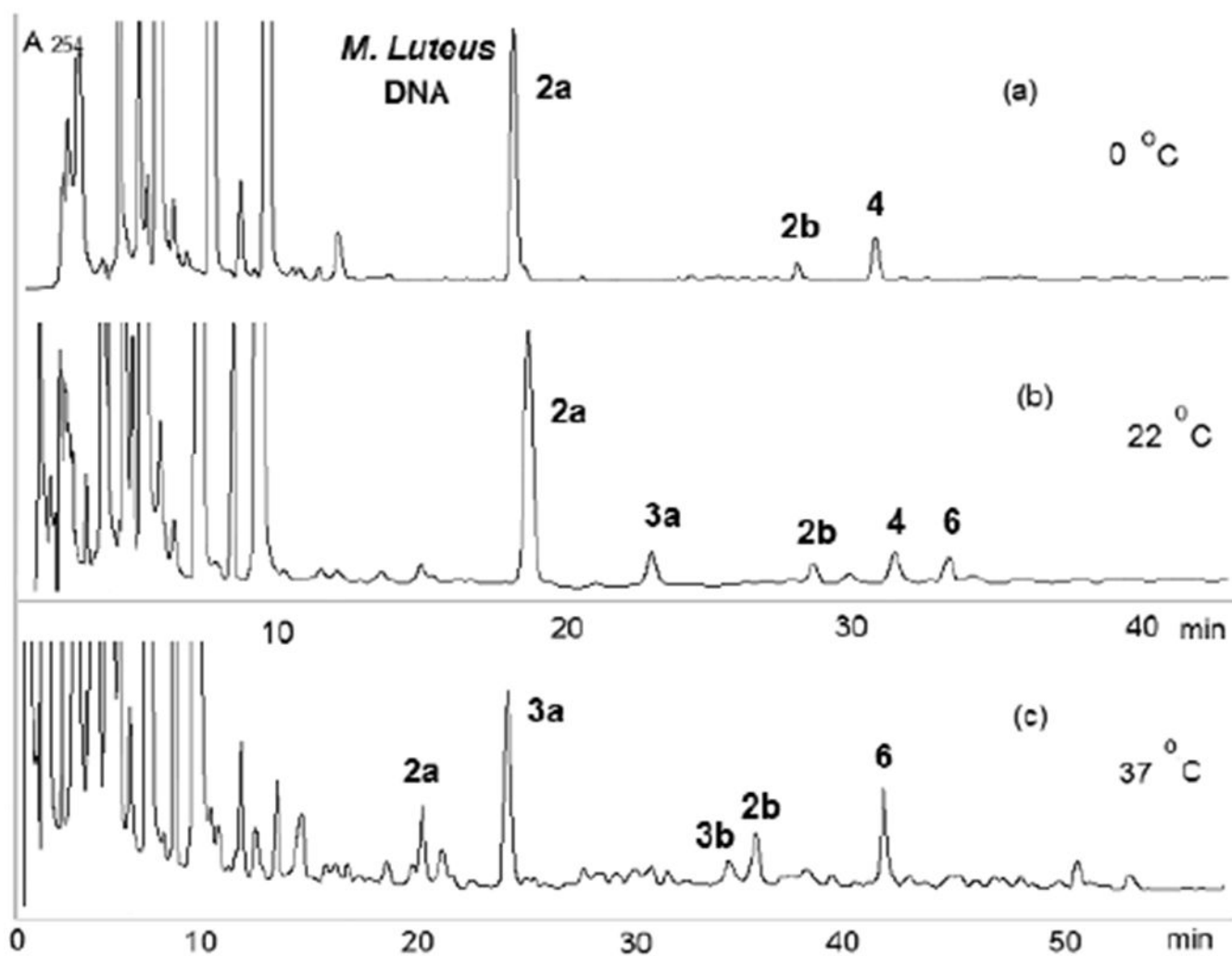


Figure 3. HPLC analysis of digests of DMC-oligonucleotide complexes formed under bifunctional conditions with *M. Luteus* DNA at various temperatures. (c) The elution gradient was modified at 37 °C (6 to 18% acetonitrile in 105 minutes) for a better separation of **3b** and **2b**.

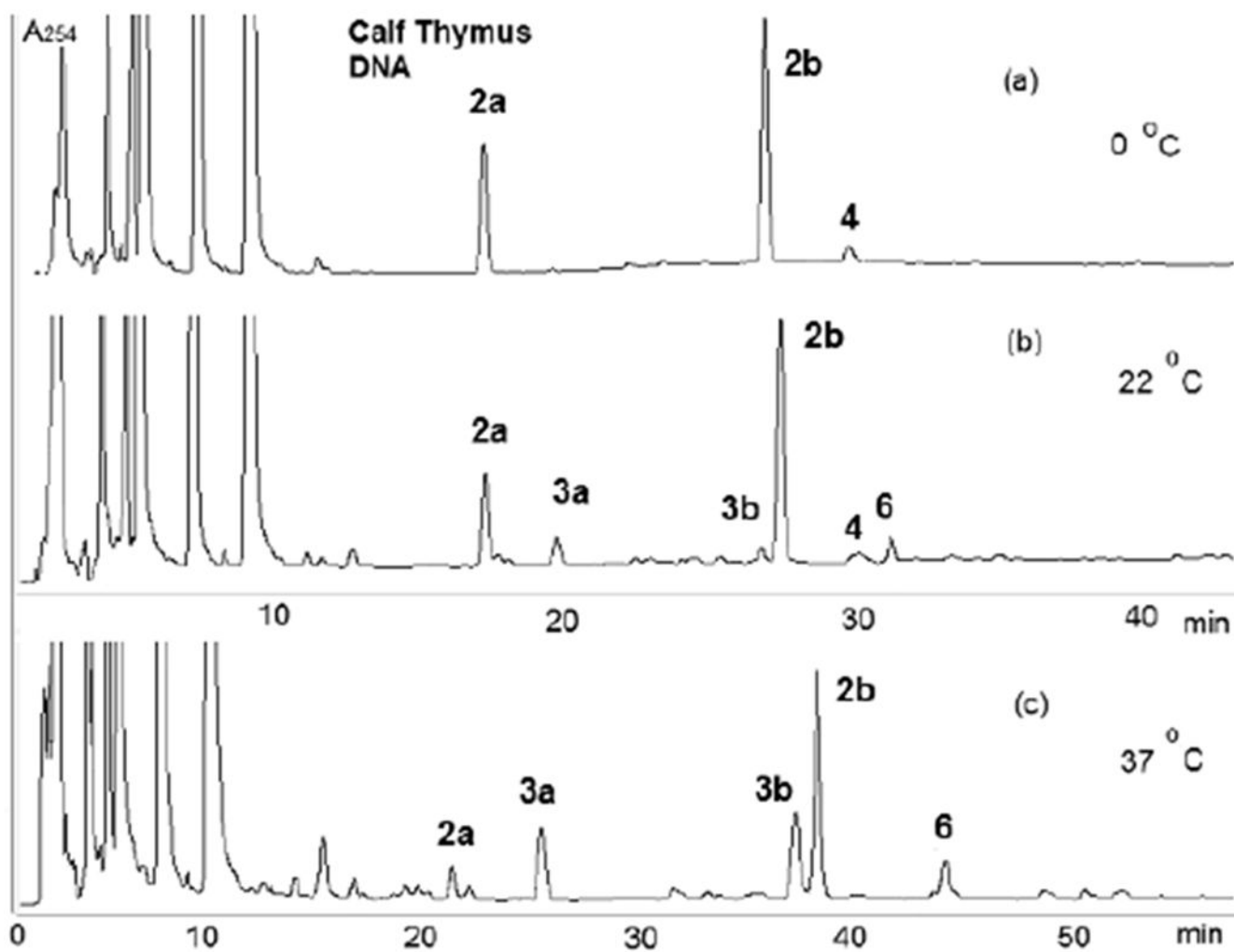


Figure 4. HPLC analysis of digests of DMC-oligonucleotide complexes formed under bifunctional conditions with calf thymus DNA at various temperatures. (c) The elution gradient was modified at 37 °C (6 to 18% acetonitrile in 105 minutes) for a better separation of **3b** and **2b**. Reprinted from reference 26.

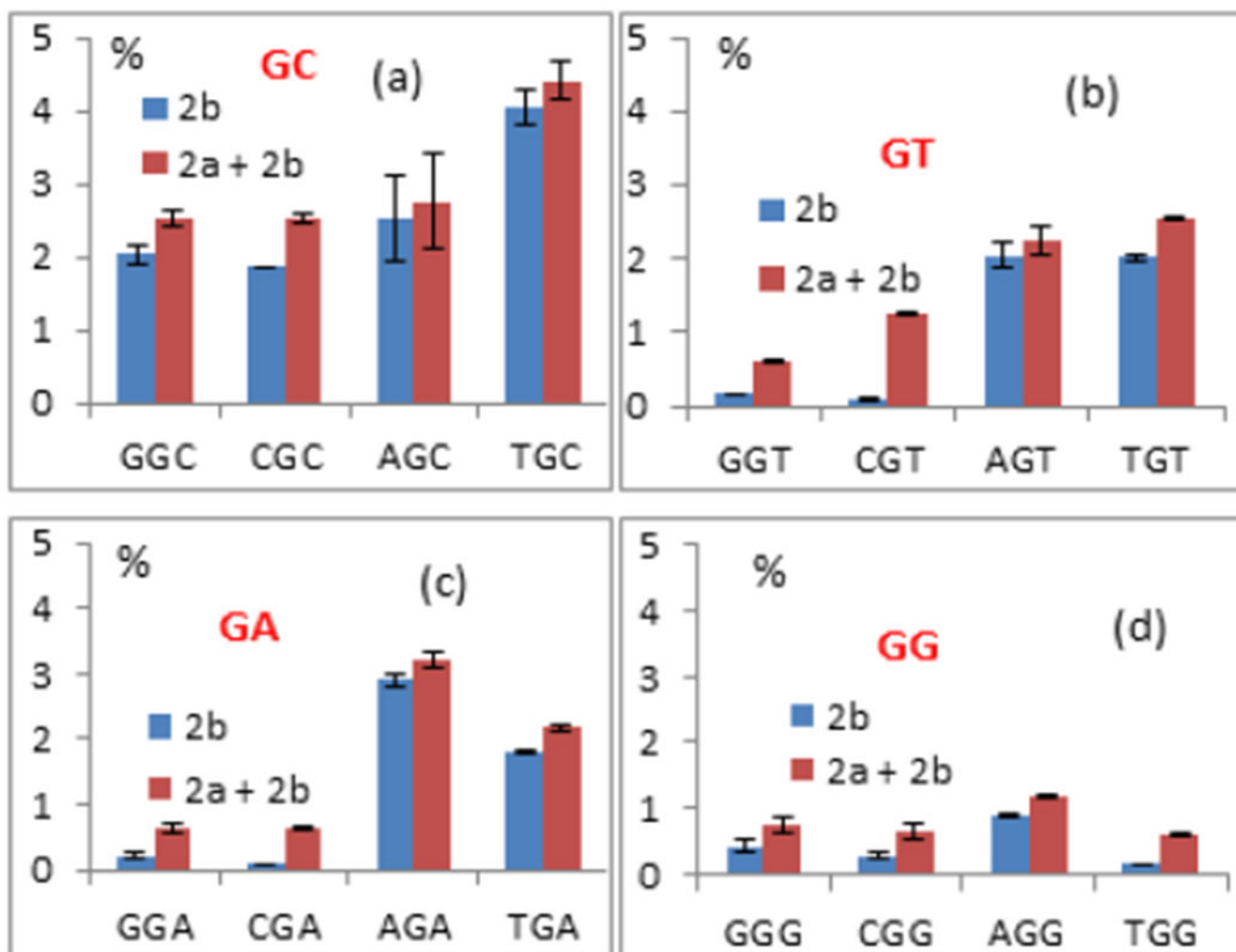


Figure 5. Figures a, b, c and d: Frequency of DMC monoadduct **2b** and of the sum of adducts (**2b** + **2a**) formed under bifunctional conditions with various oligonucleotides. The central sequence of each oligonucleotide is indicated on each horizontal axis: (a) oligonucleotides **14, 15, 16, 17**; GpC step (b) oligonucleotides **18, 19, 20, 21**; GpT step (c) oligonucleotides **22, 23, 24, 25**; GpA step (d) oligonucleotides **26, 27, 28, 29**; GpG step

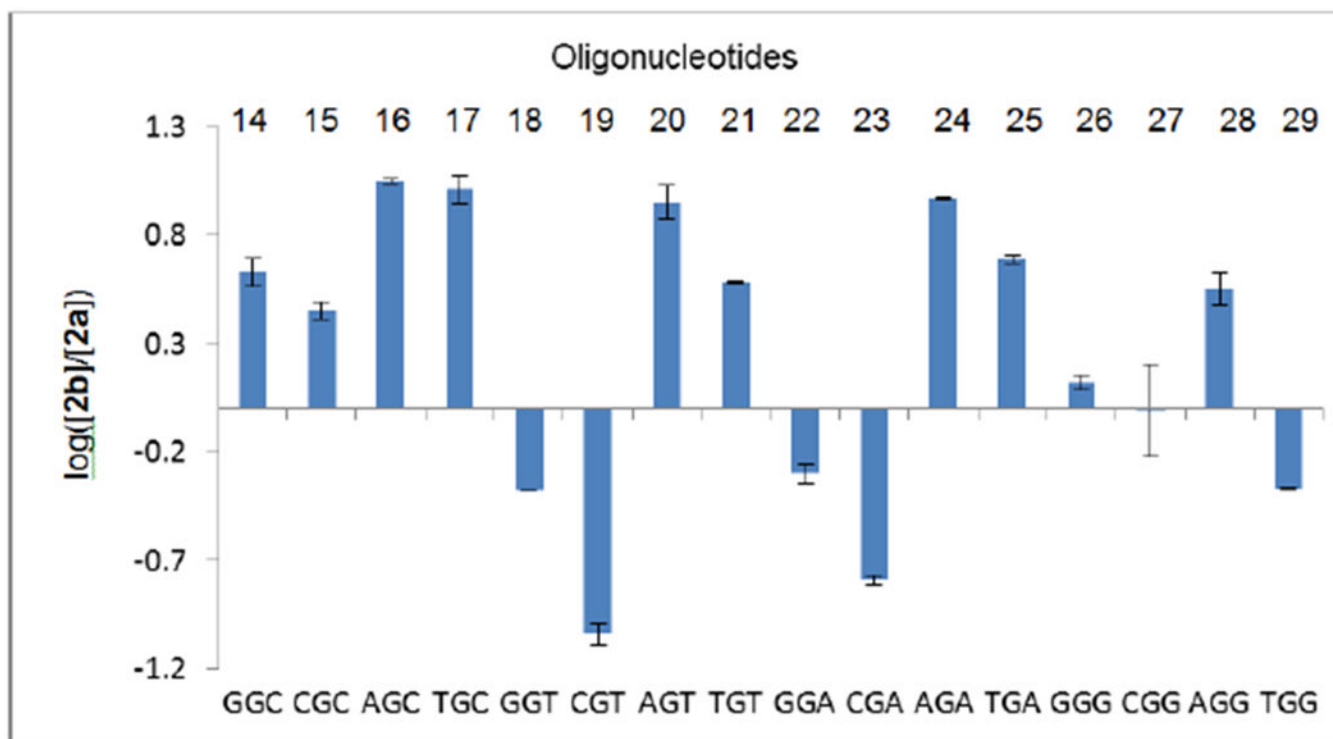


Figure 6.
Log([2b]/[2a]) in oligonucleotides 14-29 (18=29).

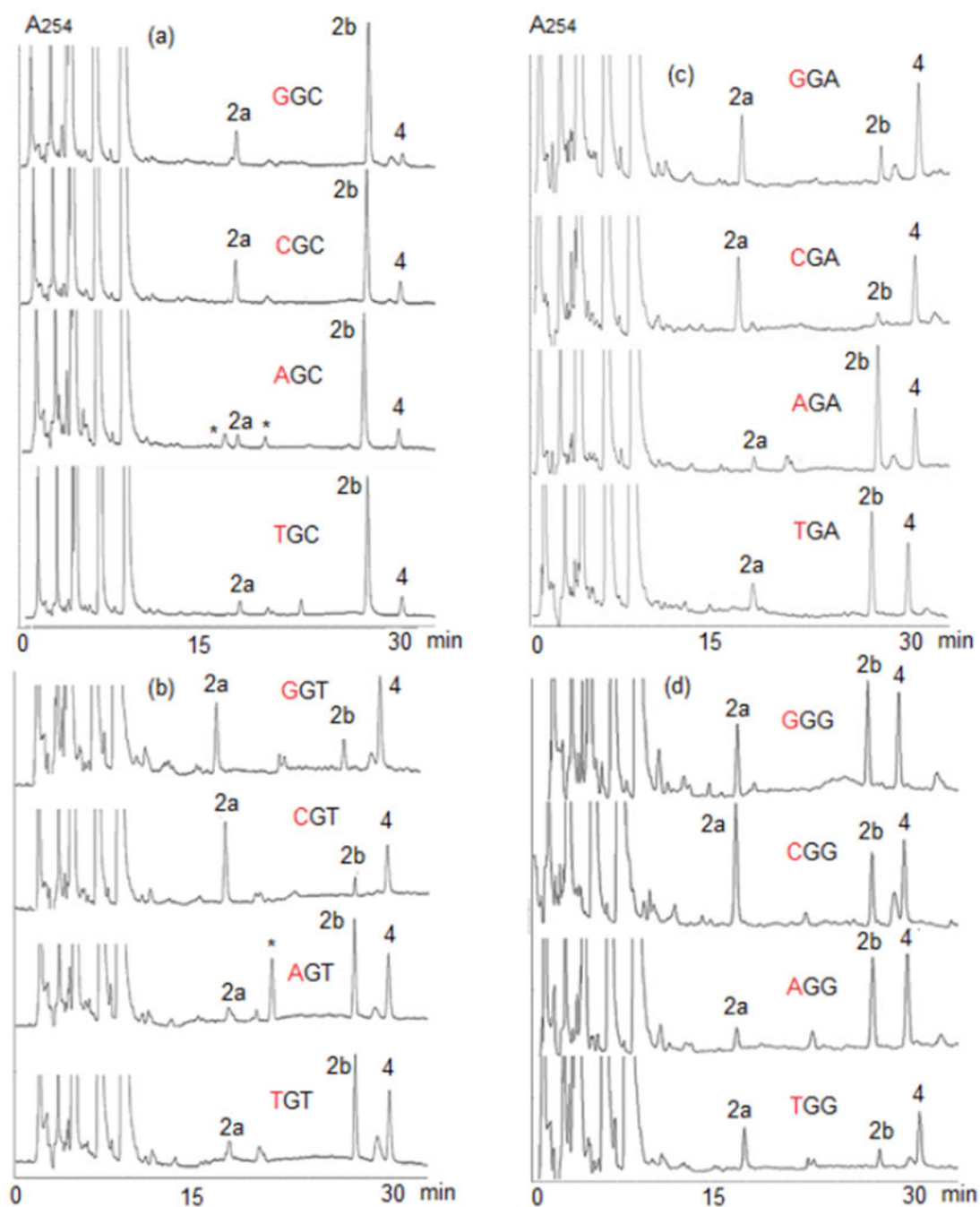


Figure 7.

HPLC of digests of DMC-oligonucleotide complexes formed under bifunctional conditions (a) GpC step; from top to bottom: oligonucleotides **14, 15, 16, 17** (b) GpT step; from top to bottom: oligonucleotides **18, 19, 20, 21** (c) GpA step; from top to bottom: oligonucleotides **22, 23, 24, 25** (d) GpG step; from top to bottom: oligonucleotides **26, 27, 28, 29=18**.

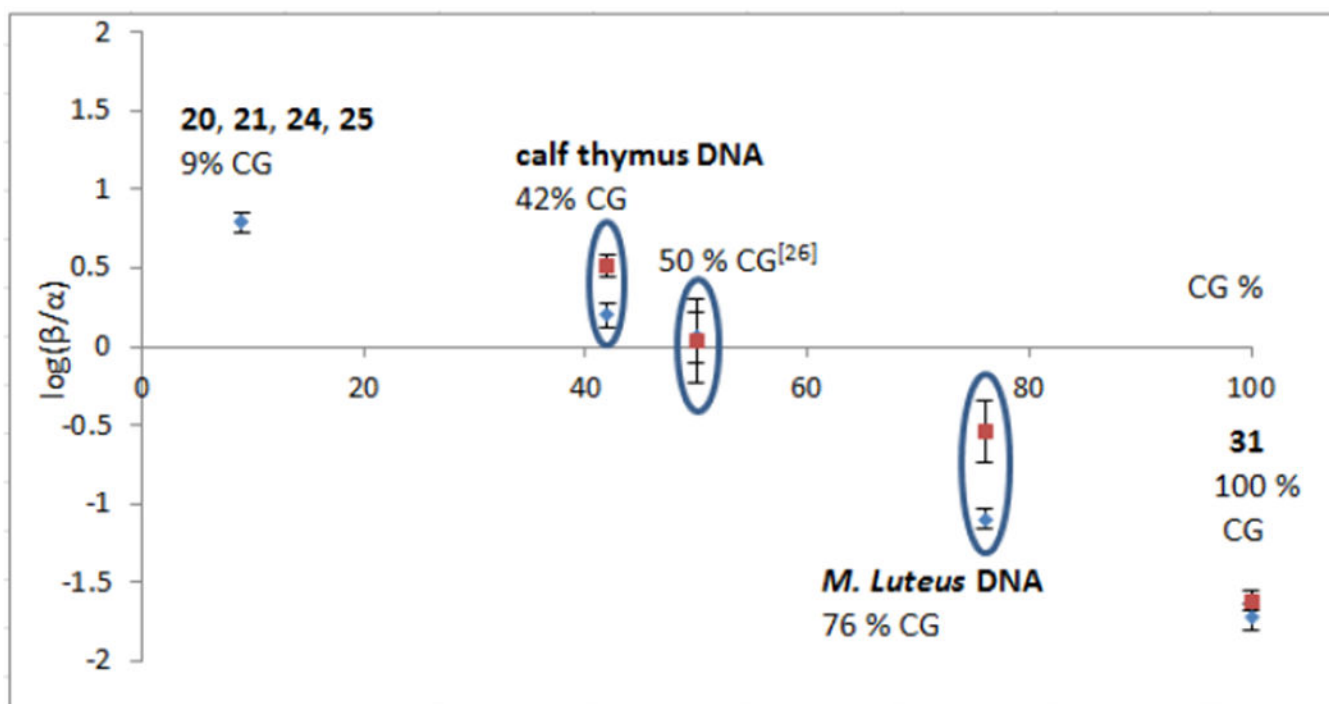
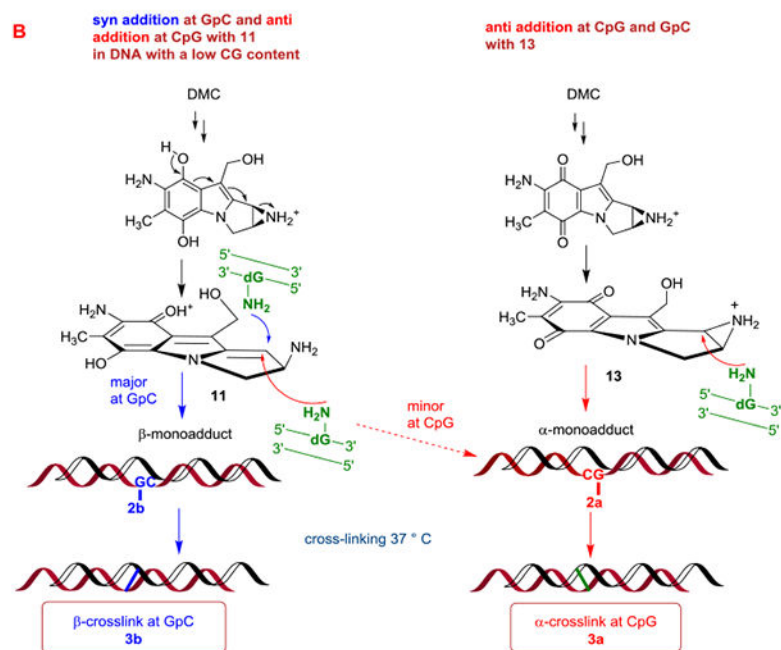
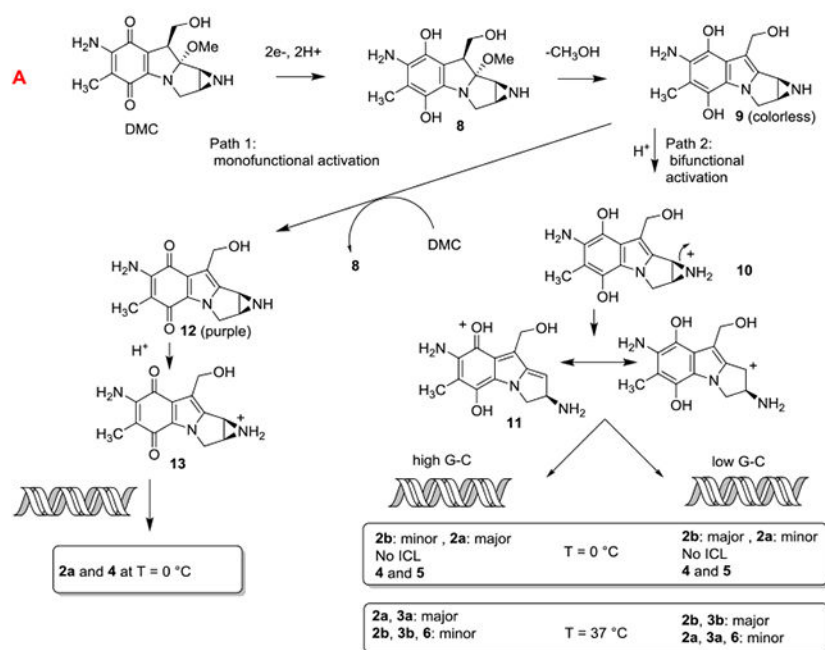
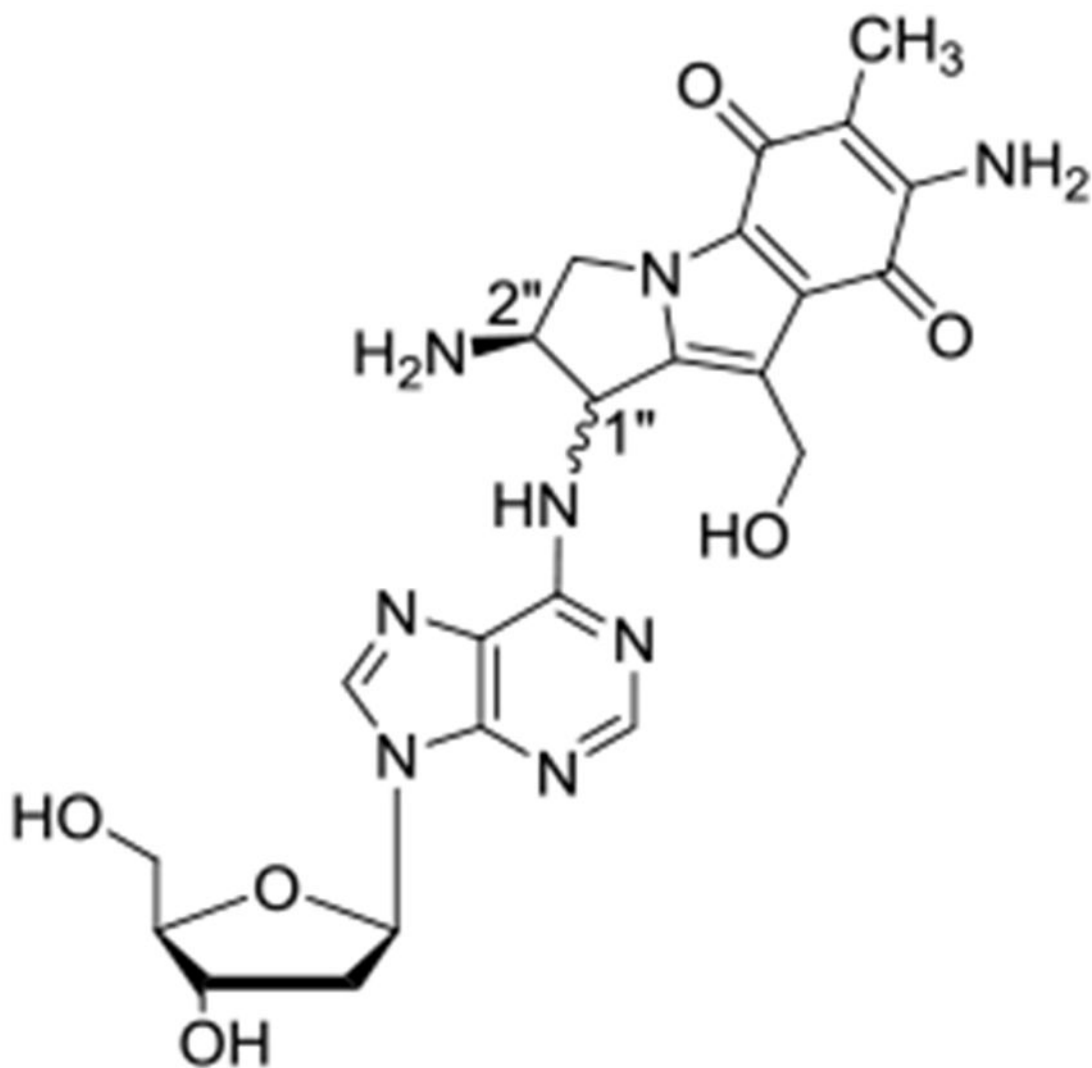


Figure 8. Variation of the ratio between DMC alpha adducts and beta adducts ($\log \left(\frac{[2a]+[3a]}{[2b]+[3b]} \right)$) formed under bifunctional conditions according to DNA C-G content. Data are taken from experiments with oligonucleotides **20**, **21**, **24**, **25** (9% C-G), with calf thymus DNA (42% C-G), oligonucleotides with a 50% CG content,²⁶ *M. Luteus* DNA (76% C-G content) and oligonucleotide **31** (100% C-G content). Blue diamonds represent the ratio **2b**/**3b** for 0 °C reactions (only monoadducts formed) and red squares represent the ratio (**2b** + **3b**)/(**2a** + **3a**) for 37 °C reactions (monoadducts and crosslinks formed).

**Scheme 1.**

(A) DMC-DNA adducts produced from the monofunctional and bifunctional activation pathways of DMC with different types of DNA, (B) syn and anti addition of dG with **11** and anti addition only with **13**.



4: trans, 1''-a dA(DMC)

5: cis, 1''- b dA(DMC)

Chart 1.

Structure of deoxyadenosine-DMC adducts detected in reactions between DMC and oligonucleotides at 0 °C.

Table 1.

Frequencies of Guanine Adducts in Single Strand DNA and Duplex DNA under Bifunctional Conditions (0.32 μ M DMC). Calculation of Percent Yields of Adducts **2a** and **2b** is Described in the Experimental Section and in the Supporting Information Section (page S8).

Single Strand DNA	2a	2b	2a+2b	2b/2a
5'-ATTAT <u>CG</u> TATT-3'	0.36 (\pm 0.04)	0.076 (\pm 0.007)	0.44	0.21
5'-ATTATT <u>GCT</u> TATT-3'	0.31 (\pm 0.02)	0.12 (\pm 0.02)	0.43	0.36
Duplex DNA				
5'-ATATA <u>GCT</u> TATAT-3'	0.12 (\pm 0.03)	1.25 (\pm 0.13)	1.38	10
5'-ATATA <u>CGT</u> TATAT-3'	0.79 (\pm 0.06)	0.37 (\pm 0.04)	1.16	0.47

Table 2

Oligonucleotides used for the Regioselectivity and Stereoselectivity of DMC-DNA monoalkylation

Sequence		Sequence	
14	5'-ATTATGGCTATT 3'-TAATACCGATAA	23	5'-ATTATCGATATT 3'-TAATAGCTATAA
15	5'-ATTATCGCTATT 3'-TAATAGCGATAA	24	5'-ATTATAGATATT 3'-TAATATCTATAA
16	5'-ATTATAGCTATT 3'-TAATATCGATAA	25	5'-ATTATFGATATT 3'-TAATAACTATAA
17	5'-ATTATIGCTATT 3'-TAATAACGATAA	26	5'-ATTATGGGTATT 3'-TAATACCCATAA
18	5'-ATTATGGTTATT 3'-TAATACCAATAA	27	5'-ATTATCGGTATT 3'-TAATAGCCATAA
19	5'-ATTATCGTTATT 3'-TAATAGCAATAA	28^[a]	5'-ATTATAGGTATT 3'-TAATATCCATAA
20	5'-ATTATAGTTATT 3'-TAATATCAATAA	29	5'-ATTATGGTATT 3'-TAATACCATAA
21	5'-ATTATIGTTATT 3'-TAATAACAATAA	30	5'-TTATIGCAATTA 3'-AATAACGTTAAT
22	5'-ATTATGGATATT 3'-TAATACCTATAA	31	5'CGCGCGCGCGCG 3'-CGCGCGCGCGCG

^[a]₂₉ = 18.

Table 3.

Frequencies of Guanine Adducts upon Replacement of the Non-Alkylated Guanine by Inosine at CpG, GpC and GpG steps in oligonucleotides **17**, **18**, and **19**.

Oligonucleotides	Frequency (%) of the major adducts detected			
	2a	2b	2a + 2b	log(2b/2a)
17 5'-ATTATT G CTATT 3'-TAATAA C GATAA	0.36 (± 0.04)	4.04 (± 0.3)	4.41	1.05
5'-ATTATT G CTATT 3'-TAATAA C IATAA	0.52 (± 0.05)	2.13 (± 0.19)	2.64	0.61
19 5'-ATTATT C GATTT 3'-TAATAA G CATAA	1.17 (± 0.03)	0.1 (± 0.01)	1.27	-1.05
5'-ATTATT C GATTT 3'-TAATAA I CATAA	0.94 (± 0.04)	0.64 (± 0.05)	1.58	-0.18
18 5'-ATTATT G GATTT 3'-TAATAA C CATAA	0.43 (± 0.03)	0.18 (± 0.005)	0.6	-0.38
5'-ATTATT I GATTT 3'-TAATAA C CATAA	0.40 (± 0.04)	1.27 (± 0.2)	1.68	0.50
5'-ATTATT G IATTT 3'-TAATAA C CATAA	0.53 (± 0.04)	0.40 (± 0.012)	0.93	-0.11