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Synthesis of Mitomycin C and Decarbamoylmitomycin C N^6 deoxyadenosine-adducts

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Abstract

Mitomycin C (MC), an anti-cancer drug, and its analog, decarbamoylmitomycin C (DMC), are DNA-alkylating agents. MC is currently used in the clinics and its cytotoxicity is mainly due to its ability to form Interstrand Crosslinks (ICLs) which impede DNA replication and, thereby, block cancer cells proliferation. However, both MC and DMC are also able to generate monoadducts with DNA. In particular, we recently discovered that DMC, like MC, can form deoxyadenosine (dA) monoadducts with DNA. The biological role played by these monoadducts is worthy of investigation. To probe the role of these adducts and to detect them in enzymatic digests of DNA extracted from culture cells treated by both drugs, we need access to reference compounds i.e. MC and DMC dA-mononucleoside adducts. Previous biomimetic methods used to generate MC and DMC mononucleoside adducts are cumbersome and very low yielding. Here, we describe the diastereospecific chemical synthesis of both C-1 epimers of MC and DMC deoxyadenosine adducts. The key step of the synthesis involves an aromatic substitution reaction between a 6-fluoropurine 2'-deoxyribonucleoside and appropriately protected stereoisomeric triaminomitosenes to form protected-MC-dA adducts with either an *S* or *R* stereochemical configuration at the adenine-mitosene linkage. Fluoride-based deprotection methods generated the final four reference compounds: the two stereoisomeric MC-dA adducts and the two stereoisomeric DMC-dA adducts. The MC and DMC-dA adducts synthesized here will serve as standards for the detection and identification of such adducts formed in the DNA of culture cells treated with both drugs.

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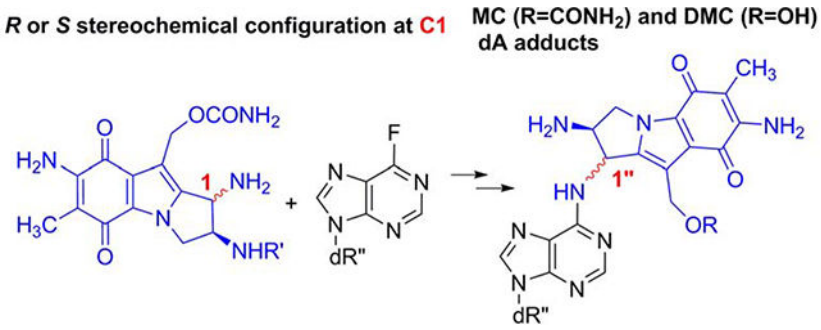
Graphical Abstract



Keywords

Mitomycin C; deoxyadenosine; nucleophilic aromatic substitution; stereoisomers; DNA-adducts

1 Introduction

Mitomycin C (MC, Fig. 1) [1–3] is a well known antineoplastic drug used to treat, among others, bladder and stomach cancers [4–7]. Its application in ophtalmology has also increased in recent years because of its modulatory effects on wound healing [8, 9]. Decarbamoylmitomycin C, (DMC, Fig. 1), is an analog of MC lacking the carbamoyl group on O10. Both MC and DMC are inert in their native state but are able to alkylate DNA upon reductive activation to generate monoadducts and Interstrand Crosslinks (ICLs), thereby inhibiting DNA synthesis [10].

The major monoadducts generated by MC and DMC result from covalent linkage between the exocyclic amino group of deoxyguanosine (dG) and the 1-position of the MC/DMC aziridine moiety (Fig. 1 and 2). These monoadducts can be converted to ICLs upon further activation of the 10-position (10-carbamate in the case of MC, and 10-hydroxyl in the case of DMC) and this results in the formation of ICLs between the exocyclic amino groups of deoxyguanosine residues located on opposing DNA strands. The major adducts identified from cancer cells treated with MC and DMC adopt an opposite stereochemical configuration at the dG-mitosene bond. For MC, the stereochemical configuration at C1” of the major adducts is always *R* (e.g. **1a**, **2a** and **3a**, Fig. 2); in contrast, for DMC, it is always *S* (e.g. **2b** and **3b**, Fig. 2) [4]. In addition, it was recently discovered that ICL **3a** (MC major ICL) is only formed at CpG sequences and ICL **3b** (DMC major ICL) is only generated at GpC steps [11, 12]. This implies that, for mitomycins, ICLs’ formation is diastereospecific and diastereodivergent. During the course of this work, it was also established that DMC is able to form two stereoisomeric deoxyadenosine (dA) adducts with DNA under certain conditions (Fig. 2, compounds **5a** and **5b**) and that single stranded DNA seems more prone to dA alkylation than duplex DNA, in contrast to what happens with dG alkylation [13]. This result indicates that unfolded single-stranded structures of nucleic acids could be targets for dA alkylation by MC and DMC in cells [13]. Such substrates would then join the growing list of cellular components targeted by Mitomycins such as rRNA [14] and Thioredoxin Reductase [15].

The current consensus is that ICLs **3a** and **3b** (Fig. 2) are the major lesions responsible for the cytotoxicity of Mitomycins because ICLs impede DNA replication and, thus, are highly harmful to dividing cells. It has been estimated that the presence of approximately 40 unrepaired ICLs can kill mammalian cells [16]. However, the biological role played by other DNA adducts generated by both drugs is worthy of investigation. It is particularly important to assess whether or not MC or DMC monoadducts may generate secondary tumors (i.e. if they are mutagenic) and if they play a role in mitomycins' cytotoxicity. Relevant to this pursuit, the bio-activity of monoadduct **1a** (Fig. 2) has been investigated and **1a** was found: 1) to be cytotoxic in *Escherichia coli* [17]; 2) to be mutagenic and to block replication in human embryonic kidney (HEK) 293T cells [18].

The identification and quantification of DNA adducts in culture cells treated by MC or DMC is performed at the nucleoside level following DNA digestion with phosphodiesterases and phosphatases, and it requires the availability of authentic standards of the adducts being identified [10–12, 19]. Therefore, in order to detect the formation of MC-dA and DMC-dA monoadducts in treated cells and investigate their role in the biological effects of mitomycins, we need to synthesize MC and DMC dA-mononucleosides so they can be used as reference compounds. One approach for the synthesis of such adducts involves the direct reaction of dA with reduced MC or DMC and we have successfully isolated adducts **5a** and **5b** using this method in the past [13]. This direct method provides the adducts in a single step, but their isolation from the reaction mixtures is cumbersome, expensive and very low yielding (0.8% yield). An alternative approach to obtain *N*-substituted-purine mononucleoside derivatives involves chemical synthesis, using a coupling reaction between an electrophilic nucleoside derivative and the corresponding amine. Our laboratory has successfully used this synthetic approach to obtain deoxyguanosine adducts of mitomycins such as N^2-dG adduct **1a** [20] (Fig. 2) as well as the N^2-dG adduct of the major Mitomycin C metabolite, 2,7-diaminomitosene [21]. Here, we present a diastereospecific chemical synthesis of the four N^6 deoxyadenosine-adducts formed by mitomycin C (**4a** and **4b)** and decarbamoylmitomycin C (**5a** and **5b**) (Fig. 2). The key step of the synthesis involves a nucleophilic aromatic substitution reaction between a ((2-trimethylsilyl)ethoxycarbonyl)-protected aminomitosene with either an *R* or *S* stereochemical configuration at C1 and a C-6 fluoropurine 2'-deoxyribonucleoside. The final deprotected MC and DMC dA adducts (**4a**, **4b**, **5a** and **5b**, Fig. 2) were obtained after treatment with fluoride-containing reagents. These reference compounds will allow the detection and quantitation of such adducts in cellular environments using techniques similar to those used previously for the detection of quantitation of other MC/DMC adducts [10–12, 19]. Two features of the chemical synthesis of these adducts are discussed in detail: (a) the efficiencies of 2'-deoxyadenosine adduct syntheses by fluorine displacement using a C-6 fluoropurine 2'-deoxyribonucleoside and the influence of the stereochemical configurations of the mitosenes on the nucleophilic substitution reaction rate and (b) the influence of the stereochemical configurations of the dA adducts on the deprotection steps.

2 Materials and methods

2.1 General Information

^{1}H NMR and ^{13}C NMR spectra were recorded using a JEOL ECX 300 (300 MHz), a Varian Inova 500 (500 MHz) or a Bruker AVANCE 500 (500 MHz) spectrometer. Spectra were recorded at 298 K and the residual solvent peak was used as the internal reference. Chemical shifts are reported in parts per million and coupling constants are in hertz (Hz). The conventional numbering system is used for the mitosene moiety and the purine carbons are numbered 1–6 also as per convention. The sugar carbons are numbered 1'−5' beginning at the anomeric carbon and proceeding *via* the carbon chain to the primary carbinol center. We will refer to mitosene derivatives with an *R* configuration at carbon 1 as "α" or *trans*, while those with an *S* configuration at carbon 1 are termed *cis* or "β".

Reagents were obtained from commercial sources and were used without further purification. All reactions were carried out under an atmosphere of Argon unless otherwise stated. Thin layer chromatographic analyses were carried out on 250 mm silica gel plates containing a fluorescent indicator. Column chromatographic purifications were performed using 200–300 mesh silica gel. Triaminomitosene precursors **6a** and **6b** and 6-fluoropurine 2'-deoxyriboside were synthesized according to previously described procedures with slight modifications, as detailed in sections 2.2 to 2.4. HRMS spectra were recorded by direct infusion using Bruker's micrOTOF-II ESI instrument at Notre Dame University Mass Spectrometry Facility.

Circular Dichroism (CD) and Ultraviolet and Visible (UV-Vis) 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 methanol (blank) was subtracted from each of the recorded spectra also in methanol. Kaleidagraph software (version 4.1.3) was used to generate the graphs presented in the manuscript.

2.2 Synthesis of 9-(2-Deoxy-β-D-erythro-pentofuranosyl)purin-6-yl-trimethylammonium chloride

This synthesis was adapted from previous work [22]. The following changes were made: The starting material, 6-chloro-9-(2-Deoxy-β-D-erythro-pentofuranosyl)purine, was dried under high vacuum while being slightly heated for 30 minutes to eliminate all moisture. The salt was stored under high vacuum (lyophilizer) until the next step. The reaction was performed under dry Argon.

2.3 Synthesis of 6-fluoro-9-(2-Deoxy-β-D-erythro-pentofuranosyl)purine

This synthesis was adapted from previous work [22]. The following changes were made: KF was dried under high vacuum while being slightly heated for 30 minutes to eliminate all moisture.

2.4 Synthesis of amine precursors 6a and 6b from mitomycin C (scheme 1)

This synthesis was adapted from our previous work [19, 20]. The following changes were made: the liquid-liquid extraction of the azide intermediate was performed using dichloromethane rather than ethyl acetate to improve recovery.

2.5 Synthesis of 7a

6-fluoro-9-(2-Deoxy-β-D-erythro-pentofuranosyl)purine (22 mg, 0.086 mmol) was dissolved in dry DMSO (95 μL). Compound **6a** (20 mg, 0.043 mmol) and diisopropylethylamine (10 μL, 7.42 mg, 0.96 mmol) were added to the reaction mixture which was incubated at 45 °C for 2 days. The resulting mixture was diluted with water (1 mL) and lyophilized. The desired product was isolated by preparative thin layer chromatography (SiO_2: NH_4OH, 1.5%; hexane, 5%; CH_3OH, 15%; CH_2Cl_2, 78.5% v/v) to give 24 mg (81 % yield) of **7a** (R_f = 0.39). ^{1}H NMR (CD_3OD, 300 MHz): δ 8.24 (s, H_{ar}, 1H), 6.41 (s, dd, *J* = 8.4, 6.4 Hz, $H_{1'}$, 1H), 5.93 (s, *br*, $H_{1''}$, 1H), 4.99 (s, $H_{10''}$, 2H), 4.76 (m, overlapping signals for $H_{2''}$ and $H_{3'}$, 2H), 4.56 (dd, *J* = 5.5, 2.5 Hz, $H_{3''a}$, 1H), 4.05 (m, overlapping signals for C*H*$_2$-O, $H_{4'}$ and $H_{3''b}$, 4H), 3.83 and 3.72 (doublets of ABq, *J* = 3.3 and 12.3 Hz, $H_{5'a}$ and $H_{5'b}$, 2H), 2.80 (app quint, *J* = 7.9 Hz, $H_{2'a}$, 1H), 2.38 (ddd, *J* = 13.4, 6.0, 2.6 Hz, $H_{2'b}$, 1H), 1.78 (s, C*H*$_3$, 3H), 0.91 (t, *J* = 8.4 Hz, C*H*$_2$-Si, 2H), −0.01 (s, Si(C*H*$_3$)$_3$, 9H). ^{13}C NMR (CD_3OD, 300 MHz): δ 178.4, 177.6, 158.0, 157.2, 154.4, 151.2, 148.4, 147.8, 140.1, 138.3, 128.9, 122.0, 120.1, 112.8, 105.1, 88.6, 85.8, 71.8, 62.8, 62.3, 60.8, 56.9, 53.3, 50.6, 40.2, 17.1, 6.8, −2.9. HRMS *m/z* calcd for $C_{30}H_{40}N_9O_9Si$ $[M + H]^+$: 698.2718, found: 698.2681.

2.6 Synthesis of 7 b

Compound **6 b** (15 mg, 0.032 mmol); 6-fluoro-9-(2-Deoxy-β-D-erythro-pentofuranosyl)purine (16 mg, 0.064 mmol) and diisopropylamine (10 μL, 7.42 mg, 0.96 mmol) were stirred in 95 μL of DMSO. The reaction mixture was incubated at 45 °C for 4 days. The resulting mixture was diluted with water (1 mL) and lyophilized. The final compound was purified by preparative thin layer chromatography (SiO_2: NH_4OH, 1.5%; hexane, 5%; CH_3OH, 15%; CH_2Cl_2, 78.5% v/v) yielding 17 mg (75 % yield) of **7b** (R_f = 0.42). ^{1}H NMR (CD_3OD, 300 MHz): δ 8.26 (s, H_{ar}, 1H), 8.22 (s, H_{ar}, 1H), 6.40 (t, *J* = 6.4 Hz, $H_{1'}$, 1H), 6.02 (s, *br*, $H_{1''}$, 1H), 5.09 (s, $H_{10''}$, 2H), 5.07 (app q, *J* = 7.2 Hz, $H_{2''}$, 1H), 4.56 (m, overlapping signals for $H_{3'}$ and $H_{3''a}$, 2H), 4.14 (dd, *J* = 12.7, 7.2 Hz, $H_{3''b}$, 1H), 4.05 (app q, *J* = 2.4 Hz, $H_{4'}$, 1H), 3.96 (app q, *J* = 8.1 Hz, C*H*$_2$-O, 2H), 3.82 and 3.73 (doublets of ABq, *J* = 3.1 and 12.4 Hz, $H_{5'a}$ and $H_{5'b}$, 2H), 2.79 (app quint, *J* = 8.4 Hz, $H_{2'a}$, 1H), 2.37 (ddd, *J* = 13.4, 5.8, 2.4 Hz, $H_{2'b}$, 1H), 1.76 (s, C*H*$_3$, 3H), 0.74 (t, *J*=8.0 Hz, C*H*$_2$-Si, 2H), −0.1 (s, Si(C*H*$_3$)$_3$, 9H). ^{13}C NMR (CD_3OD, 300 MHz): δ 178.9, 177.0, 157.0, 156.2, 154.4, 152.5, 149.2, 147.6, 140.3, 139.8, 128.9, 121.9, 120.3, 112.8, 105.2, 88.6, 84.6, 71.5, 62.5, 62.4, 56.9, 56.6, 55.6, 50.8, 19.0, 17.6, 8.9, −1.1. HRMS *m/z* calcd for $C_{30}H_{40}N_9O_9Si$ $[M + H]^+$: 698.2718, found: 698.2688.

2.7 Synthesis of 4a

TBAF (tetra-*n*-butylammonium fluoride, 1 M in THF, 36 μL, 0.036 mmol) was added to compound **7a** (5 mg, 0.0072 mmol). An additional 40 μL of THF was added. The reaction

mixture was incubated at 45 °C for 15 h. and the solvent was evaporated by Argon flow. Compound **4a** was isolated by preparative thin layer chromatography (SiO_2: NH_4OH, 3%; CH_3OH, 15%; CH_3Cl, 82% v/v; R_f=0.10). The residue was triturated with hexane and filtered to remove TBAF, giving **4a** (2.71 mg, 68%) as a pale pink solid. ^1H NMR (C_5D_5N with 20% v/v D_2O, 300 MHz) δ 8.62 (s, H_{ar}, 1H), 6.91 (t, *J* = 6.6 Hz, $H_{1'}$, 1H), 5.97 (s, *br*, $H_{1''}$, 1H), 5.67 and 5.52 (ABq, *J* = 12.4 Hz, $H_{10''a}$ and $H_{10''b}$, 2H), 5.17 (m, *br*, $H_{3'}$, 1H), 5.00–4.94 (m, *br*, $H_{3''a}$, 1H), 4.66 (app q, *J* = 3 Hz, $H_{4'}$, 1H), 4.47–4.40 (m, *br*, $H_{2''}$, 1H), 4.42 (dd, *J* = 16, 4.9 Hz, $H_{3''b}$, 1H), 4.22 and 4.15 (doublets of ABq, *J* = 3.3 and 12.3 Hz, $H_{5'a}$ and $H_{5'b}$, 2H), 3.11 (app quin, *J* = 6.2 Hz, $H_{2'a}$, 1H), 2.88 (ddd, *J* = 12.1, 4.5, 2.3 Hz, $H_{2'b}$, 1H), 2.17 (s, C*H*$_3$, 3H). ^{13}C NMR (C_5D_5N with 20% v/v D_2O, 300 MHz): δ 180.6, 179.1, 159.7, 153.9, 141.5, 140.7, 130.4, 123.6, 122.2, 115.1, 107.2, 90.3, 87.3, 77.3, 73.2, 64.5, 64.2, 59.1, 55.3, 42.1, 9.8. Note: There are three unobserved resonances hidden under the pyridine signals. HRMS *m/z* calcd for $C_{24}H_{28}N_9O_7$ $[M + H]^+$: 554.2112, found: 554.2114.

2.8 Synthesis of 4b

TAS-F (trisulfonium difluorotrimethylsilicate, 0.15 mmol, 42.5 mg) was added to 8 mg of **7b** (0.011 mmol) in 400 μL of DMF. The reaction mixture was incubated at 45 °C for 5 h. followed by dilution with 1 mL of water and lyophilization. **4b** was isolated by preparative thin layer chromatography (SiO_2: NH_4OH, 3%; CH_3OH, 15%; CH_3Cl, 82% v/v; R_f=0.12) to give 5 mg (82 % yield) of **4b**. ^1H NMR (C_5D_5N with 20% v/v D_2O, 300 MHz) δ 8.71 (s, H_{ar}, 1H), 6.84 (t, *J* = 6.8 Hz, $H_{1'}$, 1H), 6.20 (s, *br*, $H_{1''}$, 1H), 5.47 (s, $H_{10''}$, 2H), 5.12 (app t, *J* = 2.6 Hz, $H_{3'}$,1H), 4.90 (dd, *J* = 12.6, 7.2 Hz, $H_{3''a}$, 1H), 4.75 (app q, *J* = 6.9 Hz, $H_{4'}$, 1H), 4.65 (app d, *J* = 2.6 Hz, $H_{2''}$, 1H), 4.49 (dd, *J* = 12.6, 6.9 Hz, $H_{3''b}$, 1H), 4.26 and 4.20 (doublets of ABq, *J* = 4.4 and 12.4 Hz, $H_{5'a}$ and $H_{5'b}$, 2H), 3.08 (m, $H_{2'a}$, 1H), 2.93 (m, $H_{2'b}$, 1H), 2.14 (s, C*H*$_3$, 3H). ^{13}C NMR (C_5D_5N with 20% v/v D_2O, 300 MHz): δ 180.1, 179.7, 160.3, 154.3, 141.0, 140.8, 130.8, 123.3, 121.8, 115.3, 107.6, 89.9, 87.2, 77.2, 73.4, 63.9, 59.7, 58.5, 53.9, 41.9, 9.8. Note: There are three unobserved resonances hidden under the pyridine signals. HRMS *m/z* calcd for $C_{24}H_{28}N_9O_7$ $[M + H]^+$: 554.2112, found: 554.2095.

2.9 Synthesis of 5a

TAS-F (trisulfonium difluorotrimethylsilicate, 0.12 mmol, 33 mg) in 40 μL of DMF was added to compound **7a** (17 mg, 0.024 mmol). The reaction mixture was incubated at 40 °C for 5 h, diluted in 1 mL of water and lyophilized. Adduct **5a** was isolated by preparative thin layer chromatography (SiO_2: NH_4OH, 3%; CH_3OH, 15%; CH_3Cl, 82% v/v) to give 7.84 mg (63% yield) of **5a** (R_f=0.12). ^1H NMR (C_5D_5N with 20% v/v D_2O, 500 MHz): δ 8.64 (s, Ar-H_2, 1H), 8.58 (s, Ar-H_8, 1H), 6.89 (t, *J* = 6.8 Hz, $H_{1'}$, 1H), 5.91 (s, *br*, $H_{1''}$, 1H), 5.18 (m, *br*, $H_{3'}$, 1H), 5.17 and 5.14 (ABq, *J* = 14.5 Hz, $H_{10''a}$ and $H_{10''b}$, 2H), 4.99–4.92 (m, *br*, $H_{3''a}$, 1H), 4.67–4.64 (m, *br*, $H_{4'}$, 1H), 4.54–4.45 (m, *br*, $H_{2''}$, 1H), 4.46 (dd, *J* = 12.8, 3.6 Hz, $H_{3''b}$, 1H), 4.23 and 4.17 (doublets of ABq, *J* = 3.2 and 12.4 Hz, $H_{5'a}$ and $H_{5'b}$, 2H), 3.05 (app quint, *J* = 6.2 Hz, $H_{2'a}$, 1H), 2.91 (ddd, *J* = 13.5, 6.1, 2.9 Hz, $H_{2'b}$, 1H), 2.15 (s, C*H*$_3$, 3H). ^{13}C NMR (C_5D_5N with 20% v/v D_2O, 500 MHz): δ 179.5, 177.9, 154.7, 152.6, 146.8, 140.2, 137.9, 129.3, 122.1, 120.5, 106.0, 89.0, 85.8, 72.0, 63.1, 62.7, 55.7, 52.3, 53.7, 40.8,

8.5. Note: There are two unobserved resonances hidden under the pyridine signals. HRMS *m/z* calcd for $C_{23}H_{27}N_8O_6$ $[M + H]^+$: 511.2048, found: 511.2046.

2.10 Synthesis of 5b

TBAF (tetra-*n*-butylammonium fluoride, 1 M in THF, 57 μL, 0.057 mmol) was added to compound **7b** (8 mg, 0.011 mmol). An additional 64 μL of THF was added. The reaction mixture was incubated at 40 °C for 4 h and the solvent was evaporated by argon bubbling. The products were first isolated by preparative thin layer chromatography (SiO_2: NH_4OH, 3%; CH_3OH, 15%; CH_3Cl, 82% v/v). The residue was then triturated with hexane and filtered to remove TBAF, giving **5b** (6.5 mg, 63%) as a pale pink solid (R_f=0.27). ^1H NMR (C_5D_5N with 20% v/v D_2O and 0.05% v/v TMS, 500 MHz): δ 8.66 (s, Ar-H_2, 1H), 8.60 (s, Ar-H_8, 1H), 6.88 (t, *J* = 6.8 Hz, $H_{1'}$, 1H), 6.07 (s, *br*, $H_{1''}$, 1H), 5.21 and 5.15 (ABq, *J* = 13.9 Hz, $H_{10''a}$ and $H_{10''b}$, 2H), 5.16 (s, *br*, $H_{3'}$, 1H), 4.88 (dd, *J* = 12.6, 7.2 Hz, $H_{3''a}$, 1H), 4.70 (app q, *J* = 7.2 Hz, $H_{2''}$, 1H), 4.64 (app q, *J* = 3.2 Hz, $H_{4'}$, 1H), 4.47 (dd, *J* = 12.6, 7.4 Hz, $H_{3''b}$, 1H), 4.21 and 4.15 (doublets of ABq, *J* = 3.2 and 12.2 Hz, $H_{5'a}$ and $H_{5'b}$, 2H), 3.08 (app quint, *J* = 6.2 Hz, $H_{2'a}$, 1H), 2.91 (ddd, *J* = 13.3, 5.8, 2.9 Hz, $H_{2'b}$, 1H), 2.16 (s, C*H*3, 3H). ^{13}C NMR (C_5D_5N with 20% v/v D_2O, 300 MHz): δ 179.6, 178.0, 154.7, 152.6, 148.1, 140.3, 138.3, 129.4, 121.6, 120.5, 106.1, 89.1, 85.7, 72.0, 62.7, 57.8, 56.3, 52.3, 50.6, 40.9, 8.6. Note: There are two unobserved resonances hidden under the pyridine signals. HRMS *m/z* calcd for $C_{23}H_{27}N_8O_6$ $[M + H]^+$: 511.2048, found: 511.2039.

2.11 Oligonucleotide Alkylation by DMC

Self-complimentary oligonucleotide 5'-TATATATATATA (10 A_{260} unit scale; Tm: 57°C) was dissolved in 10 mM potassium phosphate buffer (pH 5.8, 355 μL) and annealed by heating (90°C, 10 min) followed by slow cooling to 0°C. The reaction mixture was put under ice and deaerated via argon bubbling (30 min) while kept at 0°C. Excess $Na_2S_2O_4$ (3.24 μmol in 20 μL of potassium phosphate buffer, 10 mM, pH 5.8) from a freshly prepared anaerobic solution was then added to the mixture quickly and immediately followed by addition of DMC (1.30 μmol). The reaction mixture was allowed to stir with argon bubbling for 1 hr and then opened to air, followed by gentle mixing until a consistent purple color was obtained. The mixture was then allowed to stir for 20 min under air and chromatographed on a 2.5*56 cm Sephadex G-25 column using 20 mM NH_4HCO_3 as eluent. Oligonucleotide containing fractions were lyophilized.

2.12 Enzymatic Digestion of Alkylated Oligonucleotides

1 A_{260} unit of oligonucleotide and 2 units of nuclease P_1 were incubated at 37°C for 2 hr in 0.8 mL of 20 mM ammonium acetate (pH 5.5); 100 mM $MgCl_2$ (20μL) was added, and the pH was adjusted to 8.2 by addition of 20 μL of 200 mM NaOH. SVD (2 units) and AP (2 units) were added and incubation was continued at 37°C for 2.5 h.

2.13 Analysis of DNA Adducts after Enzymatic Digestion

The digestion mixture was directly analyzed by HPLC using an Agilent 1200 HPLC system and a XBridge C-18 reverse phase column (5 μm, 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.

3 Results and Discussion

3.1 Synthesis of protected 2'-deoxyadenosine adducts 7a and 7b. Efficiency of nucleophilic aromatic substitutions using 6-fluoro-9-(2-Deoxy-β-D-erythro-pentofuranosyl)purine and aminomitosenes

The reaction of 2-fluoropurine or 6-fluoropurine nucleosides with amines has been used to synthesize *N*-substituted dG or dA derivatives [19–21, 23, 24]. We have previously used the coupling reaction between a 2-fluoropurine derivative and aminomitosenes to synthesize mitosene-deoxyguanosine adducts (MC-dG and DMC-dG adducts **1a** and **2b**, Fig. 2) [19, 20]. Our approach is analogous to the method developed by Harris and Rizzo to generate adducts of exocyclic amines of nucleosides [25]. In this work, a similar strategy was employed for the synthesis of MC and DMC deoxyadenosine (dA) adducts **4a**, **4b**, **5a** and **5b**. The synthesis commenced with the preparation of the isomeric protected triamino mitosenes **6a** (1,2 *trans*) and **6b** (1,2 *cis*) (Scheme 1) which can be obtained from mitomycin C according to a route that has been developed in our laboratory [19, 20].

These precursors **6a** and **6b** (Scheme 1) were then coupled to a 6-fluoropurine deoxyribonucleoside (Scheme 2, left column) to yield **7a** and **7b**. The coupling reaction showed diastereomer-dependent reactivity. The fluoride displacement reaction by the 1-*R* triaminomitosene **6a** (*trans* isomer) to produce **7a** was faster and higher yielding than in the case of the 1-*S* triaminomitosene **6b** (*cis* isomer) to give **7b** (Table 1). The same diastereomer-dependent reactivity was also observed in our previous work leading to dG adducts **9a** and **9b** (Scheme 2, right column, and Table 1) [19, 20]. This effect is probably the result of the differences in steric hindrance caused by the protected 1-amino group of **6a** and **6b** in the coupling reaction: For the 1-*S* triaminomitosene (*cis* isomer) the electrophile must approach from the same side of the bulky substituent at C2, making the reaction slower than in the case of the 1-*R* triaminomitosene (*trans* isomer), where the electrophile reacts from the less hindered opposite side.

Nucleophilic aromatic substitutions between **6a**/**6b** and 6-fluoropurines were faster and proceeded in better yields than similar reactions with 2-fluopurines (Table 1). This effect can be attributed to functional group dependence. The 2-fluoropurine derivatives contain an electron-donating substituent at C6, while the 6-fluoropurine ring has no substituents other than deoxyribose and fluoride. Differences in reactivity between the two fluoropurines have been well known in the chemical carcinogenesis field. 6-fluoropurine reacts *via* a classic S_NAr whereas 2-fluoropurine likely reacts by an extended conjugate addition followed by elimination. This could be the underlying cause for the observed differences [26].

3.2 Efficiency of deprotection methods. Influence of the stereochemical configuration of the adenine-mitosene bond

We previously found that the 2-(trimethylsilyl)ethoxycarbonyl (teoc) group was a convenient protecting group for the 2-amino group on mitosene derivatives, since its fluoride-mediated removal is compatible with the labile nature of mitosene-nucleoside and mitosene-oligonucletide adducts [19–21]. For the deprotection of the teoc group in **7a** and **7b**, we initially used tetrabutylammoniumfluoride (Scheme 3) as the source of fluoride anions.

Treatment of **7a** with excess of TBAF led to efficient teoc deprotection to give the α adduct **4a**. However, the reaction of the β isomer **7b** under the same conditions, resulted in the removal of both the teoc protecting group and the carbamoyl group at C10 to yield the β adduct **5b** (Scheme 3 and Table 2). Additionally, the rate of removal of both groups (teoc and carbamoyl) in **7b** was faster (4h) than the removal of the teoc group in **7a** (15 h) (Table 2). This difference in reactivity toward TBAF between the 2 isomers is not totally unexpected since side reactions have been commonly observed during deprotection with TBAF [27].

Therefore, in order to obtain the β (*R*) MC-dA adduct **4b**, we decided to use a milder reagent for fluoride mediated teoc deprotection. TAS-F (tris(dimethylamino)sulfonium difluorotrimethylsilicate) is a good alternative to TBAF and has the added advantage to prevent undesirable side reactions which may occur when TBAF is used [27]. Indeed, in the case of **7b**, treatment with TAS-F afforded the desired β-MC-dA adduct **4b** without decarbamoylation. The last adduct of the series, the α-DMC-dA adduct **5a**, was obtained using either TAS-F or TBAF as the source of fluoride ion, but the reaction required a much higher concentration of reactants. Under these conditions both the carbamoyl and the teoc groups were removed from **7a** in one step to afford **5a**.

3.3 Compound Characterization

All compounds synthesized were characterized by UV, CD and NMR spectroscopy. HRMS spectra confirmed the structures of diastereoisomeric pairs: **7a**/**b**; **5a**/**b** and **4a**/**b**. The UV spectra of all compounds show the presence of a deoxyadenosine chromophore (λ_{max} 267 nm) and a 7-aminomitosene chromophore (λ_{max} 313 nm). All spectra are identical to the spectra of previously isolated and characterized MC and DMC-N^6-deoxyadenosine adducts [13, 28].

Adducts **7a**, **7b**, **4a**, **4b**, **5a** and **5b** were characterized *via* NMR spectroscopy. The protected adducts **7a** and **7b** are soluble in common organic solvents such as DMSO-d_6 and Methanol-d_4 with good line resolution. However, spectra for adducts **5a**, **5b**, **4a** and **4b** could only be obtained using a mixture of D_2O and pyridine-d_6. Differences in chemical shifts of protons from the mitosene moiety between the 6 adducts are relatively small. In terms of the magnitude of the difference, the protons most affected are $H_{1''}$ and $H_{2''}$ on the tetrahydropyrole ring. The $H_{1''}$ and $H_{2''}$ protons on the *R* (α, *trans*) dA adducts **7a**, **4a** and **5a** are more shielded compared to protons on the *S* (β, *cis*) isomers **7b**, **4b** and **5b** with $|\delta\Delta|$ =0.09–0.27 ppm for $H_{1''}$ and $|\delta\Delta|$=0.22–0.31 ppm for $H_{2''}$. Regarding protons from the glycosidic moiety, the *R* (α, *trans*) and *S* (β, *cis*) isomers from each pair (**7a**/**7b**; **4a**/**4b**; **5a**/**5b**) exhibit signals at similar frequencies. In contrast, there is a marked difference in chemical shifts between the glycosidic protons from the teoc protected pair (**7a**/**7b**) and those from the deprotected pairs (**5a**/**5b**; **4a**/**4b**). Protons from the protected dA adduct pair, **7a** and **7b**, are more shielded than their analogues from both the MC-dA adducts **4a**/**4b** and DMC-dA adducts **5a**/**5b** by: $|\delta\Delta|$=0.59–0.70 ppm for $H_{4'}$; $|\delta\Delta|$=0.38–0.44 ppm for $H_{5',a}$; $|\delta\Delta|$ =0.42–0.48 ppm for $H_{5',b}$; $|\delta\Delta|$=0.40–0.51 ppm for $H_{1'}$; $|\delta\Delta|$=0.36–0.62 ppm for $H_{3'}$; $|\delta\Delta|$ =0.25–0.32 ppm for $H_{2',a}$; $|\delta\Delta|$=0.50–0.57 ppm for $H_{2',b}$. The chemical shifts of the deoxyribose ring protons, especially those at C2' are predicted to be affected by the

magnitude of the glycosidic torsion angle that results from ring current and the local magnetic anisotropy of their bases [29, 30]. Therefore, one possible explanation for the difference in chemical shifts between the glycosidic protons on teoc protected **7a**/**7b** and those on teoc deprotected **5a**/**5b**; **4a**/**4b** is that the teoc group has a direct effect on the glycosidic torsion angle.

Circular dichroism is a reliable method to assign the absolute configuration of mitosene adducts at C1" [31, 32]. The band at around 530 nm, generated by the weak and broad absorption of the mitosene chromophore (which has an extinction coefficient value of 800 at 225 nm) is diagnostic of the β or α configurations of C1" and is independent of the chemical nature of substituents at C1" and of the chemical nature of substituents on other stereogenic centers nearby (such as C2") [31, 32]. Mitosene derivatives with an α configuration at C1" display a negative cotton effect (CE) centered around 530 nm whereas those with a β configuration display a positive CE. Both the mitosene and the adenosine chromophores present absorptions in the 200–400 nm region and it has been previously hypothesized that coupled CD signals are present in this region [31]. Fig. 3 shows the CD and UV spectra of MC-protected-dA adducts **7a**/**7b** and Fig. 4 of MC-dA adducts **4a**/**4b** and DMC-dA adducts **5a**/**5b**. *Cis* and *trans* dA-adduct pairs bear a nearly mirror image relationship and all CD spectra are consistent with previously published data of mitosenes and mitosene-nucleoside adducts [13, 28, 31]. These spectra confirm the stereochemical configuration at C1" of all adducts synthesized in this work.

3.4 Use of adducts 5a and 5b as reference compounds for the detection of DMC-dA adducts formed in DNA

DMC-dA adducts **5a** and **5b**, synthesized according to the new route described above, were used as reference compounds to determine the presence of dA adducts formed during the alkylation of duplex oligonucleotide $(TATATATATATA)_2$ with DMC under bifunctional reductive acitvation conditions. The reaction is described in the experimental section and in previous work [13]. The alkylated oligonucleotide was digested to the nucleoside level using AP (alkaline phosphatase), SVD (snake venom diesterase) and nuclease P_1. HPLC analysis of the enzymatic digest showed the presence of two DMC-nucleoside adducts that eluted with retention times of 23 and 36 minutes (Fig. 5c). In order to demonstrate that the adducts detected in the digest are the same compounds as the synthesized reference compounds **5a** and **5b**, we performed 2 co-injections with the digest of the alkylated oligonucleotide: one sample spiked with pure **5a** (Fig 5d) and another one spiked with pure **5b** (Fig 5e). HPLC chromatograms of the spiked samples clearly show that the two adducts formed between duplex $(TATATATATATA)_2$ and DMC are identical to compounds **5a** and **5b**.

4. Conclusion

In conclusion, we have successfully used organic synthesis to obtain and characterize the four deoxyadenosine adducts that MC and DMC generate in their reactions with DNA. The synthesis is diastereoselective and the alkylated mononucleosides were obtained in good yields. Nucleophilic aromatic substitutions between the 6-fluoropurine derivative and triaminomitosenes to yield the protected dA adducts were faster and proceeded in better

yields than similar reactions with 2-fluoropurine derivatives leading to protected dG adducts. In the course of this synthesis, we observed differences in reactivity from the diastereomeric dA-adducts in the final deprotection reaction, with TBAF (at low concentration) removing the carbamoyl at C10 only in the case of the *cis* diastereomer.

Direct alkylation reactions between dA and MC/DMC to generate MC/DMC dA adducts are very low yielding and they did not provide sufficient quantities of the adducts for a full and rigorous characterization. The synthesis presented here allows for an easy access to such conjugates, which is crucial to investigate their role in cellular mechanisms. The newly synthesized reference compounds allowed us to determine the presence of DMC adducts when a T-A rich duplex oligonucleotide was treated by DMC. In the future, the reference compounds synthesized in this work will allow us to detect the presence of such adducts in the enzymatic digest of cellular DNA extracted from cells treated with either MC or DMC.

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Highlights

- We synthesized dA adducts generated by MC and DMC in their reactions with DNA.
- We used 6-fluoropurine 2’-deoxyribonucleosides in aromatic substitution reactions.
- We examined the influence of the stereochemical configuration at C1 on the reaction rate.
- We confirmed the stereochemical configuration at C1 by CD spectroscopy.
- We compared the efficiencies of dA adducts versus dG adducts formation.
- We used the dA adducts synthesized to detect them in DNA.

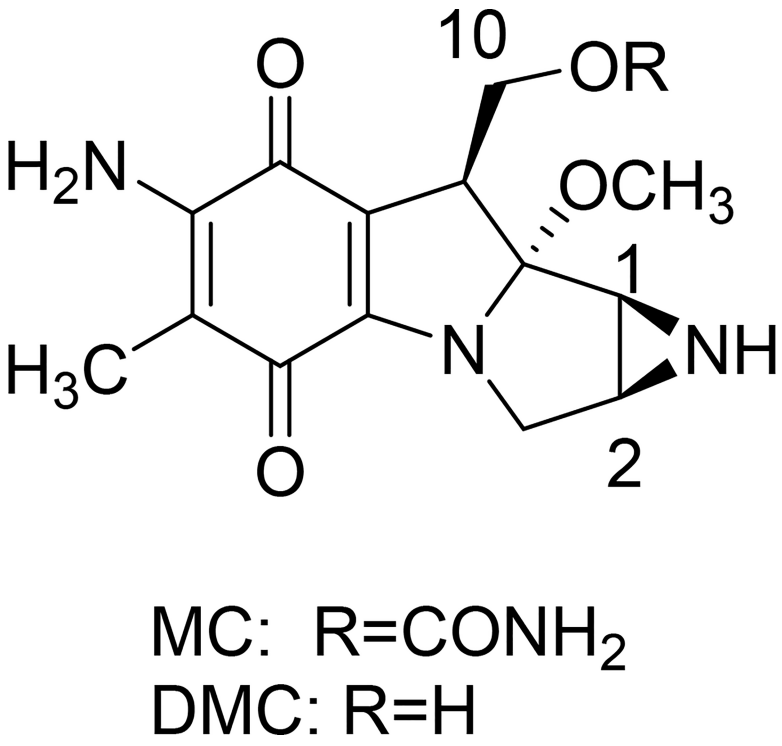


Fig. 1.
(1-column fitting image, color should not be used): Mitomycin C (MC) and Decarbamoylmitomycin C (DMC).

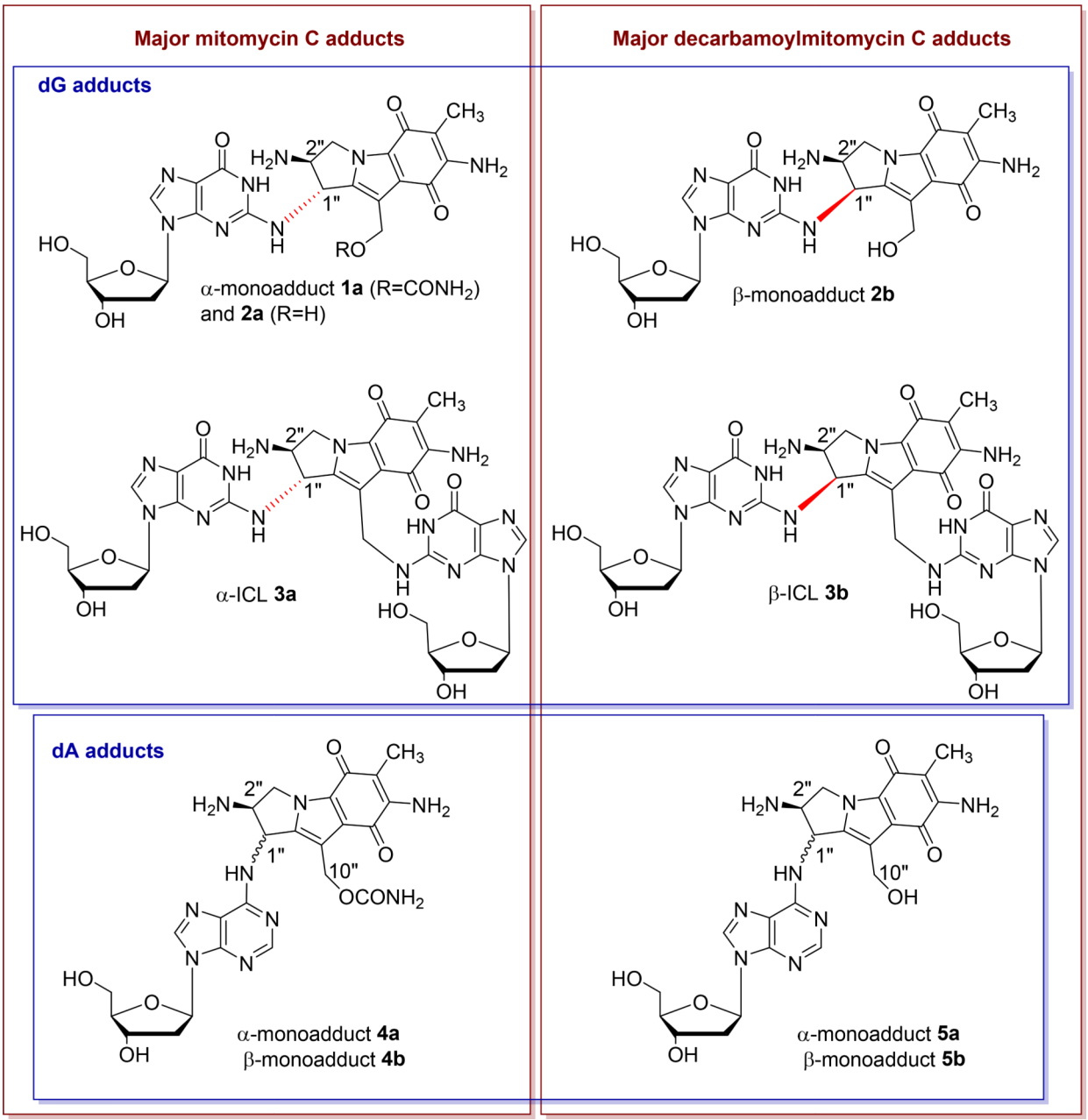


Fig. 2.

(2-column fitting image, color should be used): Major DNA adducts generated by MC and DMC in cells or purified DNA.

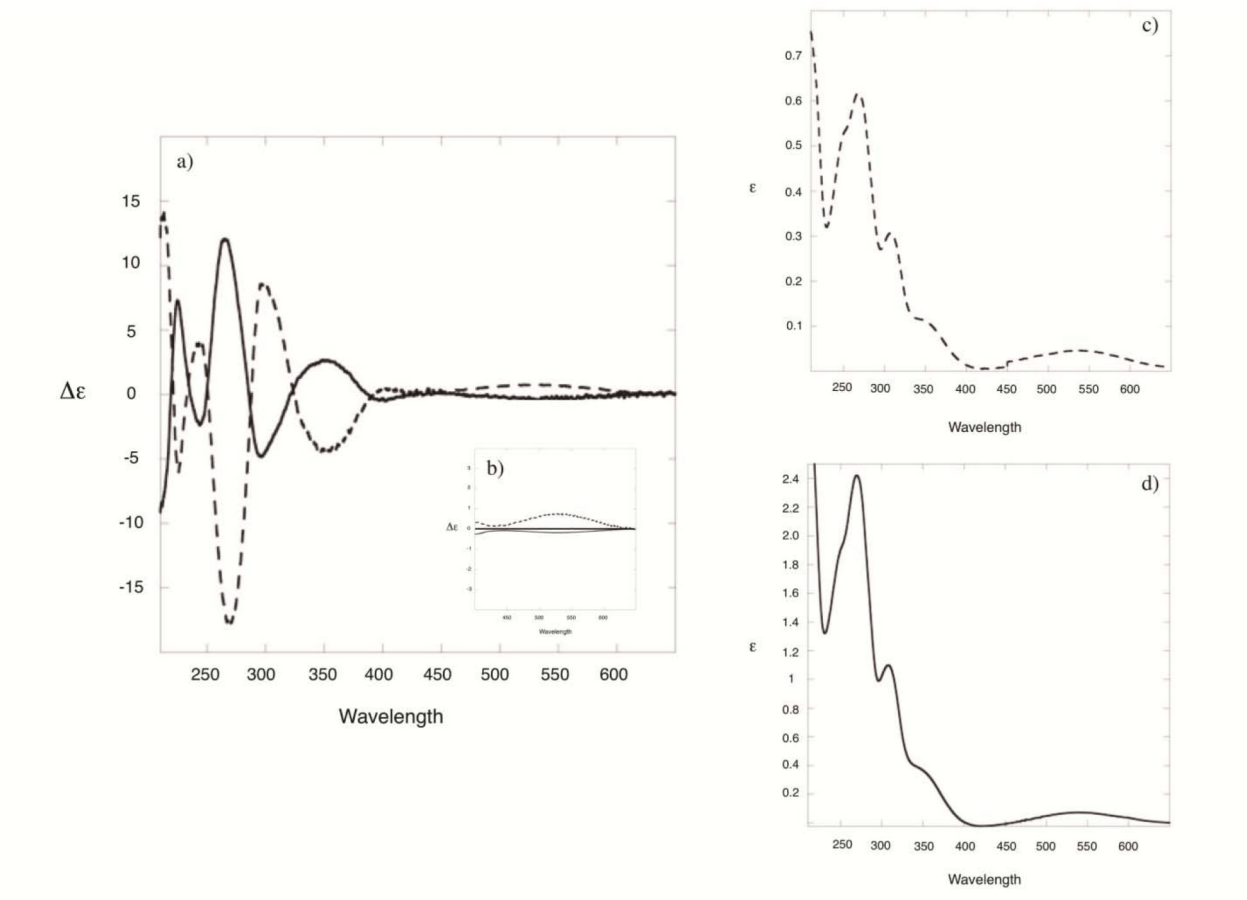


Fig. 3:

(2-column fitting image, no color should be used) (a) CD spectra of MC-protected-dA adducts **7b** (dotted line) and **7a** (full line); (b) enlargement of the area showing the cotton effect around 530 nm; (c) UV spectrum of **7b**; (d) UV spectrum of **7a**.

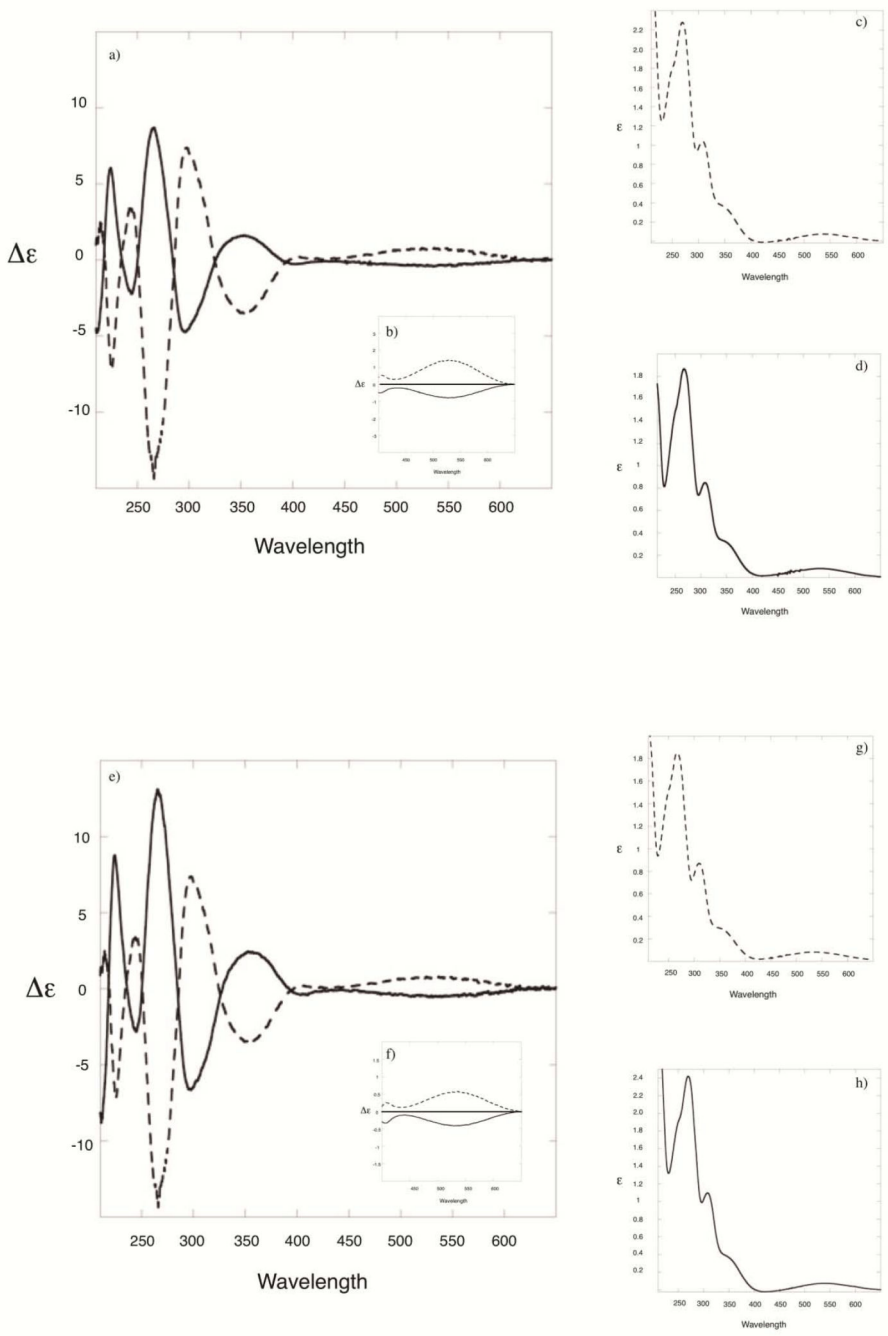


Fig. 4:

(2-column fitting image, no color should be used) (a) CD spectra of MC-dA adducts **4b** (dotted line) and **4a** (full line); (b) enlargement of the area showing the cotton effect around 530 nm; (c) UV spectrum of **4b**; (d) UV spectrum of **4a**; (e) CD spectra of DMC-dA adducts **5b** (dotted line) and **5a** (full line); (f) enlargement of the area showing the cotton effect around 530 nm; (g) UV spectrum of **5b**; (h) UV spectrum of **5a**.

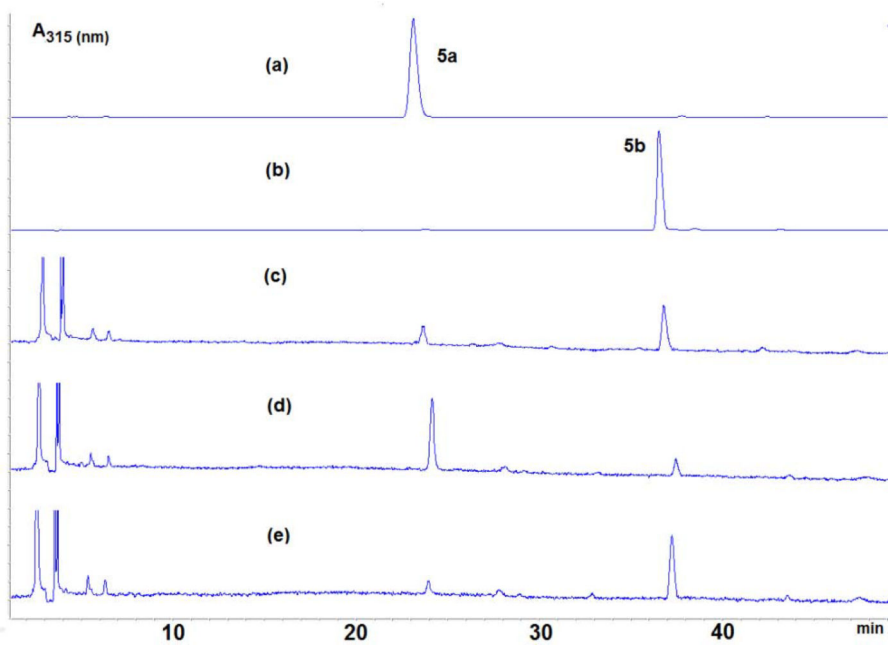
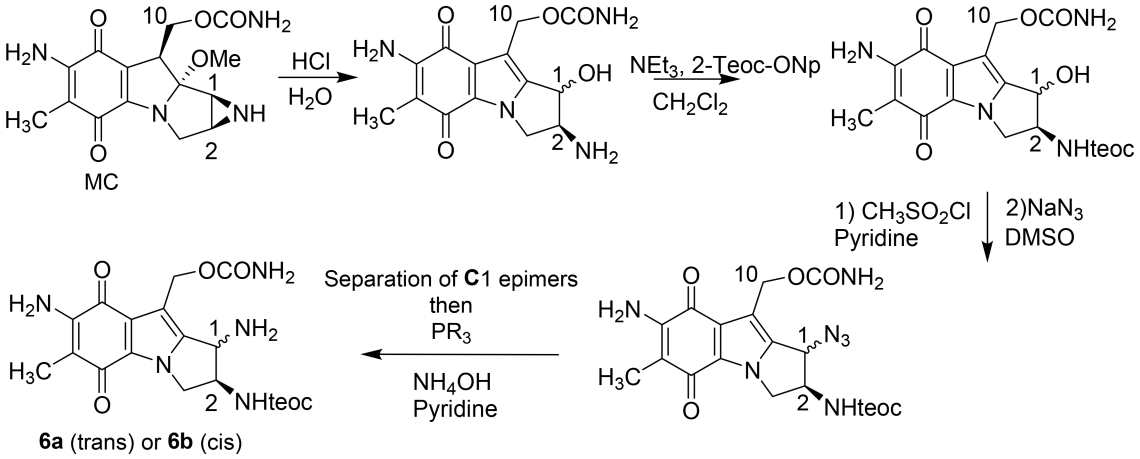


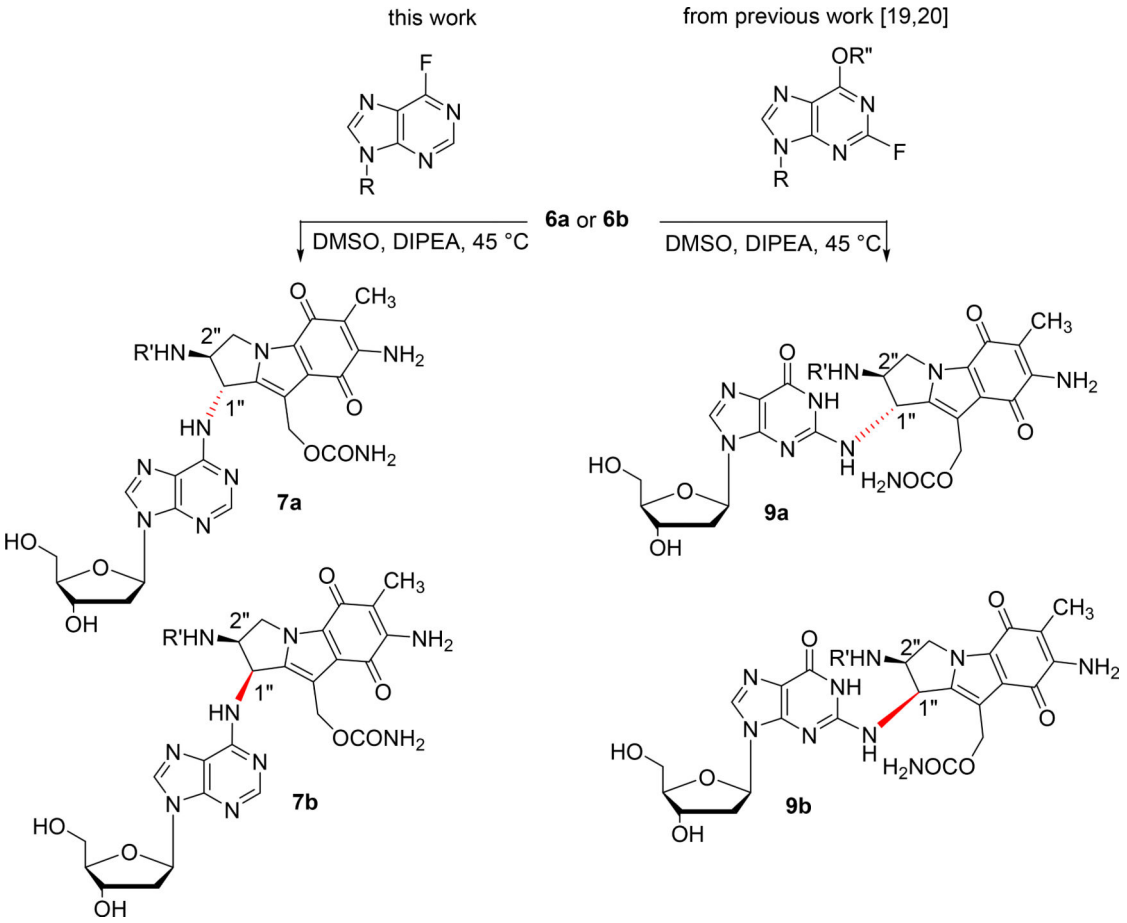
Fig. 5:

(1-column fitting image, color should be used) HPLC chromatograms: (a) synthesized adduct **5a**; (b) synthesized adduct **5b**; (c) enzymatic digest of DMC-alkylated $(TATATATATATA)_2$; (d): co-injection between (a) and (c); (e): co-injection between (a) and (c)



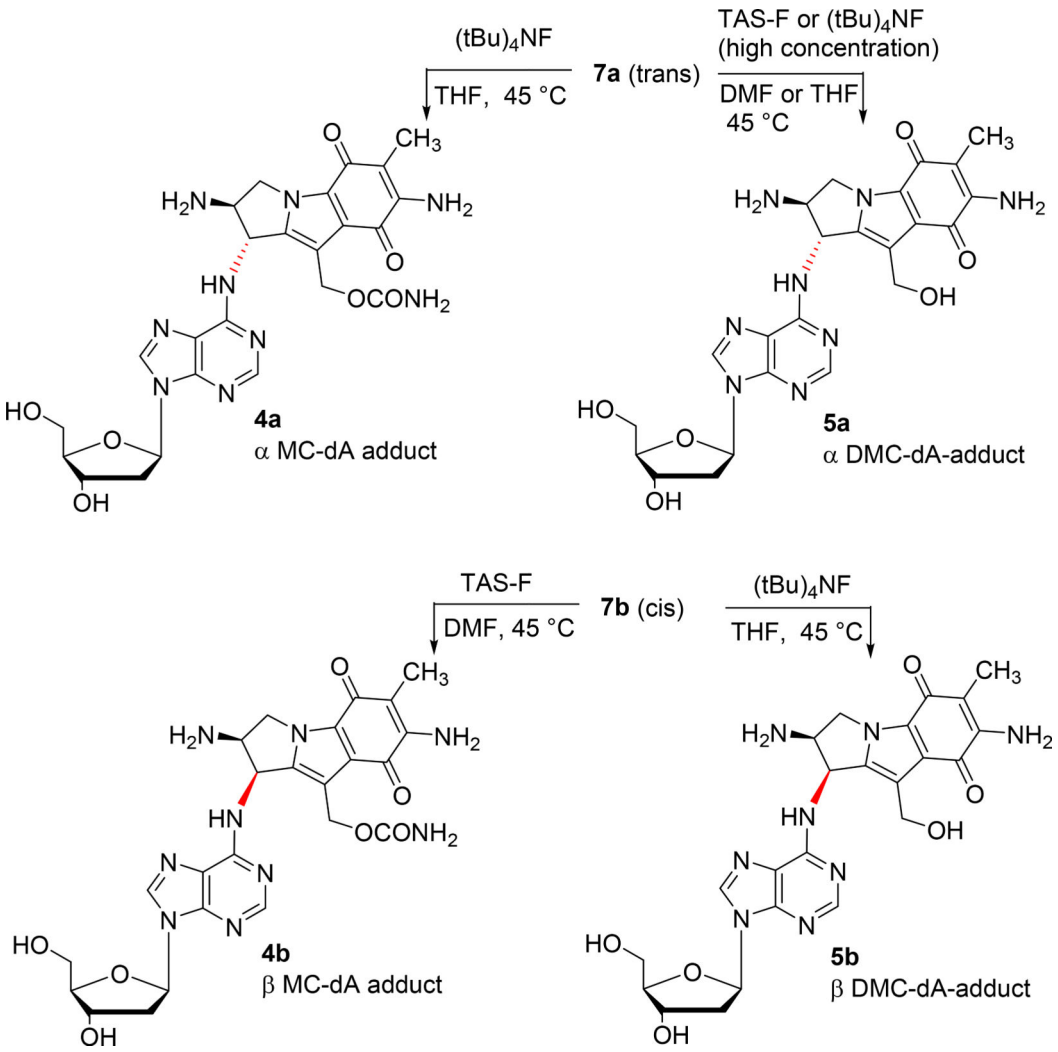
Scheme 1.

(2-column fitting image, color should not be used): Synthesis of triaminomitosenes **6a** and **6b**. R=Ph for **6a** R=Me for **6b**; Teoc=2-(trimethylsilyl)ethoxycarbonyl; Teoc-ONp=2-(trimethylsilyl)-ethyl-4-nitrophenyl carbonate.



Scheme 2.

(2-column fitting image, color should be used): Synthesis of **7a**, **7b**, **9a**, **9b** *via* nucleophilic aromatic substitution. R = deoxyribose, R' = 2-(trimethylsilyl)ethoxycarbonyl (teoc), R" = 2-*p*-nitrophenylethyl.



Scheme 3.

(2-column fitting image, color should be used): Synthesis of **4a**, **4b**, **5a**, **5b**. Fluoride mediated deprotection of the 2-(trimethylsilyl)ethoxycarbonyl (teoc) group with or without concomitant decarbamoylation.

Table 1:

Comparison between nucleophilic aromatic substitutions using 6-fluoro-9-(2-Deoxy-β-D-erythro-pentofuranosyl)purine (for the synthesis of dA adducts 7a and 7b) or 2-fluoro-O^6-(2-*p*-nitrophenylethyl)-2'-deoxyinosine (for the synthesis of dG adducts 9a [20] and 9b [19])

Coupling product	yield	Time (RT)
7a (*R*, α, dA adduct)	81%	2d
7b (*S*, β, dA adduct)	75%	4d
9a[20] (*R*, α, dG adduct)	60%	2 weeks
9b[19] (*S*, β, dG adduct)	54%	3 weeks

Table 2:

Selective conditions for the deprotection of the 2-(trimethylsilyl)ethoxycarbonyl (teoc) group with or without concomitant decarbamoylation.

Starting Material	Reagent	Equivalent	Time	Products (yield)
7a 0.6M	TAS-F or TBAF, 3 M	5	5 h	**5a** (63%)
7a 0.095M	TBAF, 0.47 M	5	15 h	**4a** (68%)
7b 0.091 M	TBAF, 0.47 M	5.2	4 h	**5b** (63%)
7b 0.028 M	TAS-F, 0.37 M	14	24 h	**4b** (82%)