Mechanism of Degradation of Purine Nucleosides by Formamide. Implications for Chemical DNA Sequencing Procedures†

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Mechanism of Degradation of Purine Nucleosides by Formamide. Implications for Chemical DNA Sequencing Procedures†

Raffaele Saladino,*,‡ Enrico Mincione,‡ Claudia Crestini,‡ Rodolfo Negri,§ Ernesto Di Mauro,§,⊥ and Giovanna Costanzo⊥

Contribution from the Dipartimento ABAC, Università degli Studi della Tuscia, Viterbo 01100, Italy, Centro di Studio per gli Acidi Nucleici, CNR, Roma, Italy, and Fondazione "Istituto Pasteur - Fondazione Cenci Bolognetti", c/o Dipartimento di Genetica e Biologia Molecolare, Università "La Sapienza", Roma, Italy

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Abstract: We describe the reaction of formamide with 2′-deoxyadenosine and 2′-deoxyguanosine to give imidazole ring opening by nucleophilic addition on the electrophilic C(8)-position of the purine ring. This information allows improvement of the one-lane chemical DNA sequencing procedure based on the base-selective reaction of formamide with DNA. The reactivity with formamide of several 7-deazapurine analogues (7-deaza-2′-deoxyinosine, 7-deaza-2′-deoxyguanosine, and 7-deaza-2′-deoxyadenosine) incorporated into polynucleotides is also described. The wide spectrum of different sensitivities to formamide displayed by these purine analogues provides the single-lane DNA chemical sequencing procedures with the possibility of wide-ranging signal intensity modulation and thus increased specificity.

Introduction

Methods for accurately establishing the order of the four bases along given DNA fragments are based on two different principles. One method uses chemical reagents that react with specific bases to break DNA preferentially at given nucleotides,[1] the other is based on the analysis of the products of DNA polymerization selectively interrupted with chain terminating deoxyribonucleotides.[2]

The ideal DNA sequencing method should yield unambiguous and complete information in a single electrophoretic lane and should be simple, rapid, economical and accurate. Compression of DNA sequencing procedures is crucial for development and improvement of automated analytical systems. Methods for partial sequence data compression have been reported,[3,4] and a single-lane sequencing procedure is currently available, based upon the dideoxy Sanger methodology, analyzed in automated sequences. This method (four fluorochromes/four bases) cannot *a priori* be compressed further. Chemical DNA sequencing analysis offers the potential of complete compression: if one could obtain unambiguous and complete sequence information in a single lane, then four different DNAs, each labeled with a different fluorochrome, could be analyzed in the same electrophoretic lane (four fluorochromes/four DNAs). The methods developed so far which aim to simplify the sequencing procedures by introducing chemical alternatives to the classical Maxam and Gilbert protocols[1] have been reviewed.[5−7]

The DNA sequencing procedure based on the selective degradation of nucleic acids by formamide provides complete sequence information in a single electrophoretic lane, by measuring intensity of the signal corresponding to each cleaved sequence position.[8−11] This procedure depends on the degradation of the purine and pyrimidine bases by formamide at high temperature (>100 °C) followed by scission of the glycosidic linkages through β-elimination reactions. In the presence of a weak base such as formamide, only the 3′-β-elimination occurs;[8] efficient β-elimination at 5′ (leading to unbiased sequencing of 5′-labeled DNAs) requires the use of piperidine[9,10] as a second reaction step. Irrespective of the specific protocol used for the phosphodiester bond breakage, the selective (i.e., base-specific) part of the reaction is carried out by formamide.[8−11]

In any sequencing protocol, the best analytical condition is one in which the difference in reactivity between the four bases is homogeneous and large. The order of sensitivity observed in the formamide reaction is G > A > C ≫ T, where the reactivity ratio Gs/As ranges from 1 to 1.5 depending on the

* Corresponding author: Raffaele Saladino, Dipartimento ABAC Università degli Studi della Tuscia, Via Camillo De Lellis, Viterbo 01100, Italy. Tel. + 39.761.357230. Fax. + 39.761.357242.

‡ Università degli Studi della Tuscia.

§ Centro di Studio per gli Acidi Nucleici.

⊥ Università "La Sapienza".

† Abbreviations: G, guanosine; A, adenosine; I, inosine; C, cytidine; T, thymidine; ATP, adenosine triphosphate; CTP, cytidine triphosphate; dATP, 2′-deoxyadenosine triphosphate; dGTP, 2′-deoxyguanosine triphosphate; dITP, 2′-deoxyinosine triphosphate; deazaA, 7-deaza-2′-deoxyadenosine; deazadATP, , 7-deaza-2′-deoxyadenosine triphospate; deazadGTP, 7-deaza-2′-deoxyguanosine triphosphate; deazadITP, 7-deaza-2′-deoxyinosine triphosphate; PCR, polymerase chain reaction; PE, primer extension; TLC, thin layer chromatography; EDTA, ethylenediaminetetraacetic acid; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide.

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reaction conditions; the reactivity ratio Cs/As is 0.15, and the reactivity of T_s is close to 0. The variations on the basic protocols used to solve these biases have been described.[8−11]

In order to simplify the formamide sequencing procedure and to abolish the possibility of errors in the determination of Gs and As residues, we have found the use of purine derivatives to be quite promising. These could (i) be incorporated into the DNAs to be analyzed by either the polymerase chain reaction (PCR)[12] or primer extension (PE)[7] procedures and (ii) be degraded at different rates, in order to increase the sensitivity intervals.

Knowledge of the degradation pathway of purine nucleosides with formamide is necessary to select analogues to be incorporated and then selectively degraded. Although the degradation pathway of purine nucleosides and nucleotides by the action of strong bases (usually HO^-) is widely described in literature,[13] the reaction with a weak base such as formamide has not been previously studied. In this paper we describe the mechanism of degradation of 2′-deoxyadenosine (**1**) and 2′-deoxyguanosine (**2**) with formamide. The degradation pattern of purine nucleosides, as defined by this analysis, allows us to predict the positions of the purine ring whose structural modifications may modify the sensitivity toward formamide, therefore allowing substantial improvement of the DNA chemical sequencing procedure.

Results

In order to obtain information on the degradation pathway of purine nucleosides by formamide, we have analyzed the reactions of 2′-deoxyadenosine (**1**) and 2′-deoxyguanosine (**2**) using the experimental conditions described for the formamide DNA sequencing method.[11] 2′-Deoxyadenosine (**1**) (538 mg, 2 mmol) was added to formamide (10 mL) in the presence of low amount of water (0.1 mL), and the mixture was refluxed at 110 °C until the disappearance of the substrate. Small amounts of the formamidopyrimidine nucleoside **3** and 6-amino-5-formamido-4-[*N*-(2′-deoxy-β-D-ribofuranosyl)]pyrimidine **4** (Scheme 1) were obtained in isolated form by analytical TLC purification in reverse phase (butanol saturated with water) and characterized by capillary gas chromatography-mass spectrometry (CG-MS) after silylation with *N*,*O*-bis(trimethylsilyl)-trifluoro acetamide (BSTFA),[14] ^{1}H-NMR, and elemental microanalysis. Compound **3** was found to be unstable even if recovered under nitrogen atmosphere, and it was easily converted to **4** by mild warming or by treatment with acidic SiO_2. The remaining reaction mixture was distilled under high vacuum to eliminate the excess formamide, and the crude product was purified by flash-chromatography (chloroform:methanol = 9.5:0.5 as eluent) to give 4,6-diamino-5-formamidopyrimidine **5** as the only recovered product. These data suggest that formamide reacts with **1** cleaving the adenine base through nucleophilic attack on the C(8) position of the purine ring. The attack on this position is in agreement with its electrophilic character[15] and with the chemical behavior toward DNA purine components previously described for efficient nucleophilic agents, such as hydrazine and hydroxylamine.[16]

According to the degradation pathway hypothesized (Scheme 1) the initial C(8)-adduct between formamide and 2′-deoxyadenosine (which could not be isolated, probably because of its instability) may easily undergo the C(8) imidazole ring-opening to yield the derivative **3**. Compound **3** is probably the first to form and it might be slowly transformed to **4** by loss of the initially acquired molecule of formamide. Under sufficiently vigorous conditions (i.e, removal of the formamide by distillation under vacuum) the 2′-deoxyribosyl residue may be hydrolytically detached to yield compound **5**.

It is noteworthy that in the reaction of **1** with formamide products of possible alternative degradation pathways (namely, nucleophilic additions at the C(6) and/or C(2)-positions on the purine ring) are not recovered in the reaction mixture.

2′-Deoxyguanosine (**2**) reacts faster than **1** with formamide at 110 °C, in agreement with the order of sensitivity observed for the purine bases in DNA.[8−11] The reaction was complete in only 3 h resulting in a complex mixture that was not characterized further. When the reaction was performed at 90 °C a minor conversion (15%) of the substrate was observed. In the latter case, 2,6-diamino-4-oxo-5-formamido-6-[*N*-(2′-deoxy-β-D-ribofuranosyl)]pyrimidine **6** and 2,6-diamino-4-oxo-5-formamidopyrimidine **7** were obtained (Scheme 2) after purification by TLC in reverse phase (butanol saturated by water) or by flash-chromatography (after distillation of the excess formamide). Formamidopyrimidine derivatives (FAPyr) **4**, **5**, **6**, and **7** are usually referred to as products of imidazole C(8)-ring cleavage of purine nucleosides and nucleotides by ionizing radiation[17−20] or by basic treatment after heterocyclic nitrogen alkylation.[21] On the basis of the structural analogies among the isolated degradation products it is reasonable to suggest that the degradation pathway of 2′-deoxyguanosine is similar to that described for 2′-deoxyadenosine, even if, in the first case, other degradation pathways cannot be completely excluded because of the lack of characterization of all possible intermediates.

The indication that formamide degrades purine nucleosides by C(8)-imidazole ring-opening may provide the chemical clues to predict the variation of reactivity for various purine analogues. In fact, structural modifications that reduce the electrophilic character of the C(8)-position of the purine ring might reduce its reactivity toward a weak nucleophile such as formamide. Available 7-deaza-2′-deoxypurine nucleosides, which lack the electron-withdrawing nitrogen at the adjacent C(7) position, satisfy this condition and might support nucleophilic attack less efficiently. In order to test this hypothesis and to apply this rationale to the chemical DNA sequencing procedure, we have analyzed the sensitivity to formamide of 7-deazaanalogues of purine nucleosides (both adenosine, guanosine and inosine) when inserted into polynucleotides.

Figure 1 shows in panel a the molecular construct used to synthesize double stranded DNA molecules carrying 7-deaza-purine residues. The indicated 34mer template segment (lower strand) was annealed to the 16mer oligo (upper strand) and used as a template-primer substrate for a Sequenase-driven polymerization. The resulting upper strand contains (outside the oligo)

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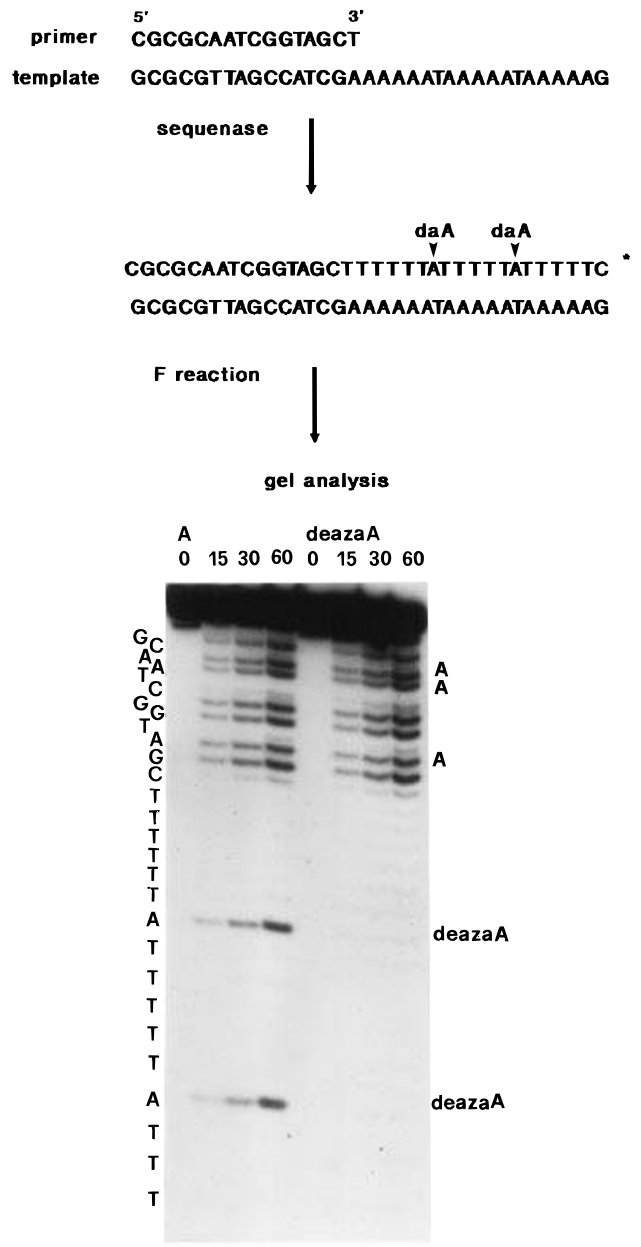

Figure 1. Degradation of 3′-labeled DNA by formamide. Comparison of the reactivity of adenosine and deazaadenosine. Panel a (top): the DNA construct used to insert the base-analogue in two defined locations (as indicated in the lower molecule, see text). The scheme of the procedure used for the analysis described in the following panels is indicated. * = labeled position. Panel b (bottom): gel analysis (18% acrylamide, 7 M urea) of the product of the attack by formamide on the DNA carrying substitutions. Each group of samples shows the products of formamide degradation of molecules in which adenosine (A) or 7-deazaadenosine (deazaA) were incorporated at positions 22 and 28, as indicated in scheme in panel a. Reactions were carried out for 0, 15, 30, and 60 min, as indicated. Left panel: adenosine; right panel: 7-deazaadenosine.

sites which incorporate A residues only at the positions 22 and 28 and was synthesized using alternatively dATP or deazaATP.

The reaction of the two differently substituted types of polynucleotides with formamide in standard conditions (110 °C, 10 min) was analyzed on a sequencing gel (Figure 1, panel b). The results show that 7-deaza modification abolishes almost completely the susceptibility to formamide attack. The sensitivity to formamide of deazaG and deazaI was analyzed with the same approach.

Figure 2 shows in panel a the molecular construct used to synthesize double stranded DNA molecules carrying G-substituted residues. The template-primer system used is similar to the one used for the analysis of the A-analogues, except for the modifications at positions number 22, 28, and 34 (see Experimental Section). The resulting upper strand contains (outside the oligo) sites which incorporate G residues only at the positions 22 and 28 and was synthesized using alternatively dGTP, dITP, deazadGTP, or deazadITP.

The reaction of four differently substituted polynucleotides with formamide in standard conditions (110 °C, 10 min) was analyzed on a sequencing gel (Figure 2 panel b). Panel c compares the reactivity of the substituted residues.

The results show that the different purine analogues tested display a remarkable difference of sensitivity to formamide attack. The following order of sensitivity is noted: I > G > deazaG > deazaI (I > G has already been reported).[11] It is noteworthy that also in this case 7-deaza modification strongly decreases susceptibility to formamide attack, to the point that deazaI is essentially inert.

Note that in the part of the molecule used as primer in this experiment (upper part of the gel lanes), G residues are present. Therefore, in the case of molecules containing substitutions, the reactivity of guanosine can be directly compared in the same molecule with that of the substituted residue. In conditions of over-reaction (at longer times), the specificity of the sequencing reaction tends to be lost (i.e., T residues become erratically cleaved).

The small difference observed in the cleavage sensitivity of A_s and G_s residues upon formamide attack is a potential source of errors in the formamide-based DNA sequencing methods.[8] This problem may be addressed by increasing the sensitivity at G positions by incorporating inosine in the corresponding positions in the PCR-amplified DNA to be sequenced.[11] An alternative, simpler solution is offered by the present observation that deazaA residues are less sensitive toward formamide degradation than A residues. Given that the incorporation rate of deazaA's is very similar to that of A's and that deazaA's do not cause chain termination (not shown), the G/A difference can be increased by incorporating a fraction (say 30%) of deazaA's instead of A's in the appropriate positions. This is achieved simply by amplifying the DNA to be analyzed with a mixture of triphosphates in which deazaAs and As are present in the appropriate concentrations.

Experimental Section

General Procedures. 2′-Deoxyguanosine, 2′-deoxyadenosine (Aldrich, Co.), 7-deaza-2′-deoxyinosine-, 7-deaza-2′-deoxyguanosine-, and 7-deaza-2′-deoxyadenosine triphosphates (Boehringer) were used without further purification. All solvents used were ACS reagent grade and were redistilled according to standard procedures. Formamide was from IBI, piperidine (puriss. p.a.) from Fluka.

^{1}H-NMR spectra were recorded with a Bruker AC-200 spectrometer (^{1}H frequency: 200.13 MHz). Data processing was performed with an Aspect 3000 computer using UXNMR software. Probe 5-mm was as follows: solvent DMSO-d_6 (Aldrich); temperature, 273 K.

The application of capillary gas chromatography-mass spectrometry (GC-MS) to chemical characterization of altered DNA bases is well established.[22,23] The relative nonvolatility of purine derivatives was improved by the use of 2′-deoxy-3′,5′-di-*O*-trimethylsilyl-β-D-ribosyl derivatives. Gas-chromatographic analysis and mass spectra were performed on samples derivatized with BSTFA[14] in pyridine at 25 °C by use of a HP GC-MS 5972 spectrometer equipped with a HP-5MS column (0.25 mm, 30 m). The spectra were recorded at 70−250 °C

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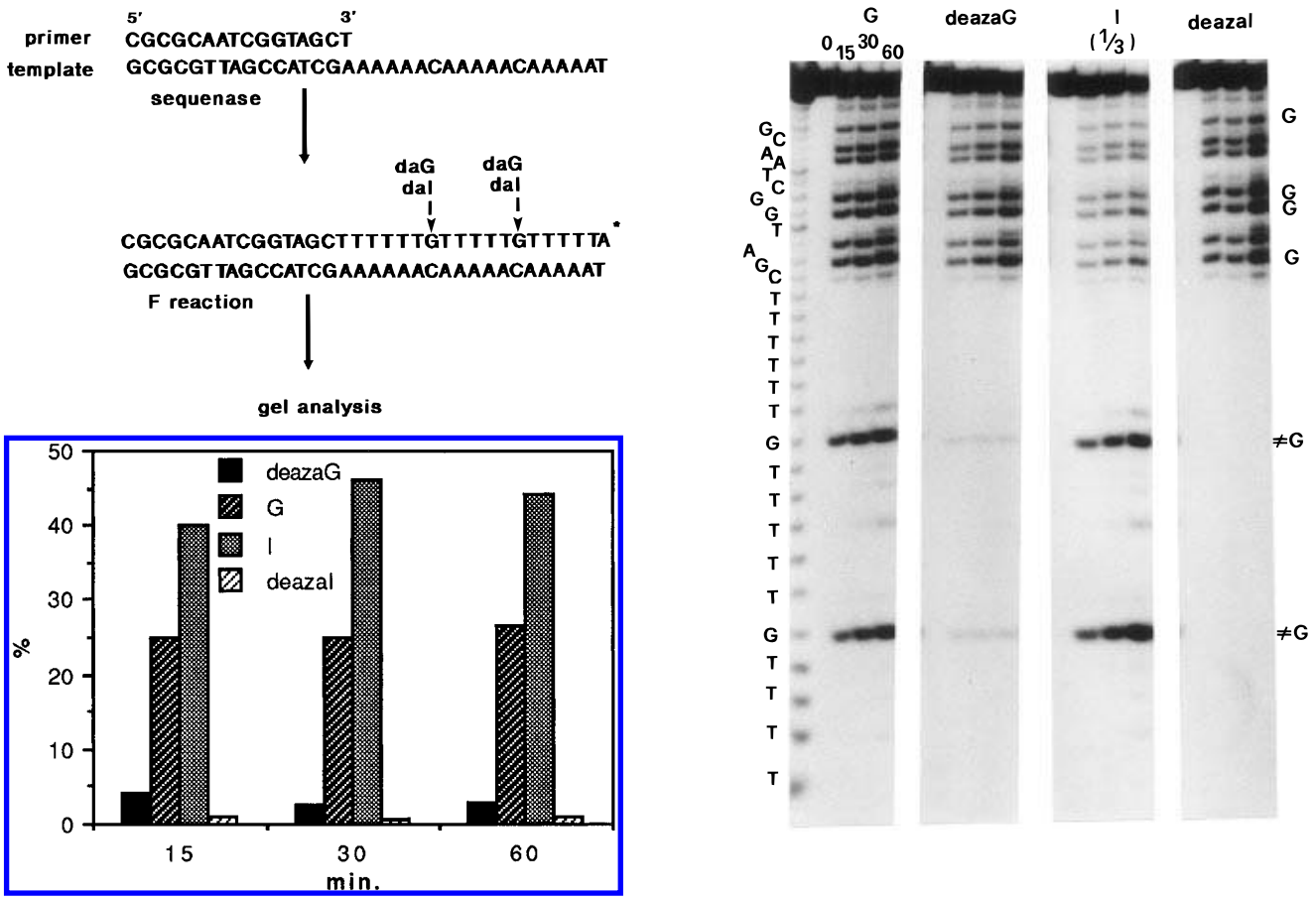
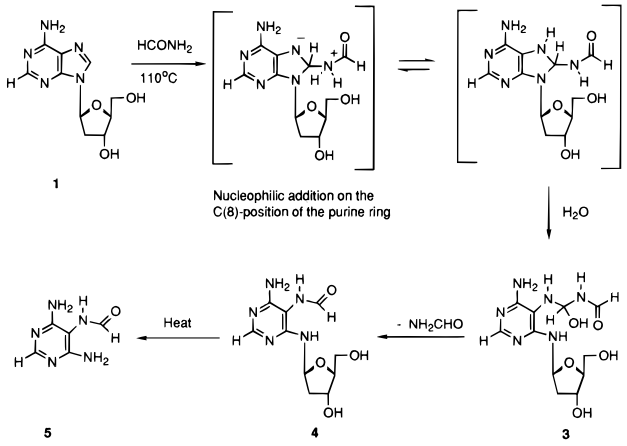


Figure 2. Comparison of the reactivity of guanosine, 7-deazaguanosine, inosine, and 7-deazainosine. Panel a (top): the DNA construct used to insert G analogues in programmed locations (positions 22 and 28, as for the construct used for A analogues). Panel b (middle): gel analysis (as in Figure 1) of the product of formamide degradation on DNAs carrying substitutions. The leftmost lane shows a whole-sequence ladder obtained by Cu-phenantroline reaction.[8,25] Each group of samples shows the products of formamide degradation of molecules in which guanosine (G), 7-deazaguanosine (deazaG), inosine (I), or 7-deazaguanosine (deazaG) were incorporated at positions 22 and 28. Reactions were carried out for 0, 15, 30, and 60 min, as indicated in the first panel. For the inosine-containing DNA, only 1/3 of the sample was loaded on the gel, to avoid overexposure effects due to the higher reactivity of the inosine residues.[11] Panel c (bottom): cleavage at the residues 22 and 28, reported as % (ordinate) of the total degradation products, as a function of the reaction time (abscissa).

Scheme 1



with the following program: isothermal at 70 °C for the first 1 min, then 10 °C/min, and finally isothermal at 250 °C for 40 min. Mass spectra were recorded on a Kratos MS80 spectrometer. Melting points were obtained on a Reichert Kofler apparatus and are uncorrected.

Elemental microanalyses were recorded on a Carlo Erba elemental analyzer EA 1108 and all were within 0.4% of calculated values for carbon, hydrogen, and nitrogen.

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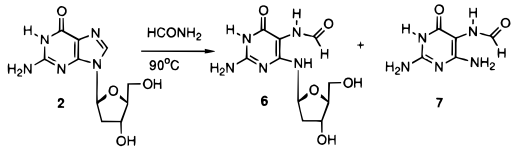
Scheme 2



Reverse phase TLC was performed using "Merck 15682 RP-8 $F_{254}S$" glass sheets (butanol saturated with water). Direct phase TLC was performed with glass sheets "Merck silica gel 60 F_{254}" (9.5:0.5 chloroform: methanol).

Degradation of 2′-deoxyadenosine (1) and 2′-Deoxyguanosine (2) with Formamide. General Procedure. 2′-Deoxypurine nucleoside (2 mmol) was dissolved in formamide (10 mL) and then heated at 110 °C (90 °C in the degradation of **2**) until disappearance of the substrate. A small portion (0.5 mL) of the crude reaction mixture was analyzed by capillary gas chromatography-mass spectrometry (GC-MS) after silylation with BSTFA and directly chromatographed on phase-reverse analytical thin layer plates using water-saturated butanol as eluent. The remaining mixture was evaporated under high vacuum to dryness, and the residue was dissolved in chloroform and purified by flash-chromatography (chloroform−methanol = 9.5:0.5).

Formamidopyrimidine Derivate 3. Mp 177−180 °C (with decomposition). IR (KBr) 3351 (OH), 3202 (NH), 2825 (CHO), 1678 (CO), 1637 (C═C), 1280, 970 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 2.05−2.25 (m, 2, H-2′, 2″), 4.62−4.83 (m, 3, H-4′, 5′, 5″), 4.89−5.21 (m, 1, H-3′), 5.40−5.87 (m, 2, H-1′, and CHOH), 6.74−6.85 (br s, 4, NH), 7.70−7.81 (m, 1, CHO), 8.25 (s, 1, H-2). Mass spectrum after silylation

with BSTFA *m*/*e*: 458 (M^+ as dimethylsilyl derivative), 413 (M − 45 [3 Me]), 384 (M − 74 [$HSi(Me)_3$]), 369 (M − 89 [NHCHOHNHCHO]), 311 (M − 147 [$HSi(Me)_3$ and $Si(Me)_3$]). Anal. Calcd for $C_{11}H_{19}N_6O_5$: C, 42.04; H, 5.77; N, 26.74. Found: C, 42.09; H, 5.78; N, 26.79.

6-Amino-5-formamido-4-[*N*-(2′-deoxy-*β*-D-ribofuranosyl)] Pyrimidine 4. Mp 158−160 °C. IR (KBr) 3351 (OH), 3202 (NH), 1680 (CO), 1637 (C═C), 1280, 970 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 2.20−2.85 (m, 2, H-2′,2″), 3.30−3.95 (m, 4, H-3′,4′,5′,5″), 6.09−6.15 (m, 1, H-1′), 6.71−7.15 (br s, 4, NH), 8.18−8.25 (br s, 1, CHO), 8.20 (s, 1, H-2). Mass spectrum after silylation with BSTFA *m*/*e*: 413 (M^+ as dimethylsilyl derivative), 358 (M − 55 [HCN and CO]), 339 (M − 74 [$HSi(Me)_3$]), 311 (M − 102 [$HSi(Me)_3$ and CO]), 265 (M − 148 [2 $HSi(Me)_3$]). Anal. Calcd for $C_{10}H_{15}N_5O_4$: C, 44.61; H, 5.61; N, 26.0. Found: C, 44.69; H, 5.62; N, 26.30.

4,6-Diamino-5-formamidopyrimidine 5. Compound **5** was identical to that reported by Denayer.[24] Mp 224−226 °C. IR (KBr) 3215 (NH), 1677 (CO), 1637 (C═C), 1280, 970 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 6.80−7.12 (br s, 5, NH and NH_2), 8.05−8.13 (br s, 1, CHO), 8.11 (s, 1, H-2). Mass spectrum *m*/*e*: 153 (M^+), 124 (M − 29 [CHO]), 109 (M − 44 [NHCHO]). Anal. Calcd for $C_5H_7N_5O$: C, 39.21; H, 4.60; N, 45.73. Found: C, 39.26; H, 4.57; N, 45.79.

2-Amino-4-oxo-5-formamido-6-[*N*-(2′-deoxy-*β*-D-ribofuranosyl)]-pyrimidine 6. Mp 180−185 °C (with decomposition). IR (KBr) 3360 (OH), 3202 (NH), 1690 (CO), 1637 (C═C), 1280, 970 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 2.28−2.85 (m, 2, H-2′, 2″), 4.05−5.20 (m, 4, H-3′,4′,5′,5″), 5.30−5.48 (m, 1, H-1′), 6.50−6.70 (br s, 2, NH_2), 6.85 (br s, 1, NH), 7.89−7.95 (br s, 1, CHO). Mass spectrum *m*/*e*: 285 (M^+), 256 (M − 29 [CHO]), 241 (M − 44 [NHCHO]). Anal. Calcd for $C_{10}H_{15}N_5O_5$: C, 42.10; H, 5.30; N, 24.55. Found: C, 42.03; H, 5.29; N, 24.67.

2,6-Diamino-4-oxo-5-formamidopyrimidine 7. Compound **7** was identical to that reported by Chetsanga and Grigorian.[20] IR (KBr) 3230 (NH), 1780 (CO), 1679 (CO), 1640 (C═C), 1290, 970 cm^{-1}. Mass spectrum *m*/*e*: 169 (M^+), 140 (M − 29 [CHO]), 125 (M − 44 [NHCHO]), 82 (M − 67 [NHCHO and HCNO]). Anal. Calcd for $C_5H_7N_5O_2$: C, 35.50; H, 4.17; N, 41.40. Found: C, 35.55; H, 4.16; N, 41.38.

The Formamide Sequencing Protocol. The DNA sequencing procedure used on 3′-radioactively labeled DNA fragments is the "formamide protocol" performed as previously described,[8] consisting essentially of heating DNA in formamide at 110 °C without further handling. Labeling, chemical treatment, and gel analysis, were performed as described.[8] The nucleotide sequence of the DNA fragments and the strategy used for the insertion of the 7-deaza derivatives into polynucleotides and for testing their sensitivity to formamide is reported in Figures 1 and 2. We describe here the procedure used in the preparation of the four DNA molecules used for the analysis of the G-analogues (the experiment reported in Figure 2). A similar procedure was used for the analysis of A-analogues. The four polynucleotides segments were synthesized as follows: 260 pmol of the 34mer template (see Figure 2, panel a) were mixed with 260 pmol of 16mer primer in 50 mM Tris HCl (pH 7.5), the annealing mixture (20 μL) was heated 5 min at 95 °C, then slowly cooled to 30 °C and divided in four aliquots. Each aliquot was incubated 30 min at 37 °C with 0.2U of Sequenase[21] (purchased from USB) in a 10 μl final volume of 20 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 250 μM TTP, and 1 μM [α-^{32}P] dATP (3000Ci/mM) and one of 250 μM dGTP, 250 μM deazadGTP, 500 μM dITP, and 500 μM deazadITP. The reaction was stopped by the addition of 20 μL of 90% formamide−10 mM EDTA, heated 2 min at 90 °C, and directly loaded on a 6% denaturing polyacrylamide gel for purification of the labeled 34mer (see Figure 2, panel a, upper strand). End-labeling was achieved by terminal selective incorporation of [α -^{32}P] ATP at the 3′ extremity in the unique A position (indicated by a star in Figure 2, panel a). A slightly different construct was used to assay for the sensitivity of 7-deaza-2′-deoxyadenosine relative to 2′-deoxyadenosine. This construct is composed of the same primer (upper strand) and of the same template (lower strand) as the one used for the analysis of the G-analogues, varying only for the presence of two T residues (instead of the Cs, as indicated in Figure 1, panel a) in position 22 and 28, thus allowing incorporation of As or deazaAs upon elongation of the upper strand (Figure 1). The K_m of Sequenase for A or deazaA does not vary appreciably (data not detailed). Another variation consists in the substitution of the last position (no. 34) of the template strand (from A to G), thus allowing specific terminal labeling of the elongated strand using [α-^{32}P] labeled CTP.

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Supporting Information Available: A detailed description (table of fragmentation, capillary gas-chromatographic profile, and schemes of fragmentations) of the reaction between 2′-deoxyadenosine and formamide (6 pages). Ordering information is given on any current masthead page.

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