DNA adducts of aristolochic acid II: total synthesis and site-specific mutagenesis studies in mammalian cells

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ABSTRACT

Aristolochic acids I and II (AA-I, AA-II) are found in all Aristolochia species. Ingestion of these acids either in the form of herbal remedies or as contaminated wheat flour causes a dose-dependent chronic kidney failure characterized by renal tubulointerstitial fibrosis. In ~50% of these cases, the condition is accompanied by an upper urinary tract malignancy. The disease is now termed aristolochic acid nephropathy (AAN). AA-I is largely responsible for the nephrotoxicity while both AA-I and AA-II are genotoxic. DNA adducts derived from AA-I and AA-II have been isolated from renal tissues of patients suffering from AAN. We describe the total synthesis, de novo, of the dA and dG adducts derived from AA-II, their incorporation site-specifically into DNA oligomers and the splicing of these modified oligomers into a plasmid construct followed by transfection into mouse embryonic fibroblasts. Analysis of the plasmid progeny revealed that both adducts blocked replication but were still partly processed by DNA polymerase(s). Although the majority of coding events involved insertion of correct nucleotides, substantial misincorporation of bases also was noted. The dA adduct is significantly more mutagenic than the dG adduct; both adducts give rise, almost exclusively, to misincorporation of dA, which leads to AL-II-dA→T and AL-II-dG→T transversions.

INTRODUCTION

Various species of Aristolochia have been used as medicinal herbs since the time of Hippocrates to treat diverse disorders including snake-bite, fever, infection, gout, diarrhea and inflammation (1). A traditional use of this herb, as its Greek name implies, has been to assist women in childbirth (2). As part of a screening program for new anti-tumor agents, Kupchan and Doskovich (3) reported that aristolochic acid I (AA-I) (1; Figure 1), a principal chemical constituent of Aristolochia indica, was highly toxic to cells in culture; in addition, the compound proved to be nephrotoxic in Phase I clinical trials (4). Development of aristolochic acid as a drug was abandoned after Mengs reported its carcinogenicity in rodents (5). Earlier reports that Aristolochia sp. might be nephrotoxic in humans was dramatically confirmed in 1993 (6). Of more than 1800 Belgian women who had been given pills that contained, by error (7), Aristolochia fangchi as part of a slimming regimen, more than 100 women later developed chronic renal failure. Shortly thereafter, Cosyns and his colleagues (8) reported that these same patients also were at risk for urothelial carcinomas. The clinical syndrome was initially termed Chinese herbs nephropathy (CHN); later, it was suggested (9) that the generic term 'aristolochic acid nephropathy' (AAN) be used in place of CHN.

These observations drew attention to an endemic disease known as Balkan nephropathy (BEN), occurring exclusively in residents of farming villages in the Danube river basin (10). In a prescient report, Ivic (11) suggested that the origins of BEN might lie in the A. clematitis that grows in the wheat fields in the endemic region. Upper urinary tract carcinomas develop in approximately 50% of BEN cases, often associated with renal insufficiency (12,13). That the histopathology and clinical features of BEN are nearly identical to those of the disease reported in Belgium was recognized by Cosyns and co-workers (14). Since then, several groups have used AA-I or a mixture of AA-I and AA-II to reproduce the main features of AAN in rodents (15–17), removing any doubt that the aristolochic acids are responsible for CHN. In areas where BEN is endemic, A. clematitis

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

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grows in the wheat fields, and its seeds, which contain significant quantities of the AAs, co-mingle with wheat grain contaminating the flour used for home-baked bread (18). Although most residents of an endemic village are potentially exposed to the AAs, <10% suffer from BEN due to differences in exposure or to the fact that a subset of the populace is resistant to the effects of AAs due to individual genetic variation. Sato and his associates (16) reported significant differences in tissue responses to various AAs among various strains of mice, a finding confirmed by Shibutani et al. (17).

The genotoxicity of AAs is supported by the finding of AA-derived DNA adducts in renal cortex of humans (19,20). These adducts were identified as 3 and 4, derived from the aristolactam (AL) metabolite of AA-I, and the corresponding adducts 5 and 6, derived from the AL metabolite of AA-II (21). In humans with AAN, AL-dA adducts are invariably more abundant than AL-dG (22). Adducts arise by the same metabolic pathways as do other aromatic nitro compounds (23), in which the intermediate N-hydroxyamines (in the cases under discussion, the N-hydroxylactams 7 and 8 or their O-acetylated

![Structure of the aristolochic acids AA-I and AA-II, the dA and dG adducts of AL-I and AL-II and related compounds discussed in the text.](image-url)
or O-sulfonylated derivatives) are the likely pro-
carcinogens. Surprisingly, C-8 purine adducts are not
formed, and only products of attack at the exocyclic
amino groups of dA and dG have been detected. 
Recently Grollman and his group (19) have shown that
‘signature’ A:T→T:A mutations predominate in the p53
tumor-suppressor gene isolated from urothelial cancers
associated with BEN. However, the molecular mechanism
by which AA-I, but not AA-II, induces proximal tubule
damage remains a mystery.

Cases of AAN have been reported in China and in other
countries where herbal remedies are widely used (24), and
the disease has been described as a problem of global
dimensions (25,26). Studies by Schmeiser and his
associates (21), Nortier et al. (27) and more recently by
Grollman and coworkers (18,19,25) strongly support the
idea that the AAs play a causative role in the upper
urinary tract carcinomas in humans exposed to these
toxins. There is a pressing need for public health
authorities to take action to reduce human exposure to
this powerful nephrotoxic carcinogen (28). Recently, in
a comprehensive review of the subject the National
Toxicology Program has designated the aristolochic
aacids as established human carcinogens (24).

In this article we describe the total synthesis, in
quantity, of the dA and dG adducts derived from AA-II
(5 and 6, respectively), allowing not only their complete
chemical characterization but also their use as standards
for the identification of AL-DNA adducts in human
issues by mass spectrometric methods and for their
site-specific incorporation into oligomeric DNA of any
designated sequence. We discuss also some of the
difficulties associated with the chemistry of the AAs and
the reasons that we adopted a ‘total synthesis’ route to the
adducts. Finally, we present the results of site-specific
mutagenesis studies in mouse embryonic cells designed
to establish the mutagenic potential and specificity of
these lesions in vivo.

MATERIALS AND METHODS

All reagents and solvents employed in this experimental
work were reagent grade and were used as such unless
otherwise specified. Melting points were taken in a
Thomas-Hoover open capillary melting point apparatus
and are uncorrected. 1H NMR spectra were recorded
using an Electrospray ionization or a Micromass Platform mass spectrometer. Samples prepared for NMR analysis were
either on a Varian Gemini 300 or a Varian NOVA 400
spectrometer. Mass spectra were recorded on either a Thermo Electron
spectrometer. Samples prepared for NMR analysis were
dissolved in CDC13 or DMSO-d6. Chemical shifts are
reported in parts per million (ppm) relative to TMS. 
Mass spectra were recorded on either a Thermo Electron
DSQ GC/MS equipped with a solid probe inlet and EI ionization or a Micromass Platform mass spectrometer
using electrospray ionization. Thin-layer chromatography
(TLC) was performed on silica gel sheets (Tiedel-deHaën,
Sleeze, Germany). After appropriate purification all new
products showed a single spot on TLC analysis in two
solvent systems: (i) 30% EtOAc in hexanes and (ii) 5%
MeOH in CH2Cl2. Components were visualized by UV
light (λ = 254 nm) or by spraying with a solution of 2%
phosphomolybdic acid in ethyl alcohol containing 5%
sulfuric acid. Flash column chromatographic separations
were carried out on 60 Å (230–400 mesh) silica gel (TSI
Chemical Co., Cambridge, MA). All experiments dealing
with moisture or air-sensitive compounds were conducted
under dry nitrogen. The starting materials and reagents,
unless otherwise specified, were the best grade commer-
cially available (Sigma-Aldrich, Milwaukee, WI or Fluka
Chemie GmbH, Sigma-Aldrich, Germany) and were used
without further purification.

7-H-Furo[3',4':4,5]benzo[1,2-d][1,3]dioxol-5-one (15)

Cuprous cyanide (115.2 g; 1.286 mol) was added to
formamide (800 ml) containing water (19.2 g), and the
mixture was heated with stirring to 100 ºC. 2-Bromopiperonyl
alcohol 14 (147 g; 0.636 mol; mp 89–90 ºC) (29), easily
obtained by the bromination of piperonyl alcohol in
methanol at 25 ºC, was then added in portions over a
period of 10 min. The temperature of the mixture was
raised to 160 ºC, and a vigorous reaction set in, the tem-
perature rising spontaneously to 178 ºC with the evolution
of ammonia and steam. Over the next 30 min the temper-

ture subsided to 170 ºC and thereafter was maintained at
165–170 ºC for 2 h. The mixture was allowed to cool to
100 ºC, poured into a solution of sodium cyanide (192 g;
3.92 mol) in water (800 ml), stirred for 30 min followed by
the addition of CH2Cl2 (2 l). The mixture was filtered
through diatomaceous earth, the organic phase was
removed and the aqueous phase again was extracted with
CH2Cl2 (400 ml). The organic extracts were
combined and dried over MgSO4. Silica gel (25 g) and
charcoal (5 g) were added to the solution while the
drying agent was still present, the mixture was stirred
for 5 min then filtered, and the filter cake was washed
with boiling CH2Cl2 (200 ml). Removal of the solvent
from the filtrate left a yellow–orange colored residue of
 crude 15 (75.6 g; yield 66.8%), mp 185–187 ºC.
Recrystallization from EtOAc/CH2Cl2 gave
two crops, 55 g and 10 g, each as a pale yellow solid,
both with mp 190–191 ºC. Combined yield of pure
lactone 15 was 65 g (57.4%). 1H NMR (CDCl3) δ 5.22
(s, 2H), 6.16 (s, 2H), 6.87 (s, 1H), 7.26 (s, 1H).

4-Nitro-7H-furo[3',4':4,5]benzo[1,2-d][1,3]dioxol-5-one (16)

Concentrated sulfuric acid (320 ml) was cooled in
water/ice bath (20 ºC), and lactone 15 (63.5 g; 35.9 mmol)
was added in small portions with stirring over 10 min
while keeping the temperature <20 ºC. The solution was
cooled to 5 ºC, and concentrated (70%) nitric acid
(24.7 ml; 37.8 mmol) was added drop-wise over a period
of 30 min (exothermic) while maintaining the reaction
temperature <5 ºC (ice-MeOH bath). Stirring was
continued for 2 h at 7–10 ºC, then the mixture was
poured on to ice (2 kg). The resulting yellow precipitate
was removed by filtration and washed with water until free
of acid. The product was air dried and recrystallized from
EtOH to give 16 as a yellow solid (70.5 g; 88%); mp 186–
187 ºC. 1H NMR (DMSO-d6) δ 7.39 (s, 1H), 6.41 (s, 2H),
5.26 (s, 2H); electrospray mass spectrometry (ESI-MS)
(M + H)+ 224.1.
4-Amino-7H-furo[3',4':5,6]benzo[1,2-d][1,3]dioxol-5-one (17)

Nitro-lactone 16 (15 g, 67.3 mmol) was dissolved in DMF (120 ml), and 5% Pd/C catalyst (1.5 g) was added under nitrogen. The mixture was then shaken in a Parr hydrogenator at 60 psi overnight. The reaction mixture was then warmed to dissolve some precipitated product, and the catalyst was removed by filtration. The filtrate was concentrated under vacuum, and the residual liquid was poured into water. The resulting solid was collected and recrystallized from EtOH to give 17 as white crystals (12 g, 92%); mp 210–214°C; 1H NMR (DMSO-d$_6$) δ 6.36 (s, 1H), 6.04 (s, 2H), 5.88 (s, 2H), 5.11 (s, 2H); EI-MS M$^+$ 193.2.

4-Amino-8-bromo-7H-furo[3',4':5,6]benzo[1,2-d][1,3]dioxol-5-one (18)

Amino-lactone 17 (3.8 g, 19.8 mmol) was dissolved in dry pyridine (100 ml), and bis(N-methyl-2-pyrrolidinone)-hydrogen tribromide (8 g, 23.7 mmol) was added under a nitrogen atmosphere with magnetic stirring. After 15 h, TLC analysis (EtOAc: hexanes/3:7) showed complete absence of the starting material. The pyridine was removed under reduced pressure. The residue, after addition of CH$_2$Cl$_2$, was washed with 10% NaHCO$_3$ solution. The organic layer was washed with water then dried to give almost pure 18. Recrystallization from isopropyl ether gave the pure material (4.6 g, 86%); mp 238–239°C; 1H NMR (DMSO-d$_6$) δ 9.94 (s, 1H), 8.08–8.03 (m, 1H), 7.73–7.66 (m, 1H), 7.42–7.39 (m, 1H), 7.30 (s, 1H), 6.13–6.09 (d, 2H), 5.20–4.88 (dd, 2H); ESI-MS (M + H)$^+$ 283.3.

8-Bromo-7H-furo[3',4':5,6]benzo[1,2-d][1,3]dioxol-5-one (19)

Amino-lactone 18 (4.9 g, 18.08 mmol) was dissolved in concentrated HCl (100 ml), and the resulting solution was diluted with cold water (200 ml) then cooled to –5°C. To this mixture sodium nitrite solution (1.2 g/180 ml) was added dropwise while maintaining the internal temperature <0°C. After the addition was completed, the mixture was stirred for 2 h at 0°C, then cold hypophosphorous acid (30%) (68 ml) was added dropwise while maintaining the internal temperature at <0°C. Thereafter, stirring was continued for 2 h, then the mixture was held at 5°C overnight. The pale pink precipitate was removed by filtration, washed with cold water and dried to give the pure desired product 19 (4.3 g, 73.7%); mp 217–217°C; 1H NMR (DMSO-d$_6$) δ 7.27 (s, 1H), 6.28 (s, 2H), 5.19 (s, 2H); EI-MS (M + H)$^+$ 257.2.

2-(7-Oxo-5,7-dihydro-furo[3',4':5,6]benzo[1,2-d][1,3]dioxol-4-yl)-benzaldehyde (21)

A solution of bromolactone 19 (10 g, 39 mmol) in dioxane (150 ml) was degassed with nitrogen for 10 min and 1,1'-bis(diphenylphosphino)ferrocene)palladium dichloride catalyst (0.85 g) was added. Degassing was continued for an additional 10 min, then a solution of Na$_2$CO$_3$ (3.5 g) in water (120 ml; previously degassed with nitrogen) was added, and nitrogen was bubbled through reaction mixture for an additional 30 min. To this mixture 2-formylphenylboronic acid 20 (7.02 g, 46.8 mmol) was added, and the mixture was refluxed for 6 h after which TLC analysis (EtOAc: hexanes/3:7) showed the reaction to be complete. The mixture was cooled, diluted with EtOAc and then filtered. Solvent removal gave a solid residue which was dissolved in CH$_2$Cl$_2$, and the solution was washed with water then dried over anhydrous Na$_2$SO$_4$. The isolated product was purified by column chromatography over silica gel (elution with hexane:EtOAc:70:30) which afforded pure compound 21 (7 g, 63.7%); mp 145–146°C; 1H NMR (CDCl$_3$) δ 9.94 (s, 1H), 8.08–8.03 (m, 1H), 7.73–7.66 (m, 1H), 7.42–7.39 (m, 1H), 7.30 (s, 1H), 6.13–6.09 (d, 2H), 5.20–4.88 (dd, 2H), ESI-MS (M + H)$^+$ 283.3.

6H-Benzof[1,3]dioxololo[4',5':4,5]benzo[1,2,3-cd]fur-an-5-one:Aristolactone II (22)

The lactonic aldehyde 21 (7.3 g, 25.9 mmol) was dissolved in anhydrous THF, and anhydrous potassium t-butoxide (5.7 g) was added under nitrogen. The mixture was refluxed with stirring for 4 h, then cooled and the solvent removed under reduced pressure. The residue was taken up in MeOH (100 ml), acidified with 12 N HCl (20 ml) and the solution was refluxed for 1 h. After cooling and removal of the MeOH, the residue was dissolved in CH$_2$Cl$_2$, and the solution was washed with water (50 ml), then with saturated NaHCO$_3$ solution (50 ml), again with water (2 x 50 ml) and finally with brine (50 ml). The solution was dried over anhydrous Na$_2$SO$_4$, filtered and evaporated to dryness. The resulting yellow solid was purified by column chromatography on silica gel. Elution with 1% MeOH in CH$_2$Cl$_2$ gave the pure desired product AA-II lactone 22 (2.3 mmol), concentrated aqueous ammonium hydroxide (8 ml), sodium sulfite (450 mg) and ammonium chloride (350 mg) dissolved in water (1 ml) was heated in a sealed tube at 140°C overnight. The lactone went into solution at 110–115°C, then gradually, as the reaction proceeded, a solid separated. The mixture was cooled, filtered and the solid product was washed with water then dried to give an almost quantitative yield of pure AL-II (23); mp 297–298°C (32); 1H NMR (DMSO-d$_6$) δ 10.72 (s, 1H), 8.60–8.58 (d, 6 Hz, 1H), 7.94–7.29 (d, 6 Hz, 1H), 7.61 (s, 1H), 7.60–7.58 (m, 2H), 7.09 (s, 1H), 6.46 (s, 1H) essentially identical with the published spectrum (32); ESI (M + H)$^+$ 264.3.
7-Bromo-6H-benzo[[f][1,3]dioxolo[4',5':4,5]benzo[1,2,3-cd]indol-5-one (11)

To a solution of 23 (1.5 g, 5.7 mmol) dissolved in glacial HOAc (10 ml) and cooled in an ice bath was added anhydrous NaOAc (500 mg, 6 mmol) followed by the drop-wise addition of bromine (1.18 g) in HOAc (5 ml). After the addition was complete, the reaction mixture was stirred for 15 min and filtered. The collected solid was washed with CH₂Cl₂, then water and dried to give compound 11 (1.9 g, quantitative) mp 301–302°C. This was virtually insoluble in any of the usual organic solvents and very sparingly soluble in hot DMSO. ¹H NMR (hot DMSO-d₆) δ 11.19 (s, 1H), 8.61–8.59 (d, 6 Hz, 1H), 7.82–7.80 (m, 3H), 6.52 (s, 2H); EI-MS M⁺ 341.2.

7-Bromo-6-(tert-butyl-dimethylsilanyloxymethyl)-6H-benzo[[f][1,3]dioxolo[4',5':4,5]benzo[1,2,3-cd]indol-5-one (24)

To a solution of sodium hydride (120 mg, 3.2 mmol; 60% suspended in mineral oil) in dry DMF (20 ml), AA-II bromolactam (11) (900 mg, 2.6 mmol) was added, and the mixture was heated at 50°C for 30 min under a nitrogen atmosphere. After cooling to ~5°C, freshly prepared (33) tert-butylchloromethoxydimethylsilane (0.5 g, 3 mmol) was added by syringe. The ice bath was removed after 10 min, and the reaction mixture was stirred at 24°C for 0.5 h when TLC analysis (EtOAc:hexanes/2:8) showed completion of the reaction. The DMF was removed under reduced pressure, and the residue taken up in CH₂Cl₂ (50 ml). This solution was washed with water, dried over anhydrous Na₂SO₄, and the solvent was removed under reduced pressure to give the crude product. This was purified by column chromatography (EtOAc: hexanes/2:8) on silica gel to afford the pure desired N-protected lactam 24 (800 mg, 63%). mp 199–200°C; ¹H NMR (CDCl₃) δ 8.62 (dd, 6H, 1H), 8.5 (dd, 6H, 1H), 7.79 (m, 2H), 7.6 (s, 1H), 6.01 (s, 2H), 5.95 (s, 2H), 0.0 (s, 9H), 5.95 (s, 6H); ESI-MS (M + H)⁺ 815.5.

7-[9-(4-Terbutyl-dimethyl-silanyloxy)-5-(tert-butyl-dimethylsilanyloxymethyl)-tetrahydro-furan-2-yl]-1,9-dihydro-purin-6-ylideneaminol-6H-benzo[[f][1,3]dioxolo[4',5':4,5]benzo[1,2,3-cd]indol-5-one (27)

An oven-dried 100 ml two-necked flask was charged with the protected dA 25 (920 mg, 1.9 mmol), cesium carbonate (1.82 g, 5.6 mmol), trisdibenzylideneacetone dipalladium catalyst (220 mg, 0.24 mmol), xantphos (460 mg, 0.79 mmol), the protected 7-bromo AL-II (24, 465 mg, 0.96 mmol) and toluene (30 ml). This mixture was stirred under a nitrogen atmosphere for 3 h and then concentrated under reduced pressure. To the residue was added aqueous 10% NaHCO₃ (25 ml) and stirred for 15 min and filtered. The collected solid was washed with water and then added to THF (10 ml) and concentrated aqueous ammonia (5 ml). This mixture was heated at 70°C in a closed vial overnight, then taken to dryness, and the remaining solid was chromatographed over silica gel using 7.5% MeOH in CH₂Cl₂ to elute the pure protected adduct 5 (0.35 g; 84%) whose UV absorbance spectrum was identical to that published by Pfau et al. (34). ¹H NMR (DMSO-d₆) δ 8.80 (s, 1H), 8.4 (br s, 1H), 8.70 (m, 2H), 8.41 (s, 1H), 8.00 (m, 1H), 7.55 (m, 3H), 6.59 (s, 2H), 6.49 (t, 1H), 5.19 (br s, 1H), 4.49 (m, 1H), 3.39 (br s, 1H), 3.75–3.51 (m, 2H), 3.21 (m, 1H), 2.83 (m, 1H); ESI-MS (M + H)⁺ 513.3.

7-[9-(5-(Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl)-4-hydroxy-tetrahydro-furan-2-yl)-1,9-dihydro-purin-6-ylideneamino]-6H-benzo[[f][1,3]dioxolo[4',5':4,5]benzo[1,2,3-cd]indol-5-one (28)

To a solution of 26 (700 mg, 0.81 mmol) in pyridine (15 ml) was added 70% HF in pyridine (2 ml) over a period of 3 min. The mixture was stirred at room temperature overnight, then poured into aqueous 10% NaHCO₃ (50 ml) and stirred for 2 h. The solid that precipitated was collected, washed with water and then added to THF (10 ml) and concentrated aqueous ammonia (5 ml). This mixture was heated at 70°C in a closed vial overnight, then taken to dryness, and the remaining solid was chromatographed over silica gel using 7.5% MeOH in CH₂Cl₂ to elute the pure deprotected adduct 5 (0.35 g; 84%) whose UV absorbance spectrum was identical to that published by Pfau et al. (34). ¹H NMR (DMSO-d₆) δ 10.80 (s, 1H), 9.83 (br s, 1H), 8.70 (m, 2H), 8.41 (s, 1H), 8.00 (m, 1H), 7.55 (m, 3H), 6.59 (s, 2H), 6.49 (t, 1H), 5.19 (br s, 1H), 4.49 (m, 1H), 3.39 (br s, 1H), 3.75–3.51 (m, 2H), 3.21 (m, 1H), 2.83 (m, 1H); ESI-MS (M + H)⁺ 513.3.
dissolved in dry CH2Cl2 (10 ml). Tetrazole (11.3 mg, 0.16 mmol) was added to the solution followed by 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (57 mg, 0.19 mmol). The reaction mixture was stirred at room temperature overnight, poured into pyridine (1.7 ml) over a period of 3 min. The mixture was stirred at room temperature overnight, poured into pyridine (8 ml) was added solid DMT chloride (200 mg; 0.59 mmol), and the mixture was stirred at room temperature for 3 h. The mixture was filtered through a pad of Celite, and the collected solids were washed with EtOAc. After vacuum evaporation of the solvent, the residue was purified by chromatography over silica gel. Elution with 5% EtOAc in CHCl3 afforded pure compound 28 as a solid (703 mg, 75%). 1H NMR (CDCl3): δ 8.75 (d, 1H), 8.13 (d, 2H), 8.04 (br s, 1H), 7.70 (s, 1H), 7.60 (m, 2H), 7.09–7.18 (m, 5H), 6.42 (s, 2H), 6.29 (t, 1H), 5.93 (s, 1H), 5.19 (s, 3H), 4.42 (m, 1H), 3.98 (m, 1H), 3.78 (m, 2H), 2.62 (m, 1H), 2.27 (m, 1H), 1.05–0.95 (m, 27H), 0.31–0.12 (m, 18H); ESI-MS (M + H)+ 991.5.

7-{6-Benzoyloxy-9-[4-(tert-butyldimethylsilyloxy)-5-(tert-butyldimethylsilyloxy)methyl]-tetrahydrofuran-2-yl}-6H-purin-2-ylamino)-6H-benzof][1,3]dioxolo[4,5-c,d][indol-5-one (30)

A dry 100 ml two-necked flask was charged with the fully protected dG derivative 29, (418 mg; 0.71 mmol), cesium carbonate (0.785 g, 2.4 mmol), 2-(4-Methoxyphenyl)phenylmethoxymethyl dipalladium catalyst (164 mg, 0.18 mmol), xantphos (343 mg, 0.59 mmol), compound 24 (450 mg, 0.93 mmol) and toluene (25 ml) under a nitrogen atmosphere. This mixture was stirred for 30 min at room temperature and then heated at 100°C for 6 h. After cooling to room temperature, the mixture was filtered, and the collected solids were washed with EtOAc. After vacuum evaporation of the solvent, the residue was purified by chromatography over silica gel. Elution with 5% EtOAc in CHCl3 afforded pure compound 30 as a solid (673 mg, 75%). 1H NMR (CDCl3): δ 8.75 (d, 1H), 8.13 (d, 2H), 8.04 (br s, 1H), 7.70 (s, 1H), 7.60 (m, 2H), 7.09–7.18 (m, 5H), 6.42 (s, 2H), 6.29 (t, 1H), 5.93 (s, 1H), 5.19 (s, 3H), 4.42 (m, 1H), 3.98 (m, 1H), 3.78 (m, 2H), 2.62 (m, 1H), 2.27 (m, 1H), 1.05–0.95 (m, 27H), 0.31–0.12 (m, 18H); ESI-MS (M + H)+ 991.5.

7-{6-Benzoyloxy-9-[4-(4-hydroxy-5-hydroxymethyl-tetrahydrofuran-2-yl)-9H-purin-2-ylamino]-6H-benzof][1,3]dioxolo[4,5-c,d][indol-5-one (31)

To an ice-cold solution of compound 30 (530 mg, 0.53 mmol) in pyridine (10 ml) was added 70% HF in pyridine (1.7 ml) over a period of 3 min. The mixture was stirred at room temperature overnight, poured into ice-cold aqueous 10% NaHCO3 solution and again stirred for 2 h. The separated solids were filtered, washed with water and added to THF (10 ml) and concentrated ammonia (5ml). The mixture was then heated overnight at 70°C in a closed vial. The solvents were removed by vacuum evaporation, and the residue was purified chromatographically over silica gel. Elution with CH2Cl2/MeOH (9:1) gave pure coupled product 31 as a glassy solid (300 mg, 91%). 1H NMR (DMSO-d6): δ 10.62 (s, 1H), 9.21 (s, 1H), 8.98 (br s, 1H), 8.19 (s, 1H), 8.05 (m, 1H), 7.79 (m, 1H), 7.59 (m, 2H), 7.02–7.18 (m, 5H), 6.45 (s, 2H), 6.20 (t, 1H), 5.20 (br s, 1H), 4.79 (br s, 1H), 4.20 (m, 1H), 3.76 (m, 2H), 2.60 (m, 1H), 2.19 (m, 1H); ESI-MS (M + H)+ 619.4.

7-{9-(4-Hydroxy-5-hydroxymethyl-tetrahydrofuran-2-yl)-6-oxo-6,9-dihydro-1H-purin-2-ylamino]-6H-benzof][1,3]dioxolo[4,5-c,d][indol-5-one (6)

To a solution of 31 (50 mg, 0.08 mmol) in methanol (50 ml) was added a 10% palladium-on-carbon catalyst. The flask was evacuated (50 torr) and flushed with hydrogen thrice. The reaction mixture was hydrogenated for 16 h at 50 psig with stirring. It was then filtered through a pad of Celite, the Celite bed was washed with DMF (20 ml), and the filtrate was concentrated under reduced pressure. The residue was diluted with water (20 ml); the separated solids was filtered and dried in a vacuum oven overnight (26 mg, 78%). 1H NMR (DMSO-d6): δ 12.04 (br s, 1H), 10.98 (br s, 1H), 10.82 (br s, 1H), 8.61 (d, 1H), 8.02 (m, 1H), 7.93 (s, 1H), 7.67–7.61 (m, 3H), 6.50 (s, 2H), 6.22 (s, 1H), 5.83 (t, 1H), 5.05 (m, 1H), 4.59 (br s, 1H), 3.98 (br s, 1H), 3.59 (m, 1H), 3.17 (br s, 1H), 2.44 (m, 1H), 1.99 (m, 1H); MALDI-MS (M + H)+ 529.1.

7-{6-Benzoyloxy-9-[5-{bis(4-methoxyphenyl)phenylmethoxymethyl]-4-hydroxy-tetrahydrofuran-2-yl}-9H-purin-2-ylamino]-6H-benzof][1,3]dioxolo[4,5-c,d][indol-5-one (32)

To a solution of compound 31 (225 mg, 0.36 mmol) in pyridine (8 ml) was added solid DMT chloride (200 mg; 0.59 mmol), and the mixture was stirred at room temperature for 3 h. TLC (CH2Cl2/MeOH 92:8) showed the reaction to be ~90% complete. The product was then worked up as in the case of 27, noted above, and purified by chromatography on silica gel. Elution with 5–10% MeOH in CH2Cl2 gave the pure DMT-protected compound 32 as a colorless glass (190 mg, 57% based on unrecovered 31; recovered 31 amounted to 99 mg). 1H NMR (CDCl3): δ 9.62 (br s, 1H), 8.60 (s, 1H), 7.90 (d, 1H), 7.83 (s, 1H), 7.56 (m, 2H), 7.38 (m, 5H), 7.28–7.12 (m, 11H), 6.77 (m, 4H), 6.36 (t, 1H), 6.22 (s, 2H), 5.48 (s, 2H), 4.55 (br s, 1H), 4.23 (m, 1H), 3.73 (s, 6H), 3.20 (m, 2H), 2.62 (m, 2H); ESI-MS (M + H)+ 921.3.

7-{9-[5-{bis(4-Methoxyphenyl)phenylmethoxymethyl]-4-hydroxy-tetrahydrofuran-2-yl}-6-oxo-6,9-dihydro-1H-purin-2-ylamino]-6H-benzof][1,3]dioxolo[4,5-c,d][indol-5-one (33)

To a solution of compound 32 (280 mg, 0.30 mmol) in EtOAc/MeOH (1:1, 20 ml) was added 10% Pd/C catalyst (50 mg). The flask was evacuated (50 torr), flushed thrice with hydrogen and then shaken under hydrogen for 16 h at 50 psi. The resulting solution was filtered through a pad of celite and concentrated under reduced pressure. The residue was purified by silica gel column chromatography. Elution with 7–10% MeOH in CHCl3 afforded pure debenzylated product 33 (220 mg, 91%). 1H NMR (CDCl3): δ 12.40 (br s, 1H), 10.62 (br s, 1H), 9.82 (br s, 1H), 8.49 (m, 1H), 8.02 (m, 1H), 7.66 (s, 1H), 7.59 (m, 1H), 7.27 (m, 6H), 6.90 (m, 5H), 6.86...
Diisopropylphosphoramidic acid, 2-[bis(4-methoxyphenyl)phenylmethoxymethyl]-5-[6-oxo-2-(5-oxo-5,6-dihydrobenzo[f][1,3]dioxolo[4,5,6]-benzol[1,2-c]indol-7-ylamino)-1,6-dihydroquin-9-yl]tetrahydrofuran-3-yl ester-2-cyano-ethyl ester (34)

The DMT derivative 33 (180 mg, 0.22 mmol) was co-evaporated with dry toluene (3 × 10 ml), and the residue was dissolved in dry CH2Cl2 (10 ml). Tetrazole (18.2 mg, 0.26 mmol) was added, followed by 2-cyanoethyl N,N,N′,N′-tetraisopropylphosphordiamidite (91.6 mg, 0.3 mmol), and the reaction mixture was stirred at room temperature for 2 h under nitrogen after which CH2Cl2 containing 2% TEA (25 ml) was added. This solution was then washed with aqueous saturated NaHCO3 solution (50 ml), dried over Na2SO4, filtered and concentrated under reduced pressure to yield the desired product 34 (245 mg, 100% yield) as a viscous oil. This was used directly in the synthesis of DNA oligomers. 1H NMR (CDCl3): δ 12.38 (br s, 1H), 10.62 (br s, 1H), 9.89 (br s, 1H), 8.62 (m, 1H), 8.42 (m, 1H), 8.02 (m, 1H), 7.99 (s, 1H), 7.82 (m, 2H), 7.42 (m, 2H), 7.40–7.05 (m, 9H), 6.66 (m, 4H), 6.37 (s, 2H), 6.22 (m, 1H), 5.60 (m, 1H), 4.62 (m, 1H), 4.02 (m, 2H), 3.83 (s, 6H), 2.97 (m, 2H), 2.60–2.31 (m, 4H), 1.07 (m, 12H); 31P NMR (CDCl3): 149.5, 149.4.

DNA synthesis

All oligomers were synthesized at the 1.0 µmol scale using an Applied Biosystems 394 DNA Synthesizer (Foster City, CA) with normal phosphoramidite reagents (Glen Research). Individual oligomers were liberated from the controlled pore glass (CPG) support by treatment with aqueous 28% ammonia at 55°C overnight which also removed all of the nucleobase-protecting groups. Purification of the DNA was accomplished in two stages. In the first the deprotected oligomers having a terminal DMT group were separated from failure stages. In the first the deprotected oligomers having a terminal DMT group were separated from failure stages. In the first the deprotected oligomers having a terminal DMT group were separated from failure stages. In the first the deprotected oligomers having a terminal DMT group were separated from failure stages. Purification of the DNA was accomplished in two stages. In the first the deprotected oligomers having a terminal DMT group were separated from failure stages. In the first the deprotected oligomers having a terminal DMT group were separated from failure stages. In the first the deprotected oligomers having a terminal DMT group were separated from failure stages.

Introduction of the modified plasmid into mouse embryonic fibroblasts and recovery of the progeny plasmid

Immortalized mouse embryo fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (100 µg/ml) under 5% CO2 at 37°C. Cells (1 × 10⁶) were plated in a 25-cm² flask and cultured overnight after which they were transfected overnight with 500 ng of a modified construct by the FuGENE6 (Roche) method according to the manufacturer’s instruction. The next day, cells were detached by treating with trypsin–EDTA, seeded in a 150-cm² flask and cultured for 4 days. Progeny plasmids were recovered by the method of Hirt (37) and analyzed for translesional events as described below.

Analysis of progeny plasmid for translesion events

To the recovered plasmids, 5 ng of pVgRXR (Invitrogen), which coded for zeocin resistance, was added. This plasmid served as an internal control for DpnI digestion. The mixture was treated with Dpn I (1 unit) for 1 h at 37°C to remove residual nonreplicated input DNA and then used to transform Escherichia coli DH10BMax electrocompetent cells (Invitrogen) by an E. coli Pulser (Bio Rad). Varying portions of a transformation mixture were plated on YT (1×) agar plates with ampicillin (100 µg/ml medium) and blasticidin S (50 µg/ml medium) or with zeocin (25 µg/ml medium). Since the adduct was located close to the blasticidin S resistance gene (36), transformants containing progeny plasmids with large deletions around the adduct site should not grow on a blasticidin-containing plate and were thus excluded from analysis. A marked reduction in the number of colonies on a zeocin-containing plate assured efficient digestion of nonreplicated plasmids by DpnI. E. coli transformants
RESULTS AND DISCUSSION

The chemistry of AA-I and AA-II and total synthesis of the dA and dG adducts 5 and 6 derived from AA-II

AA-I (1) and AA-II (2) (Figure 1) occur together naturally in many Aristolochia species and are commercially available as a mixture. However, they cannot be separated cleanly by crystallization or by simple column chromatography, and their separation by HPLC is an extremely tedious procedure applicable only to small quantities. By contrast, the corresponding methyl esters (formed by the action of diazomethane) can be separated chromatographically, albeit with difficulty, but when basic hydrolysis is attempted, <6% of the acid is recovered (39). This unusual chemistry is a reflection of the steric strain—the aromatic version of 1,3-allylic strain (40)—that exists between the nitro and carboxyl functions and parallels identically the group compression that exists in 8-nitronaphthoic acid 9. The latter acid cannot be esterified by the standard Fischer–Speier method, and its methyl ester (formed by diazomethane) undergoes hydrolysis extremely slowly under forcing conditions (41). Similarly, attempts to form the acid chloride (SOCl2) of 9 leads to 8-chloronaphthoyl chloride and other decompositions (42). Based on such aberrant chemistry, the possibility of obtaining multi-gram quantities of the individual AAs from natural sources seemed limited. We elected, therefore, to attempt to develop a new synthetic approach that would not only allow us to obtain the desired DNA adducts in quantity, but also would permit the synthesis of a wide range of AA-related substances potentially including the AAs themselves. The synthesis of AA-I had previously been accomplished by Kupchan and Wormser (43), but the method is impracticable; too many steps are involved, yields at some stages are low, the route lacks versatility and the final ester hydrolysis is extremely low-yielding as noted above. In addition the route did not seem practical for the synthesis of the 7-bromolactams 10 and 11, which we saw as the critical intermediates for the synthesis of the corresponding DNA adducts. Satisfactory synthetic routes to this class of lactam have been published by Estevez et al. (44,45) and by Couture and associates (46). The first method by the Estevez group involves a rather low-yielding benzene cyclization step, whereas both of the other approaches involve the linking of the B and D rings using a tributyltin hydride radical reaction as a critical step in the synthesis of the basic phenanthrene. In addition the latter approach utilizes an isoidoleneone intermediate (12) which is always obtained as a mixture of geometric isomers that have to be separated before conversion to the required penultimate lactam (13) is possible. These methods are suitable for the preparation of the aristolactams but not for the synthesis of the aristolochic acids themselves, one of the ultimate goals of our research. The approach that we have taken involves reversing the order of the assembly, first by
The key intermediate in the early phase of the synthetic work is the bromolactone 19, which was prepared according to Figure 3. Phthalide 15 had previously been prepared by a multi-step process (30), but in our hands was accomplished in a single step by heating 14 with cuprous cyanide at 160°C in 3% aqueous formamide. This has the advantages of (i) giving 15 directly without having to isolate the intermediate nitrile and (ii) avoiding contamination of the product with solvent by using formamide instead of DMF as the reaction medium (formamide has virtually no solubility in non-polar organic solvents). Surprisingly perhaps, electrophilic reactions of 15 lead dominantly to substitution at the 7-position (ortho to the carbonyl group), thus blocking a one-step conversion to 19. Thus, we followed literature methods that are based largely on the work of Manske and co-workers (47) in the parallel veratrole series but with modifications which made the overall route relatively efficient and manageable on a larger scale. Nitrination of 15 gave 16 (88%) which on catalytic reduction led to 17 (92%). The latter, on bromination with bis(N-methylpyrrolidin-2-one) hydrogen tribromide (48), led to compound 18 (86%), which then was converted to the desired bromolactone 19 (74%) by a standard diazotization/deamination procedure. The overall yield for the conversion of 14 to 19 was 37%, acceptable for further large-scale work.

The synthesis of the required bromolactam 11 was accomplished according to Figure 4. Coupling of the bromolactone 19 with the commercially available boronic acid 20, under Suzuki coupling conditions, afforded the biphenyl derivative 21 in 64% yield, and the latter, under the influence of potassium t-butoxide in boiling t-butanol, led to the lactonic phenanthrene 22 in 55% yield. This was converted by means of the Bucherer reaction (49) to AL-II (23: ~100%), identical in all respects with the natural product (32). Bromination of 23 then led smoothly to a quantitative yield of the desired but extremely insoluble bromolactam 11. The absence of absorption at or near 7.09 ppm in the 1H NMR spectrum of 11 (in hot DMSO-d6), characteristic of the 7-hydrogen atom in AL-II (23), confirmed that bromination had occurred at this location.

In order to couple 11 with the protected forms 25 and 29, respectively, of dA and dG (Figures 5 and 6) under Buchwald–Hartwig conditions (50), the nitrogen of the lactam ring needed to be protected by a group that not only would block any possible reaction at this site during the coupling process, but would also provide increased solubility in common solvents and be easily removable without causing collateral damage once coupling was complete. After more than a dozen attempts to accomplish this with a series of well-established protecting groups, success was finally achieved with the little-used t-butyldimethylsilyloxyethylmethyl (TBDMSOM) protecting group (51). Treatment of the bromolactam 11 with sodium hydride in dry DMF at 50°C followed by the addition of t-butyl chloromethylmethyldimethylsilyl at 0°C for 30 min gave, after chromatographic purification,
Figure 4. Synthetic scheme for the preparation of 7-bromo-6-(tetrabutyl(dimethyl)silyloxy)methyl)-6H-benzo[b][1,3]dioxolo[4',5',4,5]benzo[1,2,3-cd]indol-5-one (24). Reagents: (a) PD(dpfp)Cl₂/K₂CO₃; (b) KOBu°; (c) NaHSO₃/NH₃; (d) Br₂; (e) TBDMSOCH₂Cl/NaH/DMF and (f) HF/Pyr, then NH₃/H₂O.

Figure 5. Synthetic scheme for the preparation of AL-II-dA (5) and its 5'-DMT protected phosphoramidite (28). Reagents: (a) PdCl₂/Xantphos; (b) HF/Pyr then NH₃/H₂O; (c) DMTCl/Pyr and (d) CIP(OCH₂CH₂CN)N(i-Pr)₂/Triazole.
a 63% yield of the easily soluble, protected lactam 24. This compound when coupled (Figures 5 and 6) with either 25 or 29 under Buchwald–Hartwig conditions (50) using Xantphos as the palladium-chelating agent gave excellent yields of the protected forms 26 and 30 of the dA and dG adducts 5 and 6, respectively, of AL-II. Deprotection of the silyl-protecting groups was accomplished in two steps. Treatment with HF in pyridine removes all of the TBDMS groups but leaves a hydroxymethyl residue on the lactam nitrogen. This residue was then easily removed by heating with aqueous ammonia to give 5 in the case of 26, and 31 in the case of 30. Catalytic hydrogenolysis of the benzyl group in 31 then led to 6 almost quantitatively. The UV absorption spectrum of 5 proved to be identical with that of the published spectrum (34). Also, the $^1$HNMR spectral data of 5 was identical to those already published (34), and in agreement it contained a peak at 9.83 that was assigned by previous workers to the hydrogen at the 1 position of the purine ring. Surprisingly, however, no evidence for geometrical isomerism was noted at the N$^\text{6}$ position.

Although it proved possible to convert 5 smoothly to its DMT derivative (27) and subsequently to the desired phosphoramidite 28 by standard procedures, insolubility problems plagued adduct 6 and its 5'-O-DMT derivative could not be prepared directly. This difficulty was overcome by introducing the DMT group at an earlier stage. Reaction of 31 with DMT chloride in pyridine afforded the much more soluble 5'-O-DMT derivative 32. Removal of the benzyl group from 32 by catalytic

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**Figure 6.** Synthetic scheme for the preparation of AL-II-dG (6) and its 5'-DMT protected phosphoramidite (34). Reagents: (a) PdCl$_2$/Xantphos; (b) HF/Pyr then NH$_3$/H$_2$O; (c) DMTCl/Pyr; (d) Pd/H$_2$ and (e) CIP(OCH$_2$CH$_2$CN)/N(i-Pr)$_2$/Triazole.
hydrogenolysis followed by phosphoramidite formation then afforded the required compound.

Table 1. Sequence and mass data for the synthesized DNA oligomers

| Entry | Sequence          | Calc Mass (Da) | Meas Mass (Da) |
|-------|-------------------|----------------|----------------|
| 1     | 5’-CTC TCT A*AT ACC T-3’ | 4091           | 4090.2         |
| 2     | 5’TTC CCT CCA GAA A*CA TCC T-3’ | 5929           | 5927.3         |
| 3     | 5’-CCA TTC ACA CA*A TCC-3’ | 4702           | 4701.1         |
| 4     | 5’TIT TTA* TIT T-3’ | 3251           | 3250.3         |
| 5     | 5’-CTT TCA* CTT CTT TCC TCT CCC TTT-3’ | 7345           | 7344.2         |
| 6     | 5’TCT TCT TCT GTG GA*C TCT TCT TCT-3’ | 7439           | 7439.7         |
| 7     | 5’TCT TCT TCT GCA* GAC TCT TCT TCT-3’ | 7448           | 7447.9         |
| 8     | 5’-CGT ACG* CAT GC-3’ | 3579           | 3577.6         |
| 9     | 5’TIT* TIT-3’ | 2050           | 2048.9         |
| 10    | 5’-CTC CTC G*AT ACC T-3’ | 4107           | 4106.1         |
| 11    | 5’TTC CCT CCA GAA G8CA TCC T-3’ | 5945           | 5944.0         |
| 12    | 5’-CAA TTC ACA CG*A TCC-3’ | 4718           | 4717.6         |
| 13    | 5’TCT TCT TCT GCG*TAC TCT TCT TCT-3’ | 7439           | 7438.6         |
| 14    | 5’TCT TCT TCT GTG* CAC TCT TCT TCT-3’ | 7439           | 7436.9         |

A*, AL-II-dA; G*, AL-II-dG.

Synthesis of DNA oligomers containing adducts 5 and 6

Both DMT-phosphoramidites (28 and 34) were used successfully in the synthesis of a series of oligomers. Table 1 contains the sequences and masses obtained by ESI/MS for the oligomers containing respectively the xenonucleosides AL-II-dA (entries 1–7) and AL-II-dG (entries 8–14) adducts. All these oligomers were synthesized at the 1.0 μmol scale on an Applied Biosystems 394 DNA Synthesizer (Foster City, CA). In all cases the coupling time was 15 min for the modified deoxynucleoside phosphoramidites, and coupling efficiencies at the point of introduction varied from 93 to 98%. To verify that the modified deoxynucleosides were incorporated without further modification by reagents during DNA synthesis, two HPLC-purified oligomers were digested enzymatically to the deoxynucleosides using previously published procedures (35). The first was entry #5 in Table 1; the second was the same sequence in which the AL-II-dA was replaced by AL-II-dG. Products for both reactions were analyzed by LC/ESI/MS/MS, and in both cases the retention time and the MS/MS spectrum for the modified deoxynucleoside matched that for the synthetic standard (data not shown) indicating that AL-II-dA and AL-II-dG were stable to the conditions of DNA synthesis and were present in the oligomers.

Blocking of DNA synthesis in cells

For the biological experiments, lesions were positioned in the middle of three consecutive base mismatches. This made it possible to determine the number of progeny plasmids derived from modified and unmodified strands; the ratio of progeny reflects the degree to which DNA synthesis is blocked. In the absence of blocking, the ratio should be 50:50, as revealed with a construct containing three base mismatches without a lesion (52). DNA repair (removal of a DNA lesion and the two flanking mismatches followed by gap-filling synthesis) converts the three nucleotide sequence of the modified strand to the sequence complementary to the unmodified strand, thus losing the strand tag. Thus, DNA repair could influence the apparent blocking effect of a DNA adduct in experiments using repair-proficient MEFs. This possibility should be considered in determining the ratio of progeny. Nevertheless, translesion DNA synthesis (TLS) occurred, giving rise to a progeny plasmid from the modified strand. Both adducts block DNA synthesis strongly. When fractions of progeny for the AL-II-dG and AL-II-dA adducts were compared, the dA adduct yielded about half of the progeny produced by the dG adduct. This suggests that the dA adduct is more effective at blocking DNA synthesis than is the dG adduct.

M miscoding properties of the two adducts

In MEFs, the major coding events were the insertion of the correct nucleotides opposite the adducts: T and dC for the dA and dG adducts, respectively. However, substantial frequencies of miscorocation were observed for both adducts; 22% for the dA adduct and 9% for the dG adduct. The nucleotide mis-inserted opposite both adducts was almost exclusively dA, leading to AL-II-dG!dA transitions. In general, our site-specific mutagenesis results for the dA adduct are in accord with the findings obtained in the in-vitro system and other studies in cells, animals and humans (19,24,54–56). However, we find that the dG adduct is less miscoding than is the dA adduct in cells (Table 2).
lesions in vivo establish the mutagenic potential and specificity of these mutagenesis studies in mouse embryonic cells designed to test AL-II-dA and AL-II-dG adducts derived from AA-II and, after facile conversion to the 5′-dimethoxytrityl-protected phosphoramidites, have incorporated these adducts into DNA oligomers using automated synthesis techniques. Adducts were chemically stable to the conditions of oligomer synthesis and were isolated intact from selected oligomers by enzymatic digestion. After rigorous HPLC purification, DNA oligomers isolated intact from selected oligomers by enzymatic digestion and MS/MS analysis of two synthetic oligomers.

**Table 2.** Translesional events induced by a site-specific AL-II-dA and AL-II-dG adducts in mouse cells

| DNA adduct | No. of progeny from | Nucleotide inserted opposite adduct | MF (%) | Others |
|------------|---------------------|-------------------------------------|--------|--------|
| AAII-dA    | UMS: 429 (95)       | T                                   | 22     | 53     |
|            | MS: 22 (5)          | A                                   | 1 (0.4)| 25 (8.8)|
| AAII-dG    | UMS: 416 (91)       | C                                   | 0      | 257 (90.8)|
|            | MS: 41 (9)          | G                                   | 0      | 9      |

*Numbers were determined following removal of progeny derived from the unmodified strand by EcoRV treatment.

MF, misincoding frequency.

UMS, unmodified strand; MS, modified strand; numbers were determined before removal of progeny derived from the unmodified strand by digesting with EcoRV.

The numbers in parentheses represent percentages.

SUMMARY

The aristolochic acids I and II have been implicated in the development of urothelial cancer via DNA adduction of their metabolites. We have developed a method for the large-scale synthesis of the dA and dG adducts derived from AA-II and, after facile conversion to the 5′-dimethoxytrityl-protected phosphoramidites, have incorporated these adducts into DNA oligomers using automated synthesis techniques. Adducts were chemically stable to the conditions of oligomer synthesis and were isolated intact from selected oligomers by enzymatic digestion. After rigorous HPLC purification, DNA oligomers containing dA or dG adducts were used for site-specific mutagenesis studies in mouse embryonic cells designed to establish the mutagenic potential and specificity of these lesions in vivo. Both adducts block DNA synthesis, but the dA adduct is the more effective inhibitor. The major translesion events are the insertion of the correct nucleotides opposite the dA or dG adducts; however, misincorporation is also observed, and the nucleotide mis-inserted opposite both adducts is almost exclusively dA, leading to AL-II-dA→T and AL-II-dG→T transversions.

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