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Article

6-Nitrochrysene-Derived C8-2′-Deoxyadenosine Adduct: Synthesis of Site-Specific Oligodeoxynucleotides and Mutagenicity in *Escherichia coli*

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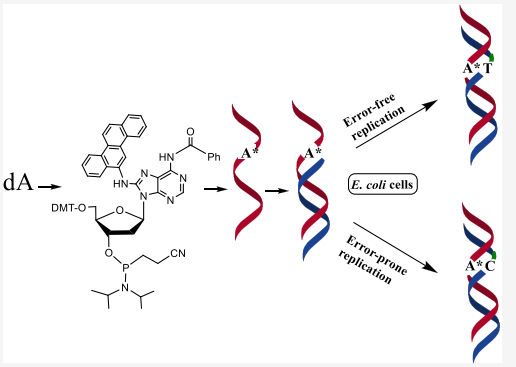
ABSTRACT: 6-Nitrochrysene (6-NC), the most potent carcinogen evaluated by the newborn mouse assay, is metabolically activated by nitroreduction and a combination of ring oxidation and nitroreduction pathways. The nitroreduction pathway yields three major DNA adducts: at the C8 and N^2 positions of 2′-deoxyguanosine (dG), *N*-(dG-8-yl)-6-AC and 5-(dG-N^2-yl)-6-AC, and at the C8 position of 2′-deoxyadenosine (dA), *N*-(dA-8-yl)-6-AC. A nucleotide excision repair assay demonstrated that *N*-(dA-8-yl)-6-AC is repaired much more slowly than many other bulky DNA adducts, including the other DNA adducts formed by 6-NC. But neither the total synthesis nor evaluation of other biological activities of this dA adduct has ever been reported. Herein, we report a convenient synthesis of the 6-NC-derived dA adduct by employing the Buchwald−Hartwig coupling strategy, which provided a high yield of the protected *N*-(dA-8-yl)-6-AC. The deprotected nucleoside showed *syn* conformational preference by NMR spectroscopy. Following DMT protection of the 5′-hydroxyl, *N*-(dA-8-yl)-6-AC was converted to its 3′-phosphoramidite, which was used to prepare oligonucleotides containing a single *N*-(dA-8-yl)-6-AC adduct. Circular dichroism spectra of the adducted duplex showed only a slight departure from the B-DNA helix profile of the control duplex. The 15-mer *N*-(dA-8-yl)-6-AC oligonucleotide was used to construct a single-stranded plasmid vector containing a single adduct, which was replicated in *Escherichia coli*. Viability of the adducted construct was ∼60% of the control, indicating slower translesion synthesis of the adduct, which increased to nearly 90% upon induction of the SOS functions. Without SOS, the mutation frequency (MF) of the adduct was 5.2%, including 2.9% targeted and 2.3% semi-targeted mutations. With SOS, the targeted MF increased 3-fold to 9.0%, whereas semi-targeted mutation increased only marginally to 3.2%. The major type of targeted mutation was A*→G in both uninduced and SOS-induced cells.



INTRODUCTION

Nitropolycyclic aromatic hydrocarbons (NO_2-PAHs) have been detected in a variety of environmental samples, including diesel exhaust.[1−3] Many of these compounds are potent mutagens and carcinogens in laboratory animals.[4−6] 6-Nitrochrysene (6-NC) is less abundant than other NO_2-PAHs in the environment. However, it is the most potent NO_2-PAH compound ever tested in newborn mouse assay.[7] 6-NC is a potent mutagen in bacteria.[8] It is an exceptionally potent carcinogen in newborn mice, and it induces mammary carcinoma in rats.[9,10] The genotoxicity of 6-NC is derived from its ability to chemically damage 2′-deoxyguanosine (dG) and 2′-deoxyadenosine (dA) in DNA to generate carcinogen−DNA adducts.[11−13] Several *in vivo* studies in mice and rats have demonstrated that 6-NC can be activated by two major pathways:[14−16] a nitroreduction and a nitroreduction−ring oxidation tethered pathway. The nitroreduction pathway involves a simple nitroreduction of 6-NC to form the corresponding *N*-hydroxy-6-aminochrysene (*N*-OH-6-AC) (Figure 1). Either *N*-OH-6-AC or the resultant nitrenium ion reacts with the nucleophilic sites of purine bases to generate DNA adducts, *N*-(dG-8-yl)-6-AC (**1**), 5-(dG-N^2-yl)-6-AC (**2**), and *N*-(dA-8-yl)-6-AC (**3**).[13] *N*-(dA-8-yl)-6-AC (**3**) is believed to give rise to the corresponding 2′-deoxyinosine adduct, but whether the process of deamination is enzymatic or non-enzymatic has never been established.[13]

Additional adducts are formed by a combination of ring oxidation and nitroreduction.[16,17] DNA lesions derived from 6-NC can play important roles in the development of human cancer if they are not repaired via cellular repair pathways prior to DNA replication.[18,19] These lesions can induce mutations in crucial sequences of cancer genes. Mutations in an oncogene, a tumor-suppressor gene such as *p53*, or a gene that controls the cell cycle can lead to uncontrolled cell growth, resulting in

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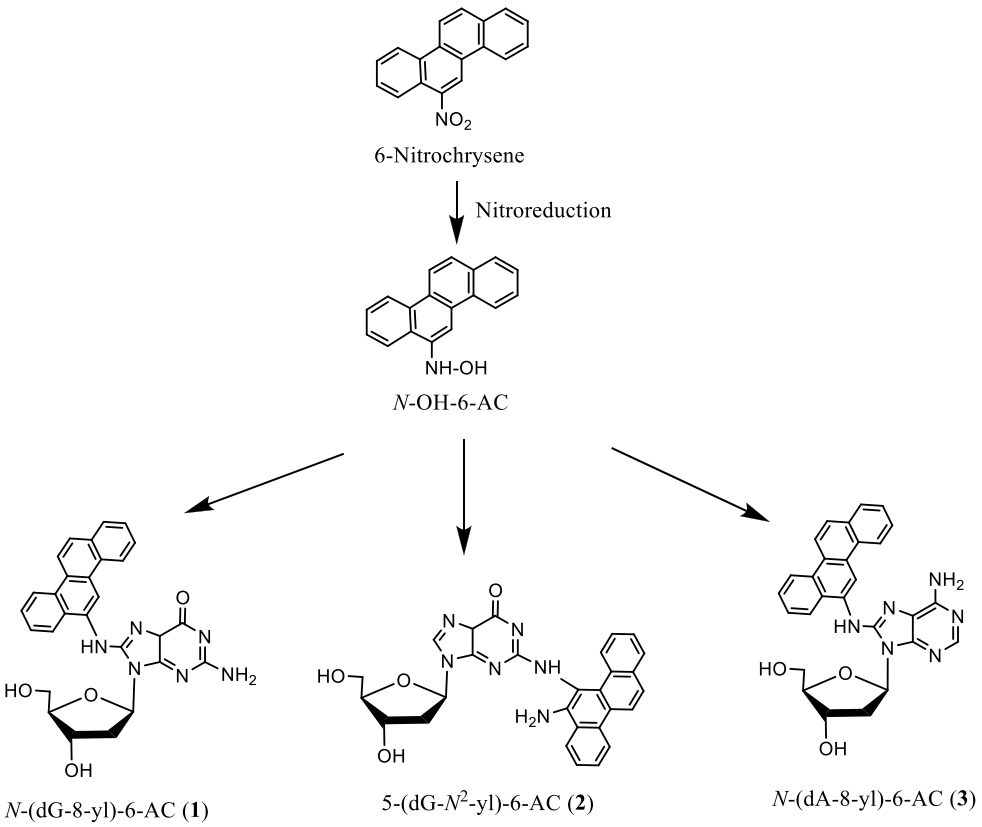

Figure 1. Metabolic activation of 6-NC and DNA adducts formed via the nitroreduction pathway.

tumorigenesis.[20,21] A large fraction of mutations in most human cancers involve mutations in the G:C base pairs of critical genes like *p53*, and a relatively smaller fraction of mutations occur at the A:T base pairs.[22,23] Indeed, carcinogen-induced mutational signatures also predominantly involve G:C base pairs.[24,25] Even so, mutations at A:T base pairs occur.[26] An example of a nitroaromatic compound-derived mutation at the A:T base pair is aristolochic acid (AA), which forms N^6-dA adducts.[27] Mutational spectra of AA are dominated by A:T→T:A transversions in the *p53* gene in urothelial tumors.[28,29]

An *in vitro* repair study by El-Bayoumy and co-workers showed that the efficiency of nucleotide excision repair (NER) of the C8-dA adduct *N*-(dA-8-yl)-6-AC is ∼8 times lower than that of the C8-dG adduct, *N*-(dG-8-yl)-6-AC.[30] In fact, *N*-(dA-8-yl)-6-AC was estimated to be more resistant to NER than all other adducts formed by 6-NC and benzo[*a*]pyrene diol epoxide.[30] A DNA adduct that is repaired slowly is a significant health concern. However, the mutagenicity of this adduct has not yet been reported, which is a goal of our work. The prior synthesis of this adduct was accomplished by treating an 11-mer oligonucleotide containing a single adenine as the only purine with *N*-hydroxy-6-aminochrysene (*N*-OH-6-AC) followed by HPLC purification.[30] In order to synthesize the adduct in any desired DNA sequence, we decided to employ a total synthesis approach. While many C8-dG adducts with different polycyclic aromatic amines and NO_2-PAHs have been synthesized and incorporated in DNA by total synthesis,[31−37] there are only a few reports on the synthesis of dA adducts. Notably, syntheses of several PAH-derived DNA adducts at the 6 position of dA have been accomplished,[33,38−40] but only Meier and co-workers have incorporated C8-arylamine-modified dA adducts in DNA by total synthesis.[41,42] They used the Pd-catalyzed Buchwald−Hartwig C−N bond-forming strategy to incorporate phenylamine derivatives and 4-amino-biphenyl at the C8 position of dA. To our knowledge, no other amino-PAH has been incorporated at the C8 position of dA. In this article, we report the synthesis of *N*-(dA-8-yl)-6-AC phosphoramidite and its incorporation in DNA. We also report its viability and mutagenicity in *E. coli*.

MATERIALS AND METHODS

All reagents were purchased from commercial chemical suppliers and used without further purification unless otherwise noted. NMR spectra were recorded on a Bruker Advance 400 MHz instrument. All ^{1}H and ^{13}C NMR data were referenced to the internal deuterated solvent relative to TMS at 0 ppm. High-resolution mass spectroscopy (HRMS) was performed on a QStar Elite electrospray/nanospray quadrupole time-of-flight mass spectrometer (AB Sciex) in positive ionization mode, and the data was analyzed using MassLynx. Flash chromatography was performed on a Teledyne ISCO CombiFlash Rf automated MPLC chromatography system with silica or aluminum oxide columns. Circular dichroism (CD) spectroscopy was performed on a Chirascan V100 spectrometer. Modified oligonucleotides were synthesized on an ABI DNA synthesizer.

8-Bromo-2′-deoxyadenosine and 8-Bromo-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (4). These compounds were prepared as previously reported.[42]

8-Bromo-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-N^6-dimethoxytrityl-2′-deoxyadenosine (5). 8-Bromo-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (**4**) (9.8 g, 17.442 mmol) and 4-dimethylaminopyrine (42.2 mg, 0.3488 mmol) were dissolved in 20 mL of anhydrous pyridine. 4,4′-Dimethoxytrityl chloride (8.9 g, 26.24 mmol) was added to the solution at room temperature. The reaction mixture was then allowed to stir at room temperature for 18 h. After this time, thin-layer chromatography (TLC) analysis indicated complete consumption of **4**. The reaction mixture was concentrated *in vacuo*, and the crude reaction mixture was purified by Al_2O_3 column

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chromatography with a step gradient of 0−12% ethyl acetate (EtOAc) in hexanes. The product was isolated as a foamy white solid (13.83 g, 92%).

HRMS (ESI$^+$): m/z calcd for $C_{43}H_{58}BrN_5O_5Si_2$ $[M+H]^+$, 860.3238; found, 860.3245.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.97 (s, 1H), 7.46−7.06 (m, 8H), 6.94−6.66 (m, 5H), 6.34 (t, J = 6.7 Hz, 1H), 4.98−4.76 (m, 1H), 4.05−3.84 (m, 2H), 3.80 (s, 7H), 3.77−3.52 (m, 2H), 2.22 (ddd, J = 13.0, 7.0, 4.3 Hz, 1H), 0.95 (s, 9H), 0.83 (s, 9H), 0.15 (s, 6H), −0.00 (s, 3H), −0.06 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 158.53, 158.41, 158.32, 152.99, 152.90, 152.56, 151.96, 151.83, 149.83, 149.52, 145.32, 145.14, 144.90, 142.33, 138.29, 137.37, 136.37, 136.11, 130.26, 130.15, 130.12, 128.83, 128.31, 128.23, 127.88, 127.76, 127.72, 126.82, 126.79, 120.05, 113.20, 113.16, 113.05, 110.22, 108.06, 87.70, 86.49, 86.24, 85.01, 72.18, 72.12, 70.66, 62.50, 59.01, 55.21, 36.89, 25.98, 25.90, 25.78, 25.74, 18.36, 18.08, −4.63, −4.68, −5.39, −5.45.

General Procedure for Optimization of Buchwald−Hartwig Cross-Coupling Reaction. 8-Bromo-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (**4**) (0.098 g, 0.1745 mmol) or 8-bromo-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-N^6-dimethoxytrityl-2′-deoxyadenosine (**5**) (0.150 g, 0.1745 mmol), 6-aminochrysene (0.064 mg, 0.263 mmol), Pd(0) catalyst (4 mol%), and *rac*-2,2′-bis-(diphenylphosphino)-1,1′-binaphthyl (BINAP) (12.5 mg, 0.02 mmol) were suspended in 5 mL of anhydrous solvent (see Table 1). The solution was degassed by purging with argon for 1 h. The reaction flask was then heated under argon at 85−100 °C for 1 h. After this time sodium *tert*-butoxide (NaOtBu) or cesium carbonate (Cs_2CO_3) (0.263 mmol) was added and the reaction stirred at 85−100 °C for an additional 1−2 h. After this time, the reaction was monitored via TLC for consumption of compound **4** or **5**. The reaction was then cooled, diluted with CH_2Cl_2, and filtered through Celite. The filtrate was allowed to evaporate *in vacuo*. For substrate **4**, the crude mixture was purified on silica gel column chromatography with a step gradient of 0−5% methanol in dichloromethane. For substrate **5**, the crude mixture was purified on Al_2O_3 column chromatography with a step gradient of 0−10% EtOAc in hexanes as the mobile phase.

8*N*-(6-Aminochrysene)-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-N^6-dimethoxytrityl-2′-deoxyadenosine (6b). 8-Bromo-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-N^6-dimethoxytrityl-2′-deoxyadenosine (**5**) (0.595 g, 0.693 mmol), 6-aminochrysene (0.250 g, 1.04 mmol), palladium(II) acetate ($Pd(OAc)_2$) (6 mg, 0.0276 mmol), and *rac*-2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP) (50 mg, 0.080 mmol) were suspended in 10 mL of anhydrous toluene. The solution was degassed by purging with argon for 1 h. The reaction flask was then heated under argon at 90 °C for 1 h. After this time cesium carbonate (Cs_2CO_3) (0.366 g, 1.04 mmol) was added and the reaction stirred at 100 °C for an additional 1 h. The reaction was cooled, diluted with ether, and filtered through Celite. The filtrate was allowed to evaporate under reduced pressure. The crude mixture was purified by aluminum oxide column chromatography with a step gradient of 0−10% EtOAc in hexanes as the mobile phase. The product was isolated as a dark green solid (0.603 g, 85%).

HRMS (ESI$^+$): m/z calcd $C_{61}H_{70}N_6O_5Si_2$ $[M+H]^+$, 1023.5025; found, 1023.5056.

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.60 (s, 1H), 8.88 (d, J = 8.2 Hz, 1H), 8.74 (d, J = 8.3 Hz, 1H), 8.70 (d, J = 9.2 Hz, 1H), 8.61 (s, 1H), 8.16 (dd, J = 8.1, 1.5 Hz, 1H), 8.03−7.92 (m, 3H), 7.81−7.54 (m, 4H), 7.42−7.35 (m, 2H), 7.33−7.29 (m, 2H), 7.28−7.17 (m, 5H), 6.85−6.70 (m, 4H), 6.65 (s, 1H), 6.53 (t, J = 7.8, 5.4 Hz, 1H), 4.23 (q, J = 3.3 Hz, 1H), 3.99 (dd, J = 11.4, 3.6 Hz, 1H), 3.91−3.80 (m, 1H), 3.77 (s, 6H), 3.21 (ddd, J = 13.4, 7.8, 5.9 Hz, 1H), 2.51 (ddd, J = 13.0, 5.5, 2.9 Hz, 1H), 0.96 (s, 8H), 0.61 (s, 8H), 0.16 (d, J = 4.6 Hz, 6H), −0.18 (s, 3H), −0.30 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 158.07, 151.59, 149.54, 149.20, 145.89, 138.18, 133.40, 132.49, 131.49, 130.09, 128.87, 128.83, 128.43, 127.73, 126.82, 126.70, 126.54, 126.51, 126.30, 126.11, 125.33, 123.99, 123.53, 121.38, 121.01, 118.71, 113.20, 113.04, 88.35, 85.60, 72.43, 70.31, 63.02, 55.16, 39.79, 25.84, 25.65, 18.24, 18.07, −4.51, −4.72, −5.71, −5.76.

8*N*-(6-Aminochrysene)-3′,5′-*O* -bis(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (6a). *Method 1.* Fully protected coupled substrate, 8*N*-(6-aminochrysene)-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-N^6-dimethoxytrityl-2′-deoxyadenosine (**6b**) (2.0 g, 1.95 mmol) was dissolved in 5.0 mL of dichloromethane, and to this was added 1 M zinc bromide solution in methanol:dichloromethane (1:1) (6 mL). The reaction was stirred for 0.5 h and monitored for completion by TLC (90/10 DCM/MeOH, v/v). The reaction was quenched with aqueous sodium bicarbonate and extracted with dichloromethane. The organic layer was combined and dried with sodium sulfate. The solvent was evaporated under reduced pressure, and crude product was purified by silica gel flash column chromatography with a step gradient of 0−10% methanol in dichloromethane to afford compound **6** as a beige powder (1.05 g, 75%).

Method 2. 8-Bromo-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (**4**) (0.098 g, 0.1745 mmol), 6-aminochrysene (0.064 mg, 0.263 mmol), tris(dibenzylideneacetone)dipalladium ($(Pd_2(dba)_3$) (6.4 mg, 0.007 mmol), and *rac*-2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP) (12.5 mg, 0.02 mmol) were suspended in 8 mL of dimethoxyethane (DME). The solution was degassed by purging with argon for 1 h. The reaction flask was then heated under argon at 85 °C for 1 h. After this time cesium carbonate (Cs_2CO_3) (86 mg, 0.263 mmol) was added and the reaction stirred at 85 °C for an 1 h. The reaction was cooled, diluted with dichloromethane, and filtered through Celite. The filtrate was allowed to evaporate under reduced pressure. The crude mixture was purified by silica gel column chromatography with a step gradient of 0−5% methanol in dichloromethane. The product **6a** was isolated as a beige powder (71 mg, 56%).

HRMS (ESI$^+$): m/z calcd $C_{40}H_{52}N_6O_3Si_2$ $[M+H]^+$, 721.37; found, 721.3729.

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.23 (s, 1H), 8.86 (d, J = 8.4 Hz, 1H), 8.74 (dd, J = 23.0, 8.6 Hz, 3H), 8.42 (s, 1H), 8.25 (d, J = 1.9 Hz, 1H), 8.16 (d, J = 7.9 Hz, 1H), 8.00 (t, J = 9.4 Hz, 3H), 7.81−7.60 (m, 5H), 6.64 (dd, J = 8.4, 5.4 Hz, 1H), 5.30 (d, J = 15.9 Hz, 1H), 4.67 (dd, J = 5.9, 2.9 Hz, 1H), 4.22 (d, J = 2.9 Hz, 1H), 4.05 (dd, J = 11.5, 3.0 Hz, 1H), 3.88 (dd, J = 11.5, 2.8 Hz, 1H), 3.12 (dt, J = 14.0, 7.1 Hz, 1H), 2.54−2.40 (m, 1H), 0.98 (s, 9H), 0.56 (s, 9H), 0.17 (d, J = 8.7 Hz, 6H), −0.24 (s, 3H), −0.33 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 158.53, 158.32, 152.99, 151.96, 145.32, 142.33, 137.37, 136.11, 130.15, 130.12, 128.83, 128.23, 127.88, 127.76, 126.82, 126.79, 120.05, 113.20, 113.16, 113.05, 110.22, 108.06, 87.70, 85.01, 72.12, 70.66, 62.50, 59.01, 55.21, 36.89, 25.90, 25.78, 25.74, 18.36, 18.08, −4.63, −4.68, −5.39, −5.45.

8*N*-(Aminochrysene)-2′-deoxyadenosine (3). 8*N*-(6-Aminochrysene)-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (**6a**) (200 mg, 0.2772 mmol) was dissolved in 3 mL of anhydrous tetrahydrofuran. To this was added 0.416 mL of 1 M tetrabutylammonium fluoride in tetrahydrofuran, and the reaction was allowed to stir for 24 h under N_2. After this time, the solvent was evaporated under reduced pressure, and the crude product was purified by silica gel column chromatography with methanol (0−5%) in dichloromethane. The product was isolated as a light brown solid (116 mg, 85%).

MS (ESI$^+$): m/z calcd $C_{28}H_{24}N_6O_3$ $[M+H]^+$, 493.53; found, 493.534.

^{1}H NMR (400 MHz, DMSO-d_6): δ 9.32 (s, 1H), 9.01 (d, J = 8.4 Hz, 1H), 8.89 (d, J = 7.4 Hz, 2H), 8.84 (d, J = 8.2 Hz, 3H), 8.19 (d, J = 8.2 Hz, 1H), 8.10 (t, J = 6.8 Hz, 2H), 8.02 (s, 1H), 7.84−7.66 (m, 4H), 4.14−3.99 (m, 1H), 3.81 (d, J = 11.9 Hz, 1H), 3.10 (td, J = 15.5, 14.4, 8.3 Hz, 1H), 2.30 (dt, J = 12.9, 6.9 Hz, 1H).

^{13}C NMR (101 MHz, DMSO-d_6): δ 153.75, 150.12, 150.02, 149.66, 135.98, 132.38, 131.56, 130.00, 128.90, 128.53, 127.64, 127.43, 127.24, 127.12, 126.32, 124.26, 124.18, 124.04, 121.74, 117.26, 116.87, 88.27, 84.32, 71.90, 62.20, 58.00, 55.38, 38.79.

N^6-Benzoyl-8*N*-(aminochrysene)-2′-deoxyadenosine (7). 8*N*-(6-Aminochrysene)-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (**6a**) (445 mg, 0.617 mmol) was dissolved in anhydrous

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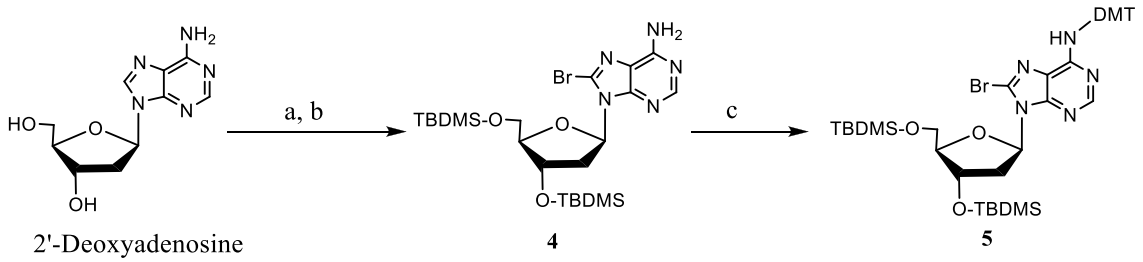
Scheme 1. Preparation of Protected Br-dA[a]



[a]Reagents and conditions: (a) Br_2, NaOAc buffer (pH 5.4), 18 h, room temperature (81%); (b) TBDMS-Cl, imidazole, pyridine, 16 h, room temperature (95%); (c) DMT-Cl, 4-DMAP, pyridine, 18 h, room temperature (92%).

pyridine (5 mL) under an atmosphere of nitrogen, and benzoyl chloride (0.108 mL, 0.9255) was added dropwise. The reaction mixture was stirred for 4 h at room temperature. It was then diluted with dichloromethane (10 mL) and washed with saturated sodium hydrogen carbonate solution, and the aqueous layer was extracted twice with dichloromethane. After removal of the dichloromethane under reduced pressure, morpholine (0.134 mL, 1.5425 mmol) was added, and the resulting mixture was stirred at room temperature for 2 h. After this time, the reaction mixture was diluted with dichloromethane (10 mL) and then washed twice with 0.5 M sodium dihydrogen phosphate solution. The aqueous layer was extracted three times with dichloromethane. After complete removal of the dichloromethane under reduced pressure, 100 mg (0.1386 mmol) of crude product was dissolved in 3 mL of anhydrous tetrahydrofuran. Next, 0.416 mL of 1 M tetrabutylammonium fluoride in tetrahydrofuran was added, and the reaction was allowed to stir for 24 h under N_2. After this time, the solvent was evaporated under reduced pressure, and the crude product was purified via silica gel column chromatography with methanol (0−10%) in dichloromethane. The product was isolated as a yellow solid (70 mg, 85%).

HRMS (ESI$^+$): m/z calcd for $C_{35}H_{28}N_6O_4$ $[M+H]^+$, 597.2250; found, 597.2234 and $[+Na]^+$.

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.89 (s, 1H), 9.74 (s, 1H), 9.61 (s, 1H), 8.99 (d, J = 7.8 Hz, 1H), 8.83 (d, J = 8.9 Hz, 2H), 8.58 (s, 1H), 8.29 (d, J = 7.6 Hz, 1H), 8.02 (t, J = 6.6 Hz, 5H), 7.79 (s, 1H), 7.59 (dt, J = 14.5, 7.4 Hz, 1H), 7.43 (dt, J = 14.4, 7.6 Hz, 3H), 6.82 (t, J = 7.1 Hz, 1H), 5.48 (d, J = 4.2 Hz, 2H), 4.57 (s, 1H), 4.12 (s, 1H), 3.91−3.59 (m, 3H), 3.27−2.97 (m, 1H), 2.42 (dd, J = 12.9, 6.0 Hz, 1H).

^{13}C NMR (75 MHz, DMSO-d_6): δ 151.51, 148.95, 145.54, 134.07, 134.00, 132.54, 132.42, 131.26, 130.18, 128.84, 128.75, 128.36, 127.60, 127.35, 127.20, 127.05, 126.96, 125.69, 124.33, 124.06, 123.33, 121.64, 115.19, 88.45, 84.85, 71.77, 62.02.

N^6-Benzoyl-8*N*-(6-aminochrysene)-5′-*O*-dimethoxytrityl-2′-deoxyadenosine. The N^6-benzoylated C8-arylamine-dA adduct (7) (0.295 g, 0.494 mmol) was dissolved in anhydrous pyridine (6 mL) under a nitrogen atmosphere, and 4,4′-dimethoxytrityl chloride (0.177 g, 0.522 mmol) and silver nitrate (0.089 g, 0.522 mmol) were added. The mixture was stirred at room temperature until the reaction was complete (18 h). The reaction mixture was then diluted with dichloromethane (10 mL) and washed with saturated sodium hydrogen carbonate solution and brine. The aqueous layer was extracted twice with dichloromethane. The organic layers were combined, dried over sodium sulfate, and filtered, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel, eluting with 0−3% methanol in dichloromethane to afford the desired DMT-protected 7 as a yellow foam (328 mg, 74%).

HRMS (ESI$^+$): m/z calcd $C_{56}H_{46}N_6O_6$ $[M+H]^+$, 899.3552; found, 899.3532.

^{1}H NMR (500 MHz, chloroform-d): δ 9.31 (s, 1H), 8.76 (dd, J = 7.5, 1.3 Hz, 1H), 8.72−8.73 (m, 1H), 8.44 (s, 1H), 8.23 (s, 1H), 8.16 (dd, J = 7.8, 1.3 Hz, 1H), 8.05−8.03 (m, 2H), 8.00−7.94 (m, 1H), 7.89 (d, J = 9.2 Hz, 1H), 7.72−7.66 (m, 2H), 7.66−7.62 (m, 2H), 7.54−7.42 (m, 3H), 7.42−7.38 (m, 2H), 7.37 (d, J = 1.4 Hz, 1H), 7.33−7.27 (m, 2H), 7.27−7.20 (m, 1H), 7.13−7.07 (m, 4H), 6.87−6.81 (m, 4H), 6.60 (ddd, J = 4.0, 2.4, 0.9 Hz, 1H), 6.30 (s, 1H), 4.23−4.18 (m, 2H), 3.82 (s, 6H), 3.68 (qd, J = 12.0, 3.1 Hz, 2H), 2.56 (dt, J = 12.5, 3.3 Hz, 1H), 2.31 (dt, J = 12.3, 5.1 Hz, 1H), 1.91 (s, 1H).

^{13}C NMR (126 MHz, $CDCl_3$): δ 165.57, 165.06, 158.21, 153.17, 152.09, 151.83, 149.38, 144.96, 144.53, 143.84, 136.17, 135.10, 135.01, 134.11, 132.71, 132.45, 132.10, 131.22, 130.09, 129.83, 129.77, 129.33, 128.49, 128.38, 128.24, 128.15, 127.68, 127.64, 127.01, 126.80, 126.73, 126.41, 125.91, 125.50, 124.80, 123.68, 123.51, 123.13, 122.01, 121.90, 121.10, 120.62, 115.11, 112.85, 110.01, 86.81, 86.48, 85.69, 71.79, 67.62, 63.37, 63.30, 54.93, 53.49, 53.07, 53.04, 53.02, 53.00, 52.93, 52.85, 52.83, 52.81, 52.79, 52.77, 40.96, 39.45, 0.01.

N^6-Benzoyl-8*N*-(6-aminochrysene)-5′-*O*-dimethoxytrityl-2′-deoxyadenosine-3′-*O*-(cyanoethyl-*N*,*N*′-diisopropylphosphoramidite (8). This reaction was performed in a glovebag. N^6-Benzoyl-8N-(6-aminochrysene)-5′-*O*-dimethoxytrityl-2′-deoxyadenosine (0.400 g, 0.444 mmol) and *N*,*N*-diisopropylethylamine (0.155 mL, 0.888 mmol) were dissolved in anhydrous dichloromethane (6 mL) under argon. 2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.105 mL, 0.444 mmol) was added and the reaction mixture stirred for 15 min. After this time, additional *N*,*N*-diisopropylethylamine (0.155 mL, 0.888 mmol) was added to the solution and the reaction stirred for an additional 0.5 h. After this time, the reaction mixture was diluted with dichloromethane (20 mL) and saturated sodium bicarbonate (25 mL). The organic layer was retained, and the aqueous layer was extracted twice with dichloromethane (2 × 15 mL). The organic layers were combined, washed with brine, and dried over sodium sulfate. The solvent was concentrated under reduced pressure (2 mL). The solution was added dropwise to a stirring solution of 220 mL of hexanes. The resulting precipitate was collected and further purified on basified silica gel flash chromatography with a step gradient of 0−5% methanol in dichloromethane containing 1% triethylamine. The product was isolated as a yellow solid (0.297 g, 61%).

HRMS (ESI$^+$): m/z calcd $C_{65}H_{63}N_8O_7P$ $[M+H]^+$, 1099.4636; found, 1099.4607.

^{31}P NMR (162 MHz, CD_2Cl_2): δ 149.06, 149.01, 148.57, 148.51.

Site-Specific Synthesis of Oligodeoxynucleotides Containing *N*-(dA-8-yl)-6-AC Adduct. Phosphoramidite **8** was used to synthesize modified oligodeoxynucleotides as per the manufacturer's instruction. However, the time of the coupling step was extended to 15 min for the incorporation of modified phosphoramidite with total coupling efficiencies of >95%. The unmodified and complementary oligodeoxynucleotide sequences were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa, USA). The modified oligonucleotides were purified by reverse-phase HPLC followed by denaturing PAGE. The homogeneity of the purified modified and unmodified (control) oligodeoxynucleotides was confirmed via phosphorylation with T4 polynucleotide kinase in the presence of [γ^{32}P]adenosine triphosphate and subsequent PAGE analysis (see SI, Figure S27). The modified oligodeoxynucleotides were further characterized by ES mass spectrometry. HRMS data are given in Table 2.

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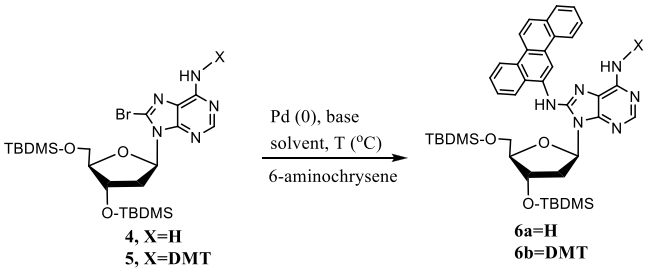
Table 1. Optimization of Buchwald–Hartwig Cross-Coupling Reaction[a]



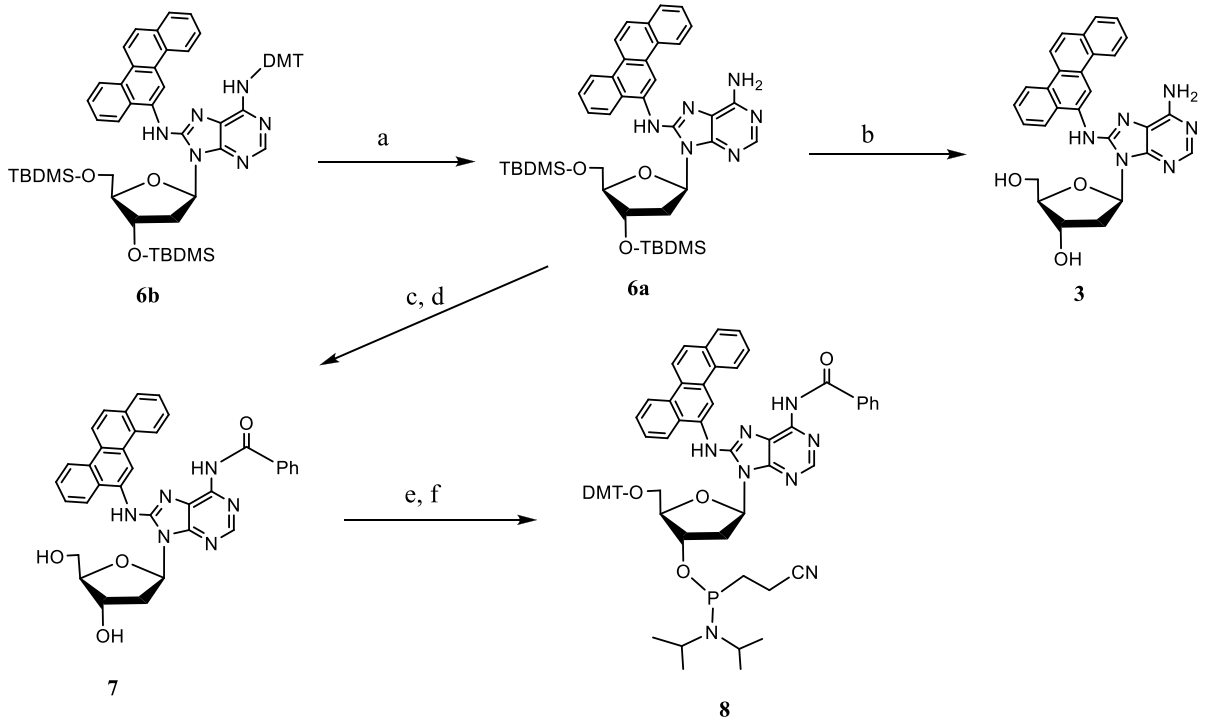
4, X=H
5, X=DMT

6a=H
6b=DMT

entry	X	catalyst (4 mol%)	base (1.5 equiv)	ligand (12 mol%)	solvent	temp (°C)	yield (%)
1	H	$Pd_2(dba)_3$	NaO*t*Bu	BINAP	toluene	100	45
2	H	$Pd(OAc)_2$	Cs_2CO_3	BINAP	toluene	100	56
3	H	$Pd_2(dba)_3$	NaO*t*Bu	BINAP	DME	85	52
4	H	$Pd(OAc)_2$	Cs_2CO_3	BINAP	DME	85	42
5	DMT	$Pd_2(dba)_3$	NaO*t*Bu	BINAP	toluene	100	75
6	DMT	$Pd(OAc)_2$	Cs_2CO_3	BINAP	toluene	100	85
7	DMT	$Pd_2(dba)_3$	NaO*t*Bu	BINAP	DME	85	68
8	DMT	$Pd(OAc)_2$	Cs_2CO_3	BINAP	DME	85	61

[a]All reactions were carried out for 3 h.

Scheme 2. Synthesis of 5′-DMT-Protected 3′-Phosphoramidite 8[a]



[a]Reagents and conditions: (a) 1 M $ZnBr_2$ in 1:1 MeOH:CH_2Cl_2 solution, 0.5 h, 75%; (b) 1 M TBAF in THF, RT, 24 h, 85%; (c) benzoyl chloride (BzCl), pyridine, RT followed by treatment with morpholine, 2 h; (d) 1 M TBAF in THF, RT, 24 h, 85%; (e) 4,4′-dimethoxytrityl chloride (DMT-Cl), pyridine, $AgNO_3$, RT, 18 h, 74%; (f) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIEA, CH_2Cl_2, RT, 1 h, 61%.

Circular Dichroism Studies. The concentration of oligonucleotides was determined using a NanoDrop 2000 spectrophotometer. Equal amounts of two complementary strands (5 nmol) were dissolved in buffer [1 mL, NaH_2PO_4 buffer (10 mM), NaCl (140 mM), EDTA (1 mM), pH 6.6], heated to 70 °C, and then slowly allowed to cool to room temperature to enable annealing of the two strands. CD measurements were carried out at 25 °C, and samples were scanned from 400 to 220 nm at 0.5 nm intervals averaged over 5 s.

Construction of Adduct-Containing Plasmid, SOS Induction, and Transformation in *E. coli*. Construction of the adduct-containing plasmid and an unmodified control pMS2 plasmid and their replication in *E. coli* AB 1157 essentially followed the protocol described in detail in refs 43 and 44. SOS induction was carried out with 50 J/m^2 of UV light (254 nm). Oligonucleotide hybridization also was performed as reported.[45,46] Lesion bypass efficiency was calculated by comparing the transformation efficiency of the *N*-(dA-8-yl)-6-AC construct with that of the control, whereas mutation frequency (MF) was calculated based on hybridization data and sequence analysis.

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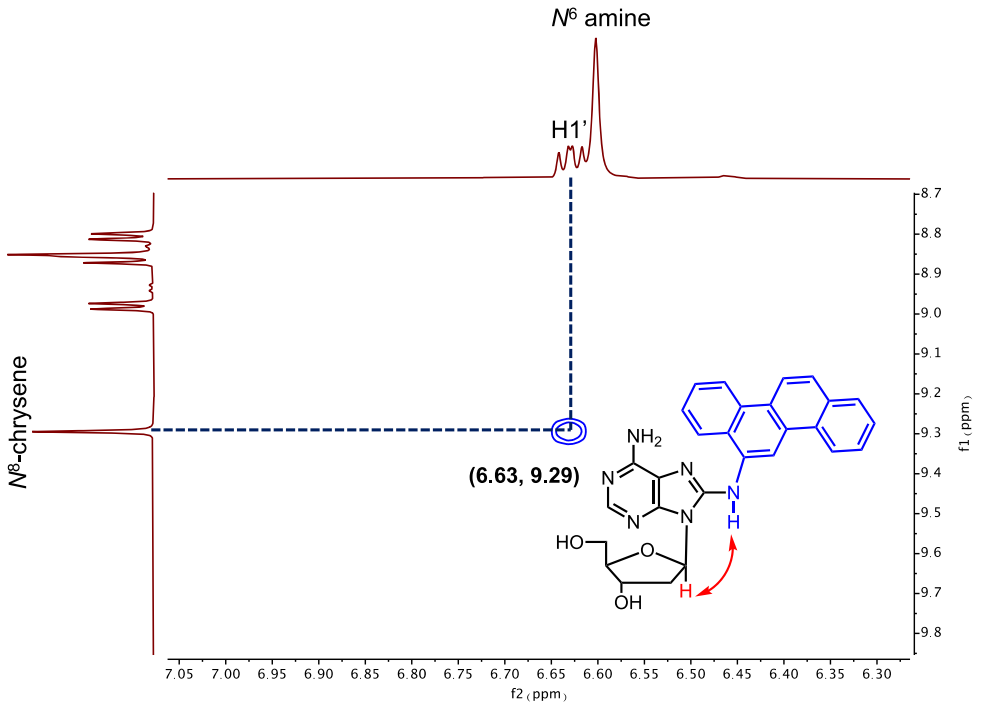

Figure 2. ROESY-NMR spectra of *N*-(dA-8-yl)-6-AC adduct **3** in DMSO-d_6 at 300 K.

RESULTS AND DISCUSSION

Protected Br-dA (**4**) was prepared by a standard method of selective bromination at the C8 position of dA using Br_2 in acetate buffer (pH 5.4) followed by TBDMS protection of the 3′ and 5′ hydroxyl groups (Scheme 1). Following Meier's strategy for coupling arylamines with dA,[41,42] we first carried out Buchwald–Hartwig cross-coupling with compound **4** and 6-aminochrysene (6-AC) using $Pd_2(dba)_3$ or $Pd(OAc)_2$ catalyst with BINAP and either NaO*t*Bu or Cs_2CO_3 as the base in toluene or DME. The C–N coupling worked in each case, but the yield remained between 42 and 56% (Table 1). We found that 12 mol% of BINAP was optimal for these reactions, and higher mol% caused partial degradation of the product. Each reaction shown here was carried out for 3 h. Longer reaction times (up to 24 h) led to significant tailing on TLC for reactions carried out at 100 °C in toluene, which is usually indicative of degradation of the product. In 1,2-DME (performed at 85 °C) this tailing was not observed, and the reactions were allowed to run overnight. However, longer reactions times did not result in improvement in reaction yields.

For the Pd-catalyzed coupling of protected dG with polyaromatic amines, Gillet and Schärer showed that N^2-DMT-protected dG provides an excellent yield of the adduct,[34] which we have used successfully for the synthesis of dG-1-aminopyrene adduct.[47] So, to improve the coupling efficiency, the N^6 exocyclic amine was protected using 4,4′-dimethoxytrityl chloride (DMT-Cl) in pyridine with catalytic amounts of 4-dimethylaminopyridine (DMAP), and then the N^6DMT-protected Br-dA nucleoside **5** was subjected to the Pd-catalyzed coupling. We are pleased to report better coupling efficiency for each reaction, and the best coupling yield of 85% was achieved with $Pd(OAc)_2/Cs_2CO_3$/BINAP combination in toluene at 100 °C for 3 h (Table 1). For the DMT-protected Br-dA nucleoside **5** also longer reaction time did not improve the yield. We believe that the improved yields with N^6-DMT-protected substrate in the Buchwald–Hartwig cross-coupling reactions can be attributed to the increased solubility of the substrate in toluene (and DME), facilitated by the nonpolar DMT group.

With the optimized conditions to access coupled product **6b**, the DMT group on N^6-excocyclic amine was deprotected using a 1 M solution of $ZnBr_2$ to furnish **6a** (Scheme 2). The N^6-amine functionality was then reprotected with a base-labile benzoyl group using benzoyl chloride in pyridine followed by treatment with morpholine to afford N^6-benzoylated product, and desilylation of the 5′- and 3′-hydroxyls was achieved using tetrabutylammonium fluoride (TBAF) in THF to furnish **7**. The 5′-hydroxyl was protected with the acid-labile DMT group and used to prepare the 3′-phosphoramidite using 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite to give the phosphoramidite monomer **8** (Scheme 2).

***Syn*/*Anti* Conformational Preference of *N*-(dA-8-yl)-6-AC Adduct 3.** NMR data on C8-arylamine-dA adducts showed that, with a single phenyl ring, the preferred conformation of the *N*-glyosidic bond is *anti*, but addition of an *N*-acetyl group replacing the amino hydrogen rotates it to *syn* conformation.[42] To determine *syn* or *anti* preference of *N*-(dA-8-yl)-6-AC, its conformation was investigated using ROESY-NMR spectroscopy. Figure 2 shows a cross-peak between the H1′ proton of the sugar and 6-AC amine tethered to the C8 site of dA, indicating a preference for *syn* conformation. Furthermore, no cross-peaks were observed between the H2 proton on the nucleobase and the H1′ proton on the sugar, typically observed for the *anti* conformation. These results indicate a preference for *N*-(dA-8-yl)-6-AC to exist in the *syn* conformation of the *N*-glyosidic bond, which was also reported for other bulky C8-arylamine-modified nucleosides.[48,49]

Synthesis of Oligodeoxynucleotides of *N*-(dA-8-yl)-6-AC Adduct. The protected *N*-(dA-8-yl)-6-AC phosphoramidite **8** was used to synthesize 12- and 15-mer oligonucleotides,

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and the coupling efficiency was more than 95% for the modified dA in each case. The oligonucleotides were deprotected at 55 °C with ammonium hydroxide for 24 h. During deprotection, 0.25 M 2-mercaptoethanol was added to avoid oxidative degradation. The modified oligodeoxynucleotides were purified by reverse-phase HPLC followed by polyacrylamide gel electrophoresis (PAGE) and characterized by high-resolution electrospray ionization mass spectrometry in negative mode (Table 2).

Table 2. HPLC-ESI HRMS (Negative Mode) Data for Modified and Unmodified (Control) Oligodeoxynucleotides

	m/z (Da) (charge)	
oligodeoxynucleotides[a]	calculated	observed
5′-GTGCAT GTT TGT-3′	1225.8680 (−3)	1225.9200 (−3)
5′-GTGCA*T GTT TGT-3′	1306.3212 (−3)	1306.2352 (−3)
5′-GCCCTCAACAAGATG-3′	1134.8941 (−4)	1134.9442 (−4)
5′-GCCCTCAA*CAAGATG-3′	1195.2252 (−4)	1195.2252 (−4)

[a]A* represents *N*-(dA-8-yl)-6-AC.

Circular Dichroism (CD) Studies. The 15-mer and 12-mer unmodified and *N*-(dA-8-yl)-6-AC-modified oligodeoxynucleotides were allowed to anneal to their complementary strand, and each of the resultant duplexes was evaluated on a CD spectrometer (Chirascan V100). As shown in Figure 3, the CD spectra confirm overall B-type DNA conformation of the unmodified control duplex and the *N*-(dA-8-yl)-6-AC-containing duplex. For the control duplex a positive Cotton effect was observed at 280 nm along with a negative one at 245 nm typically found in B-type DNA. However, the CD curve of the *N*-(dA-8-yl)-6-AC-containing duplex shows a slight deviation in the B-type DNA helical structure of the unmodified duplex. Nevertheless, the positive and negative Cotton effects observed were consistent with canonical B-type DNA structures.

Mutational Analyses of *N*-(dA-8-yl)-6-AC in *E. coli*. The DNA sequence of the 15-mer was chosen from *TP53* gene codon 129−133, because crops contaminated with another nitroaromatic carcinogen aristolochic acid (AA) caused A→T mutation in codon 131 in patients with urothelial tumors.[23,50] While many polyaromatic amine and nitroaromatic compounds form adducts at the C8 position of dG, which induce frameshifts and G→T transversions as the dominant mutations,[43,51,52] the major AA adducts are formed at the N^6 position of dA.[27] The 15-mer oligonucleotide (5′-GCCCTCAA*CAAGATG-3′) was incorporated into a scaffolded single-stranded pMS2 plasmid as reported for the construction of other DNA lesions, after which the scaffold was enzymatically removed.[53,54] An unmodified control plasmid was also prepared using the same approach. The *N*-(dA-8-yl)-6-AC-containing and control plasmids were then replicated in uninduced and SOS-induced *E. coli* AB1157 cells. In uninduced cells, the number of progeny colonies from the adduct-containing construct was reduced to ∼60% to that of the control (Figure 4A and SI, Table S1). This suggests that though replication was inhibited, *N*-(dA-8-yl)-6-AC is less toxic than the C8 dG adduct of 1-nitropyrene, which gives less than 30% progeny.[52,55] Upon induction of SOS, viability of the adducted genome increased to nearly 90%, indicating significantly more facile translesion synthesis (TLS) of *N*-(dA-8-yl)-6-AC by the SOS proteins (Figure 4A and SI, Table S1). It would be of interest to determine in the future which SOS proteins participate in the increased TLS of this adduct by using *E. coli* strains with appropriately knocked out TLS polymerase(s).[56]

Without SOS, 5.2% of the replicates were mutants, which included 2.9% targeted (i.e., mutations at the modified A) and 2.3% semi-targeted mutations (Figure 4B), the latter being defined as mutations near the lesion site. Of the targeted mutations, 80% were A*→G transitions. With SOS, MF increased to 12.2%, showing 3-fold increase of the targeted

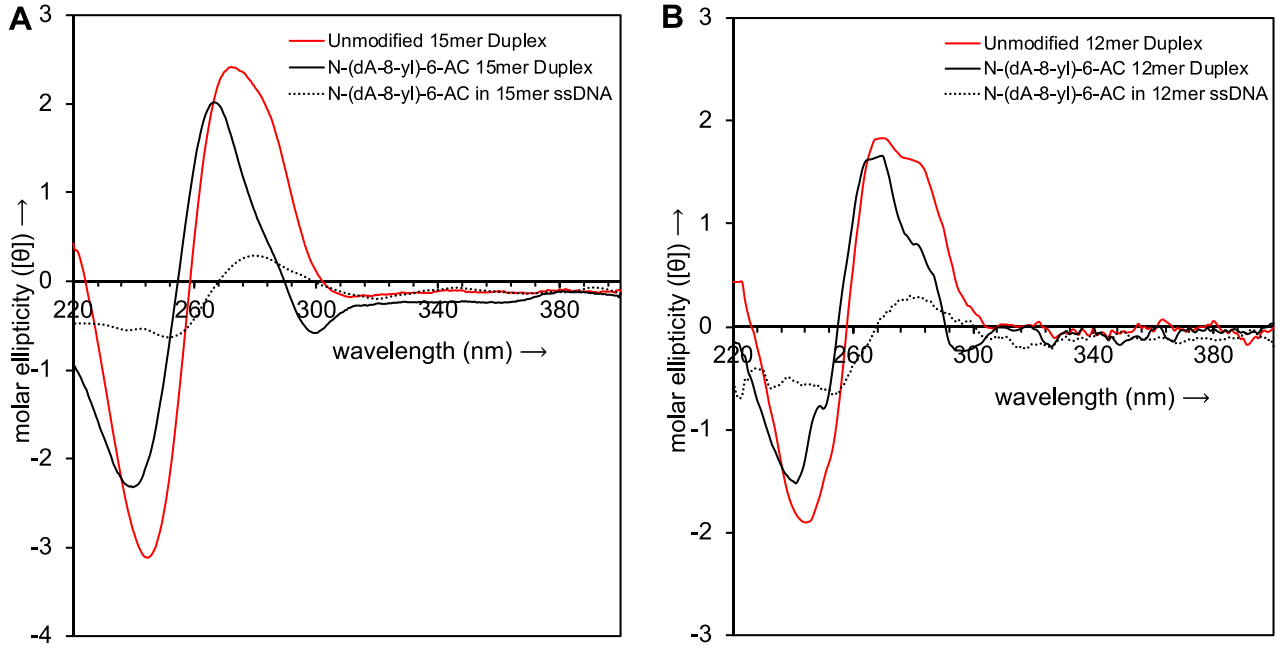

Figure 3. CD spectra of unmodified (red solid line) and modified (black solid line) 15-mer (A) and 12-mer (B) oligonucleotides (sequence shown in Table 2) after annealing with their complementary strand. The CD spectra of the single-stranded oligonucleotides are shown in black dotted lines.

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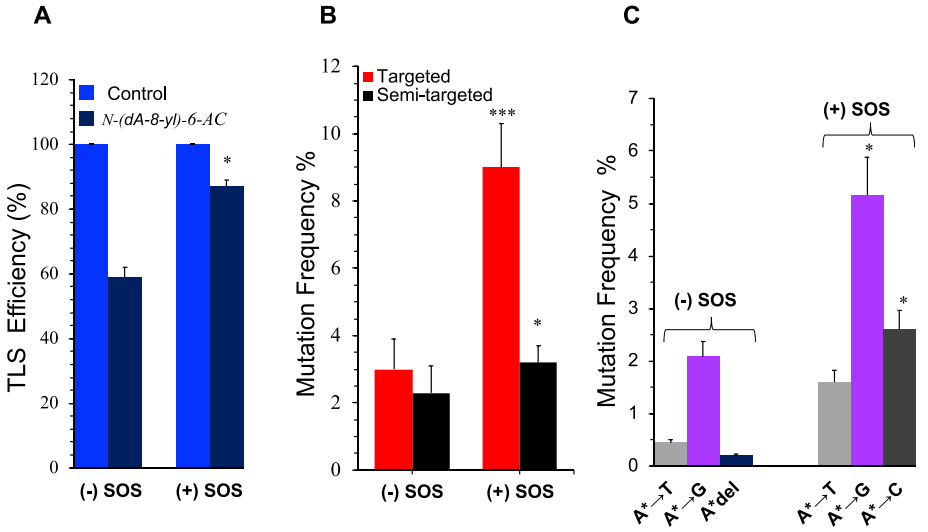

Figure 4. (A) Bypass efficiencies in *N*-(dA-8-yl)-6-AC construct in *E. coli* (−) SOS (WT) and (+) SOS cells. The data represent the means and standard errors of results from three independent experiments. (B) Frequencies of total targeted and semi-targeted mutations and (C) frequencies of specific types of targeted mutations induced in progeny from *N*-(dA-8-yl)-6-AC construct in (−) SOS (WT) and (+) SOS *E. coli* cells. The data represent the means and standard errors of results from three independent experiments. $^{*}P < 0.05$; $^{***}P < 0.001$. The *P*-values were calculated by using unpaired two-tailed Student's *t*-test.

mutations to 9% frequency, whereas semi-targeted mutation increased only marginally to 3.2% (Figure 4C and SI, Table S1). Although A*→G was also the prevalent (55%) targeted mutation with SOS, nearly half as many A*→C was detected.

Although the predominant conformation of *N*-(dA-8-yl)-6-AC is *syn*, it is not known if the same conformation is maintained in DNA. But many bulky polyaromatic moieties at the C8 position of dG in DNA predominantly rotate the base to *syn* conformation.[49] It was speculated that *syn* conformation of an adduct may be more repair-prone than others, but mutagenic relevance to these conformational preferences is significantly more complex.[49] Additional structural studies will be necessary to determine if the conformation of *N*-(dA-8-yl)-6-AC played a role in the observed mutagenesis. Even so, it is likely that the TLS polymerases are involved in the error-prone bypass of *N*-(dA-8-yl)-6-AC, as reflected in an increase in the targeted MF with SOS.

In conclusion, an efficient strategy for the synthesis of *N*-(dA-8-yl)-6-AC has been developed. The 6-aminochrysene moiety was introduced at the C8 position of dA using Buchwald−Hartwig palladium-catalyzed cross-coupling chemistry. This optimized strategy provided efficient and convenient access to this adduct. Using ROESY-NMR spectroscopy, we determined that the adducted purine prefers *syn* conformation of its glyosidic bond of *N*-(dA-8-yl)-6-AC in solution. The 3′-phosphoramidite of *N*-(dA-8-yl)-6-AC was used to site-specifically incorporate the dA-adduct into oligonucleotides by solid-phase DNA synthesis. The CD spectra of the 15-mer duplex were consistent with B-DNA, although it showed small deviations from the control duplex, suggesting a less structured helix. In terms of the biological effects of *N*-(dA-8-yl)-6-AC, it stalls DNA synthesis in *E. coli*, but TLS increased to ∼90% with SOS. The predominant mutation induced by *N*-(dA-8-yl)-6-AC in *E. coli* was A→G transitions, which increased significantly with SOS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.9b00429.

NMR and MS of the synthesized compounds, an autoradiogram of denaturing polyacrylamide gel electrophoresis of the modified oligonucleotide in comparison to their unmodified counterparts, and a table of TLS efficiency and MF (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

NO_2-PAH, nitropolycyclic aromatic hydrocarbons; 6-NC, 6-nitrochrysene; *N*-(dG-8-yl)-6-AC, *N*-(deoxyguanosin-8-yl)-6-aminochrysene; 5-(dG-N^2-yl)-6-AC, 5-(deoxyguanosin-N^2-yl)-6-aminochrysenene; *N*-(dA-8-yl)-6-AC, *N*-(deoxyadenosin-8-yl)-6-aminochrysene; AA, aristolochic acid; NER, nucleotide excision repair; PAH, polynuclear aromatic hydrocarbon; BINAP, *rac*-2,2′-bis(diphenylphosphino)-1,1′-binaphthyl; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; MF, mutation frequency; TLS, translesion synthesis

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