Synthesis and Application of Nitro-Substituted Nucleosides and Nucleotides

Tomohiko Kawate, Bing H. Wang,[1] Charles R. Allerson,[2] Jia Liu Wolfe*

Center for Computational and Integrative Biology, Massachusetts General Hospital, 185 Cambridge St., Boston, MA 02114, USA
Fax +1(617)6433328; E-mail: jwolfe@ccib.mgh.harvard.edu

Received 28 April 2006

Abstract: Protocols for the synthesis of C^7-nitropurine nucleosides and nucleotides are described. Each of these nucleotides can completely replace its naturally occurring counterpart in polymerase chain reaction (PCR), generating amplified duplex DNA containing nitropurines. As predicted, the electron-withdrawing power of the nitro group rendered these modified oligonucleotides susceptible to chemoselective cleavage at the modified bases. Treatment with secondary amines at elevated temperatures produced selective DNA scission at nitropurine sites. Interestingly, this Maxam–Gilbert type of DNA cleavage reaction was facilitated by the addition of tris(2-carboxyethyl)phosphine (TCEP), a strong reducing agent and nucleophile. Mass spectrometry analysis not only confirmed that DNA cleavage occurred at the nitropurines, but also revealed that the fragmented DNA products are appended by a TCEP moiety at its 3′-end. This surprising result provided new insights into the mechanisms of such DNA cleavage reactions.

Key words: cleavage, nucleotides, oligonucleotides, nucleophilic additions, regioselectivity

Chemically modified nucleosides and nucleotides that are substrates of DNA and RNA polymerases are useful in many different applications, including DNA sequencing[3] and genotyping,[4] sequence-specific protein-DNA interaction analysis,[5] and functional aptamer generation and selection.[6] These DNA and RNA building blocks are used in a variety of different capacities, including that of introducing fluorescent labels,[7,8] mass tags,[9] and diverse chemical functional groups into polynucleotides of interest.[10,11]

Nitro modification has recently been introduced into purine and purine nucleotides in the forms of 7-deaza-2′-deoxy-7-nitroadenosine (7-NO_2-dA) and 7-deaza-2′-deoxy-7-nitroguanosine (7-NO_2-dG).[12] They were designed to mimic the effect of N^7-methylation (Figure 1) on 2′-deoxyguanine (dG) that weakens the glycosidic bond due to the electron-withdrawing N^{7+}-CH_3 moiety. At elevated temperatures, such methylated DNA can be cleaved at the modified dG by piperidine treatment.[13]

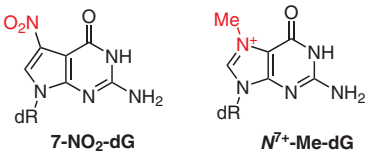


Figure 1 7-NO_2-dG and N^{7+}-Me-dG

SYNTHESIS 2006, No. 19, pp 3280–3290
Advanced online publication: 04.09.2006
DOI: 10.1055/s-2006-950218; Art ID: C04106SS

The strong electron-withdrawing power of the nitro group at C^7 position had indeed rendered 7-NO_2-dA and 7-NO_2-dG similar lability to basic conditions, which conferred sequence-specific cleavage of DNA at the modified bases.[14] Thus, the nitropurine nucleotides are stable enough to withstand high temperatures during PCR amplification, and yet are labile enough to be cleaved by alkaline treatments, under conditions similar to that used by classical Maxam–Gilbert DNA sequencing methods.[15]

The site of nitro modification is positioned at C^7 in 7-NO_2-dATP and 7-NO_2-dGTP. Substituents on C^7 of 7-deazapurines point toward the major groove of DNA, such that they do not interfere with the formation of Watson–Crick base pairs with thymidine (T) or 2′-deoxycytidine (dC) (Figure 2). As a result, these nitro-modified nucleotides can be readily incorporated into DNA by PCR, completely displacing dA and dG, respectively.[14] Because 7-deaza-2′-deoxy-7-nitroinosine is also capable of forming Watson–Crick base pair with dC, its 5′-triphosphate (7-NO_2-dITP) was also synthesized and tested.

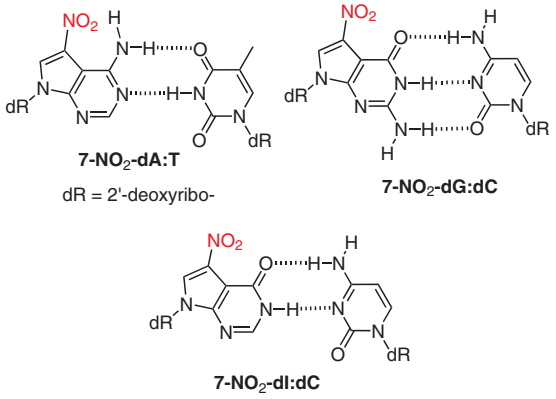


Figure 2 Watson–Crick base pairs 7-NO_2-dA:T, 7-NO_2-dG:dC and 7-NO_2-dI:dC

Competing electronic effects from neighboring and/or conjugating functional groups often hamper the efforts of regioselective electrophilic substitutions of 7-deazapurines and their nucleosides. The process of identifying a suitable synthetic protocol often involves trial-and-error rather than rational design. Seela's group has reported on 7-deaza-dA modifications, and has shown that judicious selection of protection groups could exert remarkable influence on the outcome of the reactions.[16,17] The relative stability of postulated intermediate σ complexes resulted from an electrophilic attack at C^7 or C^8 was used to explain the experimental results.

A systematic examination of differently protected 7-deazapurine derivatives revealed that, to achieve regioselective nitration, chemically and electronically distinctive analogues of dA and dG are required. Figure 3 compares the mesomeric stabilization of electrophilic addition at C^7 of 6-NH_2-protected 7-deaza-dA and the attack at C^8 of unprotected 7-deaza-dA, through the formation of σ-complexes **3A** and **3B**, respectively.

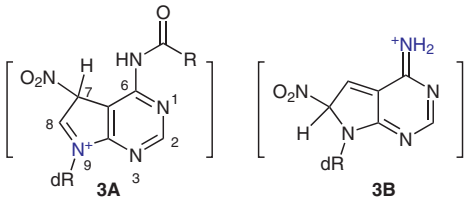


Figure 3 σ-Complexes **3A** and **3B**

Similar analyses on various 7-deaza-dG analogues, on the other hand, suggested that 6-chloro-substitution (in **4A**) would promote C^7 selectivity, whereas the presence of unprotected 2-NH_2 (in **4B**) or 6-OMe (in **4C**) favors electrophilic attack at C^8 (Figure 4). In addition, because 2′-deoxyguanosine is known to be more susceptible to deglycosylation than 2′-deoxyadenosine, and a positive charge is expected to generate on N^9 by electrophilic attack at C^7 (**4A**), 7-deazaguanine should be a better starting compound than 2′-deoxyguanosine. Analogues of purines or purine nucleosides that promote selective C^7 electrophilic substitution were thus selected accordingly for nitration reactions.

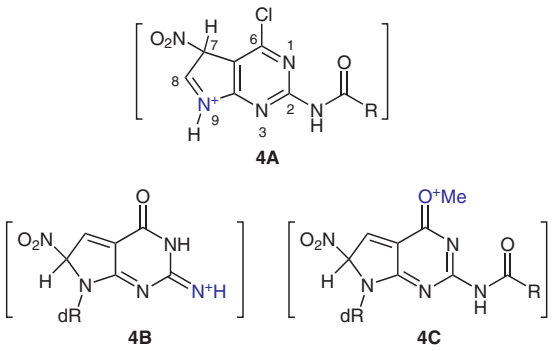
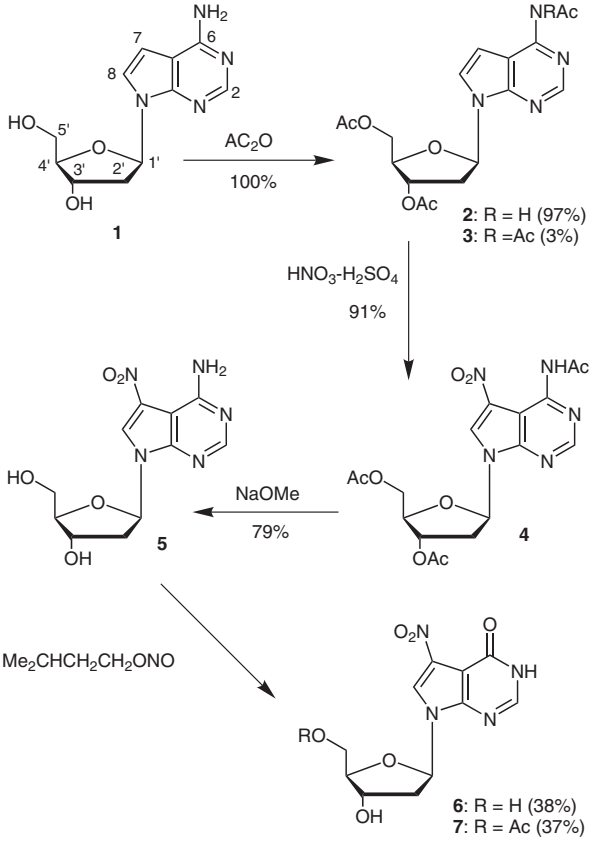


Figure 4 Various 7-deaza-dG analogues for transition-state analyses

Regioselective C^7 nitration (Scheme 1) was accomplished by using N^6-acetylated dA **2/3** as starting material, which was obtained quantitatively from 7-deaza-dA (**1**) through treatment with a large excess of acetic anhydride. Nitration with 1:1 fuming nitric acid and concentrated sulfuric acid afforded compound **4** in 91% yield as a single regioisomer. Deprotection at N^6 afforded desired 7-NO_2-dA (**5**) in 71% yield after column chromatography. Compared to a previously reported nitration reaction starting from unprotected 7-deaza-dA,[18] the protection of exocyclic amino group greatly improved both yield and regioselectivity.[19] These results are in agreement with predictions based on



Scheme 1

the relative stability of the intermediate σ-complexes shown in Figure 3.

The high efficiency of 7-NO_2-dA (**5**) synthesis prompted our interest in the feasibility of preparing 7-NO_2-dI (**6**) directly from **5**. Derivatives of adenosine can be converted to corresponding inosines in a single step by hydrolytic deamination, which has been achieved by using adenosine deaminase[20,21] or alkyl nitrites.[21,22] Alkyl nitrites have been very useful in replacing the 6-amino group of adenosine with various different functional groups, presumably through a 6-diazonium intermediate. Treatment of triacetylated adenosine with *tert*-butyl nitrite has been reported to produce similarly protected inosine in good yields.[23]

To convert 7-NO_2-dA (**5**) to 7-NO_2-dI (**6**), compound **5** was dissolved in a 1:1 mixture of acetic acid and water and treated with isoamyl nitrite at room temperature. After reaction with a total of 2 molar equivalents of isoamyl nitrite over a period of 40 hours, TLC analysis showed that the conversion was about 50% complete. Longer reaction time with additional isoamyl nitrite caused the production of side products, a significant portion of which was identified as 5′-acetyl-7-NO_2-dI (**7**, 37%) after column chromatography. The desired 7-NO_2-dI (**6**) was isolated in a yield of 38%. This result suggests that the nitro substitution is compatible with the alkyl nitrite dA to dI conversion reaction, and that avoiding acetic acid in the reaction mixture may improve the conversion yield. However, this

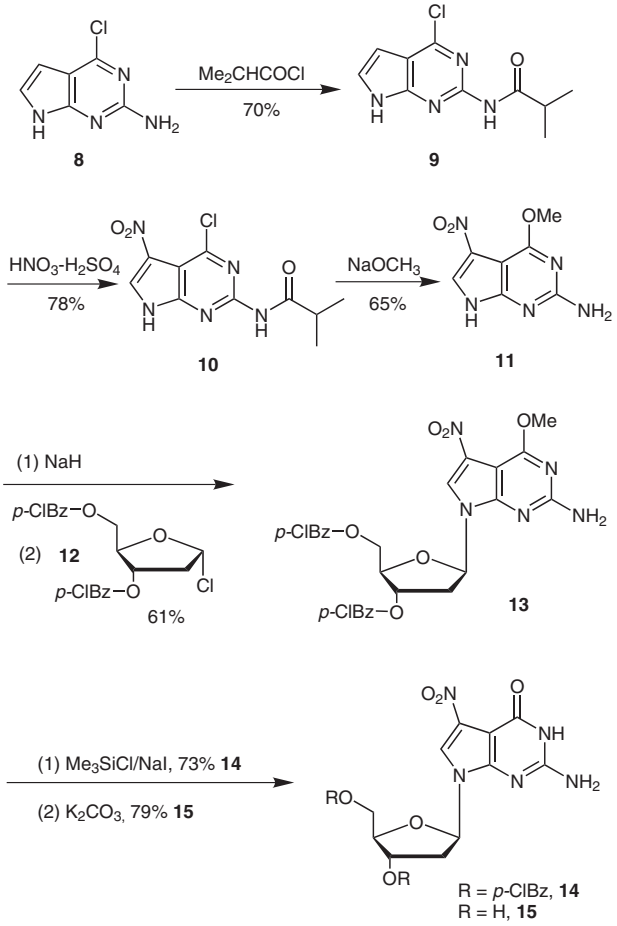
direction was not pursued further as the focus was turned to 7-NO_2-dG preparation.

7-NO_2-dG (**15**) was prepared according to Scheme 2, a seven-step procedure that afforded **15** in good yields. The key step was the nitration reaction, in which the regioselectivity can be dramatically influenced by the choice of starting material. When 2-NH_2 protected 7-deaza-2′-deoxy-6-methoxyguanosine was used, a complex mixture of 7- and 8-NO_2 derivatives of 7-deaza-dG was generated that was very difficult to purify.[18] By simply rearranging the synthetic sequence and using an electron-withdrawing protecting group at C^6 (Cl), the nitration regioselectivity was markedly improved to the extent that only the desired C^7 nitration was observed. These results are consistent with the transition state analyses depicted in Figure 4, i.e. a C^8 electrophilic attack on the C^6-methoxy analogue is stabilized by the formation of σ-complex **4C**, whereas a C^7 attack on the C^6-Cl analogue resulted in an electronically favorable intermediate **4A**.

Specifically as shown in Scheme 2, 6-chloro-7-deazaguanine (**8**) was first protected at 2-NH_2 by reaction with isobutyryl chloride to genereate **9** in an isolated yield of 70%. Treatment of **9** with fuming nitric acid provided C^7-selective nitration product **10** in 78% after silica chromatography. Deprotection at N^2 and Cl substitution by OMe (65%), glycosylation with **12** (61%), followed by demethylation at O^6 (73%) and ribose deprotection (79%), provided the desired product 7-NO_2-dG (**15**). Because of the relative instability of glycosidic bond in dG derivatives, as well as the need of transiently using an electron-withdrawing substituent in place of O^6 to alter its electronic character to favor C^7 nitration, the preparation of 7-NO_2-dG (**15**) was much more lengthier than that of 7-NO_2-dA (**5**).

With these nitro-modified nucleosides in hand, the 5′-triphosphates of 7-NO_2dA, 7-NO_2-dI, and 7-NO_2-dG were prepared in 20–30% yields using a standard synthetic procedure with several modifications. As reported, 7-NO_2-dATP (**16**) and 7-NO_2-dGTP (**17**) were each used in place of dATP and dGTP, respectively, in PCR and generated DNA products that contain each of these nitropurines. 7-NO_2-dITP (**18**) was similarly incorporated into DNA by PCR, where it replaced guanine as base-pairing partner for cytosine (see Figure 2).

To examine the feasibility to sequence-specifically cleave these modified DNA at the incorporated nitropurine bases, the PCR products were fragmented under alkaline conditions and the products analyzed by gel electrophoresis and/or mass spectrometry. In initial cleavage experiments, DNA products were treated with the standard conditions used for Maxam–Gilbert DNA sequencing, i.e., heating in 1 M aqueous solution of piperidine at 90 °C.[15] In these experiments, although DNA cleavage was evident and seemed to occur at selected bases, the cleavage was incomplete and many different discrete bands were observed by polyacrylamide gel electrophoresis (PAGE) analysis (data not shown).



Scheme 2

Considering that the nitropurine triphosphates can survive PCR conditions and are much less labile than N^{7+}-CH_3-dGTP, it is not entirely surprising that the standard Maxam–Gilbert piperidine treatment did not completely cleave these bases. To address the possibility that piperidine might lose its activity during the course of the reaction through *N*-oxide formation, we added DTT (dithiothreitol) or TCEP, two commonly used reducing agents in molecular biology, to the cleavage reaction mixtures. To our surprise, preliminary data indicated that, although DTT did not produce any obvious effect, addition of TCEP altered the cleavage pattern and generated cleaner cleavage products.

To investigate this unexpected phenomenon more closely, the human transferrin receptor (TR) gene, which contains a polymorphic nucleotide A or G at position 424, was chosen as a model system.[14] As depicted in Figure 5, Primer I and Primer II were designed to generate three 7-NO_2-dA-containing PCR products (82 base pairs) using TR DNA templates of three genotypes: A/A homozygote, G/G homozygote, or A/G heterozygote. Primer I was designed to hybridize near the polymorphic base, such that the 7-NO_2-dA specific cleavage would generate an oligonucleotide product of 25nt and/or 28nt in length, depending on which alleles are present in a particular PCR product. Complete site-specific cleavage at 7-NO_2-dA residues is expected to

generate following primer-containing fragments: a 22nt fragment comprising Primer II from the cleavage of the bottom strand DNA, which should be present in all three samples, a Primer I-containing 25nt fragment from A/A homozygote and A/G heterozygote samples, and a Primer I-containing 28nt fragment from G/G and A/G samples (Figure 5). In addition, an 11nt fragment, which contains the complementary polymorphic base (T or C) on the bottom strand of DNA, will be generated by cleavage at 7-NO_2-dA as well, as indicated by green arrows in Figure 5.

For PAGE experiments, a small portion of the A/A genotype PCR product was labeled at 5′-end using [γ-^{32}P]-ATP and T4 kinase. The labeled sample was treated with various cleavage reagents, and the products were purified/desalted and then analyzed by denaturing PAGE along with ^{32}P-labeled control samples and oligonucleotide markers. Lanes 5–8 of Figure 6 show the cleavage products using four different mixtures of reagents as indicated, either at 95 °C for 1 hour (lanes 5, 7, and 8), or at 70 °C for 2 hours for the use of low boiling ammonia (lane 6). Tris base (5 equiv) was added to each cleavage reaction to neutralize the acidic TCEP. In this experiment piperidine/Tris seemed to have cleaved 7-NO_2-dA with higher efficiency than previously observed with 1 M piperidine at 90 °C, probably due to the presence of Tris base and the higher reaction temperature (lane 8). However, slower migrating DNA bands are still visible just above the two major product bands. In contrast, lane 5 did not show any incomplete cleavage products, suggesting that the treatment with a mixture of 1 M piperidine, 0.2 M TCEP, and 0.5 M Tris generated complete cleavage products. Interestingly, these products migrated slightly slower than the major cleavage products in lane 8. Surprisingly, with the formation of minor incomplete cleavage products notwithstanding, treatment with NH_4OH/TCEP/Tris produced similar results (lane 6 vs. lane 5). Furthermore, cleavage by pyrrolidine/TCEP/Tris produced two sets of cleavage products: one set corresponds to the piperidine cleavage products, and the other to that from piperidine/TCEP/Tris. It is noteworthy that the slower migrating products were only present when TCEP is in the cleavage mixture.

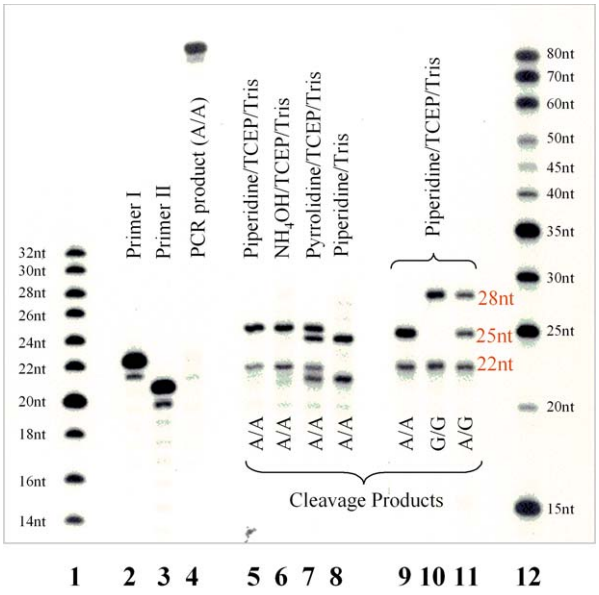


Figure 6 Lane 1: 2nt oligonucleotide ladder containing oligonucleotides of 32nt–14nt in length. Lane 2: Primer I. Lane 3: Primer II. Lane 4: 82bp 7-NO_2-dA-modified PCR product with AA genotype. Lanes 5–8: cleavage products from the sample in Lane 4 under the conditions indicated. Lanes 9–11: cleavage products from AA, GG, or AG genotypes, using the reagents indicated. Lane 12: 5nt oligonucleotide ladder containing oligonucleotides of 15nt–80nt in length.

After these exploratory cleavage experiments, piperidine/TCEP/Tris mixture was selected as the cleavage reagents for treating three PCR products corresponding to A/A, G/G, or A/G genotypes. Each cleavage product was desalted and divided into two portions. A small sample was labeled at 5′-end using [γ-^{32}P]-ATP and T4 kinase, and then analyzed by denaturing PAGE. The remainder of each sample was saved for mass spectrometry analysis. As shown in lanes 9–11 of Figure 6, two or three distinctive product bands were observed, in agreement with expected genotypes of each sample: 25nt represents the presence of A allele in samples A/A and A/G, 28nt represents the presence of G allele in the samples G/G and A/G, with a common 22nt in all three samples. This experiment demonstrated the feasibility to genotype a DNA sample using the analogue incorporation and chemical cleavage approach, specifically (1) using 7-NO_2-dATP in place of dATP during

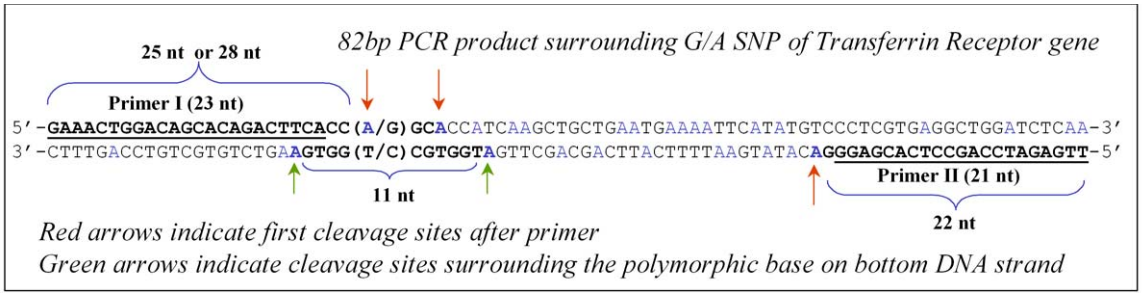


Figure 5 DNA sequence of 82bp PCR products containing 7-NO_2-dA, which is denoted as blue-colored A in the sequence. Polymorphic bases are shown in parentheses. Red arrows indicate 7-NO_2-dA residues that are positioned closest to the primers. Green arrows indicate 7-NO_2-dA residues that surround the internal fragment containing a polymorphic base. bp: base pairs; nt: nucleotides.

PCR amplification, (2) fragmenting the PCR product using piperidine/TCEP/Tris mixture, and (3) analyzing the cleavage products by denaturing PAGE.

To understand the cleavage mechanism and definitively assign the chemical structures of the cleaved products, and to investigate the feasibility to utilize this incorporation-cleavage genotyping strategy in mass spectrometry platform, the remaining samples of each PCR product was similarly cleaved and then analyzed using MALDI-TOF mass spectrometry. The resulting mass spectrum from the heterozygote G/A sample is shown in Figure 7A, with the expected and observed major fragments listed in Figure 7B. As shown in Figure 7A, three fragments were observed in the mass region between 7000 and 9000 Daltons. By using internal mass calibration, the three species were determined to be 7189 Da, 8057 Da, and 9005 Da, respectively. Based on a previously proposed mechanism[15] of piperidine-induced, Maxim-Gilbert DNA scission, the cleavage products expected from such cleavage are 3′-phosphorylated DNA (3′-P-DNA) fragments, including **Primer II**-G-PO_3^-, **Primer I**-CC-PO_3^-, and **Primer I**-CCGGC-PO_3^- (see Figure 5 and Scheme 3). The expected *m*/*z* of these 3′-phosphorylated species in positive ion mode are 6841 Da, 7709 Da, and 8657 Da, respectively. Thus, there is an extra 348 Da in our piperidine/TCEP/Tris cleaved fragments.

Because our experimental data showed an excellent correspondence among the observed higher masses, the slower electrophoresis mobility, and the presence of TCEP in the cleavage reaction mixtures, it seemed reasonable to postulate that the cleavage products contain a TCEP-generated moiety. Because the molecular weight of TCEP ($C_9H_{15}O_6P$) is 250 Da and that of dideoxyribose moiety ($C_5H_{10}O_2$) is 102 Da, the fusion of the two with 3′-phosphorylated DNA should provide the observed extra mass of 348 Da.

Based on the Maxam–Gilbert cleavage mechanism of N^{7+}-Me-dG-DNA,[24] a piperidine cleavage mechanism for 7-NO_2-dA-DNA is proposed in Scheme 3. As illustrated, the electron-withdrawing capacity of nitro group facilitates attack by hydroxide on the neighboring carbon and yields a carbinolamine, which further reacts with hydroxide to open the five-membered ring of the purine. The resulting riboside carbinolamine can open to form a Schiff base, which is susceptible to attack by piperidine to form a piperidinium ion. Depending on reaction conditions, this key intermediate of the cleavage reaction can lead to several different products. First, as shown in Scheme 3, a general base attacks the activated α-hydrogen, resulting in an E2 reaction with loss of the β-phosphate group to generate DNA strand scission at what used to be 3′-phosphate. A second E2 reaction causes the loss of γ-hydrogen and δ-phosphate, releasing what was the 5′-end of the DNA chain. The final oligonucleotide product is the 3′-phosphorylated DNA.

The second plausible fate of the activated piperidinium intermediate is shown at the top panel of Scheme 4. It could be hydrolyzed before any E2 reactions could occur, resulting in the formation of an apurinic DNA. It has been shown that apurinic DNA is susceptible to β-elimination catalyzed by N-terminal β-amino group of peptides, through the formation of a Schiff base intermediate similar to piperidinium intermediate.[25] Conversely, it seems reasonable to hypothesize that an inadequate availability of free amines such as piperidine could stall the DNA cleavage reaction at the apurinic DNA stage.

When TCEP is added to the cleavage reaction, the otherwise stagnant apurinic DNA is driven by β-elimination of what used to be 3′-phosphate (Scheme 4), generating a Michael acceptor that can be readily attacked by the phosphine on TCEP, a strong nucleophile. The resulting Michael addition product is a 3′-phosphodideoxyribosylate-DNA modified by a phosphonium moiety at the 3′-position of the basic ribose. This proposed product is in excellent agreement with the observed mass spectrometry data, adding 348 Da mass to 3′-phosphorylate-DNA. Because piperidine moiety is not observed in the final product, the piperidinium intermediate must have been hydrolyzed before Michael addition. This also lends additional support to the idea that the incomplete cleavage reaction was stalled at the stage of apurinic DNA species in the absence of TCEP.

In short, two competitive cleavage pathways exist for the piperidinium intermediate: β-elimination/hydrolysis,

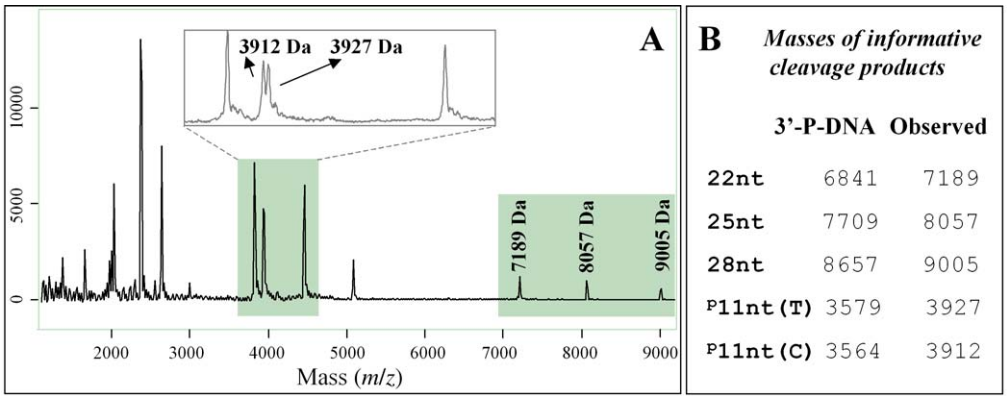
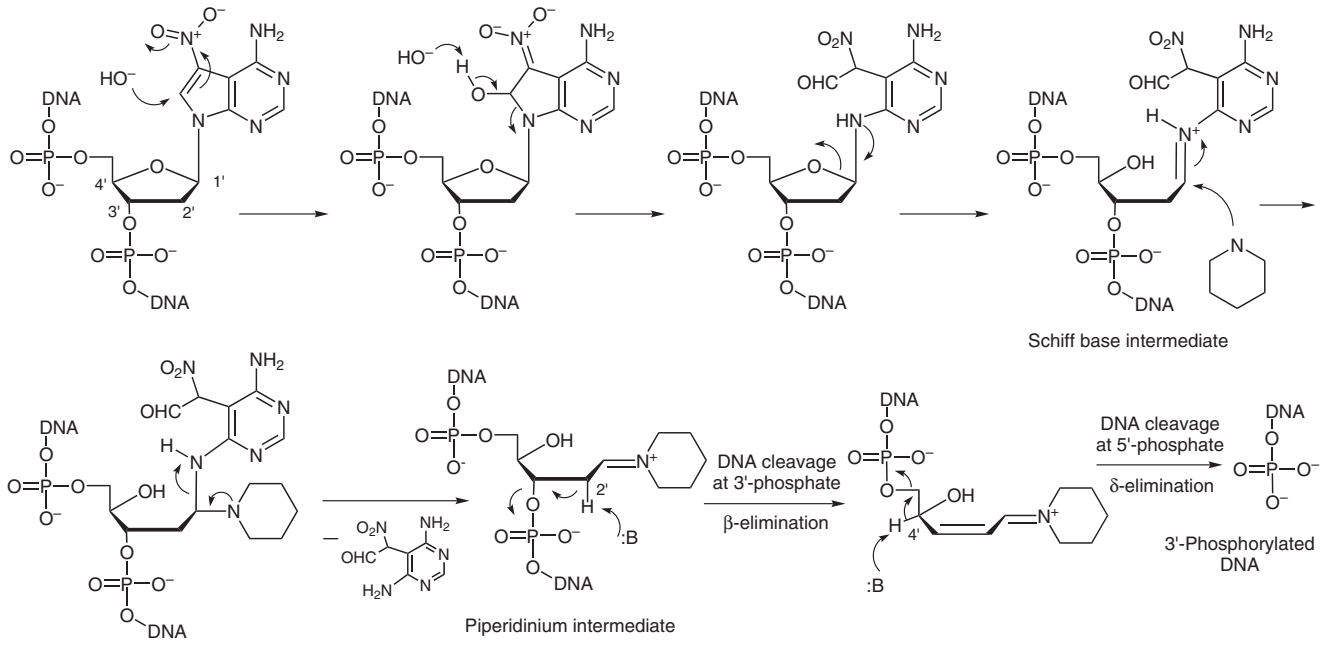
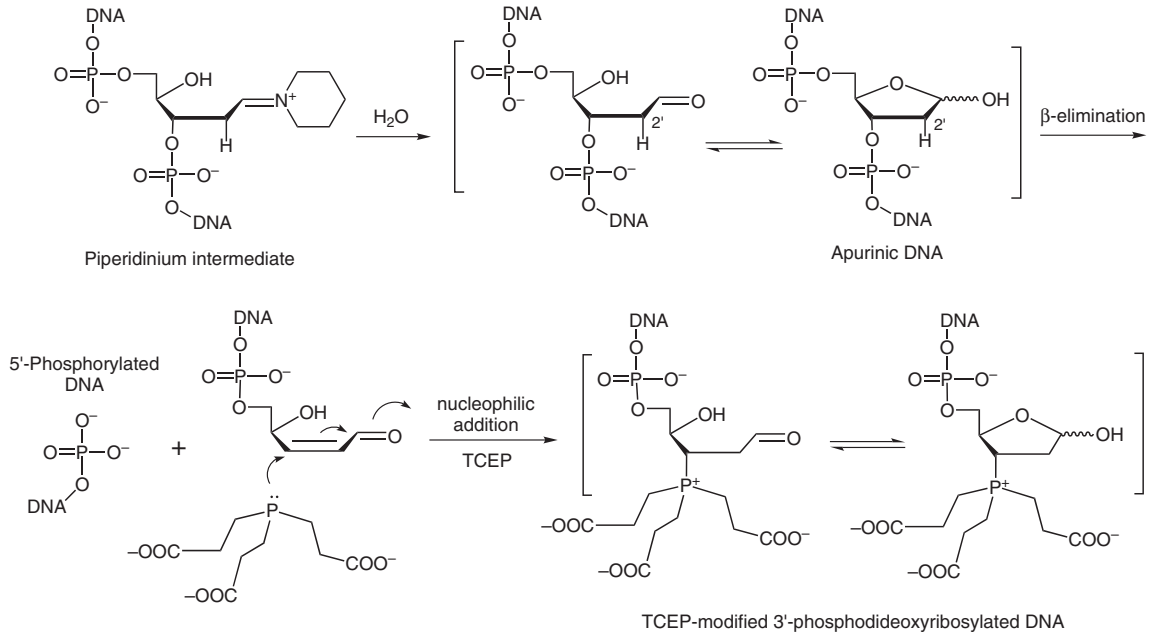


Figure 7 Mass spectrum of a heterozygote G/A sample



Scheme 3

which lead to δ-elimination followed by formation of 3′-phosphorylated DNA (Scheme 3), or apurinic DNA formation followed by Michael addition of a strong nucleophile (TCEP) to yield a 3′-phosphodideoxyribosylated DNA adduct (Scheme 4). These proposed pathways predict that the final cleavage outcome could be influenced by the strength of basicity or nucleophilicity of the reagents used; giving rise to 3′-phosphorylate-DNA and/or the TCEP adduct of 3′-phosphoribosylated-DNA. Indeed, in the mixture of pyrrolidine/TCEP/Tris where piperidine is replaced by pyrrolidine (lane 7 of Figure 6), two sets of distinctive cleavage products were observed. One set with the same mobility as the piperidine cleavage products, the other the same as the cleavage products by piperidine/TCEP/Tris. The higher pK_a of pyrrolidine likely promoted the formation of pyrrolidinium intermediate, ensuring subsequent β- and δ-elimination to yield 3′-phosphate-DNA product, which was absent when TCEP/piperidine/Tris is used (lane 5 in Figure 6). NH_4OH, on the other hand, with a lower pK_a than piperidine and pyrrolidine, could not cleave 7-NO_2-dA completely (lane 6 in Figure 6). However, the fact that NH_4OH/TCEP/Tris actually could cleave 7-NO_2-dA-DNA quite efficiently suggests that the formation of piperidinium or pyrrolidinium



Scheme 4

intermediates can be bypassed; the hydrolysis of Schiff base intermediate could directly yield apurinic DNA, which is a good Michael acceptor for phosphine.

Mass spectrometry analysis (Figure 7) not only provided exact mass of each major cleavage fragments that led to the proposal of cleavage mechanisms, but also provided information on the smaller, internal cleavage fragments that are genotype-specific. Four *m*/*z* peaks were observed in the *m*/*z* region of 3500–5000 (Figure 7A and inset therein), corresponding to one 11nt fragment from top strand DNA (located near Primer II in Figure 5), one 13nt fragment from bottom strand DNA (located near Primer I), and two 11nt fragments that contained genotype information on SNP 424 (highlighted in Figure 5). These 11nt long fragments clearly showed the mass difference of 15 Da, in agreement with expected mass difference between T allele and C allele. The observed *m*/*z* values, 3912 Da and 3927 Da, are consistent with molecular structures of a protonated 5′-PO_3H_2-TGG(**T**/**C**)GCTGGTG-PO_2H-dideoxyribosylate-TCEP. This result provides additional support for the cleavage mechanism shown in Scheme 4, which suggests the formation of 5′-phosphorylated C-terminus cleavage fragment.

The proposed cleavage mechanisms also suggest that the cleavage efficiency could be improved using reagents and conditions with increased basicity and nucleophilicity. To this end, pyrrolidine was first used to cleave 7-NO_2-dA-DNA at 95–98 °C and resulted in better cleavage than piperidine. Further improvements were accomplished with the use of 3-pyrrolidinol, a pyrrolidine derivative with high boiling point, excellent water miscibility, and presumably comparable pK_a to piperidine. Because of its high boiling point, reagent loss during the cleavage reaction was minimized, which both improves reaction efficiency and eliminates the unpleasant odor typical of amines. Due to its high miscibility with water, it can be used at higher concentrations than piperidine and pyrrolidine. Eventually it was found that a 1.46 M aqueous solution of 3-pyrrolidinol could consistently induce complete cleavage at 7-NO_2-dA by heating at 98 °C for 1 hour. Consequently, these conditions were used in the proof-of-concept experiments for a mass spectrometry based genotyping methodology.[14]

In summary, we have developed synthetic methods that support regioselective nitration of purine derivatives based on electronic considerations of transition states. Different synthetic strategies were developed for the nitration of 7-deazaadenosine and 7-deaza-guanine, with excellent yields and regioselectivity.[12] 7-NO_2-dA can be converted to 7-NO_2-dI by hydrolytic deamination, although the procedure has not been optimized. Corresponding nucleotide triphosphates were prepared and can be incorporated into DNA by PCR, substituting corresponding natural nucleotides. Such modified DNA is susceptible to chemical cleavage under alkaline conditions that are similar to what has been used by Maxam–Gilbert DNA sequencing.[15] Although the resulting DNA resisted complete cleavage by piperidine, it was discovered that addition of a potent nucleophile such as TCEP could drive the cleavage reaction of 7-NO_2-dA-DNA to completion. Quite unexpectedly, a different end product, i.e., a TCEP modified 3′-phosphodidepxyribosylate DNA species, was obtained. The identity of the cleavage products was confirmed by MALDI-TOF mass spectrometry analysis, and a cleavage mechanism was proposed based on the product structure and the previously articulated mechanism for N^{7+}-Me-dG-DNA cleavage. Based on the proposed mechanism, additional cleavage reagents were tested and found to be superior in generating complete cleavage products. Nitropurines turned out to be an excellent choice for PCR incorporation and chemical cleavage.[14] The facility with which the TCEP adduct was formed during cleavage suggests that this reaction may be useful for introducing other functional groups, such as fluorescent labels, into cleaved DNA in situ.

Reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO) or J. T. Baker (Phillipsburg, NJ) unless otherwise specified. The ^1H and ^{31}P NMR spectra were recorded on a Bruker DPX-400 NMR spectrometer using either TMS as an internal standard (^1H spectra) or 80% H_3PO_4 as an external standard (^{31}P spectra).[26] Chemical shifts (δ) are reported in ppm downfield from TMS or H_3PO_4. Column chromatography was performed with silica gel 60 (230–400 mesh, Merck 9385) using standard flash methods. Analytical TLC was carried out on Merck silica gel 60 F_{254} precoated plates.

3′,5′,N^6-Triacetyl-7-deaza-2′-deoxyadenosine (2) and 3′,5′,N^6,N^6-Tetraacetyl-7-deaza-2′-deoxyadenosine (3)

2′-Deoxy-7-deazaadenosine (**1**; 2.092 g, 8.36 mmol, from ChemGenes) and 4-dimethylaminopyridine (30 mg, 0.36 mmol) were placed in a 100-mL flask. Anhyd pyridine (20 mL) and Ac_2O (20 mL, 0.21 mol) were added and the mixture was stirred at r.t. for 7.5 h. The mixture was evaporated to near dryness and the residue was chromatographed on silica gel (Merck 9385, 50 g, 2% MeOH–CH_2Cl_2) to give a mixture of acetylated 7-deaza-dA (3.328 g, quantitative). The ratio of triacetylated 7-deaza-dA (**2**: R = H) and tetraacetylated 7-deaza-dA (**3**: R = Ac) was 34:1 based on ^1H NMR spectroscopy. The following spectral data were obtained from each isolated material.

Compound 2

^1H NMR (400 MHz, $CDCl_3$): δ = 9.09 (br s, 1 H, NH), 8.54 (s, 1 H, H-2), 7.31 (d, *J* = 3.9 Hz, 1 H, H-8), 7.01 (d, *J* = 3.5 Hz, 1 H, H-7), 6.77 (dd, *J* = 5.9, 8.6 Hz, 1 H, H-1′), 5.37 (td, *J* = 2.0, 6.2 Hz, 1 H, H-3′), 4.32 (m, 3 H, H-4′, H-5′), 2.68 (ddd, *J* = 6.2, 8.6, 14.1 Hz, 1 H, H-2′b), 2.52 (ddd, *J* = 2.0, 5.9, 14.1 Hz, 1 H, H-2′a), 2.34 (s, 3 H, $COCH_3$), 2.13 (s, 3 H, $COCH_3$), 2.12 (s, 3 H, $COCH_3$).

Compound 3

^1H NMR (400 MHz, $CDCl_3$): δ = 8.87 (s, 1 H, H-2), 7.49 (d, *J* = 3.9 Hz, 1 H, H-8), 6.77 (dd, *J* = 5.9, 8.6 Hz, 1 H, H-1′), 6.47 (d, *J* = 3.9 Hz, H-7), 5.39 (td, *J* = 2.0, 6.6 Hz, 1 H, H-3′), 4.36 (m, 3 H, H-4′, H-5′), 2.74 (ddd, *J* = 6.8, 8.6, 14.1 Hz, 1 H, H-2′b), 2.58 (ddd, *J* = 2.0, 5.9, 14.1 Hz, 1 H, H-2′a), 2.32 (s, 6 H, 2 × $COCH_3$), 2.15 (s, 3 H, $COCH_3$), 2.11 (s, 3 H, $COCH_3$).

ES$^+$-MS: *m*/*z* [M + Na^+] calcd for $C_{19}H_{22}N_4O_7$ + Na: 441; found: 441.35.

3′,5′,N^6-Triacetyl-7-deaza-2′-deoxy-7-nitroadenosine (4)

A mixture of acetylated 7-deaza-dA (**2**/**3**, 3.325 g, ~8.36 mmol, prepared as above) was dissolved in CH_2Cl_2 (400 mL, HPLC grade) in

a 1-L flask. The solution was cooled in an ice-bath and stirred vigorously. A mixture of fuming HNO_3 (6.2 mL) and concd H_2SO_4 (6.2 mL) was prepared by dropwise addition of H_2SO_4 to ice-cooled and stirred HNO_3, and immediately added dropwise to the CH_2Cl_2 solution of **2/3** over a 10 min period. After stirring for another 20 min, the reaction was quenched by the addition of chilled 20% aq K_2CO_3 (150 mL). The resulting organic phase was saved, and the aqueous phase was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic solutions were washed with H_2O (100 mL) and brine (100 mL) and dried (Na_2SO_4). After rotary evaporation, the residue was chromatographed on silica gel (Merck 9385, 50 g, 2% MeOH–CH_2Cl_2] to give **4**; yield: 3.209 g (91% from **1**).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.67 (br s, 1 H, NH), 8.74 (s, 1 H, H-8), 8.53 (s, 1 H, H-2), 6.73 (t, *J* = 6.6 Hz, 1 H, H-1′), 5.36 (m, 1 H, H-3′), 4.42 (m, 3 H, H-4′ and H-5′), 2.75 (ddd, *J* = 3.1, 6.2, 14.4 Hz, 1 H, H-2′b), 2.59 (td, *J* = 7.0, 14.4 Hz, 1 H, H-2′a), 2.54 (s, 3 H, $COCH_3$), 2.19 (s, 3 H, $COCH_3$), 2.15 (s, 3 H, $COCH_3$).

7-Deaza-2′-deoxy-7-nitroadenosine (5)

Compound **4** (3.205 mg, 7.61 mmol) was dissolved in anhyd MeOH (38 mL) and cooled in an ice bath. A solution of NaOMe in MeOH (0.5 M, 76 mL) was added under ice cooling. The ice bath was removed and the mixture was stirred for 5 h at r.t. After neutralization with AcOH (~1.2 mL) to pH ~8, silica gel (10 g) was added. The solvent was evaporated and the residue was loaded on to a silica gel column (Merck 9385, 40 g). The column was eluted with 7.5–10% MeOH–CH_2Cl_2 and fractions containing 7-NO_2-dA (**5**) were combined and evaporated. The yellow residue was triturated in MeOH (10 mL) and the solid was collected by filtration. After washing with MeOH (2 × 3 mL) and drying under vacuum overnight, compound **5** was obtained as light yellow powder (1.778 mg, 79%). Evaporation of the filtrate produced additional 212 mg of product as yellow caramel (9%), which was almost as pure as the yellow powder.

^{1}H NMR (400 MHz, CD_3OD): δ = 8.72 (s, 1 H, H-8), 8.19 (s, 1 H, H-2), 6.59 (t, *J* = 6.4 Hz, 1 H, H-1′), 4.53 (td, *J* = 3.1, 6.2 Hz, H-3′), 4.04 (td, *J* = 3.1, 3.1 Hz, 1H, H-4′), 3.85 (dd, *J* = 3.1, 12.1 Hz, 1 H, H-5′b), 3.76 (dd, *J* = 3.5, 12.1 Hz, 1 H, H-5′a), 2.79 (td, *J* = 6.6, 13.2 Hz, 1 H, H-2′b), 2.45 (ddd, *J* = 3.5, 6.2, 13.3 Hz, 1 H, H-2′a).

1D NOE between H-1′ and H-8 signals: irradiating at δ = 6.59 (H-1′) caused 4% enhancement on the signal at δ = 8.72 (H-8); irradiating at δ = 8.72 caused 5% enhancement on the signal at δ = 6.59.

7-Deaza-2′-deoxy-7-nitroinosine (6)

Solid **5** (61.6 mg, 0.21 mmol) was dissolved in a mixture of AcOH (4 mL) and H_2O (4 mL). To the solution, isoamyl nitrite (140 μL, 1.04 mmol) was added. After stirring at r.t. for 15 h, the mixture was analyzed using TLC by developing in 5:1 CH_2Cl_2–MeOH. To the mixture an additional amount of isoamyl nitrate (140 μL) was added, and the resulting mixture was allowed to react for 25 h. TLC analysis suggested that the product contained a mixture of ~1:1 7-NO_2-dA (**5**) and 7-NO_2-dI (**6**). To this mixture an additional amount of isoamyl nitrite (140 μL) was added and the mixture stirred for 3 days. TLC results indicated the formation of side products in addition to compound **6**. The mixture was concentrated to dryness, and the residue was purified using preparative TLC. Beside the recovered starting material **5**, compound **7** was obtained as a side product (26.2 mg, 37%). The desired product **6** was obtained as yellow caramel (23.4 mg, 38%).

Compound 6

^{1}H NMR (400 MHz, CD_3OD): δ = 12.4 (br s, 1 H, NH), 8.56 (s, 1 H, H-8), 8.02 (s, 1 H, H-2), 6.63 (t, *J* = 6.6 Hz, 1 H, H-1′), 4.52 (m, 1 H, H-3′), 4.02 (m, 1 H, H-4′), 3.83 (dd, *J* = 3.5, 12.1 Hz, 1 H, H-5′b), 3.76 (dd, *J* = 3.9, 12.1 Hz, 1 H, H-5′a), 2.54 (m, 1 H, H-2′b), 2.46 (ddd, *J* = 3.5, 6.2, 13.7 Hz, 1 H, H-2′a).

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.58 (s, 1 H, H-8), 8.07 (s, 1 H, H-2), 6.49 (t, *J* = 6.6 Hz, 1 H, H-1′), 5.34 (br s, 1 H, OH), 5.17 (br s, 1 H, OH), 4.36 (m, 1 H, H-3′), 3.87 (m, 1 H, H-4′), 3.64 (dd, *J* = 3.9, 11.7 Hz, 1 H, H-5′b), 3.57 (dd, *J* = 3.4, 11.7 Hz, 1 H, H-5′a), 2.47 (m, 1 H, H-2′b, overlapped with DMSO), 2.30 (ddd, *J* = 3.4, 5.9, 13.2 Hz, 1 H, H-2′a).

Compound 7

^{1}H NMR (400 MHz, CD_3OD): δ = 8.36 (s, 1 H, H-8), 8.03 (s, 1 H, H-2), 6.61 (t, *J* = 6.2 Hz, 1 H, H-1′), 4.48–4.56 (m, 1 H, H-3′), 4.34 (m, 2 H, H-5′), 4.16 (m, 1 H, H-4′), 2.42–2.63 (m, 2 H, H-2′), 2.13 (s, 3 H, $COCH_3$).

6-Chloro-7-deaza-*N*^{2}-isobutyrylguanine (9)

6-Chloro-7-deazaguanine (**8**; 6.7228 g, 39.88 mmol, from ChemGenes) was dissolved in anhyd pyridine (280 mL). The solution was chilled in an ice/water bath. To this solution was added freshly distilled isobutyryl chloride (43.87 mmol, 4.6 mL, 1.1. equiv, from ICN) dropwise with stirring. After stirring for 20 min at 0 °C, the reaction was quenched with MeOH (600 μL), followed by an additional 10 min of stirring. The mixture was concentrated in vacuo to give a yellow solid, and then resuspended in Et_2O (20 mL). The solid was collected by vacuum filtration, washed with ice-cold 80% MeOH–20% Et_2O (50 mL), ice-cold MeOH (10 mL), and then Et_2O (10 mL). Drying in vacuo yielded 6.6794 g (70%) of **9** as pale yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.34 (br s, 1 H, NH), 10.54 (br s, 1 H, NH), 7.49 (dd, *J* = 2.4 Hz, 3.2 Hz, 1 H, H-8), 6.49 (dd, *J* = 1.6 Hz, 3.2 Hz, 1 H, H-7), 2.78 [sept, *J* = 6.8 Hz, 1 H, C*H*$(CH_3)_2$], 1.08 [d, *J* = 6.8 Hz, 6 H, CH$(CH_3)_2$].

^{13}C NMR (100 MHz, DMSO-d_6): δ = 174.7, 152.8, 151.2, 150.3, 127.1, 112.9, 98.9, 34.4, 19.3.

6-Chloro-7-deaza-2-isobutyrylamino-7-nitroguanine (10)

Solid **9** (6.2651 g, 26.25 mmol) was added to stirred, ice-chilled fuming HNO_3 (25 mL). This solution was stirred under ice-bath cooling for 2 h, during which time the reaction took on a bright yellow color. This solution was then added dropwise to stirred ice water (600 mL). Within 5 min, a pale-yellow precipitate formed. This solution was allowed to warm to r.t., with continued stirring, for 3 h. The precipitate was collected by filtration using a fine-fritted filter funnel, washed with H_2O until the filtrate was no longer acidic, and then dried in vacuo to yield 5.8055 g (78%) of **10** as pale yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 13.69 (br s, 1 H, NH), 10.87 (br s, 1 H, NH), 8.81 (s, 1 H, H-8), 2.79 [sept, *J* = 6.8, 1 H, C*H*$(CH_3)_2$], 1.09 [d, *J* = 6.8Hz, 6 H, CH$(CH_3)_2$].

^{13}C NMR (100 MHz, DMSO-d_6): δ = 174.9, 152.9, 152.6, 151.0, 132.1, 127.2, 104.7, 34.6, 19.2.

Compound **10** was methylated at *N*^{9} position and the resulting compound was analyzed by 1D NOE experiments between H^8 and *N*^{9}-CH_3 signals.

Methylated 10

^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.93 (1 H, s, NH), 8.92 (1 H, s, H-8), 3.82 (3 H, s, NCH_3), 2.83 (1 H, m, COCH), 1.10 (6 H, d, *J* = 6.8 Hz, 2 CH_3).

Irradiation at δ = 8.92 (H-8) caused 5% enhancement on the signal at δ = 3.82 (NCH_3); irradiation at δ = 3.82 caused 4% enhancement on the signal at δ = 8.92.

7-Deaza-*O*^{6}-methyl-7-nitroguanine (11)

A solution of **10** (4.6497 g, 16.39 mmol) in 0.5 M NaOMe in MeOH (100 mL) was refluxed for 8 h. Following the removal of the heating source, the mixture was cooled to 0 °C in an ice-water bath,

quenched with glacial AcOH (2 mL), and then evaporated onto silica gel. Silica gel flash chromatography (~10% MeOH in CH_2Cl_2) yielded 2.2183 g (65%) of **11** as a bright yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.44 (br s, 1 H, NH), 8.18 (s, 1 H, H-8), 6.57 (br s, 2 H, NH_2), 3.95 (s, 3 H, OCH_3).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 162.4, 160.5, 154.7, 128.5, 125.8, 89.6, 53.4 (OCH_3).

3′,5′-Bis[*O*-(4-chlorobenzoyl)]-7-deaza-2′-deoxy-*O*^{6}-methyl-7-nitroguanosine (13)

A solution of **11** (1.4765 g, 7.059 mmol) in anhyd DMF (14 mL) was added dropwise to a stirred, ice-water-chilled suspension of NaH (60% w/w mineral oil dispersion, 1.3 equiv, 9.275 mmol, 371 mg) in anhyd DMF (10 mL). During and after the addition, the vigorous evolution of gas (H_2) was observed. After stirring for 1 h, this solution was drawn into a dry syringe, and then was added dropwise to a ice-chilled, stirring suspension of **12** (3.179 g, 7.398 mmol, 1.05 equiv) in anhyd MeCN (150 mL). This suspension was warmed to r.t. and stirred for an additional 2 h. The mixture was then poured into CH_2Cl_2 (I L) and the CH_2Cl_2 layer was washed with H_2O (2 × 300 mL), 10% (w/v) aq LiBr (2 × 200 mL), and brine (400 mL). The resulting organic layer was dried (Na_2SO_4), and then evaporated. During concentration, a yellow precipitate formed. This precipitate was collected by filtration, washed with Et_2O, and proved to be pure product by TLC (1:2:7 EtOAc–hexane–CH_2Cl_2) and ^{1}H NMR spectroscopy. The remaining solution was evaporated onto silica gel and flash-chromatographed with EtOAc–hexane–CH_2Cl_2 (1:2:7) to isolate the remaining product. The combination of filtration and chromatography yielded 2.5973 g (61%) of **13**.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.46 (s, 1 H, H-8), 8.04 (d, J = 8.6 Hz, 2 H_{arom}), 7.96 (d, J = 8.6 Hz, 2 H_{arom}), 7.65 (d, J = 8.6 Hz, 2 H_{arom}), 7.57 (d, J = 8.6 Hz, 2 H_{arom}), 6.83 (br s, 2 H, NH_2), 6.56 (dd, J = 6.4, 8.0 Hz, 1 H, H-1′), 5.72 (m, 1 H, H-3′), 4.68 (dd, J = 7.4, 14.1 Hz, 1 H, H-5′b), 4.57 (m, 2 H, H-5′a and H-4′), 3.96 (s, 3 H, OCH_3), 3.09 (td, J = 7.8, 14.4 Hz, 1 H, H-2′b), 2.73 (ddd, J = 2.3, 6.2, 14.4 Hz, 1 H, H-2′a).

3',5'-Bis[*O*-(4-chlorobenzoyl)]-7-daza-2'-deoxy-7-nitroguanosine (14)

To a suspension of **13** (2.2519 g, 3.738 mmol) and NaI (1.12 g, 7.476 mmol, 2 equiv) in anhyd MeCN (150 mL) was added freshly distilled (from K_2CO_3) chlorotrimethylsilane (0.72 mL, 1.5 equiv). After refluxing the resulting suspension for 4 h, the mixture was cooled to 0 °C with ice-water, quenched with MeOH (2 mL), and then evaporated to dryness. The residue was redissolved in CH_2Cl_2 (800 mL) washed with half-saturated NaCl (200 mL), 10% (w/v) aq Na_2SO_3 (200 mL), and brine (200 mL), dried (Na_2SO_4), and then evaporated onto silica gel. Silica gel flash chromatography with CH_2Cl_2–MeOH (19:1) gave 1.6121 g (73%) of **14** as a bright yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.87 (br s, 1 H, NH), 8.25 (s, 1 H, H-8), 8.03 (d, J = 8.2 Hz, 2 H_{arom}), 7.98 (d, J = 8.2 Hz, 2 H_{arom}), 7.65 (d, J = 8.2 Hz, 2 H_{arom}), 7.58 (d, J = 8.2 Hz, 2 H_{arom}), 6.68 (br s, 2 H, NH_2), 6.47 (dd, J = 6.2, 8.2 Hz, 1 H, H-1′), 5.70 (m, 1 H, H-3′), 4.66 (dd, J = 6.6, 13.3 Hz, 1 H, H-5′b), 4.56 (m, 2 H, H-4′ and H-5′a), 3.02 (m, 1 H, H-2′b), 2.72 (ddd, J = 2.3, 6.2, 14.4 Hz, 1 H, H-2′a).

7-Deaza-2′-deoxy-7-nitroguanosine (15)

To a solution of **14** (161.6 mg, 0.2747 mmol) in anhyd CH_2Cl_2 (10 mL) was added a 50 mM solution of K_2CO_3 (20 mL) in anhyd MeOH. This solution was stirred at r.t. for 1 h, during which time it turned bright orange. The reaction was neutralized by the addition of Dowex 50WX-100 (H^+ form), which was later removed by vacuum filtration. The solution was evaporated to dryness, and the residue was resuspended in MeOH (30 mL) and diluted with H_2O (150 mL). This solution was washed with Et_2O (3 × 100 mL), and then evaporated to afford **15** as a bright yellow solid (79%).

^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.80 (br s, 1 H, NH), 8.25 (s, 1 H, H-8), 6.65 (br s, 2 H, NH_2), 6.34 (dd, J = 5.9, 7.3 Hz, 1 H, H-1′), 5.27 (m, 1 H, OH), 5.04 (m, 1 H, OH), 4.32 (m, 1 H, H-3′), 3.82 (m, 1 H, H-4′), 3.58 (m, 2 H, H-5′a and H-5′b), 2.38 (ddd, J = 5.9, 7.3, 13.2 Hz, 1 H, H-2′b), 2.20 (ddd, J = 2.9, 5.9, 13.2 Hz, 1 H, H-2′a).

7-Deaza-2′-deoxy-7-nitroadenosine 5′-*O*-Triphosphate (16)

Compound **5** (32.3 mg, 0.109 mmol) was co-evaporated twice from anhyd pyridine and dried overnight under high vacuum. Its suspension in trimethyl phosphate (0.25 mL) was sonicated for 20 sec and cooled in an ice-bath. To the mixture was added $POCl_3$ (12 μL, 0.129 mmol) and stirred for 3.5 h at 0 °C. To this mixture was added simultaneously a 0.5 M solution of $(Bu_3NH)_2H_2P_2O_7$ in DMF (1 mL, 0.5 mmol) and Bu_3N (0.13 mL, 0.54 mmol). After stirring vigorously for 1 h at 0 °C, the mixture was poured into ice-cold 1.5 M triethylammonium bicarbonate (TEAB, pH 7.5, 10 mL) and lyophilized. The yellow residue was dissolved in H_2O (10 mL) and purified by ion-exchange chromatography [DEAE Sephadex A25, 9 × 2.5 cm, linear gradient of TEAB (0.05 to 1.5 M, 1500 mL), flow 5 mL/min]. The triphosphate fractions, eluted at 0.6–0.75 M TEAB, were pooled and lyophilized. The residue was purified by reversed phase HPLC [Hamilton PRP-1, ϕ 10 mm × l 250 mm, flow 4 mL/min] with a gradient of MeOH mixed with 0.1 M triethylammonium acetate (TEAA, pH 8.5). The triphosphate product was eluted at 39–41% MeOH. Lyophilization (3 times from H_2O) gave triethylammonium salt of **16** as a white solid. UV quantitation indicated that 33 μmoles of product was obtained, corresponding to 30% yield. ^{1}H NMR showed this material contains ~3 moles of Et_3N per mole of triphosphate.

Compound 16·3N(CH_2CH_3)$_3$

^{1}H NMR (400 MHz, D_2O): δ = 8.63 (s, 1 H, H-2), 8.16 (s, 1 H, H-8), 6.57 (t, J = 5.6 Hz, 1 H, H-1′), 4.76 (m, 1 H, H-3′, overlapped with HOD), 4.28 (br, 1 H, H-4′), 4.18 (m, 2 H, H-5′), 3.18 (q, J = 7.1 Hz, 18 H, NCH_2), 2.70 (m, 1 H, H-2′), 2.59 (m, 1 H, H-2′), 1.26 (s, 27 H, NCH_2CH_3).

^{31}P NMR (162 MHz, D_2O): δ = –10.9 (m, γ), –11.4 (d, J = 17.2 Hz, α), –23.3 (m, β).

The sample was reconstituted in H_2O (~1 mL) and treated with Dowex-50W × 8 resin (Na^+ form, 600 mg) to exchange the counter ion to Na^+. The resin was removed by filtration and washed with H_2O (600 μL total). The combined aqueous solution was lyophilized to afford **16**.

Compound 16

^{1}H NMR (D_2O, 400 MHz): δ = 8.68 (s, 1 H, H-2), 8.21 (s, 1 H, H-8), 6.64 (t, J = 6.7 Hz, 1 H, H-1′), 4.77 (m, 1 H, H-3′, overlapped with HOD), 4.28 (m, 1 H, H-4′), 4.18 (m, 2 H, H-5′), 2.70 (m, 1 H, H-2′), 2.58 (m, 1 H, H-2′).

^{31}P NMR (162 MHz, D_2O): δ = –9.7 (m, γ), –11.4 (d, J = 20.8 Hz, α), –22.9 (t, J = 18.9 Hz, β).

7-Deaza-2′-deoxy-7-nitroguanosine 5′-*O*-Triphosphate (17)

A mixture of Proton-Sponge® [1,8-bis(dimethylaminonaphthalene, 34.7 mg, 151 μmol] and **15** (25.1 mg, 81 μmol) in $PO(OMe)_3$ (250 μL) was sonicated for 1 min and cooled in an ice-bath. $POCl_3$ (12 μL, 129 μmol) was added and the mixture was stirred under ice cooling for 5 h. A solution of $(Bu_3NH)_2H_2P_2O_7$ in DMF (0.5 M, 0.81 mL, 405 μmol) and Bu_3N (96 μL, 403 μmol) were added and the whole was stirred for another 1 h. The mixture was poured into 1 M TEAB (pH 7.5, 20 mL) and the mixture was vortexed for 2 min and kept for 1 h at r.t.. The resulting solution was washed with Et_2O (3 × 5 mL) and lyophilized. The residue was dissolved with H_2O

(30 mL) and fractionated on a Sephadex-A-25 DEAE column (ϕ 2.5 cm × l 10 cm, 10 g resin, ca 4 mL/min) eluted with H_2O (200 mL) followed by 0 → 1 M linear gradient of TEAB (pH 7.5, 1000 mL). Fractions corresponding to 0.69–0.82 M TEAB were pooled and lyophilized. The residue was purified with reversed phase HPLC (Hamilton PRP-1, ϕ 10 mm × l 250 mm, flow 4 mL/min) with a gradient of MeOH mixed with 0.1 M TEAA, pH 8.5. The triphosphate product was eluted at 38–50% MeOH. Fractions containing triphosphate were pooled and lyophilized. The residue was lyophilized repeatedly from H_2O to give a triethylammonium salt of **16** as yellow solid. ^{1}H NMR showed this material contains ~2 moles of Et_3N per mole of triphosphate.

Compound 17·2N(CH_2CH_3)$_3$

^{1}H NMR (400 MHz, D_2O): δ = 8.28 (s, 1 H, H-8), 6.45 (t, *J* = 6.9 Hz, 1 H, H-1′), 4.76 (m, 1 H, H-3′), 4.24 (br, 1 H, H-4′), 4.17 (m, 2 H, H-5′), 3.18 and 3.06 (q, *J* = 7.2 Hz, 12 H, NCH_2), 2.70 (m, 1 H, H-2′), 2.47 (m, 1 H, H-2′), 1.26 and 0.94 (s, 18 H, NCH_2CH_3).

The sample was reconstituted in H_2O (~1 mL) and treated with Dowex-50W × 8 resin (Na^+ form, 600 mg) to exchange the counter ion to Na^+. The resin was removed by filtration and washed with H_2O (600 μL total). The combined aqueous solution was lyophilized to afford **17** (14.6 mg, 28%) as orange-yellow foam.

Compound 17

^{1}H NMR (400 MHz, D_2O): δ = 8.32, (s, 1 H, H-8), 6.48 (t, *J* = 6.8 Hz, 1 H, H-1′), 4.80 (m, 1 H, H-3′, overlapped with HOD), 4.17–4.32 (m, 3 H, H-4′ and H-5′), 2.72 (m, 1 H, H-2′b), 2.50 (ddd, *J* = 3.9, 6.2, 14.1 Hz, 1 H, H-2′a).

^{31}P NMR (162 MHz, D_2O): δ = –6.22 (d, *J* = 18.3Hz, γ), –10.9 (d, *J* = 19.5 Hz, α), –21.91 (t, *J* = 19.5 Hz, β).

7-Deaza-2′-deoxy-7-nitroinosine 5′-*O*-Triphosphate (18)

A mixture of Proton-Sponge® (9.3 mg, 43.4 μmol) and 7-NO_2-dI (**6**, 6.5 mg, 21.9 μmol) was suspended in H_2O (0.5 mL) and lyophilized. The residue was dissolved in $PO(OMe)_3$ (100 μL) and cooled in an ice-bath. $POCl_3$ (2.5 μL, 26.8 μmol) was added to the yellow colored solution, and the resulting purple colored mixture was stirred under ice cooling for 3 h. A solution of $(Bu_3NH)_2H_2P_2O_7$ in DMF (0.5 M, 0.22 mL, 110 μmol) and Bu_3N (26 μL, 109 μmol) were added and the mixture was stirred under ice cooling for another 1 h. The mixture was poured into 1 M TEAB (pH 7.5, 10 mL) and the mixture was stirred for 1 h at r.t.. The resulting solution was washed with Et_2O (3 × 10 mL) and lyophilized. The residual white solid was dissolved with H_2O (15 mL) and fractionated on a Sephadex-A-25 DEAE column (ϕ 2.5 cm × l 10 cm, 10 g resin, ca. 3.5 mL/min), eluted with H_2O (150 mL) followed by 0 → 1 M linear gradient of TEAB (pH 7.5, 1000 mL). Fractions corresponding to 0.65–0.82 M TEAB were pooled and lyophilized. The residue was purified with reversed phase HPLC (Hamilton PRP-1, ϕ 10 mm × l 250 mm, flow 4 mL/min) with a gradient of MeOH mixed with 0.1 M TEAA, pH 8.5. The triphosphate product was eluted at 38–39% MeOH; the corresponding fractions were pooled and lyophilized. The residue was lyophilized again from H_2O (3 mL) to remove triethylamine salts. The sample was reconstituted in H_2O (0. 4 mL) and treated with 400 mg of Dowex-50W × 8 resin (Na^+ form) to exchange the counter ion to Na^+. The resin was removed by filtration and washed with H_2O (3 × 400 μL). The combined aqueous solution was lyophilized to afford **18** as tetrasodium salt (4.66 mg, 34%).

^{1}H NMR (400 MHz, D_2O): δ = 8.61 (s, 1 H, H-8 or H-2), 8.19 (s, 1 H, H-2 or H-8), 6.67 (t, *J* = 6.6 Hz, 1 H, H-1′), 4.80 (m, 1 H, H-3′, overlapped with H_2O), 4.30 (m, 1 H, H-4′), 4.17–4.26 (m, 2 H, H-5′), 2.74 (m, 1 H, H-2′b), 2.59 (ddd, *J* = 4.3, 6.6, 14.1 Hz, 1 H, H-2a′).

^{31}P NMR (162 MHz, D_2O): δ = –6.27 (d, *J* = 19.5 Hz, γ), –10.95 (d, *J* = 19.5 Hz, α), –21.85 (t, *J* = 19.5 Hz, β).

PCR Incorporation of 16, 17 or 18

Primers were purchased from Sigma-Genosys (Woodlands, TX). Nucleotide dNTPs were from Amersham Biosciences (Piscataway, NJ), AmpliTaq Gold polymerase, 10 × Taq buffer with 15 mM $MgCl_2$ were from Applied Biosystems (Foster City, CA). Typical PCR reactions (20 μL or 500 μL) contained 1 × PCR buffer; 1.5 mM $MgCl_2$; 200 μM 7-NO_2-dATP (**16**), 7-NO_2-dGTP (**17**), or 7-NO_2-dITP (**18**); 200 μM each remainder three unmodified nucleotides, i.e. d(CGT)TPs or d(ACT)TPs; 1 μM Primer I, 1 μM Primer II, and 2 ng/μL plasmid DNA template.[14,27] A typical PCR cycling protocol was one cycle of (94 °C, 120 s) followed by 40 cycles of (94 °C, 60 s; 55 °C, 60 s; 72 °C, 120 s) followed by one cycle of (72 °C, 600 s). Each amplified DNA sample was purified using Sephadex G50 spin columns (Amersham). The collected eluates were dried on a Savant speedvac (Farmingdale, NY), followed by reconstitution in 50 μL H_2O.

Chemical Cleavage

Each DNA sample (25 μL out of the 50 μL of purified PCR product, lyophilized) was subject to chemical cleavage at 95 °C for 60 min in a thermocycler with heated lid. A typical alkaline cleavage reaction mixture contained DNA sample in 100 μL of 1 M piperidine, 1 M pyrrolidine, or 1 M 3-pyrrolidinol. A piperidine/TCEP/Tris cleavage reaction contained a DNA sample in 100 μL of 1 M piperidine, 0.2 M TCEP [tris(2-carboxyethyl)phosphate], and 0.5 M Tris base [tris(hydroxymethyl)aminomethane]. A pyrrolidine/TCEP/Tris cleavage reaction contained a DNA sample in 100 μL of 1 M pyrrolidine, 0.2 M TCEP, and 0.5 M Tris base. An NH_4OH/TCEP cleavage reaction contained a DNA sample in 100 μL of 0.2 M TCEP and ~30% NH_4OH. The cleavage samples were concentrated to dryness using speedvac, and each was dissolved in 500 μL of 0.2 M TEAA (triethylammonium acetate). The resulting aqueous solutions were desalted using OASIS® columns from Waters (Milford, MA), using procedures recommended by the manufacturer. The collected MeCN eluates containing DNA were concentrated to dryness, and then dissolved in 100 μL of H_2O.

PAGE Analysis of ^{32}P-Labeling of Primers, PCR Products, and Cleavage Products

T4 DNA polynucleotide kinase and 10 × T4 kinase buffer were from New England Biolabs (Beverly, MA). [γ-^{32}P]-ATP was from PerkinElmer (Boston, MA). A typical labeling reaction contained 1 μL 10 × T4 kinase buffer, 1 μL 20 μM primer (or 1 μL purified PCR product, or 1 μL cleavage product), 0.5 μL 1.67 μM [γ-^{32}P]-ATP (6000 Ci/mmol), 6.5 μL distilled H_2O, and 1 μL 10 U/μL T4 kinase. After incubation at 37 °C for 60 min, to each labeling reaction mixture was added 20 μl of 1 × TE (10 mM Tris/1 mM EDTA) pH 8 buffer. The resulting aqueous solutions were purified using Sephadex G50 columns and the resulting eluates were adjusted to 50 μL using distilled water. Each ^{32}P-labeled sample (~1 μL) was mixed with formamide loading dye, separated on a 12% denaturing polyacrylamide gel, and subjected to autoradiography.

MALDI-TOF Mass Spectrometry Analysis

The remainder of each purified cleavage sample (~99 μL) was concentrated to dryness. After resuspending in 3 μL of 3-hydroxypicolinic acid/diammonium citrate matrix solution a 0.6 μL aliquot of the sample was deposited on a stainless steel target plate and allowed to air dry. The sample was analyzed with a Voyager DE Linear mass spectrometer equipped with a 337 nm nitrogen laser (Applied Biosystems). Analysis was carried out in the positive ion mode with delayed extraction. The voltage for acceleration was 20 kV, while the grid voltage was at 92.5%. The guide wire voltage was set at 0.1% and the delay time was 250 ns. Typically 20 individual spectra were added to generate a summed spectrum.

Acknowledgment

This research was supported by Variagenics, Inc. We thank our colleagues at Variagenics for their assistance and helpful discussions.

References

(1) Present address: B.H. Wang, Genzyme Corp., Waltham, MA 02451, USA.
(2) Present address: C. R. Allerson, ISIS Pharmaceuticals, Carlsbad, CA 92008, USA.
(3) Metzker, M. L. *Genome Res.* **2005**, *15*, 1767.
(4) Sobrino, B.; Brion, M.; Carracedo, A. *Forensic Sci. Int.* **2005**, *154*, 181.
(5) Verdine, G. L.; Norman, D. P. *Annu. Rev. Biochem.* **2003**, *72*, 337.
(6) Proske, D.; Blank, M.; Buhmann, R.; Resch, A. *Appl. Microbiol. Biotechnol.* **2005**, *69*, 367.
(7) Brakmann, S.; Lobermann, S. *Angew. Chem. Int. Ed.* **2002**, *41*, 3215.
(8) Ruparel, H.; Bi, L.; Li, Z.; Bai, X.; Kim, D. H.; Turro, N. J.; Ju, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5932.
(9) Fei, Z.; Smith, L. M. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 950.
(10) Kato, Y.; Minakawa, N.; Komatsu, Y.; Kamiya, H.; Ogawa, N.; Harashima, H.; Matsuda, A. *Nucleic Acids Res.* **2005**, *33*, 2942.
(11) Endo, M.; Mitsui, T.; Okuni, T.; Kimoto, M.; Hirao, I.; Yokoyama, S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2593.
(12) Kawate, T.; Allerson, C. R.; Wolfe, J. L. *Org Lett.* **2005**, *7*, 3865.
(13) Hayashibara, K. C.; Verdine, G. L. *J. Am. Chem. Soc.* **1991**, *113*, 5104.
(14) Wolfe, J. L.; Kawate, T.; Sarracino, D. A.; Zillmann, M.; Olson, J.; Stanton, V. P. Jr.; Verdine, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11073.
(15) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499.
(16) Ramzaeva, N.; Seela, F. *Helv. Chim. Acta* **1995**, *78*, 1083.
(17) Seela, F.; Peng, X. *Synthesis* **2004**, 1203.
(18) Storek, M. J.; Ernst, A.; Verdine, G. L. *Nat. Biotechnol.* **2002**, *20*, 183.
(19) Seela, F.; Rosemeyer, H.; Zulauf, M.; Chen, Y.; Kastner, G.; Reuter, H. *Liebigs Ann./Recl.* **1997**, 2525.
(20) Ciuffreda, P.; Alessandrini, L.; Terraneo, G.; Santaniello, E. *Eur. J. Org. Chem.* **2003**, *24*, 4748.
(21) Wnuk, S. F.; Stoeckler, J. D.; Robins, M. J. *Nucleosides Nucleotides* **1994**, *13*, 389.
(22) Nair, V.; Richardson, S. G. *J. Org. Chem.* **1980**, *45*, 3969.
(23) Francom, P.; Janeba, Z.; Shibuya, S.; Robins, M. J. *J. Org. Chem.* **2002**, *67*, 6788.
(24) Streitwieser, A.; Heathcock, C. H.; Kosower, E. M. *Introduction to Organic Chemistry*; 4th ed., MacMillan: New York, **1992**, 1169.
(25) Kurtz, A. J.; Dodson, M. L.; Lloyd, R. S. *Biochemistry* **2002**, *41*, 7054.
(26) The spectra were recorded by Dr. Jin Hong, 77 Pine Ridge Drive, Ayer, MA 01432, 978-772-6939, jinhong@gis.net.
(27) McClelland, A.; Kuhn, L. C.; Ruddle, F. H. *Cell* **1984**, *39*, 267.