Articles

Synthesis and Biophysical and Biological Properties of Oligonucleotides Containing 2-Aza-2′-Deoxyinosine

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The preparation of oligonucleotides containing 2-aza-2′-deoxyinosine is described. Protection of the 2-azahypoxanthine moiety with the photolabile 2-nitrobenzyl group enabled us to obtain the phosphoramidite derivative and oligonucleotides containing protected 2-aza-2′-deoxyinosine. After purification, photolysis of the oligonucleotides containing the protected analogue provided the desired oligonucleotides in good yields. Melting curves of duplexes containing 2-azahypoxanthine paired with the four natural bases at pH 6 and pH 8 proved that 2-azahypoxanthine base pairs were less stable than perfectly matched duplexes but showed little variation among different bases.

Synthetic oligonucleotide probes have proven to be very useful in the detection of cloned DNA sequences. When a partial protein sequence is available, the design of oligonucleotide probes is complicated by the degeneracy of the genetic code. To circumvent this problem, a sustained effort has been devoted to the design of base analogues that can potentially base pair with any of the four natural bases. 2′-Deoxyinosine was the first compound studied and it is the most used.[1] Its ribonucleoside derivative, inosine, was found in *t*-RNA at the third position of the anticodon, capable of pairing with the A, C, and U bases of the codon. Abasic and phenyl analogues have been prepared but base pairing was significantly less stable than the normal Watson–Crick base pairing.[2] The preparation of oligonucleotides containing 2′-deoxyinosine derivatives: allopurinol 2′-deoxyriboside and 7-deaza-2′-deoxyinosine have also been described.[3] Oligonucleotides containing 5-fluorouracil have been reported to form stable base pairs with adenine and guanine.[4] Similarly, it has been shown that oligonucleotides containing pyrimidine analogues that have an amino–imino tautomeric constant near unity, pair equally well with A and G. The same holds for a purine analogue that is able to form strong base pairs with T and C.[5] Very recently, oligonucleotides containing 3-nitropyrrole 2′-deoxyriboside at several sites have been used as primers for sequencing and polymerase chain reaction (PCR).[6a] This analogue is designed to maximize base stacking, minimizing hydrogen-bonding interactions. Also, the 5-nitroindole derivative has proved superior to the 3-nitropyrrole in terms of duplex stability.[6b]

Another possible approach is to modify 2′-deoxyinosine searching for better base pairing properties. Introduction of a hydrogen bond acceptor in position 2 yields a purine analogue derived from 2′-deoxyinosine that contains the configuration of hydrogen bonding groups: acceptor–donor–acceptor. This configuration permits the drawing of base-pairing structures with two hydrogen bonds for the four natural bases. The preparation of synthetic oligonucleotides containing xanthine, the first base analogue of this type, has been reported.[7] Xanthine-containing oligonucleotides were found to have a very low melting temperature at neutral pH, probably due to the ionization of xanthine at neutral pH ($pK_a$of xanthosine 5.5). It was also shown that at pH 5.5 xanthine bases pairs become more stable.[7a]

Substitution of the keto group at position 2 of xanthine by a nitrogen gives a base analogue, 2-azahypoxanthine (Figure 1), that has the same acceptor-donor-acceptor configuration, but has a higher pK_a($pK_a$of 2-azainosine 6.8).[8] Attempts to synthesize oligonucleotides containing 2-aza-2′-deoxyinosine protected with the *N,N*-diphenylcarbamoyl group failed, due to an expected side-reaction

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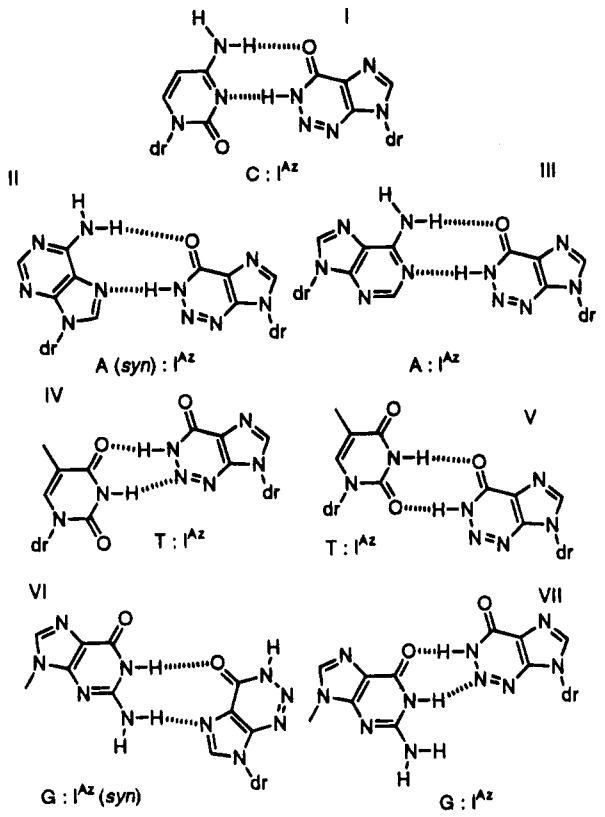

Figure 1. Possible base-pairing structures for 2-azahypoxanthine. Structures I, II, III, V, and VI have been observed in hypoxanthine base pairs. Structures IV and VII are only possible in 2-azahypoxanthine.

on the protected derivative during the final ammonia treatment.[9] In the present communication we describe the preparation and use of a photolabile derivative of 2-aza-2′-deoxyinosine for the synthesis of oligonucleotides containing 2-aza-2′-deoxyinosine as well as the base pairing properties of this analogue.

Results and Discussion

Previous work has shown that 2-aza-2′-deoxyinosine was very sensitive to depurination.[9,10] In this respect 2-aza-2′-deoxyinosine resembles 2′-deoxyxanthosine more closely than 2′-deoxyinosine.[7] As it was shown for 2′-deoxyxanthosine[7] and 2-aza-2′-deoxyinosine,[9] the protection of the lactam group stabilizes 2′-deoxynucleosides making their isolation and characterization possible. Once incorporated into DNA, the 5′ and 3′ phosphate bonds stabilize the nucleoside toward depurination.[7]

It has also been reported than the best route to obtain 2-aza-2′-deoxy derivatives starts from the ribonucleoside 2-azainosine that can be easily prepared from commercially available 5-aminoimidazole-4-carboxamide riboside (AICA-riboside).[8,9] Conversion of ribonucleoside to 2′-deoxynucleoside could be done by the method described by Robins,[11] that implies protection of 5′ and 3′ alcohol groups, conversion of 2′ alcohol to thionoester, reduction of the thionoester by tin hydride, and removal of 3′ and 5′ protecting groups with fluoride ions.

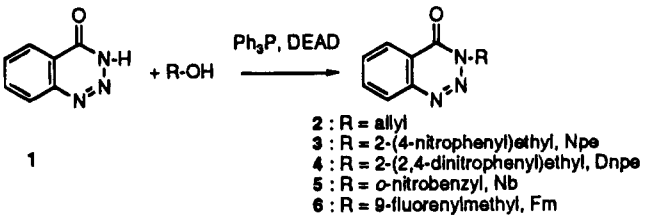

Figure 2. Preparation of N^3-protected benzotriazinone derivatives.

For these reasons, different groups were tested for the protection of the 2-azahypoxanthine ring, having the following criteria in mind: (a) the protecting group should be incorporated into the 2-azahypoxanthine ring in good yield, (b) the protecting group should be removed in good yield after assembly of the oligonucleotide sequence, and (c) the protecting group should be stable during the preparation of the protected phosphoramidite and during oligonucleotide synthesis.

Selection of the Protecting Group. In order to find the most suitable protecting group for 2-azahypoxanthine, the model compound 1,2,3-benzotriazen-4(3*H*)-one **1** was selected. Previous work has demonstrated that this compound is a good model for the 1,2,3-triazen-4-one system of 2-azahypoxanthine.[9] Several protecting groups have been described for the protection of position 6 of guanosine and inosine.[12] Among them, the base-labile 2-(4-nitrophenyl)ethyl (Npe)[13] was selected. Two similar base-labile groups, 2-(2,4-nitrophenyl)ethyl (Dnpe)[14] and 9-fluorenylmethyl (Fm),[15] were also tested together with the allyl group[16] and the photolabile 2-nitrobenzyl (Nb).[17] With the exception of the Npe group, any one of these groups was used for the protection of position 6 of guanine ring.

Mitsunobu reaction with compound **1** and the appropriate alcohol gave the desired protected 1,2,3-benzotriazen-4-one derivatives **2-6** (see Figure 2) in good yield (70−90% except for the Fm derivative which was only 47%). We also tried (a) activation of position 4 with $POCl_3$/pyridine to form the pyridinium derivative and subsequent displacement with the appropriate alcohol and DBU as described for inosine[18] and (b) formation of the sodium salt of **1** with NaH and subsequent reaction with the appropriate bromide as has also been described for inosine.[19] These methods were not appropriate for the base-labile (Npe, Dnpe, and Fm) groups, and for the other groups yields were very low. Thus, the Mitsunobu

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reaction was found to be the best choice for introduction of the protecting groups in the 1,2,3-benzotriazen-4-one system. In principle, the protecting group could be introduced in four different positions: at the O in position 4, and at the nitrogen in positions 1, 2, or 3. For guanine it has been described that Mitsunobu reaction introduces the protecting group at the oxygen in position 6[13,20] (equivalent to position 4 in **1**), while in inosine a mixture of N-alkylated and O-alkylated products was obtained.[21] In benzotriazinone **1**, only one product was obtained. Protected derivatives **2-6** prepared by the Mitsunobu reaction had a carbonyl band in IR (1670–1690 cm^{-1}), and the ^{13}C chemical shift of the carbon bonded to the benzotriazinone ring is between 49-53 ppm. This indicates that the only product formed is a product coming from N-acylation. On the basis of previous data on the reactivity of 1,2,3-benzotriazin-4-one systems,[9,22] we concluded that the protecting group was attached to position N-3 (equivalent to N-1 position in purine nucleosides).

Removal of protecting groups in N-alkylated compounds was more difficult than in O-alkylated compounds. The allyl group in compound **2** was completely stable in the conditions described to remove allyloxycarbonyl groups.[16] When Npe-, Dnpe-, and Fm-protected compounds **3-5** were treated with a 0.5 M 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) solution in dry dichloromethane, the solution became colored but only a very small amount of deprotected **1** was observed in TLC, even after heating at 50 °C overnight. On the other hand, Dnpe (**4**)- and Fm (**5**)-protected compounds were completely deprotected by an overnight treatment with a concentrated aqueous ammonia at 50 °C. Npe-protected compound (**3**) was stable to the ammonia treatment. As described for inosine,[21] N-protected derivatives start being deprotected by DBU, but the olefin thus generated reacts back by a Michael addition with the unprotected molecule to yield the starting protected derivative. When ammonia was used, the excess of ammonia acted as scavenger of the olefin allowing the complete deprotection of compounds **4** and **5**. The Npe group in compound **3** was not removed because ammonia was not strong enough to produce the β-elimination reaction. Finally, removal of Nb group of compound **6** was performed by photolysis[17] providing the desired benzotriazinone **1** in good yield.

Stability of protecting groups to fluoride ions was tested because this reagent is needed to deprotect the alcohol groups of the sugar after Barton reduction.[11] Only compounds **2** (R = allyl) and **6** (R = Nb) were stable. All the base labile groups were rapidly removed with 1 equiv of tetrabutylammonium fluoride in tetrahydrofuran.

In conclusion, among the protecting groups studied only the photolabile Nb group accomplished the conditions needed for protection of the 2-azahypoxanthine ring.

Preparation of Nb-Protected 2-Aza-2′-deoxyinosine Phosphoramidite. The preparation of protected phosphoramidite derivative of 2-aza-2′-deoxyinosine needed for DNA synthesis was performed following two routes. First, 5′ and 3′ alcohol functions were protected with the tetraisopropyldisiloxanyl (TIPDS) group, and the 2′-OH was reacted with phenoxythiocarbonyl chloride (PTC-Cl). Tri-*n*-butyltin hydride (Bu_3SnH) reduction yielded TIPDS-2-aza-2′-deoxyinosine (**10**) as the major product together with small amounts of TIPDS-2-azainosine. The product was immediately reacted with 2-nitrobenzyl alcohol under Mitsunobu reactions,[23] and silyl groups were removed with tetra-*n*-butylammonium fluoride (Bu_4NF). Nb -protected 2-aza-2′-deoxyinosine **11** was obtained in a 40% overall yield from compound **10**. As for benzotriazinone one only regioisomer was obtained, and spectroscopic analysis (IR and ^{13}C-NMR) was in agreement with the N^1-substitution.

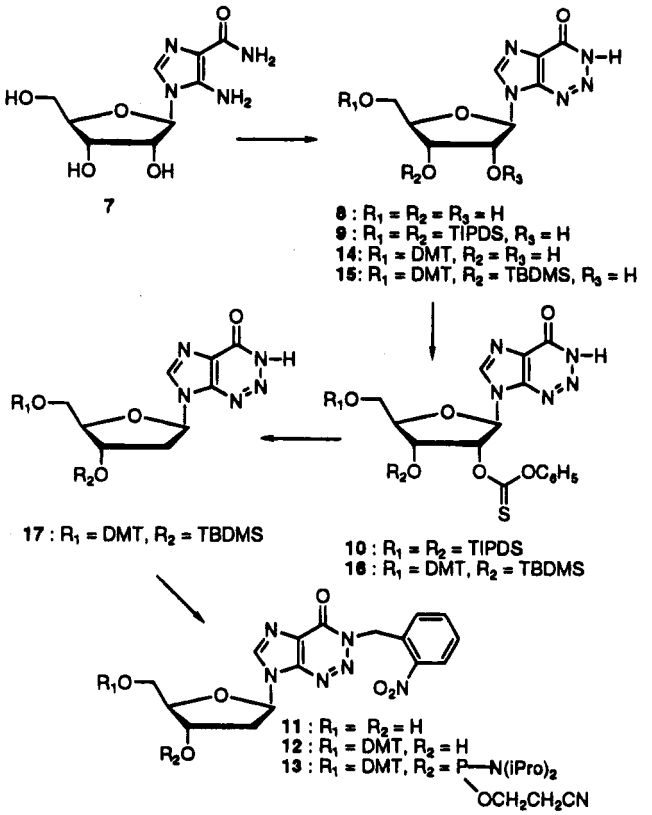

Figure 3. Preparation of the phosphoramidite derivative of N^1-(*o*-nitrobenzyl)-2-aza-2′-deoxyinosine.

Second, the 5′-OH of 2-azainosine was protected with the dimethoxytrityl (DMT) group, and the resulting DMT-derivative was reacted with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) and imidazole in DMF. A 3:2 mixture of 5′-DMT-3′-TBDMS and 5′-DMT-2′-TBDMS isomers was obtained from where the desired 5′-DMT-3′-TBDMS isomer was obtained by purification on a silica gel column.[24] Formation of the 2′-*O*-thiono ester followed by Barton reduction yielded the 2′-deoxy-2-azainosine derivative **17**, that was protected immediately with the 2-nitrobenzyl group. Removal of the TBDMS group directly yielded 5′-*O*-DMT-N^1-Nb-protected 2′-deoxy-2-azainosine **12**. Overall yield in this second route was 8% (from 2-azainosine, five steps) that was slightly higher than the overall yield in the first route (6%, from 2-azainosine, five steps). Phosphoramidite derivative **13** was prepared by reaction of compound **12** with (2-cyanoethoxy)-(N,N-diisopropylamino)chlorophosphine following standard protocols.[25] Compound **12** was con-

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verted to the 3′-*O*-hemisuccinate derivative and reacted with amino-containing solid support (LCAA-CPG)[25,26] to yield a solid support loaded with 2-aza-2′-deoxyinosine.

N^1-Nb-2-aza-2′-deoxyinosine **11** was irradiated for 6 h with a EYE H125 BL 125 W black-light lamp (350 nm) in water/acetonitrile. Small aliquots were taken to follow the progress of the photolysis reaction by HPLC. In the first minutes, a product having a similar retention time (28.9 min, λ_{max} = 276 nm) to that of the starting product (29.7 min, λ_{max} = 287 nm) was observed, and this product was slowly converted to the desired 2-aza-2′-deoxyinosine (10.4 min, λ_{max} = 290 nm). During the progress of the reaction, the solution acquired the red-brown coloration of the azo compounds known to appear from decomposition of 2-nitrosobenzaldehyde formed during the removal of Nb group.[27] All these data indicated that the photolysis of the Nb group was going on in a similar fashion as for the benzotriazinone. At longer times, depurination of 2-aza-2′-deoxyinosine was observed to yield 2-azahypoxanthine (6.2 min, λ_{max} = 282 nm), and after 10 h 2-azahypoxanthine was the major product of the photolysis reaction. The lability of 2-aza-2′-deoxyinosine was in agreement with previous work.[9,10]

Oligonucleotide Syntheses. The protected derivative of 2′-deoxy-2-azainosine was introduced into hexamers A 5′CCCAAAI^{Az}3′ and B 5′GTCAAI^{Az}3′ and pentadecamer C 5′ TAGAGGI^{Az}TCCATTGC 3′ (I^{Az} being 2-azahypoxanthine) sequences, using an automated DNA synthesizer. Coupling yield of the 2-aza-2′-deoxyinosine phosphoramidite was similar to the commercial phosphoramidites. Treatment of oligonucleotide-supports with concentrated ammonia followed by HPLC purification gave the desired oligonucleotides containing protected N^1-(2-nitrobenzyl)-2-azahypoxanthine as judged by HPLC analysis of enzymatic digestion of oligonucleotides. After HPLC purification, oligonucleotides were dissolved in water/acetonitrile and irradiated for several hours with UV light at 350 nm. Photochemical reactions were performed by placing the oligonucleotide solutions either under a 125 W black-light lamp or inside a Rayonet apparatus equipped with black light tubes. In these conditions, the 2-nitrobenzyl group was removed yielding the desired oligonucleotide containing unprotected 2-azahypoxanthine that had a shorter retention time on HPLC (see Figure 4). The presence of 2-aza-2′-deoxyinosine was confirmed by enzyme digestion and HPLC analysis of nucleosides of hexamer A (see Figure 5). 2-Aza-2′-deoxyinosine has the same retention time as dG, which means that in guanine-containing oligomers (sequences B and C) 2-aza-2′-deoxyinosine was covered by dG. Also, mass spectrometry analysis of purified pentadecamers C before and after photolysis confirmed the expected molecular masses. Longer irradiation times in oligomer C gave two products that had a shorter retention time than the desired 2-azahypoxanthine oligomer, and they were characterized for their nucleoside composition as the breakdown products of the oligonucleotide at the 2-aza-2′-deoxyinosine site. This indicates that, at longer times of photolysis, depurination may occur at the 2-aza-2′-deoxyinosine site and the resulting product breaks into two shorter oligonucleotides following the known β-elimination process of phosphates. For these reasons, optimal time for photolysis of oligonucleotides were found to be 4 h using the 125 W lamp and 3 h in the Rayonet apparatus.

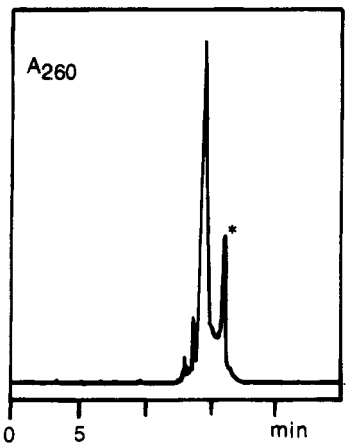

Figure 4. HPLC profile of pentadecamer C after 3 h of irradiation in a Rayonet apparatus equipped with 12 black-light fluorescent tubes (350 nm). The peak marked with an asterisk corresponds to starting Nb-protected pentadecamer C.

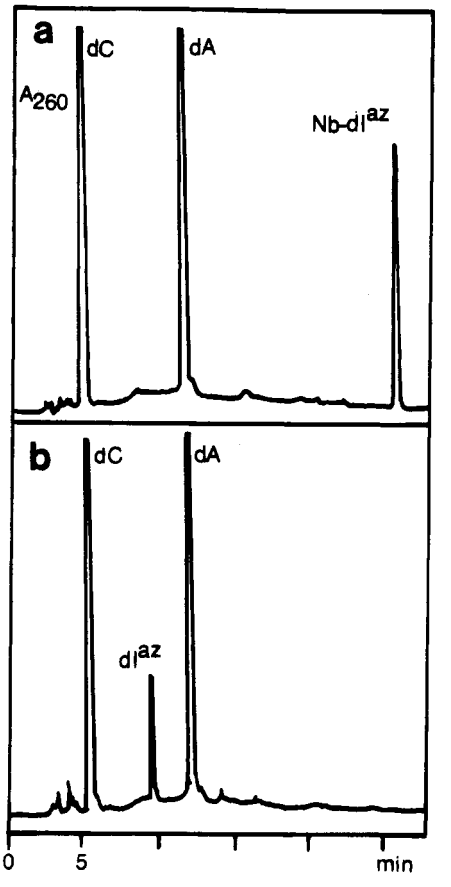

Figure 5. HPLC profile of the enzyme digestion of hexamer 5′CCCAAAI^{Az}3′. (a) Purified 2-nitrobenzyl protected hexamer, before photolysis. (b) Purified hexamer after photolysis.

Melting Experiments. Duplexes having the 2-azahypoxanthine and hypoxanthine base pairs with the four natural bases were analyzed. Melting temperatures at pH 6.0 and pH 8.0 are shown in Table 1. At pH 8.0 the most stable 2-azahypoxanthine base pairs were I^{Az}.T (51.0 °C) and I^{Az}.A (50.5 °C). Surprisingly the least stable was I^{Az}.C (48.0 °C). At pH 6.0 most of the I^{Az} base pairs were more stable, the most stable base pairs being I^{Az}.C

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Table 1. Melting Temperatures (°C) of 2-Azahypoxanthine Duplexes

5' TAGAGGXTCCATTGC 3'

3' ATCTCCYAGGTAACG 5'

	pH 8.0	pH 6.0	Δ(pH8.0 − pH6.0)
X = G, Y = C	62.0	62.0	0
X = A, Y = T	58.0	58.0	0
X = G, Y = T	54.0	53.0	−1
X = A, Y = C	48.0	52.0	+4
X = I^{Az}, Y = T	51.0	50.0	−1
X = I^{Az}, Y = A	50.5	52.0	+1.5
X = I^{Az}, Y = G	49.0	50.5	+1.5
X = I^{Az}, Y = C	48.0	52.5	+4.5
X = I, Y = C	58.0	56.0	−2
X = I, Y = A	56.5	56.5	0
X = I, Y = G	55.0	55.0	0
X = I, Y = T	54.0	52.5	−1.5

(52.5 °C) and I^{Az}.A (52.0 °C). Little variation between different I^{Az} base pairs were observed (at pH 8.0, 3°; and at pH 6.0 2.5°). However, when compared with perfectly matched duplexes (G.C 62.0 °C, A. T 58.0 °C) and dI base pairs, 2-aza-2'-deoxyinosine base pairs were substantially less stable. The low stability of the I^{Az}.C base pair compared with I. C at pH 8.0 and the large increase of stability when going to pH 6.0 (+4.5°) may indicate that, at pH 8.0, 2-azahypoxanthine is in the anionic form. This is in agreement with a pK_a of 6.8 described for 2-azainosine.[8] At pH 6.0, most of the 2-azahypoxanthine molecules should be in the neutral form, and thus the base pair stability is increased. Unfortunately, in spite of the increased stability at pH 6.0, the substitution of an A by 2-azahypoxanthine in the 15-mer duplex causes a destabilization of 7°. When these results are compared with the recently described nitroazole derivatives,[6] the order of duplex stability for the analogues appears to be 5-nitroindole > 4-nitroindole > 6-nitroindole ≥ 2-azahypoxanthine > 3-nitropyrrole.

Antiviral and Cytotoxicity Activities. 2-Azainosine and related compounds have been shown to possess some antiviral and cytotoxic properties.[8,10] Compounds **8** and **11** were tested for antiviral activity and cytotoxicity, together with N^1-(*o*-nitrobenzyl)-2-azainosine (**20**) and its triacetyl derivative **19**. These compounds **8**, **11**, **19**, and **20** were tested for inhibition of the following viruses: cytomegalovirus (strains AD-169 and Davis), varicella-zoster virus (strains OKA, YS, 07/1, YS/R), polio virus-1, coxsackie virus B4, vesicular stomatitis virus, herpes simplex virus 1 (strain KOS) and 2 (strain G), vaccinia virus, parainfluenza-3 virus, reovirus-1, sindbis virus, semliki forest virus. No appreciable antiviral activity was observed with any of the compounds against any of the viruses tested. Nor did the compounds prove toxic to the host cells [human embryonic lung (HEL) cells, human embryonic skin-muscle (ESM) fibroblasts, HeLa or Vero cells] at the highest concentration tested (400 μg/mL for ESM, HeLa and Vero cells; 50 μg/mL for HEL cells).

Experimental Section

Abbreviations: AIBN: α,α'-azoisobutyronitrile, ACN: acetonitrile, AICA: 5-aminoimidazole-4-carboxamide, Bu_4NF: tetra-*n*-butylammonium fluoride, Bu_3SnH: tri-*n*-butyltin hydride, CPG: controlled pore glass, DBU: 1,8-diazabicyclo-[5.4.0]undec-7-ene, DCM: dichloromethane, DEAD: diethyl azodicarboxylate, DMAP: (dimethylamino)pyridine, DMF: dimethylformamide, DMT: 4,4'-dimethoxytrityl, Dnpe: 2-(2,4-dinitrophenyl)ethyl, Et_3N: triethylamine, Fm: 9-fluorenylmethyl, LCAA: long-chain amino alkyl, MeOH methanol, Nb: 2-nitrobenzyl, Npe: 2-(4-nitrophenyl)ethyl, PTC: phenoxythiocarbonyl, TBDMS: *tert*-butyldimethylsilyl, THF: tetrahydrofuran, TIPDS: tetraisopropyldisiloxane-1,3-diyl.

General Methods. All reactions were carried out in oven-dried glassware, under a nitrogen or argon atmosphere, unless specified otherwise. Before use, starting materials were dried by evaporation with the dry solvent that will be used for the reaction. 1,2,3-Benzotriazen-4(3*H*)-one (**1**) was prepared as described previously.[9] 2-Azainosine[8] (**8**) and 2'-*O*-PTC-3',5'-*O*-TIPDS-2-azainosine[9] (**9**) were prepared according to previously described protocols. Reagents for oligonucleotide synthesis were from Cruachem Ltd. Dry solvents were from SDS. HPLC grade solvents were from E. Merck. AICA-riboside was from Sigma. Snake venom phosphodiesterase (from *Crotalus durissus*) and alkaline phosphatase were from Boehringer Mannheim. The rest of the reagents were from Aldrich and Fluka and were used without further purification. Analytical TLC was run on aluminum sheets coated with silica gel 60 F254 from E. Merck.

3-N-Substituted-1,2,3-benzotriazen-4-one (2-6). 1,2,3-Benzotriazinone[9] (**1**) was dissolved with dioxane, and 1.5 equiv of the appropriate alcohol and 1.6 equivalents of Ph_3P were added. To this solution was added 1.6 equiv of DEAD dropwise. After 2 h of magnetic stirring at room temperature, the solution was concentrated to dryness and the product was purified on a silica gel column eluted with DCM.

Compound **2** (R = allyl). Yield 79%. Mp 65−70 °C. Elemental analysis: C 64.14%, H 4.95%, N 22.33% (expected C 64.17%, H 4.81%, N 22.45%). TLC (DCM) R_f 0.4. UV (ACN) λmax: 287 nm (8200). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.3−7.7 (4H, m), 6.1 (1H, m), 5.3 (2H, m), 5.0 (2H, m). ^{13}C-NMR ($CDCl_3$) δ (ppm): 155.1, 144.2, 137.7, 132.2, 128.1, 124.9, 131.4, 119.8, 119.1, 51.9. IR (film, cm^{-1}): 1685 (CO str).

Compound **3** (R = Npe). Yield 90%. Mp 200−205 °C. Elemental analysis: C 60.58%, H 4.12%, N 18.32% (expected C 60.81%, H 4.05%, N 18.91%). TLC (DCM) R_f 0.36. UV (ACN) λmax: 278 nm (6800). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (2H, 2H, dd), 7.3 (2H, dd), 8.3−7.7 (4H, m), 4.79 (2H, t), 3.3 (2H, t), 3.3 (2H, t). ^{13}C-NMR ($CDCl_3$) δ (ppm): 155.4, 146.9, 145.2, 144.1, 135.0, 132.5, 128.4, 125.5, 129.7, 123.8, 119.5, 50.0, 34.6. IR (film, cm^{-1}): 1676 (CO str), 1508 (NO_2 str asym), 1351 (NO_2 str sym).

Compound **4** (R = Dnpe). Yield 79%. Mp 201−205 °C. Elemental analysis: C 53.13%, H 3.26%, N 19.87% (expected C 52.78%, H 3.22%, N 20.52%). TLC (DCM) R_f 0.39. UV (ACN) λ_{max}: 276 nm (10600). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.8−7.3 (7H, m), 4.8 (2H, t, J = 6.8Hz), 3.6 (2H, t, J = 6.6 Hz). ^{13}C-NMR ($CDCl_3$) δ (ppm): 155.5, 149.2, 146.8, 144.0, 140.0, 135.1, 132.7, 128.5, 125.0, 133.8, 127.1, 120.2, 119.4, 14.1, 32.6. IR (film, cm^{-1}): 1681 (CO str), 1537, 1521 (NO_2 str asym), 1346 (NO_2 str sym).

Compound **5** (R = Nb). Yield 70%. Mp 178−182 °C. Elemental analysis: C 59.67%, H 3.62%, N 19.65% (expected C 59.57%, H 3.54%, N 19.85%). TLC (DCM) R_f 0.34. UV (ACN) λ_{max}: 281 nm (10000). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.3−7.7 (4H, m), 7.5−7.1 (4H, m), 6.0 (2H, s). ^{13}C-NMR ($CDCl_3$) δ (ppm): 155.4, 148.3, 144.2, 135.1, 132.7, 128.5, 125.2, 133.6, 128.9, 128.7, 125.2, 131.2, 119.7, 50.1. IR (film, cm^{-1}): 1693 (CO str), 1527, 1521 (NO_2 str asym), 1338 (NO_2 str sym).

Compound **6** (R = Fm). Yield 43%. Mp 148−151 °C. Elemental analysis: C 77.70%, H 4.64%, N 12.85% (expected C 77.52%, H 4.61%, N 12.92%). TLC (DCM) R_f 0.65. ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.4−7.7 (4H, m), 7.4−7.2 (8H, m), 4.8−4.7 (3H, m). ^{13}C-NMR ($CDCl_3$) δ (ppm): 155.8, 144.2, 143.9, 141.1, 135.0, 132.5, 128.4, 125.2, 127.8, 127.1, 124.9, 120.1, 119.7, 53.3, 45.9. IR (film, cm^{-1}): 1683 (CO str).

3',5'-*O*-Tetraisopropyldisiloxane-1,3-diyl-2-aza-2'-deoxyinosine (9). Compound **8**[9] (2.3 g, 8.5 mmol) was dissolved in 130 mL of DMF and 9 mL of pyridine. A 1.1 equiv (9.4 mmol) amount of TIPDS-Cl_2 was added dropwise to the solution, and the reaction mixture was stirred for 4 h at room temperature. Solvents were evaporated, and the residue was dissolved in ethyl acetate. The solution was washed with water, cold 1 N HCl, and saturated $NaHCO_3$ and NaCl aqueous

solution. The organic phase was dried and evaporated to dryness. The residue was purified by column chromatography on silica gel eluted by a 2–6% MeOH gradient in DCM. Yield: 50%. TLC (10% MeOH/DCM) R_f 0.47. UV (ACN) λ_{max}: 283 nm (4600). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (1H, s), 6.1 (1H, d), 4.8 (1H, m), 4.5 (1H, m), 4.1 (3H, m), 1.1 (28H, m). ^{13}C-NMR ($CDCl_3$) δ (ppm): 154.7, 141.8, 137.8, 125.2, 90.2, 82.5, 75.6, 69.9, 61.0, 16.8–17.1, 12.5–13.0. IR (film, cm^{-1}): 3420 (OH), 2945–2870 (CH st), 1714 (CO str), 1043–1087 (SiOC, SiO str sym), 854 (SiC str).

3',5'-*O*-Tetraisopropyldisiloxane-1,3-diyl-2'-*O*-(phenoxythiocarbonyl)-2-azadeoxyinosine (10). Compound **9** (1 g, 1.9 mmol) and 5 equiv (9.5mmol) of DMAP were dissolved with 35 mL of CH_3CN. A 1.1 equiv (2.1 mmol) amount of PTC-Cl were added dropwise, and the reaction mixture was stirred for 3 h at room temperature. The solvent was evaporated, and the residue was treated as described above for compound **9**. Yield: 1.13 gr (89%). Elemental analysis: C 51.21%, H 6.37%, N 10.45%, S 4.61% (expected C 51.91%, H 6.38%, N 10.81%, S 4.95%). TLC (5% MeOH/DCM) R_f 0.4. UV (ACN) λ_{max}: 285 nm (5200). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.3 (1H, s), 7.2–7.5 (5H, m), 6.5 (1H, d), 6.2 (1H, d), 5.1 (1H, m), 4.1 (3H, m), 1.0 (28H, m). IR (film, cm^{-1}): 2890–2970 (CH st), 1725 (CO str), 1200 (CS st) , 1050–1089 (SiOC, SiO str sym).

N^1-(2-Nitrobenzyl)-2-aza-2'-deoxyinosine (11). Compound **10** (0.8 g, 1.2 mmol) was dissolved in 40 mL of toluene and 0.15 mmol of AIBN was added. A 2.4 mmol amount of Bu_3SnH was added dropwise with a syringe, and the reaction mixture was heated to 75 °C for 3 h. The solution was cooled, and solvents were evaporated. The residue was partially purified by column chromatography eluted with 2.5% MeOH in DCM. Yield 60%. TLC (10% MeOH/DCM) R_f 0.64. ^{1}H-NMR shows the absence of aromatic protons from the PTC group.

The product obtained (0.5 g, 1 mmol) was reacted with 1.5 mmol of 2-nitrobenzyl alcohol, 1.6 mmol of Ph_3P, and 1.6 mmol of DEAD as described for compounds **2–6**. The product was partially purified by column chromatography on silica gel eluted with a 2–4% MeOH gradient in DCM. TLC (5% MeOH/DCM) R_f 0.65. ^{1}H-NMR showed the presence of the aromatic protons and the singlet at 6.1 ppm of the 2-nitrobenzyl group.

The product obtained (0.3 gr, 0.47 mmol) was dissolved in THF, and 0.7 mL of a 1 M solution of Bu_4NF in THF was added. The reaction mixture was stirred for 15 min at room temperature, and solvents were evaporated. The residue was purified by column chromatography on silica gel eluted by a 5–10% MeOH gradient in DCM. Overall yield (from compound **10**): 39%. Elemental analysis: C 46.96%, H 4.65%, N 20.36% (expected for C_{16} $H_{16}N_6O_6$. H_2O C 47.29%, H 4.47%, N 20.68%). TLC (10% MeOH/DCM) R_f 0.35. UV (ACN) λ_{max}: 283 nm (7700). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.6 (1H, s), 8.1 (1H, m), 7.5–7.1 (3H, m), 6.6 (1H, t, J = 6.6 Hz), 6.0 (2H, s), 4.8 (1H, m), 4.2 (1H, m), 3.9 (2H, m), 2.8 (1H, m), 2.7 (1H, m).^{13}C-NMR ($CDCl_3$) δ (ppm): 155.1, 149.1, 146.8, 144.3, 134.9, 130.6, 130.0, 126.4, 131.8, 126.9, 86.8, 83.9, 71.8, 62.4, 51.5, 40.2. IR (film, cm^{-1}): 3389 (wide, OH), 1714 (CO str), 1525 (NO_2 str asym), 1357 (NO_2 str sym).

5'-*O*-(Dimethoxytrityl)-N^1-(2-Nitrobenzyl)-2-aza-2'-deoxyinosine (12). From Compound 11. 0.1 g (0.25 mmol) amount of compound **11** was dissolved in 10 mL of pyridine, and 0.13 g (0.37 mmol) of DMT-Cl was added. After 5 h of magnetic stirring at room temperature, 0.2 mL of methanol was added and the solvents were evaporated. The residue was dissolved in DCM and the solution washed with 5% $NaHCO_3$ solution and saturated NaCl. The organic phase was dried and concentrated to dryness. The residue was purified by column chromatography on silica gel eluted with a 0–5% MeOH gradient in DCM. Yield 35%. TLC (10% MeOH/DCM) R_f 0.65. ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (1H, s), 8.1 (1H, m), 6.7–7.5 (16H, m), 6.5 (1H, t, J = 6.4 Hz), 6.0 (2H, d, J = 4.6 Hz), 4.7 (1H, m), 4.2 (1H, m), 3.7 (6H, s), 3.4 (2H, m), 2.6 (2H, m).

From Compound 17. A 0.67 g (0.91 mmol) amount of compound **17** was reacted with 2-nitrobenzyl alcohol, Ph_3P, and DEAD as described for compounds **2–6**. The resulting product was partially purified on silica gel eluted with a 1–2.5% MeOH gradient in DCM. Fractions that gave a blue spot with the diphenylamine reagent (TLC 2%MeOH/DCM R_f= 0.18) were pooled and concentrated to dryness. The product was contaminated with triphenylphosphine oxide and it was used in the next step without further purification.

The product obtained in the previous reaction was treated with 1 M Bu_4NF solution in THF as described for the preparation of compound **11**. The resulting product was purified on silica gel eluted with 1–4% MeOH gradient in DCM. Yield (from compound **17**): 45%. Elemental analysis: C 62.43%, H 4.93%, N 11.47% (expected for $C_{37}H_{34}N_6O_9 \cdot H_2O$ C 62.71%, H 5.12%, N 11.86%). TLC (10% MeOH/DCM) R_f 0.65. UV (EtOH) λ_{max}: 271 nm (8000), 281 nm (6200). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (1H, s), 8.1 (1H, m), 7.5 - 6.7 (16H, m), 6.5 (1H, t, J = 6.4 Hz), 6.0 (2H, d, J = 4.6 Hz), 4.7 (1H, m), 4.2 (1H, m), 3.7 (6H, s), 3.4 (2H, m), 2.6 (2H, m).^{13}C-NMR ($CDCl_3$) δ (ppm): 158.5, 153.8, 148.1, 144.3, 144.2, 141.9, 135.4, 133.7, 131.0, 129.9, 128.9, 128.7, 127.9, 127.8, 127.1, 126.9, 125.3, 113.1, 86.1, 85.6, 71.9, 63.3, 55.1, 50.3, 41.1.

3'-*O*-[(2-Cyanoethoxy)-(*N*,*N*-diisopropylamino)phosphinyl]-5'-*O*-(dimethoxytrityl)-N^1-(2-nitrobenzyl)-2-aza-2'-deoxyinosine (13). Compound **12** (0.2 g, 0.29 mmol) was dissolved in DCM, and 0.2 mL (1.16 mmol) of ethyldiisopropylamine was added. The reaction mixture was cooled with ice, and 102 mg (0.43 mmol) of (2-cyanoethoxy)-(*N*,*N*-diisopropylamino)chlorophosphine was added with a syringe. After 30 min of magnetic stirring at room temperature, the reaction was stopped by adding 1 mL of MeOH, and solvents were evaporated. The residue was dissolved in ethyl acetate, and the solution was washed with saturated solutions of $NaHCO_3$ and NaCl. The organic phase was dried and concentrated to dryness. The residue was purified on silica gel eluted with ethyl acetate/$CHCl_3$/Et_3N (47:47:5) solution. Yield: 55%. Elemental analysis: C 62.84%, H 6.05%, N 12.02% (expected C 62.01%, H 5.77%, N 12.58%). TLC (ethyl acetate/$CHCl_3$/EtN_3 47:47:5): 0.70. ^{31}P-NMR ($CDCl_3$) δ (ppm): 149.6 and 149.5 (two diastereoisomers).

5'-*O*-(Dimethoxytrityl)-2-azainosine (14). A 2 g (7.4 mmol) amount of 2-azainosine[8] (**8**) was dissolved in 30 mL of pyridine. A 3.0 g (8.9 mmol) amount of DMT-Cl was added to the solution. After 2 h of magnetic stirring a further 2.5 g (7.4 mmol) of DMT-Cl was added and the reaction was left overnight. The mixture was treated as described for compound **12** from componud **11**. Product was purified by silica gel chromatography (2–10% MeOH gradient in DCM). Yield 62%. Elemental analysis: C 60.63%, H 5.16%, N 11.49% (expected for $C_{30}H_{31}N_5O_8$. H_2O C 61.11%, H 5.30%, N 11.88%). TLC (10% MeOH/DCM) R_f 0.47. UV (EtOH) λ_{max}: 268 nm (5000), 275 nm (5100) ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (1H, s), 7.3– 6.7 (13H, m), 6.2 (1H, d, J = 2.4 Hz), 4.8 (1H, m), 4.5 (1H, m), 4.3 (1H, m), 3.6 (6H, s), 3.4 (2H,m).^{13}C-NMR ($CDCl_3$) δ (ppm): 158.3, 154.8, 144.7, 144.3, 141.0, 135.5, 130.0, 128.0, 127.8, 126.7, 126.6, 113.1, 89.9, 86.6, 84.2, 75.2, 70.6, 63.0, 55.0.

3'-*O*-(*tert*-Butyldimethylsilyl)-5'-*O*-(dimethoxytrityl)-2-azainosine (15). Compound **14** (2.9 g, 5.1 mmol) was dissolved in 35 mL of anhydrous DMF, and 1.36 g (20 mmol) of imidazole was added, followed by 0.90 g (6 mmol) of TBDMS-Cl. After 16 h of magnetic stirring at room temperature, the reaction mixture was evaporated to dryness. The residue was purified by column chromatography on silica gel eluted with a 0–5% MeOH gradient in DCM. Two regioisomers were obtained.

5',3'-Isomer (slow isomer): Yield 55%. TLC (10% MeOH/DCM) R_f 0.55. UV (EtOH) λ_{max}: 268 nm (6000), 274 nm (5800). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (1H, s), 7.3– 6.7 (13H, m), 6.1 (1H, d, J = 4.6 Hz), 4.6 (1H, t, J = 4.8 Hz), 4.4 (1H, t, J = 5.2 Hz), 4.1 (1H, m), 3.7 (6H, s), 3.4 (1H,m), 3.2 (1H, m), 0.79 (9H, s), 0.02 and 0.09 (6H, 2s).^{13}C-NMR ($CDCl_3$) δ (ppm): 158.4, 155.1, 145.0, 144.1, 141.8, 135.2, 129.9, 127.9, 127.8, 127.5, 126.9, 113.1, 89.8, 86.6, 84.7, 75.3, 71.7, 62.4, 55.1, 25.6, 17.9, −4.8, −4.9.

5',2'-Isomer (fast isomer): Yield 38%. TLC (10% MeOH/DCM) R_f 0.71. UV (EtOH) λ_{max}: 268 nm (6300), 274 nm (6200). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (1H, s), 7.4–6.7 (13H, m), 6.2 (1H, d, J = 5.0 Hz), 4.8 (1H, t, J = 4.9 Hz), 4.3 (2H, m), 3.7 (6H, s), 3.5 (2H,m), 0.8 (9H, s), 0.02 and 0.09 (6H, 2s).^{13}C-NMR

($CDCl_3$) δ (ppm): 158.4, 154.7, 144.9, 144.2, 141.1, 135.2, 129.8, 127.8, 127.3, 126.8, 113.1, 88.9, 86.6, 84.4, 76.9, 71.2, 63.0, 55.0, 25.3, 17.7, −5.1, −5.3.

3′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)-2′-(phenoxythiocarbonyl)-2-azainosine (16). Compound **15** (1.2 g, 1.7 mmol, 5′,3′-isomer) was reacted with 1.6 g (8.7 mmol) of DMAP and 0.32 g (1.9 mmol) of PTC-Cl in CH_3CN as described for compound **10**. The product was purified on silica gel (1−2.5% MeOH gradient in DCM). Yield 81%. Elemental analysis: C 57.84%, H 5.71%, N 7.27%, S 3.60% (expected for $C_{42}H_{47}N_5O_8SiS{\cdot}CH_2Cl_2$: C 57.71%, H 5.52%, N 7.83%, S 3.58%). TLC (10% MeOH/DCM) R_f 0.61. UV (EtOH) λ_{max}: 275 nm (6500), 281 nm (6400). ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (1H, s), 7.4- 6.7 (18H, m), 6.5 (1H, d, J = 6.4 Hz), 6.2 (1H, t, J = 6.0 Hz), 4.8 (1H, m), 4.2 (1H, m), 3.7 (6H, s), 3.4 (2H, m), 0.8 (9H, s), 0.02 and 0.08 (6H, 2s).^{13}C-NMR ($CDCl_3$) δ (ppm): 193.9, 158.6, 154.5, 153.2, 145.3, 144.0, 141.8, 135.2, 130.0, 129.6, 128.0, 127.9, 127.3, 127.0, 126.9, 121.5, 113.2, 87.1, 86.7, 86.2, 83.3, 70.6, 62.6, 55.2, 25.6, 18.0, −4.6, −4.8.

3′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)-2-aza-2′-deoxyinosine (17). Compound **16** (1.1 g, 1.3 mmol) was treated with Bu_3SnH and AIBN, as described for the preparation of compound **11**. The product was purified on silica gel (1−2.5% MeOH gradient in DCM). The purified product contained a small amount (15%) of starting material, and it was used in the next reaction without further purification. Yield: 55%. TLC (10% MeOH/DCM): 0.51. ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.3 (1H, s), 7.4− 6.7 (13H, m), 5.8 (1H, t), 4.8 (1H, m), 4.2 (1H, m), 3.7 (6H, s), 3.6 (2H, m), 2.8 (2H, m), 0.8 (9H, s), 0.02 and 0.08 (6H, 2s).

2′,3′,5′-Tri-*O*-(acetyl-2-azainosine (18). A 3.5 g amount of 2-azainosine (**8**, 13 mmol) was reacted with acetic anhydride (83 mmol) in pyridine (50 mL) for 24 h at 60 °C. The dark reaction mixture was allowed to cool to room temperature, and 8 mL of hot MeOH was added. The mixture was stirred for a few minutes and filtered. The filtrate was concentrated to dryness, and the residue was heated with ethanol for 20 min. The mixture was filtered, and the filtrate was maintained at 0 °C overnight. A white precipitate was formed that was separated by filtration. The filtrate was concentrated to dryness, and the residue was purified by silica gel chromatography eluted with a 2−10% MeOH gradient in DCM. Yield: 3.4 g (64%). Spectroscopic properties as previously described.[8]

2′,3′,5′-Tri-*O*-(acetyl-*N*^{1}-(2-nitrobenzyl)-2-azainosine (19). A 3.3 g (8.3 mmol) amount of 2′,3′,5′-tri-*O*-acetyl-2-azainosine (**18**) was reacted with 2-nitrobenzyl alcohol (12 mmol), Ph_3P (13 mmol), and DEAD (13 mmol) in dioxane as described for compounds **2-6** for 3 h. Silica gel chromatography eluted first with 3:1 hexane/DCM and afterwards with pure DCM. Yield: 80%. TLC (2% MeOH/DCM): 0.32. ^{1}H-NMR (Cl_3CD) δ (ppm): 8.2 (1H, s), 8.1 (1H, dd), 7.6 (2H, m), 7.1 (1H, dd), 6.3 (1H, d, J = 5.2 Hz), 6.1 (2H, s), 5.8 (1H, t, J = 4.9 Hz), 5.6 (1H, t, J = 3.5 Hz), 4.4 (3H, m), 2.16 (3H, s), 2.14 (3H, s), 2.11 (3H, s). ^{13}C-NMR (Cl_3CD): 170.3, 169.3, 169.3, 153.1, 148.1, 147.2, 141.8, 133.8, 129.2, 128.9, 125.3, 133.1, 123.1, 87.4, 80.5, 73.6, 70.2, 62.1, 50.5, 20.7, 20.4, 20.3. IR (film) cm^{-1}: 1724, 1749, 1527, 1320.

***N*^{1}-(2-Nitrobenzyl)-2-azainosine (20).** The product described above was treated with a solution containing methanol (15 ml), dioxane (15 mL), and 15% ammonia aqueous solution (30 mL) at room temperature overnight. Following removal of solvents, the residue was purified by silica gel chromatography (0−15% gradient of MeOH in DCM). Yield 2.2 g (81%). TLC (10% MeOH/DCM): 0.23. ^{1}H-NMR (d_6-DMSO) δ (ppm): 8.2 (1H, s), 8.1 (1H, dd), 7.6 (2H, m), 7.2 (1H, dd), 6.6 (1H, wide s), 6.1 (1H, d, J = 4.6 Hz), 5.9 (1H, wide s), 5.3 (1H, wide s), 5.1 (1H, t, J = 5.2 Hz), 4.5 (1H, m), 4.2 (1H, m), 3.7 (2H, m). ^{13}C-NMR (d_6-DMSO): 155.4, 149.9, 146.2, 144.6, 134.9, 130.5, 130.1, 126.1, 132.2, 127.9, 91.3, 87.5, 76.8, 71.6, 62.4, 51.5. IR (film) cm^{-1}: 1710, 1525, 1350.

Deprotection and Stability Studies. Deprotection of benzotriazinone derivatives carrying Npe (**3**), Dnpe (**4**), and Fm (**6**) were studied in two different conditions. First, compounds **3**, **4**, and **6** (30−50 mg) were dissolved in DCM and 1 equiv of DBU dissolved in DCM was added. Aliquots of the resulting solutions were analyzed by TLC (DCM). No deprotection was observed after 24 h at room temperature. Larger excess of DBU was also tried with the same negative result. Second, 30−50 mg of compounds **3**, **4**, and **6** were dissolved in dioxane, and 1 equivalent of ammonia was added by addition of the corresponding amount of a 32% ammonia aqueous solution. After 5 h at room temperature 50% of compounds **4** and **6** were deprotected. If the reaction was performed at 55 °C, deprotection of compounds **4** and **6** was completed. Compound **3** was not deprotected in these conditions.

Deprotection of *N*^{3}-allylbenzotriazinone (**2**) was studied in two different conditions. First, 0.2 g of compound **2** was dissolved in 8 mL of wet DCM, and 0.02 equiv of tetrakis(triphenylphosphine)palladium(0) together with 1.2 equiv of Bu_3SnH were added. No reaction was observed by TLC (DCM) after 5 h at room temperature. Second, 10 mg of compound **2** was dissolved in 3 mL of THF, and 1.5 equiv of tris(dibenzylidenacetone) palladium(0)−chloroform adduct, 25 equivalents of Ph_3P, and 3 mL of a 1.2 M solution of (1:1) *n*-butylamine/formic acid were added. No reaction was observed after 1 h at room temperature; the reaction mixture was heated to 50 °C with the same negative result.

N^3-(2-Nitrobenzyl)benzotriazinone (**5**, 200 mg) was dissolved in DCM, and the solution was placed under an EYE H125 BL 125 W black-light lamp (350 nm). After 5 h of photolysis, the major part of compound **5** was deprotected yielding benzotriazinone (**1**) that precipitated from the solution and 2-nitrosobenzaldehyde that was decomposed to yield the colored azo-derivatives.

In order to study the stability of the benzotriazinone derivatives to fluoride, compounds **3-6** were treated with 2 equivalents of a 1 M Bu_4NF solution in THF. Compounds carrying Npe (**3**), Dnpe (**4**), and Fm (**6**) were completely deprotected in less than 15 min at room temperature. The Nb derivative **5** was completely stable.

Oligonucleotide Syntheses. The following sequences: A 5′CCCAAAI^{Az}3′, B 5′GTCAAI^{Az}3′, and C 5′TAGAGGI^{Az}TCCATTGC3′ were prepared on an Applied Biosystems automatic DNA synthesizer using standard 2-cyanoethyl phosphoramidites and the modified phosphoramidite. For the preparation of sequences A and B, a solid support containing Nb-protected 2-aza-2′-deoxyinosine was used. Complementary pentadecamers containing natural bases were prepared using commercially available chemicals and following standard protocols.[25]

Oligonucleotide-supports were treated with a 32% aqueous ammonia at 50 °C for 16 h. Ammonia solutions were concentrated to dryness, and the products were purified by reverse phase HPLC. Oligonucleotides A and B were prepared on a 0.2 μmol scale and without the last DMT group at the 5′ end. Oligonucleotide C was prepared in 4 μmol scale and the last DMT group was left during the ammonia treatment in order to facilitate the HPLC purification. All syntheses presented a major peak that was collected and analyzed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis of the nucleosides (conditions A).[9] Yield (OD units at 260 nm): heptamer A (0.2 μmol synthesis): 8 OD; hexamer B (0.2 μmol): 6 OD; pentadecamer C (4 μmol): 90 OD. HPLC conditions: In all cases solvent A was 20 mM triethylammonium acetate (pH 7.8) and solvent B was a 1:1 mixture of water and CH_3CN. For analytical runs the following conditions were used. Column: Nucleosil 120C18, 250 × 4 mm, flow rate: 1 mL/min. (A) 5−95% B linear gradient in 40 min. (B) 5−50% B linear gradient in 20 min. For semipreparative runs the following conditions were used: Columns: Nucleosil 120C18, 250 × 10 mm. Flow rate: 3 mL/min. A 25−80%B linear gradient in 20 min (DMT on), or a 5−50% B linear gradient in 30 min (DMT off). The retention time of the nucleosides obtained after enzyme digestion were in conditions A: dC 4.9 min, dG 9.8 min, T 10.5 min, dA 12.6 min, Nb-protected dI^{Az} 28.8 min.

Photolysis of 2-Nitrobenzyl Groups. Aliquots (1−10 OD) of purified oligonucleotides A-C containing Nb-protected azainosine were dissolved in 2 mL of 5: 2 water/CH_3CN mixture. The resulting solutions were located either under

an EYE H125 BL 125 W black-light lamp or inside the reaction vessel of a Rayonet apparatus equipped with 12 × 25 W black-light fluorescent tubes, and they were irradiated. After different times, aliquots of the solutions were taken and analyzed by HPLC (conditions A). In all cases the starting oligonucleotides were converted to a more polar compound. Retention times: oligonucleotide A, before photolysis 18.0 min, after 13.9 min; oligonucleotide B, before photolysis 18.6 min, after 14.0 min; oligonucleotide C, before photolysis 15.7 min, after 12.5 min. The optimal time for the irradiation using the 125 W lamp was 4 h, and for the Rayonet apparatus it was 3 h. At these times conversion of Nb-protected oligonucleotide to the desired oligonucleotide were approximately 70–80%. At longer times of irradiation of pentadecamer C with the 125 W lamp two more polar products appeared. These products were collected and analyzed and they had the nucleoside composition that suggested that they were breakdown products of the oligonucleotide at the 2-azahypoxanthine site. For that reason it is not convenient to prolong the irradiation for more than 4 h to drive the reaction to completion.

Purified unprotected oligonucleotides were analyzed by phosphodiesterase degradation (see above). In all cases a correct nucleoside composition was found together with the absence of Nb-protected dI^{Az}. 2-Aza-2'-deoxyinosine had the same retention time as dG. Oligomers B and C had a higher content in dG corresponding to the addition of one molecule of dI^{Az}. Oligonucleotide A had no G, and the presence of dI^{Az} was clearly seen. Mass spectra (electrospray): pentadecamer C protected with 2-nitrobenzyl group: found M 4728.8, M + Na 4750.9 (expected 4729); pentadecamer C after photolysis: found M 4583.6 (expected 4583).

Melting Experiments. Pentadecamer duplexes were made by mixing equimolar amounts of two pentadecamer strands dissolved in two different solutions: Solution pH 8.0 = 50 mM tris·HCl buffer pH 8.0, 0.15 N NaCl; solution pH 6.0 = 0.1 M citric acid, 0.2 M sodium phosphate. Duplexes were annealed by slow cooling from 80 °C to 4 °C. UV absorption spectra and melting curves (absorbance vs temperature) were recorded in 1-cm path-length cells using a Shimadzu 2100 spectrophotometer having a temperature controller with programmed temperature increase of 1°/min. Melts were run on duplex concentrations of 3–6 μM at 260 nm.

Antiviral and Cytoxicity Studies. The antiviral activity and cytotoxicity assays were carried out according to well-established procedures.[28]

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Supporting Information Available: Copies of ^1H NMR spectra of compounds **17** and **19**, and ^{13}C NMR spectra of compounds **9**, **15**, and **20** (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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