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An Efficient Route to N^6 Deoxyadenosine Adducts of Diol Epoxides of Carcinogenic Polycyclic Aromatic Hydrocarbons

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Abstract—Polycyclic aromatic hydrocarbons are metabolized to a wide variety of oxidized derivatives, including highly reactive diol epoxides which alkylate DNA. The reaction lacks regio- or stereospecificity but occurs primarily at the exocyclic amino groups of deoxyguanosine and deoxyadenosine. An efficient route to N^6 adducts of deoxyadenosine is described using as examples those arising from *trans* opening of the *anti*-tetrahydrodiol epoxides of naphthalene, benzo[*a*]pyrene, and benzo[*c*]phenanthrene. The adducts were synthesized in 50–92% yields by reaction of 6-fluoropurine 2'-deoxyriboside with aminotriols formed by *trans* opening of racemic dihydrodiol epoxides using liquid NH$_3$. The diastereomeric adducts were separated by HPLC and their absolute configurations were assigned by circular dichroism. ^{1}H NMR studies revealed significant differences in conformation of the tetrahydroaromatic ring between the sterically unrestricted naphthalene derivative and the sterically congested derivatives of benzo[*a*]pyrene and benzo[*c*]phenanthrene. These differences may have a bearing on the higher carcinogenicity shown by the latter hydrocarbons. Undecadeoxyoligonucleotides bearing regio- and stereochemically defined adenine N^6-*anti-trans*-benzo[*a*]pyrene adducts have been prepared.

Introduction

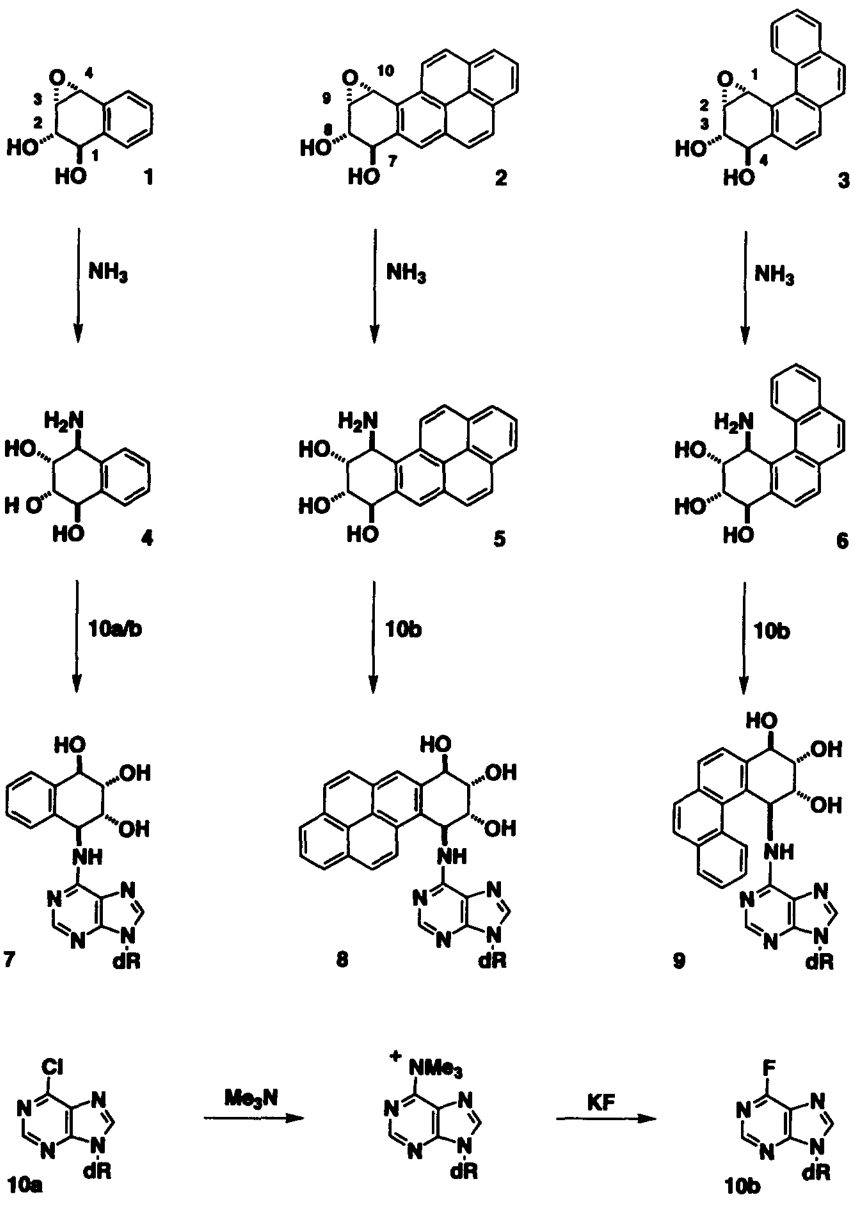
Polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, benzo[*a*]pyrene, and benzo[*c*]phenanthrene are widespread in the environment as products of combustion. In living cells they are converted to a variety of oxygenated derivatives,[1] including dihydrodiol epoxides (e.g. **1–3** in Scheme 1). The covalent binding of PAH metabolites to DNA is believed to play an important role in mutagenesis and in the initiation of the carcinogenic process. The major DNA adducts result from attack of the exocyclic amino groups of deoxyguanosine and deoxyadenosine on the benzylic position of the epoxides with both *trans* and *cis* ring opening being observed.[2]

There is considerable interest in the preparation of PAH adducts of nucleosides and oligonucleotides for use in chemical and biological studies. Several groups have prepared modest quantities of the nucleoside adducts by biomimetic procedures, i.e. reaction of dihydrodiol epoxides of PAHs with deoxyguanosine or deoxyadenosine or with DNA followed by enzymatic degradation.[3,4] Frequently, the yields are low due to competing hydrolysis of the epoxides.

The preparation of oligonucleotides bearing structurally defined adducts presents formidable problems. Reactions of PAH diol epoxides with oligonucleotides frequently give complex mixtures of adducts. Reactions at multiple sites and mixtures of *trans* and *cis* adduction products are observed. Epoxides **2** of benzo[*a*]pyrene react primarily at the N^2 position of guanine. Geacintov has studied extensively the reactions of these epoxides with oligonucleotides.[5] Yields can be quite high in cases where the oligomer contains only a single guanine but are strongly sequence dependent.

Minor adducts of this diol epoxide at the N^6 position of adenine appear in some cases to have disproportionately important biological impact. An important example involves mutations seen at an adenine residue located at the second position of the 61st codon of the ras proto-oncogene by Vousden *et al.* in tumors resulting from treatment of laboratory animals with racemic epoxide **2** of benzo[*a*]pyrene.[6] These mutations are over-represented relative to the yield of this minor adduct. The limitations of Geacintov's method are most evident for situations where multiple guanine residues are present or where adducted oligomers containing minor regioisomers or stereoisomers are desired. The region of ras codon 61 typifies this problem; not only is the inherent reactivity of the benzo[*a*]pyrene diol epoxides at the adenine N^6 position low but the sequence surrounding the adenine in question, 5'-C-GGA-CAA-GAA-G-3', contains four adenine and four guanine residues.

An alternative approach for synthesis of adducted oligonucleotides involves incorporation of adducted nucleosides into oligonucleotides. Both chemical and enzymatic syntheses have been employed with other carcinogen adducts. For PAH adducts, significant success has been observed with the chemical approach, in spite of the fact that protection of the PAH hydroxy



Scheme 1.

groups is required during DNA synthesis.[7] A major shortcoming of the procedure is that it is wasteful of the expensive diol epoxides since they are introduced into the synthetic pathway at an early stage and oligonucleotide syntheses are normally carried out with the phosphoramidite reagents as the excess reagent.

Herein we report a synthesis of PAH adducts at the N^6 position of deoxyadenosine which provides full control of stereospecificity and overcomes the problems of yields. The method should have a high degree of generality. In addition, an example is presented of an equally efficient application of this strategy to the synthesis of the above mentioned ras-61 oligonucleotide which provides complete control of regiospecificity and introduces the PAH moiety near the end of the synthesis.

Results and Discussion

This route for the synthesis of adducted nucleosides involves reversal of the nucleophile–electrophile relationship of the nucleoside and PAH.[8] Thus, the halogen atom of 6-halopurine 2'-deoxyriboside (**10**) is displaced via an aromatic nucleophilic substitution reaction by an aminotriol derived from the diol epoxide. The configurations of the substituents in the aminotriol determine the configurations of the substituents in the adduct.

A similar approach is also being employed by others involved in this research area, notably by Lakshman and by Seidel.[7] However, rather than focusing on the synthesis of protected PAH adducts for use in oligonucleotide synthesis, we desired a synthesis of unprotected adducts which could be accomplished with a minimum of steps (preferably no protection–deprotection) and which would be very economical in its use of valuable diol epoxides. As our targets we chose adducts derived from *trans* opening of diol epoxides of an unhindered hydrocarbon (naphthalene), a bay-region hydrocarbon (benzo[*a*]pyrene) and a fjord-region PAH (benzo[*c*]phenanthrene).

The success of this strategy is, in no small part, dependent on having an effective synthesis of the aminotriols. Smith *et al.* described a two-step synthesis of naphthalene aminotriol (**4**) from the corresponding *anti* dihydrodiol epoxide by S_N2 epoxide ring opening with trimethylsilylcyanide to give an isonitrile, hydrolysis of which gave the aminotriol.[9] Benzo[*a*]pyrene aminotriol (**5**) has been synthesized from (±)-*anti*-dihydrodiol epoxide by Lehr and by Meehan by two-step procedures using azide ring opening followed by reduction.[10]

We have found that the conversion of *anti*-dihydrodiol epoxides to the *trans*-aminotriols can be achieved efficiently in a single step by treatment of the diol epoxides with liquid ammonia for ~24 h at 70–100 °C in a Parr high pressure reactor. Aminolysis has been carried out with racemic *anti*-dihydrodiol epoxides of naphthalene, benzo[*a*]pyrene, and benzo[*c*]phenanthrene to yield (±)-aminotriols **4**–**6**. The reactions are S_N2 processes and give exclusively *trans* opening products in yields that are near quantitative. It is noteworthy that even severely hindered bay-region epoxides react readily with ammonia under these conditions. Lakshman *et al.*[11] in a similar study with benzo[*c*]phenanthrene diol epoxide detected, in addition to the major isomer **6**, a minor isomer resulting from *trans* opening at the non-benzylic end of the epoxide.

Racemic naphthalene aminotriol (**4**) reacted with 6-chloropurine 2'-deoxyribonucleoside (**10a**)[12] to give the diastereomeric N^6 adducts **7ab** of deoxyadenosine in ~60% yield. Condensation with benzo[*a*]pyrene aminotriol (**5**) under the same conditions gave only ~12% of adducts **8ab**, reflecting steric retardation of the condensation reaction. To improve the adduction yield, the 6-chloropurine nucleoside was converted to the fluoro analog **10b** by displacement with trimethylamine followed by fluoride ion.[7b,c,13] The more reactive, but much more unstable, 6-fluoropurine deoxyribonucleoside was treated with naphthalene aminotriol (2.0 eq). The reaction, monitored by TLC and HPLC, was complete within 1.5 days at 55 °C with no detectable side reactions. Preparative HPLC gave a 93% yield of **7ab**. Under the same conditions the reaction with benzo[*a*]pyrene aminotriol (**5**) required 5 days to reach completion (78% yield) and the reaction with benzo[*c*]phenanthrene aminotriol **6** (50% yield) was not finished after 5 days.

The diastereomeric products were separated by reversed-phase HPLC. Each diastereomeric adduct was analyzed by NMR and CD spectroscopy. Adducts of (±)-**2** at the N^6 position of deoxyadenosine have previously been prepared by Jeffrey *et al.* using poly(deoxyadenosine) and Cheng *et al.* from calf thymus DNA and deoxyadenosine 5'-phosphate by reaction with diol epoxide followed by enzymatic hydrolysis and HPLC isolation.[3] The adducts from (+) and (–) forms of *syn* and *anti*-benzo[*c*]phenanthrene diol epoxides have been synthesized by Jerina *et al.* from 2'-deoxyadenosine 5'-phosphate by reaction with the enantiomeric diol epoxides followed by dephosphorylation and HPLC isolation.[4] The N^6-adducts of *anti*-benzo[*c*]phenanthrene diol epoxide have been prepared in protected form by Lakshman *et al.* by a non-biomimetic strategy similar to ours.[11]

The CD spectrum of the more mobile diastereomer prepared from benzo[*a*]pyrene aminotriol **5** was identical to that reported by Cheng *et al.* for the *trans* adduct of (+)-**2** (*S* configuration at the benzylic epoxide carbon, i.e. 10*S*), while the slower isomer corresponded to the *trans* adduct of (–)-**2** (10*R*). In the case of benzo[*c*]phenanthrene, the more mobile diastereomer (**9b**, 4*S* stereochemistry) is also the one derived from the diol epoxide with *R* stereochemistry at the benzylic epoxide carbon 4 [(–)-**3**].[4] CD spectra for the adducts derived from naphthalene aminotriol (**4**) (Fig. 1) have not been reported previously but the stereochemical assignments could be made by analogy with previously analyzed spectra;[3,4,14] i.e. the faster eluting isomer exhibited a positive CD band in the 270–280 nm region and was assigned the *S* configuration at the N-substituted carbon.

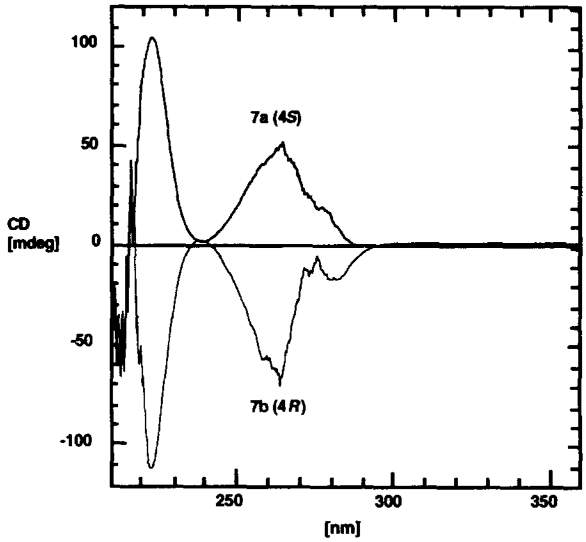


Figure 1. Circular dichroism spectra of naphthalene diol epoxide N^6-deoxyadenosine adducts **7a** (4*S* isomer, upper spectrum) and **b** (4*R* isomer, lower spectrum). Spectra were obtained in methanol.

The ^{1}H NMR spectra (Table 1) of the deoxyadenosine adducts were assigned by a combination of COSY and NOE difference spectroscopy. ^{1}H NMR spectra of peracetylated derivatives of the deoxyadenosine adducts of diol epoxides of benzo[*a*]pyrene and benzo[*c*]phenanthrene have been reported earlier;[3,4] however, it was of interest to examine the non-acylated adducts to determine if there are conformational differences induced by acetylation. For both PAHs the coupling constants in the tetrahydroaromatic ring were only slightly affected by acetylation (for example, in acetylated **8a** $J_{7,8}$ was 9.3, $J_{8,9}$ was 2.3 and $J_{9,10}$ was ~3 Hz), confirming the conclusion reached by Cheng *et al.*[3a] (based on a comparison of acetylated and unacetylated tetrols) that no drastic changes are induced by acetylation. The diastereomeric pairs had nearly identical chemical shifts, although there is a slight upfield shift of the 2' and 3' protons in the isomer with the *S* configuration at the linkage site. However, a comparison of the ^{1}H NMR spectra of the adducts provides insight into the effect of steric constraints imposed by bay-region hydrocarbons as compared with non-bay-region. With the naphthalene adduct, the coupling constant between the N-substituted benzylic CH and the vicinal CH is 7.9 Hz. In contrast, the corresponding coupling constants in the benzo[*a*]pyrene and benzo[*c*]phenanthrene adducts are only 3.3 and 4.2 Hz, respectively. In addition, there is a significant downfield shift of the adenine H-2 proton in the benzo[*a*]pyrene and benzo[*c*]phenanthrene derivatives. The differences in coupling constants and chemical shifts reflect the fact that the adenine moiety is forced out of the plane of the aromatic ring much more severely in the case of the bay region derivatives. The difference in nucleoside conformation can be expected to occur in PAH-adducted DNA as well and may contribute to the greater carcinogenicity of bay region PAH diol epoxides.

The adducts were also characterized by mass spectrometry using thermospray and fast atom bombardment. Thermospray gave much cleaner spectra with weak but detectable MH$^+$ ions. In all cases extensive fragmentation occurred leading to formation of ions corresponding to protonated adenine and

Table 1. ^{1}H NMR data for deoxyadenosine-N^6-PAH adducts[a]

Proton	7a/b	8a	8b	9a	9b
Adenine					
2	8.23 s br	8.53 s br	8.53 s br	8.54 s	8.54 s
8	8.24 s	8.18 s	8.18	8.18 s	8.18 s
Sugar					
1'	6.44 dd	6.44 ~t br	6.44 ~t br	6.44 ~t	6.44 ~t
	J = 6.0, 8.0				
2'	2.82 ddd	2.85 ddd	2.83 ddd	2.83 ddd	2.81 ddd
2"	2.42 ddd	2.42 ddd	2.43 ddd	2.42 ddd	2.42 ddd
3'	4.58 ddd	4.59 ddd	4.58 ddd	4.59 ddd	4.58 ddd
4'	4.08 ~q	4.08 ~q	4.08 ~q	4.07 dd	4.08 dd
5'	3.84 dd	3.85 dd	3.87 dd	3.84 dd	3.87 dd
5"	3.74 dd	3.74 dd	3.76 dd	3.74 dd	3.75 dd
Hydrocarbon					
1	4.75 d	8.155 dd	8.15 dd	6.56 d	6.56 d
	$J_{1.2}$ 5.6	$J_{1.2}$ 7.7	$J_{1.2}$ 7.7	$J_{1.2}$ 4.2	$J_{1.2}$ 4.2
2	4.10 dd	7.97 t	7.97 t	4.67 dd	4.68 dd
	$J_{2.3}$ 2.3	$J_{2.3}$ 7.7	$J_{2.3}$ 7.7	$J_{2.3}$ 2.5	$J_{2.3}$ 2.5
3	4.28 dd	8.19 dd	8.19 dd	4.24 dd	4.24 dd
	$J_{3.4}$ 7.9	$J_{1.3}$ 1.2	$J_{1.3}$ 1.2	$J_{3.4}$ 7.8	$J_{3.4}$ 7.8
4	5.71 br	8.04 d	8.04 d	4.96 d	4.99 d
	$J_{4.5}$ small	$J_{4.5}$ 9.1	$J_{4.5}$ 9.1		
5	~7.28 dd	8.12 d	8.12 d	8.01 dd	8.00 dd
	$J_{5.6}$ 7.9			$J_{5.6}$ 7.8	$J_{5.6}$ 7.8
	$J_{5.7}$ 1.9				
6	7.24 ddd	8.57 d	8.57 d	8.01 dd	8.00 dd
	$J_{6.7}$ 6.7	$J_{6.7}$ 1.0	$J_{6.7}$ 1.0		
	$J_{6.8}$ 1.4				
7	7.31 dddd	5.24 dd	5.24 dd	7.75 dd	7.75 dd
	$J_{7.8}$ 7.6	$J_{7.8}$ 9.0	$J_{7.8}$ 9.0	$J_{7.8}$ 8.8	$J_{7.8}$ 8.8
8	7.50 dd	4.25 dd	4.25 dd	7.75 dd	7.75 dd
	$J_{1.8}$ <1.0	$J_{8.9}$ 2.2	$J_{8.9}$ 2.2		
9		4.56 dd	4.56 dd	7.85 d	7.85 d
		$J_{9.10}$ 3.3	$J_{9.10}$ 3.3	$J_{9.10}$ 7.6	$J_{9.10}$ 7.6
				$J_{9.11}$ 1.4	$J_{9.11}$ 1.4
10		~6.49 br	~6.49 br	7.42 t	7.42 t
				$J_{10.11}$ 6.1	$J_{10.11}$ 6.1
11		8.09 d br	8.09 d br	7.10 t	7.11 t
		$J_{11.12}$ 9.4	$J_{11.12}$ 9.4	$J_{11.12}$ 8.7	$J_{11.12}$ 8.7
12		8.03 d	8.03 d	8.65 d	8.65 d

[a] Spectra were measured at 400 MHz in methanol-d_4 at 300 K with 0.1 Hz/pt resolution.

aminotriol as well as ions arising from loss of water and ammonia from the nucleoside adduct. Protonated adenine (m/z 136) was the base peak in the spectra of **8** and **9**; in **7** the base peak was protonated aminotriol.

The aminotriol method should be fully applicable to preparation of nucleosides of diol epoxides of other PAHs and other stereoisomers of the diol epoxides. With minor adaptation the synthetic strategy should provide a convenient route to the adducted nucleotides which are useful as standards for chromatographic detection of PAH adducts in biological samples using the very sensitive post-labeling procedures developed by Randerath.[15] At present, PAH-deoxyadenylate derivatives are not commercially available.

The application of this electrophile–nucleophile reversal strategy to preparation of adducted oligonucleotides offers the potential for substantial benefits over the previously employed direct adduction or the assembly of oligonucleotides from adducted nucleosides, both in terms of structural specificity and economics. The cost advantage lies in the fact that introduction of the PAH residue would be delayed until the end of the synthesis and excess aminotriol used to promote reaction could be recovered for use in subsequent syntheses. However, for the extension of this strategy to oligonucleotides to be successful, several formidable problems had to be overcome. Key amongst them was management of the somewhat labile 6-fluoropurine residue during oligonucleotide synthesis.

This post-oligomerization or 'convertible nucleoside' strategy, to use the term coined by Verdine, has been employed by Verdine and others but for different purposes.[16] However, none of the previously reported methods used 6-fluoropurine as the functionalized unit. The fluoro substitutent is probably more active than the ones used previously but is required with the hindered and relatively non-nucleophilic PAH amino triols.

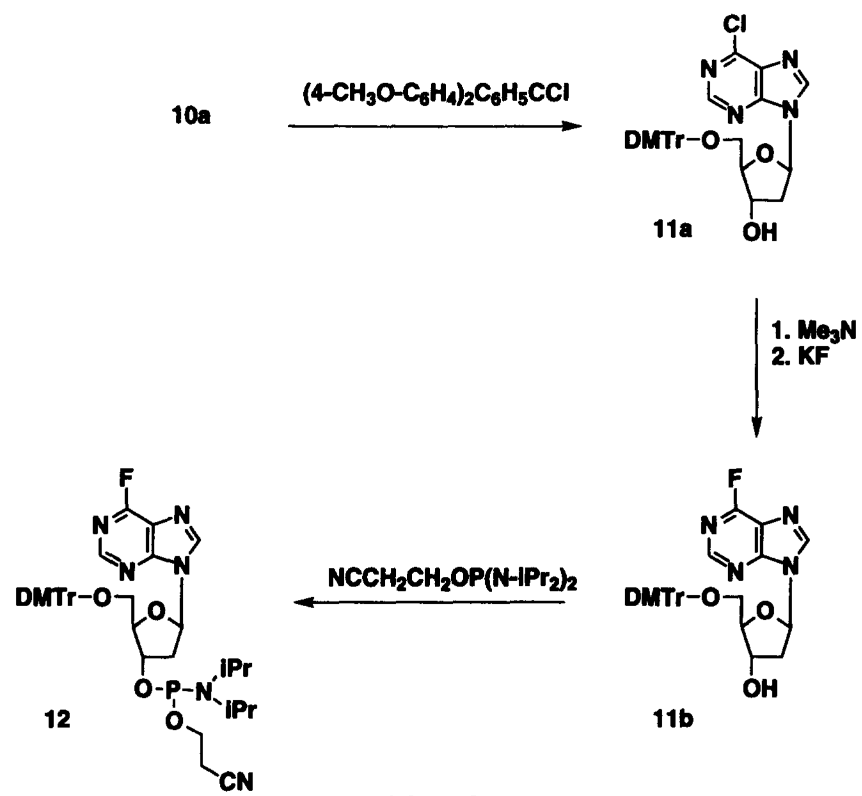
In conventional synthesis of oligonucleotides the oligomer is assembled on a solid support. The 4,4'-dimethoxytrityl group is released from the 5' position of the terminal deoxyribose by brief treatment with an acidic reagent and the protective groups, i.e. the acyl groups on the exocyclic amino groups and cyanoethyl on the phosphate, and the succinate linkage to the solid support are cleaved with ammonia or other nucleophiles. It was immediately apparent that fluoropurine **10b** would not be stable to ammonia treatment, thus presenting a serious block to the application of a post-oligomerization nucleophile–electrophile reversal strategy to the synthesis of oligonucleotides bearing adenine N^6 PAH adducts.

By judicious choice of conditions it might be possible to prepare oligonucleotides containing chloropurine nucleoside **10a**. However, the nucleoside studies had shown that **10a** was not sufficiently reactive to give satisfactory yields with hindered amines. We considered the possibility of preparing the oligonucleotide containing **10a** at the target site and then converting it to the 6-fluoro analog immediately before reaction with the amine. However, conditions could not be found to achieve this transformation efficiently. As a consequence, we chose to carry out the aminotriol adduction reaction on immobilized oligonucleotide while the protective groups were still in place on exocyclic amino sites and phosphate groups. This procedure offers the advantage that unreacted aminotriol is readily recovered from reaction mixtures but suffers from the inherent difficulties of solid phase reactions: i.e. (1) the immobilized reagent cannot be purified before the adduction reaction; (2) progress of the adduction reaction is difficult to monitor; (3) the 6-fluoropurine could have reduced reactivity in the solid support.

For assembly of fluoro phosphoramidite **12**, several possibilities presented themselves as to the timing of introduction of the fluoro substituent during assembly of the trityl-protected phosphitylated nucleoside. After some experimentation, the most dependable of these appeared to be conversion of chloro nucleoside **10a** to 4,4'-dimethoxytrityl derivative **11a**[17] followed by replacement of the chlorine by fluorine to give **11b** (Scheme 2). Phosphoramidite reagent **12** is then prepared by treatment of **11b** with 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphoradiamidite in the presence of diisopropylammonium tetrazolide.[17] Purification was achieved by flash chromatography on silica gel. A small amount of triethylamine was added to the eluting solvent since gradual release of HF by adventitious moisture causes precipitous destruction of the reagent. The reagent when pure can be stored at low temperature if protected from moisture.

Oligonucleotide synthesis was carried out by standard solid-phase procedures to prepare 5'-d(C-GGA-CXA-GAA-G)-3' where X is fluoronucleoside **10b** (Scheme 3). After the final detritylation, the immobilized oligonucleotide was carefully washed with acetonitrile and dried to remove occluded acid and moisture. Treatment with racemic aminotriol **5** was carried out at 65 °C for 5 days using approximately a 10-fold excess of the aminotriol. Our earlier studies had employed dimethylacetamide as the solvent but dry dimethylsulfoxide containing diisopropylethylamine has been found to give cleaner reactions and higher yields. Subsequently, the cyanoethyl and acyl protecting groups were removed by treatment with concentrated NH_4OH (60 °C, 7.5 h) to give benzo[*a*]pyrene-adducted oligonucleotides **13ab**. The diastereomeric mixtures resulting from use of racemic aminotriol were purified by reversed-phase HPLC (Fig. 2). The lipophilicity of the PAH moieties facilitated separation from unadducted oligonucleotides. The diastereomers present in **13** separated readily in spite of the fact that the diastereoisomerism reflects only a small portion of the oligonucleotide structures.

Oligonucleotides **13ab** were obtained in a combined yield of 13%. The oligonucleotides were further purified by polyacrylamide gel electrophoresis for use in



Scheme 2.

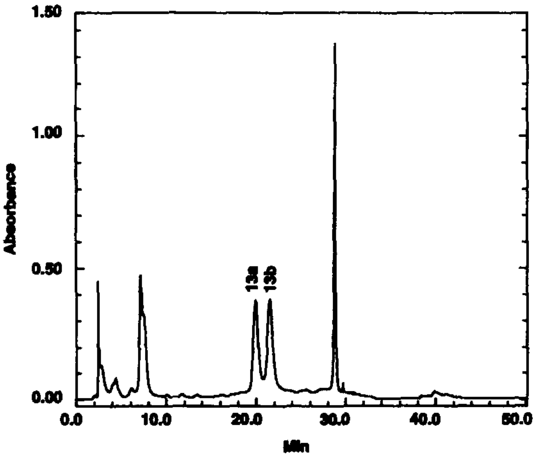


Figure 2. HPLC elution profile of oligonucleotides **13a** and **b** (CGGAC-$A^{N6\ (anti\text{-}trans\ BP)}$-AGAAG). PRP-1 column, 55 °C, linear gradient, 10 mM ethylenediamine acetate, pH 7.45, 0–20% acetonitrile over 20 min.

mutagenesis and *in vitro* polymerase studies.[18] The purified oligonucleotides were characterized by circular dichroism (Fig. 3), capillary gel electrophoresis (Fig. 4), and enzyme digestion (Fig. 5). In our preliminary report we were unable to obtain complete hydrolysis with snake venom phosphodiesterase and alkaline phosphatase;[8] this is in accord with results obtained by others.[19] Complete digestion was accomplished with a combination of nuclease P1 hydrolysis followed by treatment with snake venom phosphodiesterase and alkaline phosphatase. The nucleoside ratios for both **13a** and **b** were dC, 2; dG, 3.9; dA, 3.8; BP-nucleosides **8a/b**, 1.1 (unmodified oligonucleotide gave values of dC, 2; dG, 4.0; dA, 4.8). Stereochemical assignments for the BP moiety in **13a** and **b** were made from the CD spectra by comparison with CD spectra of **8a** and **b**;[3a] confirmation was obtained by HPLC comparison of the adducted nucleosides from the enzyme digestion with **8a** and **b** (Fig. 6); the first eluted oligonucleotide, **13a**, had the same BP stereochemistry as **8b**, i.e. 10*R*. Detailed high field NMR studies on the oligonucleotides duplexed with their complementary strands are in progress.

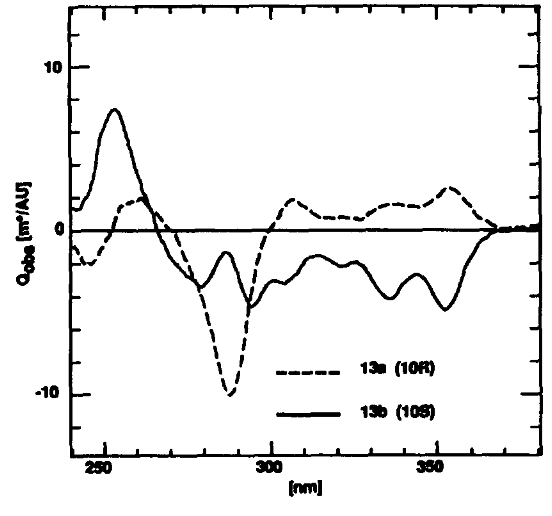
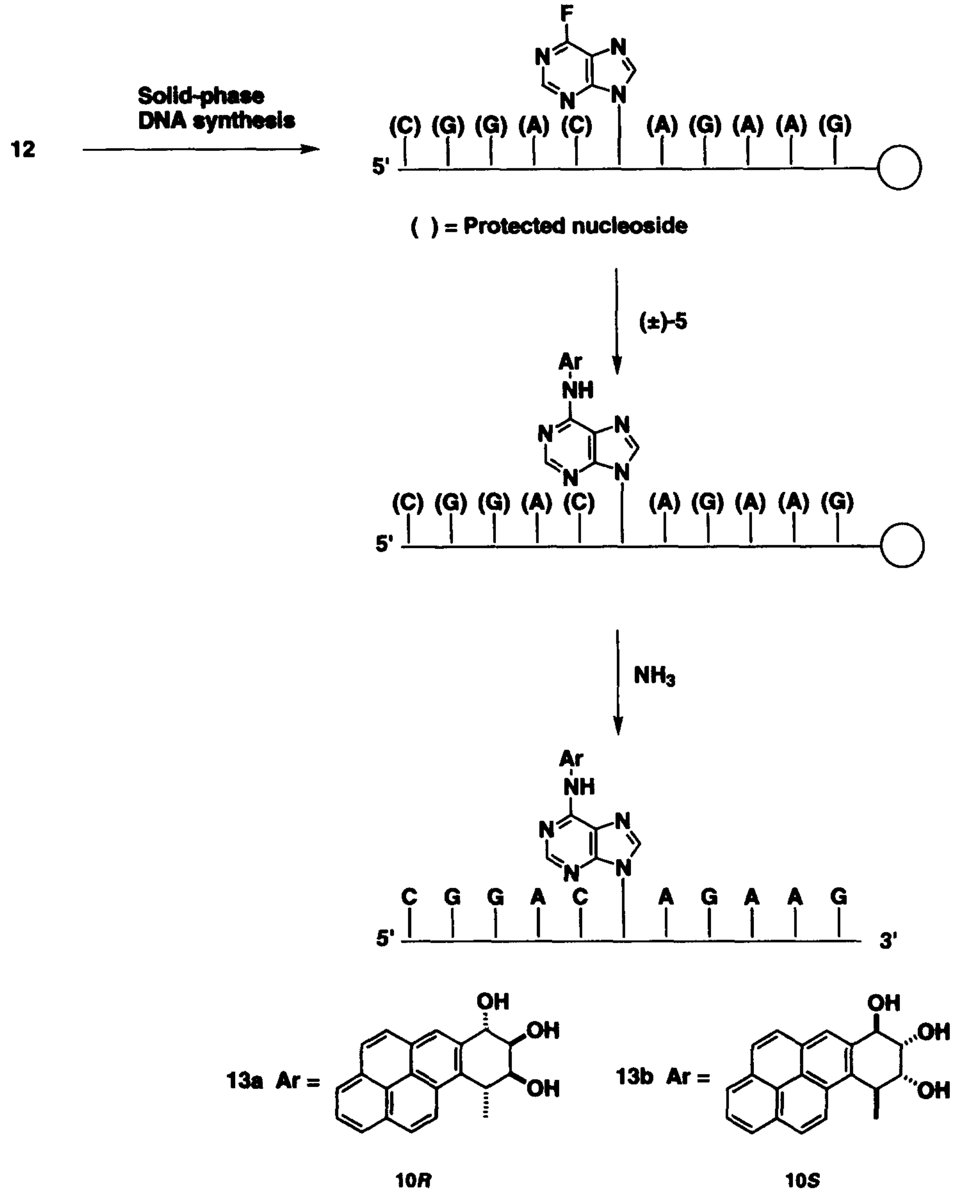


Figure 3. Circular dichroism spectra of oligonucleotides **13a** (dashed line) and **b** (solid line) in 20 mM sodium phosphate, pH 7.0. Spectra are normalized to 1.0 absorbance at 260 nm.

The synthetic method provides a versatile procedure for the preparation of oligonucleotides bearing structurally defined adducts of polycyclic aromatic hydrocarbons at



Scheme 3.

the N^6 position of adenine. The success of the method is independent of sequence; i.e. the sequence chosen for the present study contained a large number of other target sites, which if the adducts had been prepared by direct adduction would have led to an extremely complex mixture of products. The strategy should be equally applicable to other PAHs and other stereochemical arrangements of functional substituents.

We have yet to demonstrate that the method will be as useful for preparation of oligonucleotides bearing adducts on the N^2 position of guanine. Halogen substituents at the C-2 of purines are substantially less reactive with nucleophiles than those at C-6. While displacements occur readily with small, sterically unencumbered nucleophiles, bay-region aminotriols of PAHs may be too sterically hindered to give satisfactory yields.

An inherent limitation in the post-oligomerization strategy for preparation of PAH adducted oligonucleotides is that clean separation of diastereomeric adducts will not in all cases be feasible by HPLC. Indeed, in examples which will be reported elsewhere, we have seen separations which are inadequate to completely resolve the diastereomers. The use of individual enantiomers would circumvent this problem but the high cost and poor availability of optically pure species are problems which need to be overcome. We are currently developing an enantioselective route to PAH diol epoxides.

Experimental

CAUTION. The diol epoxides of polycyclic aromatic hydrocarbons are potent carcinogens.

Spectroscopic Methods

NMR spectra were obtained at 300 and 400 MHz on Bruker spectrometers. Mass spectral data were obtained

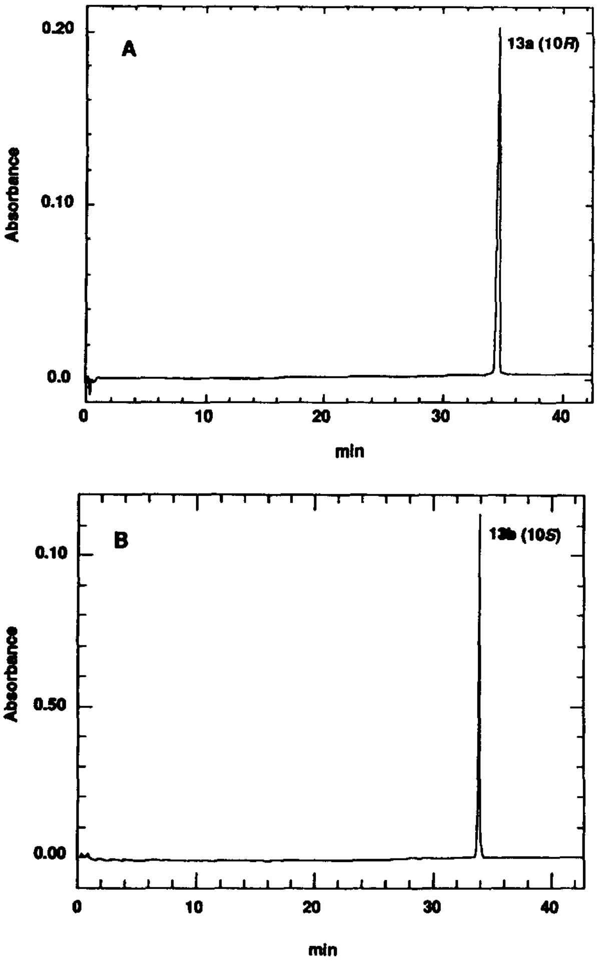


Figure 4. Capillary gel electrophoresis of oligonucleotides **13a** (A) and **b** (B). Samples were run on a 40 cm column at 30 °C in Tris–borate–urea buffer.

on a Nermag R30-10 triple quadrupole mass spectrometer. The spectra were collected and processed using a SIDAR data system. Thermospray ionization mass spectra were obtained using a thermospray interface based on the Vestec design. The photomultiplier detector voltage after the third quadrupole (Q3) was set at 600 V to obtain the thermospray mass spectra. The HPLC system consisted of a Hitachi L-6200 intelligent pump and a Valco C6W injector. The mobile phase was methanol:0.01 M ammonium acetate (20:80, v/v) at a flow rate of 1.5 mL min^{-1}. Direct loop injections were used for obtaining the spectra. All spectra were obtained using 0.5 µg of sample. HRMS were obtained with a VG 70-250 instrument using a probe and a standard Ion-Tech saddle field gun producing xenon atoms of 8 kV energy. Circular dichroism spectra were measured on a Jasco Model J-720 spectropolarimeter.

HPLC

HPLC analyses and isolations of nucleosides were carried out on a Nicolet LC/9560 HPLC equipped with a Varian Polychrom 9060 diode array detector using gradients generated from (A) 9:1, MeOH:H_2O and (B) H_2O. Analytical runs were performed on a C-18 reversed-phase column (4.6 x 250 mm) at a flow rate of 1.0 mL min^{-1}; preparative scale isolations were carried out on 10 x 250 mm C-18 column at a flow rate of 2.0 mL min^{-1}.

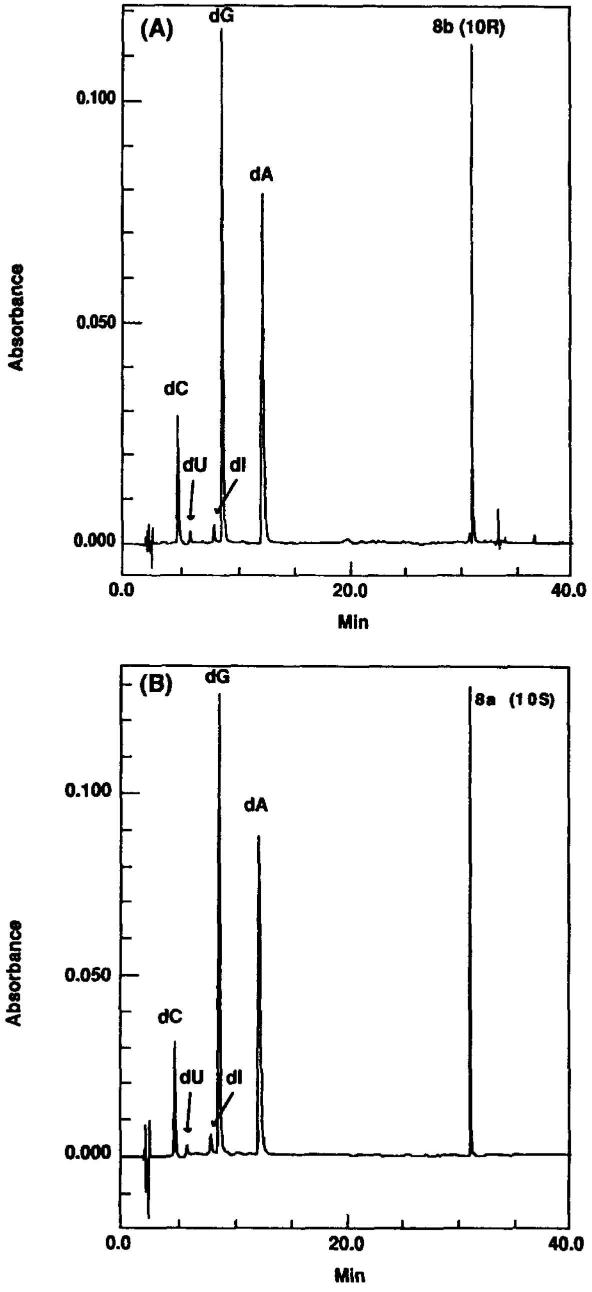


Figure 5. HPLC elution profile of enzyme digests (nuclease P1, snake venom phosphodiesterase and, alkaline phosphatase) of oligonucleotides **13a** and **b**. Reverse phase, 0.01 M ammonium formate, pH 5.90/acetonitrile from 1 to 10% acetonitrile over 15 min followed by 10–99% acetonitrile over 20 min.

General procedure for synthesis of aminotriols 4–6

Diol epoxide (1–3) was weighed into a glass container (1.5 x 10 cm) containing a 1 cm magnetic stirring bar. Liquid ammonia (6–7 mL) was added slowly. The glass tube was immediately sealed in a Parr high pressure reactor (38 x 114 mm). The reactor was heated in a oil bath and stirred via a magnetic mixer. After completion of the reaction, the reactor was cooled to room temperature and then cautiously vented. After several hours the final traces of ammonia were removed under vacuum. The residue was dissolved in anhydrous DMSO and transferred to a small flask. The aminotriols were obtained as powders after evaporation of the solvent under high vacuum.

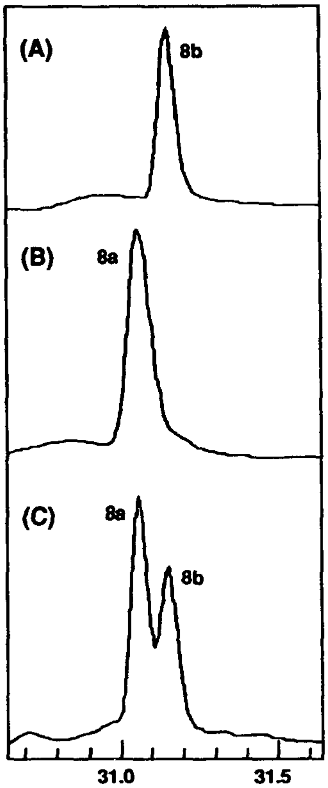


Figure 6. Expansion of 30–32 min region of chromatogram in Figure 5. (A) N^6-BP nucleoside from oligonucleotide **13a**; (B) N^6-BP nucleoside from oligonucleotide **13b**; (C) N^6-BP-deoxyadenosine standards **8a** (10*S*) and **b** (10*R*).

(±)-4β-Amino-1,2,3,4-tetrahydronaphthalene-1β,2α,3α-triol (4). (±)-1β,2α-Dihydroxy-3α,4α-epoxy-1,2,3,4-tetrahydronaphthalene (**1**, 50 mg), synthesized from 1,2-dihydronaphthalene,[20] was stirred in liquid ammonia (6–7 mL) under ~250 psi in the Parr reactor at 70 °C for 20 h. Aminotriol **4** was formed in essentially quantitative yield; no other products were detected either on TLC (R_f 0.3, silica gel, 8:2 acetonitrile:H_2O) or by NMR analysis. ^{1}H NMR (DMSO-d_6/D_2O) δ 7.48 (*d*, 1H, Ar), 7.30 (*d*, 1H, Ar), 7.18 (*m*, 2H, Ar), 4.42 (*d*, 1H, H-4), 3.84 (*dd*, 1H, H-3), 3.73 (*d*, 1H, H-1), 3.66 (*dd*, 1H, H-2); $J_{1,2}$ = 7.2, $J_{2,3}$ = 2.1, $J_{3,4}$ = 5.1 Hz; MS (electrospray) $[MH]^+$ 196. The aminotriol was converted to the *N*-acetylated derivative by treatment with excess acetic anhydride in methanol. ^{1}H NMR (DMSO-d_6/D_2O): δ 8.16 (*d*, 1H, N–H), 7.36 (*m*, 1H, Ar), 7.22 (*m*, 2H, Ar), 7.10 (*m*, 1H, Ar), 4.97 (*dd*, 1H, H-4), 4.50 (*d*, 1H, H-1), 3.84 (*m*, 2H, H-2, H-3); $J_{1,2}$ = 5.3, $J_{3,4}$ = 6.8, $J_{4,N-H}$ = 8.2 Hz.

(±)-10β-Amino-7,8,9,10-tetrahydrobenzo[a]pyrene-7β,8α,9α-triol (5). (±)-7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene (**2**, 10 mg, purchased from Midwest Research Institute or from Chemsyn) was converted to aminotriol **5** by the general procedure (70 °C, 24 h). Aminotriol **5** was obtained as a yellow powder in essentially quantitative yield (TLC; R_f 0.45, silica gel, 8:2 acetonitrile:H_2O). ^{1}H NMR (d_6-acetone + H^+): δ 8.57 (*s*, 1H, H-6), 5.67 (*d*, 1H, H-10), 5.25 (*d*, 1H, H-7), 4.24 (*dd*, 1H, H-8), 4.06 (*dd*, 1H, H-9); $J_{7,8}$ = 8.6, $J_{8,9}$ = 2.0, $J_{9,10}$ = 3.2 Hz; (DMSO-d_6): δ 4.89 (*d*, 1H, H-7), 4.77 (*d*, 1H, H-10), 4.15 (*dd*, 1H, H-8), 4.09 (*dd*, 1H, H-9); $J_{7,8}$ = 8.6, $J_{8,9}$ = 2.1, $J_{9,10}$ = 3.2 Hz. UV (MeOH): 342 (33,000), 326 (22,000), 312 (9300), 278 (34,000), 266 (20,600), 244 (60,000), 237 nm (36,800). The CD spectrum of optically active material prepared from (+)-**2** showed negative Cotton effects at 346 and 328 nm and a positive Cotton effect at 282 nm. MS (FAB^+): Calcd for $C_{20}H_{17}NO_3$ + H^+: 320.1286. Observed: 320.1298. The aminotriol was converted to the *N*-acetyl derivative: MS (FAB^+): Calcd for $C_{22}H_{19}NO_4$ + H^+: 362.1392. Observed: 362.1396. ^{1}H NMR (acetone-d_6) δ 8.53 (*s*, 1H, Ar), 8.0–8.28 (*m*, 7H, Ar), 7.52 (*d*, 1H, N–H), 6.03 (*dd*, 1H, H-10), 5.20 (*d*, 1H, H-7), 4.34 (*dd br*, 1H, H-9), 4.07 (*dd*, 1H, H-8); $J_{7,8}$ = 8.6, $J_{8,9}$ = 2.2, $J_{9,10}$ = 3.2, $J_{10,N-H}$ = 8.0 Hz.

(±)-3β,4α-Dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[c]phenanthrene (3). The procedure of Harvey and co-workers[21] was followed to synthesize 1,2-dihydrobenzo[*c*]phenanthrene except that *t*-butyllithium was used for the coupling reaction of 2-(2-naphthyl)ethyl iodide and 1,5-dimethoxycyclohexa-1,4-diene.[22] The dihydro compound was converted to 3,4-dihydrodiol benzo[*c*]phenanthrene via the Prévost reaction followed by bromination with NBS and dehydrohalogenation with DBN.[20,23] The dihydrodiol was converted to *trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene by oxidation with *m*-chloroperbenzoic acid in anhydrous THF.[20,22]

(±)-1β-Amino-1,2,3,4-tetrahydrobenzo[c]phenanthrene-2α,3α,4β-triol (6). The general procedure was followed with **3** to give a good yield of aminotriol **6** as a brown solid: ^{1}H NMR (DMSO-d_6/D_2O): δ 9.79 (*d*, 1H, H-12) 8.0–7.64 (*m*, 7H, Ar), 4.74 (*d*, 1H, H-1), 4.60 (*d*, 1H, H-4), 4.35 (*d*, 1H, H-3), 4.03 (*br*, 1H, H-2); MS (electrospray) $[MH]^+$ 296; *N*-Acetyl derivative: ^{1}H NMR (acetone-d_6): δ 8.61 (*d*, 1H, H-12), 8.0–7.5 (*m*, 8H, Ar, N–H), 6.06 (*dd*, 1H, H-1), 4.94 (*d*, 1H, H-4), 4.44 (*dd*, 1H, H-2), 4.13 (*dd*, 1H, H-3); $J_{1,2}$ = 4.5, $J_{2,3}$ = 2.4, $J_{3,4}$ = 7.4 Hz; MS (electrospray) $[MH]^+$ 338.

Syntheses of aminotriol adducts of deoxyadenosine. (1α,2β,3β,4α)-2'-Deoxy-N⁶-(1,2,3,4-tetrahydro-1,2,3-trihydroxynaphthalen-4-yl)-adenosine (7a and b). 6-Fluoro-2'-deoxyriboside **10b**[13] (5.82 mg, 22.9 μmol) and racemic aminotriol **4** (6.7 mg, 34.3 μmol) were weighed into a conical vial. Triisobutylamine (distilled from CaH_2) (11 μL, 45.5 μmol) and *N*,*N*-dimethylacetamide (100 μL) were added, the vial was sealed, and the reaction was stirred at 55 °C. The reaction was monitored by TLC (R_f **10b**: 0.63, **7ab**: 0.2, **4**: 0.02, 8:2, CH_2Cl_2:MeOH). The reaction was complete within 1.5 days. The diastereomeric products were collected together on HPLC using a linear gradient of 60:40 B:A to 80% A over 30 min. 9.12 mg (93%) of (±) **7a–b** was obtained after evaporation of the solvents. Isomers **7a** and **b** were separated by HPLC using a linear gradient of 90:10 A:B to 100% A over 30 min (flow rate 1.0 mL min^{-1}). Isomer **7a** was assigned 4*S* stereochemistry and **7b** as the 4*R* isomer (see discussion). ^{1}H NMR (see Table 1) MS (thermospray) 430 [MH]$^+$, 314 [protonated modified base], 196 [base peak, protonated aminotriol]. HRMS (FAB$^+$, glycerol/DMSO/PEG 200–600) Calcd for $C_{20}H_{24}N_5O_6$ [M + H] 430.1727; Found 430.1735. UV (MeOH) 274 (14,300), 269 (14,400), 264 nm (sh, 13,400).

(7α, 8β, 9β,10α)-2'-Deoxy-N^6*-(7,8,9,10-tetrahydro-7,8,9-trihydroxybenzo[*a*]pyren-10-yl)-adenosine (**8a** and **b**).* 6-Fluorodeoxyriboside **10b** (5.4 mg, 21.3 μmol) and racemic aminotriol **5** (4.47 mg, 14.0 μmol) were weighed into a conical vial. Triisobutylamine (6.8 μL, 28 μmol) and *N,N*-dimethylacetamide (100 μL) were added and the reaction was stirred at 55 °C. The reaction mixture was monitored by TLC (R_f **10b**: 0.65, **8ab**: 0.35, **5**: 0.15 using 9:1 CH_3CN:H_2O). The reaction appeared to be complete within 5 days. The diastereomeric products were collected together on HPLC (flow rate: 2.0 ml min^{-1}) using a linear gradient of 60:40 B:A to 100 % A over 30 min. 5.86 mg (76%) of **8ab** was obtained after evaporation. Isomers **8a** (10*S* isomer) and **b** (10*R* isomer) were separated using a gradient of 80:20 B:A to 100% A over 30 min (flow rate 2.0 mL min^{-1}). ^{1}H NMR (see Table 1); MS (thermospray) 554 [MH]$^+$, 438 [protonated modified base], 320 [protonated **5**], 303 [**5**–NH_3], 285 [**5**–NH_3–H_2O], 136 [base peak, protonated adenine]. HRMS (FAB$^+$, glycerol/DMSO/PEG 200–600) Calcd. for $C_{30}H_{28}N_5O_6$ [M + H] 554.2040; Found 554.2040. UV (MeOH) 344 (41,000), 328 (26,900), 314 (11,400), 280 (58,000), 269 (36,000), 245 (66,000) nm.

(1α,2β,3β,4α)-2'-Deoxy-N^6*-(1,2,3,4-tetrahydro-2,3,4-trihydroxybenzo[*c*]phenanthren-1-yl)-adenosine (**9a** and **b**).* 6-Fluorodeoxyriboside (**10b**) (4.6 mg, 18.1 μmol) and crude (±)-1β-amino-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene-2α,3α,4β-triol (**6**) (10.7 mg, 36.3 μmol) were weighed into a conical vial. Triisobutylamine (8.7 μL, 36 μmol) and *N,N*-dimethylacetamide (100 μL) were added and the reaction was stirred at 55 °C. The reaction mixture was monitored by TLC (silica gel, 9:1, CH_3CN:H_2O; R_f, **10b**: 0.65, **9ab**: 0.46, **6**: 0.35). The reaction was stirred for 5 days (a small amount of **6** was still visible). The diastereomeric products were purified together on HPLC using a linear gradient of 60:40 B:A to 100% A over 30 min. 4.8 mg (50 %) of **9ab** was obtained after evaporation. Diastereomers **9a** (1*S* isomer) and **9b** (1*R* isomer) were separated by preparative HPLC using a gradient of 60:40 B:A to 100% A over 40 min at a flow rate of 2.0 mL min^{-1}. ^{1}H NMR (see Table 1); MS (thermospray) 530 [MH]$^+$, 414 [protonated modified base], 296 [protonated **6**], 252 [protonated deoxyadenosine], 136 [base peak, protonated adenine]. HRMS (FAB$^+$, glycerol/DMSO/PEG 200–600) Calcd. for $C_{28}H_{28}N_5O_6$ [M + H] 530.2040; Found 530.2025. UV (MeOH) 299 (7200), 287 (sh, 11,800), 279 (15,000), 268 (19,200), 252 (39,000) nm.

*6-Chloro-9-[5'-O-(dimethoxytrityl)-2'-deoxy-β-D-erythro-ribofuranosyl]-9H-purine (**11a**).* Nucleoside **10a**[12] (174 mg, 0.64 mmol, dried at 35 °C/0.25 mm, 24 h) was evaporated from dry pyridine two times and suspended in dry pyridine (2.5 mL) in a 50-mL round-bottomed flask. *N,N*-Diisopropylethylamine (0.168 mL, 0.96 mmol) was added, followed by 4,4'-dimethoxytrityl chloride (304 mg, 0.90 mmol). The reaction was followed by TLC; it was complete after 5 h. MeOH (1 mL) was added followed after 10 min by H_2O (18 mL). The reaction mixture was extracted with CH_2Cl_2. The CH_2Cl_2 extracts were combined, washed with 10% K_2CO_3, dried and evaporated to yield 847 mg of orange solid which was purified by silica gel chromatography with elution by MeOH–CH_2Cl_2 mixtures containing 1% *N,N*-diisopropylethylamine to give 317 mg (86%) of DMTr derivative **11a**. ^{1}H NMR (CD_3OD): δ 8.60 (*s*, 1H, H-2/H-8), 8.20 (*s*, 1H, H-8/H-2), 7.37 (*m*, aromatic, 2), 7.30–7.15 (*m*, 7H, aromatic), 6.77 (*m*, 4H, aromatic), 6.42 (*t*, 1H, H-1'), 4.62 (*m*, 1H, H-3'), 4.10 (*m*, 1H, H-4'), 3.74 (*s*, 6H, OCH_3), 3.40–3.30 (*m*, 2H, H-5', H-5"), 2.80 (*m*, 1H, H-2'), 2.50 (*m*, 1H, H-2').

*5'-O-(Dimethoxytrityl)-6-fluoropurine-2'-deoxyriboside (**11b**).* 5'-DMTr-6-chloropurine-2'-deoxyriboside (**11a**) (202 mg, 0.35 mmol) was dried by evaporation with dry benzene (2 × 10 mL) in a 25-mL flask. Anhydrous ethylene glycol dimethyl ether (10 mL) was added and the flask was equipped with a Dry Ice condenser. Gaseous Me_3N was introduced until ~1.5 mL liquid condensed; the condenser was removed and the yellow solution was stirred at room temperature for 30 min. The solvents were removed under aspirator vacuum and the residue was dried under high vacuum to leave a yellow foam. Anhydrous DMF (10 mL) and excess anhydrous KF (spray-dried, dried at 125 °C overnight) were added to the flask and the mixture was stirred at room temp. for 40 min. After evaporation of the DMF *in vacuo* the residue was taken up in CH_2Cl_2 (heterogeneous mixture) and applied to a silica gel column (2.5 x 20 cm). Elution with EtOAc:CH_2Cl_2:Et_3N, 40:60:0.1 (all solvents dried) gave **11b** as a white solid (100 mg, 51%). ^{1}H NMR ($CDCl_3$): δ 8.55 (*s*, 1H, H-8/H-2), 8.23 (*s*, 1H, H-2/H-8), 7.37 (*m*, 2H, aromatic), 7.21–7.30 (*m*, 7H, aromatic), 6.81 (*m*, 4H, aromatic), 6.51 (*t*, 1H, H-1'), 4.71 (*m*, 1H, H-3'), 4.16 (*m*, 1H, H-4'), 3.78 (*s*, 6H, OCH_3), 3.44 (*m*, 2H, H-5', H-5"), 2.85 (*m*, 1H, H-2'), 2.62 (*m*, 1H, H2"). ^{19}F NMR ($CDCl_3$, ref.: $CFCl_3$ = 0): δ –69.69. MS (FAB$^+$, CH_2Cl_2/glycerol): 557 [M + H]$^+$; TLC (silica gel, EtOAc), R_f 0.5.

*5'-O-(Dimethoxytrityl)-3'-O-[(N,N-diisopropylamino) (2-cyanoethoxy)phosphinyl]-6-fluoropurine-2'-deoxyriboside (**12**).* In a 50-mL, 3-necked flask under nitrogen a slurry of diisopropylammonium tetrazolide was prepared from 23.7 mg (0.34 mmol) vacuum-dried tetrazole and 47.4 μL (0.34 mmol) diisopropylamine (distilled from CaH_2).[17b] DMTr nucleoside **11b** (157 mg, 0.28 mmol), dissolved in 1 mL of dry CH_2Cl_2 was added to the salt mixture; the purine-containing flask was rinsed with two 1-mL portions of CH_2Cl_2 which were added to the slurry. The phosphitylating reagent, 2-cyanoethyl-*N*,*N*,*N'*,*N'*-tetraisopropylphosphoradiamidite (135 μL, 0.424 mmol) was added to the mixture by syringe. The reaction was stirred at room temperature under nitrogen for 2 h. The mixture was poured with stirring into saturated $NaHCO_3$ (35 mL); the resulting mixture was extracted with 3 × 40 mL portions of CH_2Cl_2. The organic extracts were combined, dried (K_2CO_3), evaporated and purified by flash chromatography with elution by CH_2Cl_2:EtOAc:Et_3N (14:6:1) to give 167 mg (79%) of **12** as a yellowish foam. TLC (silica gel, EtOAc:hexane:Et_3N, 100:100:1) two spots, R_f 0.43 and 0.33; ^{31}P NMR (CD_3CN, ref.: external 5% aq H_3PO_4 = 0): δ 151.05, 151.16. ^{19}F NMR ($CDCl_3$, ref.: $CFCl_3$ = 0): δ –69.96, –70.02. MS (FAB^+, CH_2Cl_2/3-nitrobenzoic acid): 757 $[M + H]^+$.

Synthesis of oligonucleotides

The oligonucleotide 5'-d(C-GGA-CXA-GAA-G)-3', where X is nucleoside **10b**, was synthesized by automated solid-phase synthesis (Applied Biosystems, Model 391 synthesizer) using the standard 1 μmol protocol. After assembly of the sequence, the final 4,4'-dimethoxytrityl group was removed, the beads were washed thoroughly with acetonitrile, removed from the cassette and dried *in vacuo* overnight.

In a typical synthesis of adducted oligonucleotide, the beads from two 1-μmol cassettes were placed in a 3-mL conical vial; freshly distilled DMSO (0.4 mL) and diisopropylethylamine (10 μL) were added followed by vacuum-dried racemic aminotriol **5** (6 mg, ~20 μmol). The vial was capped and heated in a heating block with occasional shaking for 5 d at 65 °C. The reaction mixture was cooled to room temp. and the supernatant was decanted. The beads were washed with several small portions of MeOH until the wash was colorless. The beads were air-dried, transferred to a 3-dram screw-capped vial and treated with conc NH_4OH (2.5–3.0 mL) at 60 °C for 7.5 h. The vial was cooled to room temp., cautiously opened and allowed to stand for several hours. Following transfer of the supernatant to a 50-mL polypropylene centrifuge tube, the beads were rinsed with H_2O and the rinsings were added to the supernatant which was then lyophilized.

Chromatographic separation of oligonucleotides

Before HPLC purification, the crude oligonucleotide mixture was passed through a column of Sephadex G-15 (elution by H_2O) to remove small-molecule impurities. The diastereomeric benzo[*a*]pyrene-adducted oligonucleotides were separated on a PRP-1 column (7 × 305 mm, Hamilton) at 55 °C using a linear gradient of 10 mM ethylenediamine acetate, pH 7.45, containing 0–20% CH_3CN over 20 min, flow rate 3.0 mL min^{-1}. The chromatographic separations were monitored using a Varian Polychrom 9060 diode array detector. The benzo[*a*]pyrene-containing oligonucleotides **13a** and **b** eluted between 16 and 18 min to give a combined yield of 13% (16.8 ODs of the faster moving peak; 25.4 ODs of the slower). The oligonucleotides could be repurified by HPLC under conditions similar to those used for the original purification (linear gradient 3–20% CH_3CN over 30 min); the fractions were lyophilized and desalted on a Sephadex G-15 column (1.5 x 40 cm) with elution by H_2O.

PAGE

For capillary gel analysis and enzyme digestion the oligonucleotides were further purified by denaturing polyacrylamide gel electrophoresis (Hoeffer SE 620 vertical slab , 18% polyacrylamide, 7 M urea, 14 x 32 cm gels, 1.5 cm thick). After detection by UV shadowing the bands were cut out and eluted overnight at room temperature with 0.05 M triethylammonium acetate, pH 7.0.

Capillary gel electrophoresis

Capillary gel electrophoresis was performed on an Applied Biosystems Model 270 instrument, using gel-filled capillaries (Applied Biosystems) and the manufacturer's Micro-Gel buffer or on a Beckman PACE 2000 system with the manufacturer's e-CAP gel-filled capillary and Tris–borate–urea buffer. Samples were applied at –5 kV and run at –15 kV at 30 °C.

Enzyme digestions

The oligonucleotides (0.2–0.6 ODs), lyophilized in a 1.5-mL microfuge tube, were digested in a two-stage process. In the first step buffer (20 μL, 0.01 M Tris–HCl, 0.01 M $MgCl_2$, pH 7.00) was added followed by nuclease P1 (4 μL, Sigma N-8630, 1.0 mg dissolved in 0.5 mL H_2O) After digesting for 3–6 h at 37 °C the second digestion step was performed. Tris–HCl buffer (20 μL, 0.1 M, pH 9.00) was added followed by snake venom phosphodiesterase (Sigma 5785, 0.04 units) and alkaline phosphatase (Sigma P-4282, 3.24 μL). Digestion was continued at 37 °C for 3–6 h. Before HPLC analysis, H_2O (100 μL) was added to each sample. Following centrifugal filtration the digests were analyzed by HPLC (C-18 column with the following gradient: 0.01 M ammonium formate, pH 5.90/CH_3CN from 1 to 10% CH_3CN over 15 min followed by 10–99% CH_3CN over 20 min at a flow rate of 1.5 mL min^{-1}. Under these conditions the diastereomeric PAH-adducted nucleosides eluted at 30–32 min. Peak identification was made by UV comparison and co-injection with adducted nucleosides of known stereochemistry.

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