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PREPARATION OF OLIGONUCLEOTIDES CONTAINING dAICA USING AN UNEXPECTED SIDE-REACTION OBSERVED ON A PROTECTED DERIVATIVE OF 2-AZA-2'-DEOXYINOSINE.

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ABSTRACT : Attempts of synthesis of oligonucleotides containing 2-aza-2'-deoxyinosine protected with the N,N-diphenylcarbamoyl group are described. An unexpected behaviour of the protected nucleoside can be used for the introduction of dAICA in synthetic oligonucleotides.

Recent advances in nucleic acid chemistry are making possible the preparation of oligonucleotides containing biollogically relevant base analogues. These site-specifically modified oligonucleotides are important tools for the determination of the structural and biological properties of these non-natural bases in DNA. The study of these properties has a strong impact on several fields such as chemical carcinogenesis and DNA repair (1) as well as increases the applications of synthetic oligonucleotides in Molecular Biology (2-4).

We are interested on the preparation of oligonucleotides with purine analogues that contain the configuration of hydrogen bonding groups : acceptor-donor-acceptor because that configuration permits to draw a two hydrogen bond structure for the four natural bases (5). Some time ago, we and others described the preparation of synthetic oligonucleotides containing xanthine, the first base analogue of this type (5, 6). Xanthine containing oligonucleotides were found to have a very low melting temperature at neutral pH probably due to a destabilizing effect of the negative charge present on xanthine at neutral pH (pKa of xanthine 5.5). It was also shown that at pH 5.5 xanthine base pairs were more stable (5).

Substitution of the keto group at position 2 of xanthine by a nitrogen atom gives a base analogue, 2-azahypoxanthine (figure 1), that has the same acceptor-donor-acceptor configuration than xanthine but instead it has a higher pKa (pKa of 2-azainosine 6.8 (7)). Based on these data we have attempted the incorporation of 2-aza-2'-deoxyinosine into synthetic

oligonucleotides to measure the stabilities of duplexes containing this analogue base-paired with the natural bases. In this communication, we describe the preparation of a protected derivative of 2-aza-2'-deoxyinosine that can be incorporated into synthetic oligonucleotides but on the deprotection undergoes to decomposition. We also show that this side reaction can be directed to yield oligonucleotides containing 5-amino-1-(β-D-2'-deoxyribofuranosyl) imidazole-4-carboxamide (dAICA), the 2'-deoxy derivative of the key intermediate in the *de novo* biosynthesis of purine ribonucleotides.

RESULTS AND DISCUSSION

Preparation of the phosphoramidite derivative of 2-aza-2'-deoxyinosine.

The preparation of 2-aza-2'-deoxyinosine was described in 1975 by enzymatic deamination of 2-aza-2'-deoxyadenosine (8). Chemical deamination of 2-aza-2'-deoxyadenosine was not successful due to depurination of 2-aza-2'-deoxyinosine in the acidic media used during the deamination reaction (8). On the other hand, the ribonucleoside 2-azainosine can be easily prepared by direct diazotization of commercially available 5-amino-1-(β-ribofuranosyl) imidazole-4-carboxamide (AICA riboside, compound 1, figure 1) (7, 9).

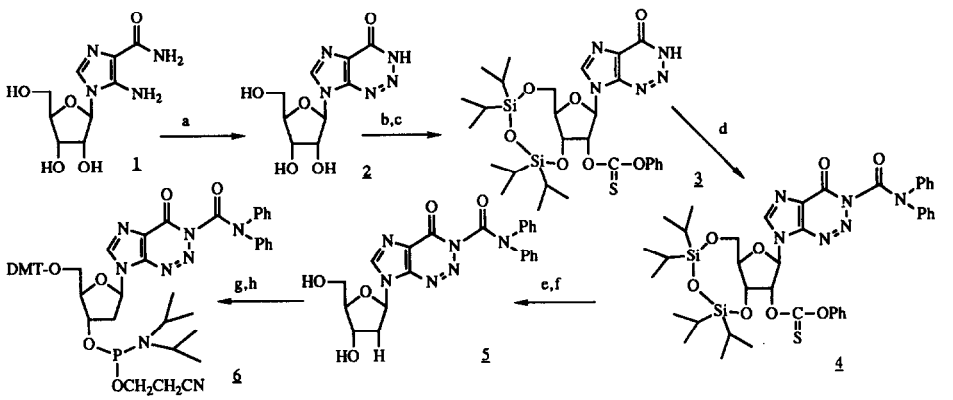

Figure 1 : Synthetic scheme used for the preparation of the phosphoramidite derivative of 2-aza-2'-deoxyinosine.a) $NaNO_2$, HCl; b) $TPDSCl_2$, pyridine; c) PTC-Cl, dimethylaminopyridine; d) diphenylcarbamoyl chloride, diisopropylethylamine; e) Bu_3SnH, AIBN; f) Bu_4NF; g) DMT-Cl, pyridine; h) 2-cyanoethyl-N,N-diisopropylaminochlorophosphine, Et_3N.

Starting from 2-azainosine, prepared from AICA-riboside, we have tried the preparation of 2-aza-2'-deoxyinosine using the method described by Robins et al. for the conversion of ribonucleotides to 2'-deoxyribonucleotides (10). We found that 2-aza-2'-deoxyinosine was obtained but during the work-up this product undergoes spontaneous depurination. A similar behaviour has been shown for 2'-deoxyxanthosine and it was overcome by protection of the lactam

function with the p-nitrophenylethyl group (5, 6). In our case, we selected the N,N-diphenylcarbamoyl protecting group because the nitrophenyl group could be reduced with tin hydride used for the 2'-deoxygenation.

Using AICA-riboside as starting material we developed the synthetic scheme shown in figure 1, obtaining protected 2-aza-2'-deoxyinosine (5) in good yields. It has been described that acylation of guanosine with N,N-diphenylcarbamoyl chloride yields the O-6 guanine derivative (11). In our case we were not able to distinguish from spectroscopic data (IR, ^{1}H and ^{13}C-NMR) whether the acyl group was attached at N-1 or O-6 position. Below we will show that the behaviour of this product in conc. ammonia indicates that the acyl group is at N-1 position.

Protected 2-aza-2'-deoxyinosine (5) was converted to the phosphoramidite derivative (6) needed for oligonucleotide synthesis by treatment with 4,4'-dimethoxytrityl chloride followed by reaction of the resulting 5'-O-DMT derivative with chloro-(N,N-diisopropylamino) 2-cyanoethoxy phosphine.

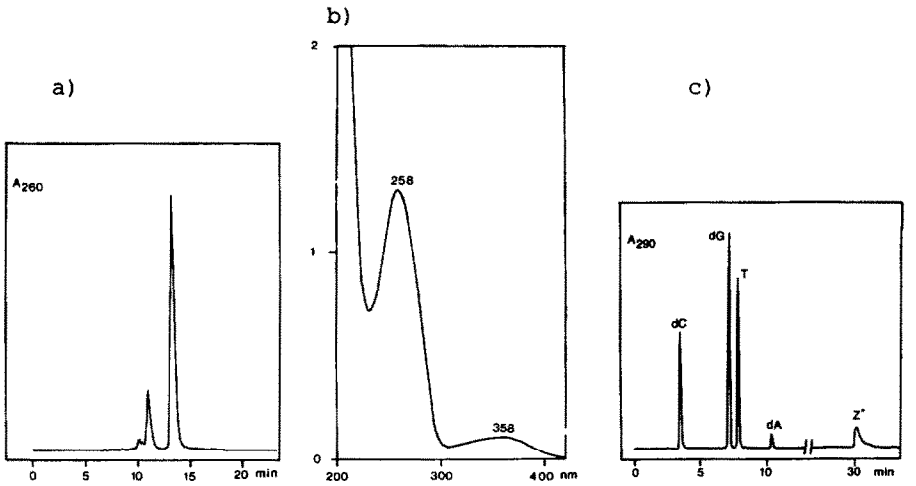

Figure 2. Purification and characterization of 5'CGATGTTAZ*TACATGAGAC 3'. a) Analytical HPLC chromatogram after detritylation. b) UV spectrum of the purified product. c) HPLC chromatogram of the enzymatic digestion monitored at 290 nm.

Following standard phosphite-triester methodology (12) we have prepared the sequence: 5'CGATGTTAZTACATGAGAC 3' where Z stands for 2-aza-2'-deoxyinosine. At the end of the synthesis, the DMT-oligonucleotide-resin was treated with concentrated ammonia at 40°C for 2 days. The resulting DMT-oligonucleotide was purified by reversed-phase HPLC, detritylated and repurified by HPLC. The purified product was homogeneous by analytical HPLC and gel electrophoresis but, unexpectedly, the oligonucleotide had an extra absorption peak at 358 nm (see figure 2). Furthermore, HPLC analysis of the

snake venom phosphodiesterase and alkaline phosphatase digestion of the oligonucleotide showed the absence of 2-aza-2'-deoxyinosine (retention time 6 min) and instead a product with a large retention time (30.1 min) and a strong absorption maxima at 358 nm was observed (figure 2). On the other hand, when protected 1-N-(N',N'-diphenylcarbamoyl)-2-aza-2'-deoxyinosine (5) was treated with concentrated ammonia we observed the formation of two main products : the desired 2-aza-2'-deoxyinosine (10%, HPLC retention time 6 min) and the same product found in the enzymatic digestion (90%, HPLC retention time 30.1 min). This side product was purified by semipreparative HPLC. Proton NMR showed the presence of the 2'-deoxyribose protons, the imidazole proton and the phenyl protons of the N,N-diphenylcarbamoyl group. Fast-atom bombardment mass spectra gave a molecular mass of 466 that corresponds to a triazene imidazole derivative that could be formed by nucleophilic attack of ammonia to the C-6 carbonyl group followed by ring opening of 2-aza-2'-deoxyinosine (figure 3). Fragmentation patterns and UV spectrum confirm the presence of the triazene imidazole system.

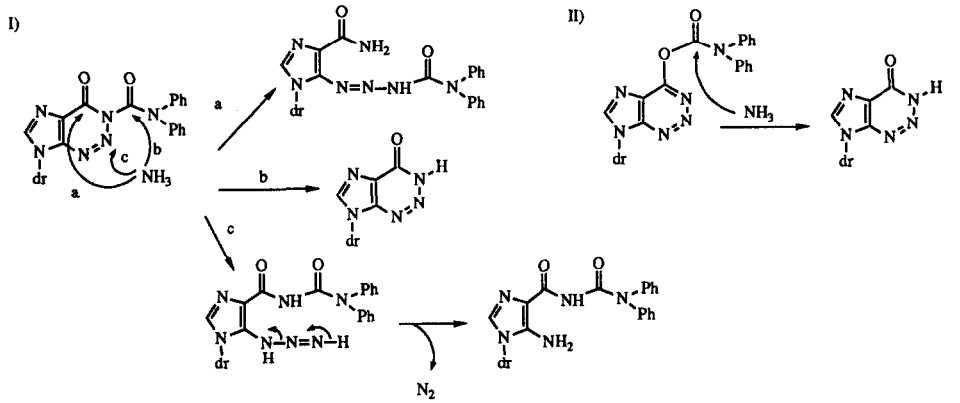

Figure 3. Deprotection products that can be expected from the ammonia treatment of I) 1-N-(N',N'-diphenylcarbamoyl)-2-aza-2'-deoxyinosine and II) 6-O-(N,N-diphenylcarbamoyl)-2-aza-2'-deoxyinosine.

The formation of the triazene imidazole derivative as the major product of the ammonia treatment shows that the N,N-diphenylcarbamoyl group was at N-1 position instead of at O-6 position described for guanosine derivatives (11). In order to confirm the predominance of N-acylation in 1,2,3-triazen-4-one systems we have prepared the model compound 1,2,3-benzotriazen-4(3H)-one (figure 4). That compound was treated with N,N-diphenylcarbamoyl chloride in the same conditions that we used on the acylation of 2-aza-2'-deoxyinosine derivative. X-ray diffraction studies on the substituted benzotriazenone showed the presence of the N,N-diphenylcarbamoyl group at the N-3 position of the benzotriazenone (figure 5) demonstrating the preference for N-

substitution over O-substitution. Finally, we checked that N-3 protected benzotriazenone behaved like the 2-aza-2'-deoxyinosine derivative when it was treated with concentrated ammonia obtaining the corresponding phenyltriazene derivative that was purified and characterized.

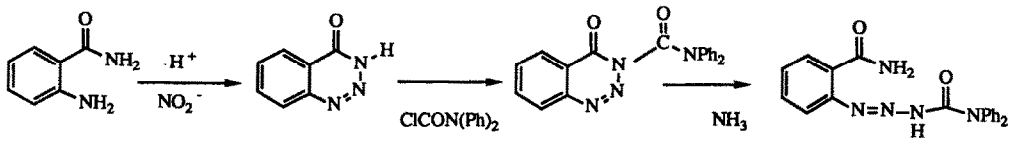

Figure 4. Preparation of N,N-diphenylcarbamoyl derivative of 1,2,3-benzotriazen-4(3H)-one. The ammonia treatment gave the corresponding phenyl triazene derivative.

We determined the melting temperatures (Tm) of the duplex containing the triazene imidazole derivative in front of cytosine (5'-GTCTCATGTA**C**TAACATCG paired with 3'-CAGAGTACAT**Z***ATTGTAGC where Z* stands for 5-N-(N',N'-diphenylcarbamoyl)triazeneimidazole-4-carboxamide) at low salt concentration (0.15 M NaCl, 50 mM sodium phosphate pH 7.5). As it was expected for the bulkiness of the modified nucleoside, the Tm of the duplex was very low (Tm= 48°C) compared with the perfectly matched duplex (Tm= 60°C). After the melting experiment we observed that the absorption maximum at 358 nm of the modified oligonucleotide had disappeared indicating the decomposition of the imidazole triazene derivative during the melting conditions. It has been described that disubstituted 5-triazeneimidazole-4-carboxamide derivatives undergo photochemical decomposition giving diazoimidazole derivatives than can yield 2-azahypoxanthine (13). But, on the other hand, N-substituted imidazole derivatives (like in our case with the 2-deoxyribofuranosyl group) do not react in this way because they can not form the diazo intermediate. We have checked by gel electrophoresis in denaturating conditions (20% acrylamide, 8M urea) that no crosslinking takes place during the melting conditions, suggesting that diazoimidazole derivatives are not formed during the decomposition of the (N,N-diphenylcarbamoyl)triazene imidazole derivative.

For a better understanding of the behaviour of triazene imidazole containing oligonucleotides during the melting conditions, the 2'-deoxyribosyl derivative obtained by ammonia treatment of 1-N-(N',N'-diphenylcarbamoyl)-2-aza-2'-deoxyinosine was heated at 75°C inside the spectrophotometer with the UV lamp on. As shown in figure 6, the UV spectra of the irradiated compound changed until total disappearance of the absorption at 358 nm in 5-7 hrs. A similar behaviour was found when the product was heated at 75°C with the UV lamp off, but it took longer time for

completion (about 12-16 hrs). Analytical HPLC of the irradiated and non-irradiated samples showed the complete conversion of the starting material to a major peak with a much shorter retention time. This new product had the same retention time and coeluted with a known sample of 5-amino-1-(ß-D-deoxyribofuranosyl)imidazole-4-carboxamide (dAICA).

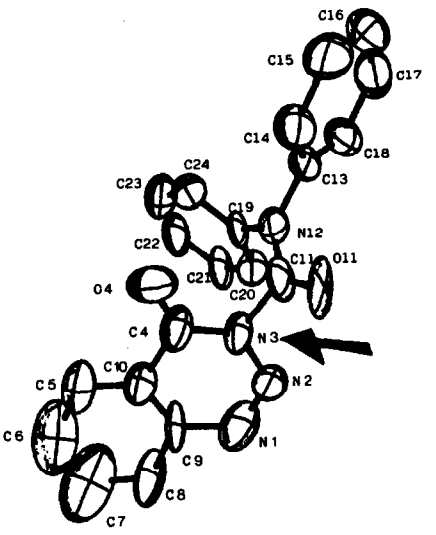

Figure 5. Structure of the N,N-diphenylcarbamoyl derivative of 1,2,3-benzotriazen-4-one as deduced from X-Ray diffraction studies.

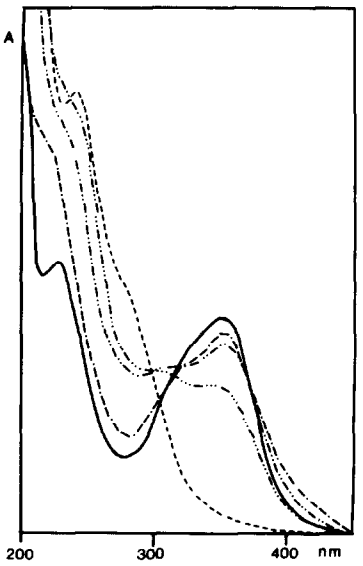

Figure 6. Decomposition of 1-(N',N'-diphenylcarbamoyl)-2-aza-2'-deoxyinosine at 75°C as seen by UV spectrophotometry. — t=0; ——·t=45 min —··t=1,5 hr; —···t=3 hr; ——t=7 hr.

The oligonucleotide containing the triazene imidazole derivative was irradiated at 75°C in a similar way and the progress of the reaction was monitored by HPLC. After 6 hrs of treatment, a major peak of shorter retention time was observed (see figure 7a). The product was purified by HPLC and digested with snake venom phosphodiesterase and alkaline phosphatase. The resulting mixture was analyzed by HPLC (figure 7b) showing that the oligonucleotide contained dAICA.

CONCLUSIONS

Our initial objective of the present work was to prepare oligonucleotides containing 2-aza-2'-deoxyinosine. During the preparation of this analogue we found that the desired 2'-deoxynucleoside was very sensitive to depurination. In this aspect, 2-aza-2'-deoxyinosine resembles 2'-deoxyxanthosine more than to 2'-deoxyinosine. As it was shown for 2'-deoxyxanthosine (5, 6), the protection of the lactam group stabilizes the 2'-deoxynucleoside making

possible its isolation and characterization. We have demonstrated that the N,N-diphenylcarbamoyl protecting group is incorporated into the N-1 position of 2-aza-2'-deoxyinosine. It has been postulated that the N,N-diphenylcarbamoyl group is incorporated in the O-6 position of guanosine (11) due to the bulkyness of the protected amino group in position 2. There is no data in the literature on the use of this protecting group on inosine, but the preference of N-acylation over O-acylation of 2-azainosine is not surprising based on the known reactivity of inosine. We also have demonstrated than , after DNA synthesis, and during the ammonia treatment ammonia nucleophillic attack takes place on position 6 of the N,N-diphenylcarbamoyl derivative of 2-azainosine rather than on the carbamoyl group. This reactivity produces ring-opening and the subsequent formation of a triazene imidazole derivative easily detected by its strong yellow color. This unexpected side-reaction precludes the use of the N,N-diphenylcarbamoyl derivative for the preparation of oligonucleotides containing 2-azainosine, so, in the future, a new way has to be developed for the protection of 2-aza-2'-deoxyinosine.

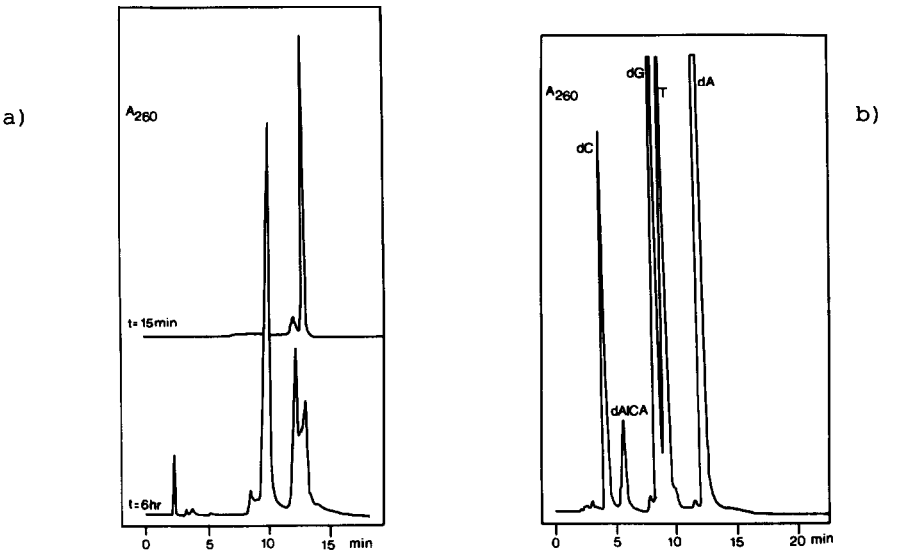

Figure 7. a) Decomposition of the triazene imidazole derivative inside a oligonucleotide (19 mer) followed by HPLC. b) HPLC chromatogram of the enzymatic digestion of the major product obtained after irradiation of the 19 mer containing the triazene derivative.

On the other hand, we have found that the triazene imidazole derivative is transformed to dAICA when this side-product is heated, and the reaction is catalyzed by UV light. To our aknowledgement, this type of reaction has not been previously described in the literature and we have demonstrated than

it can be used for the transformation of the oligonucleotide with the triazene imidazole side product to an oligonucleotide containing dAICA. So, the derivative of 2-aza-2'-deoxyinosine prepared in this work could be used to prepare oligonucleotides containing dAICA. As a mather of fact, the chemical preparation of oligonucleotides containing dAICA has not been yet described due to the difficulty to prepare a suitable protected derivative of dAICA. It has been shown that protection of the amino group in position 5 of dAICA is difficult because of concomitant dehydration of the carboxamido group is observed (14, 15). Only recently, it has been described the preparation of oligonucleotides containing dAICA using dAICA triphosphate and terminal deoxynucleotidyltransferase (14). The synthetic scheme for the preparation of dAICA triphosphate was based on the simultaneous protection of carboxamido and amino groups by using an inosine derivative that was ring-oppened with a strong base treatment that can be used only at the nucleoside level. The synthetic protocol described in this paper relies in similar ideas but the ring opening and deprotection to form dAICA are done in conditions compatible with chemical stability of DNA.

EXPERIMENTAL SECTION

Abbreviations : ACN : acetonitrile, AIBN :α,α'-azoisobutyronitrile, AICA : 5-aminoimidazole-4-carboxamide, DCM : dichloromethane, DMF : N,N-dimethylformamide, DMT : 4,4'-dimethoxitrityl, MeOH : methanol, PTC : phenoxythiocarbonyl, THF : tetrahydrofuran, TPDS : tetraisopropyldisiloxanyl.

Phenoxythiocarbonyl chloride was prepared from thiophosgene and phenol as described in ref. 16. 2-azainosine was prepared according previously described protocols (7, 9). Reagents for oligonucleotide synthesis were from Applied Biosystems, USA. All other solvents and reagents were reagent grade and they were used without further purification. Oligonucleotide synthesis was performed on an Applied Biosystems 380A automatic DNA synthesizer. UV absorption spectra, melting studies and photochemical reactions were done using a Perkin-Elmer Lambda 5 spectrophotometer equipped with a temperature controller. Enzymatic digestions of oligonucleotides were performed as described in reference 5.

Preparation of 5-amino-1-(β-D-2'-deoxyribofuranosyl)imidazole-4-carboxamide (dAICA).

3',5'-O-tetraisopropyldisiloxanyl-5-amino-1-(β-ribofuranosyl)imidazole-4-carboxamide.

To 1 g (4 mmols) of dried AICA riboside suspended in 60 mL of anhydrous DMF, was added 4 mL of dry pyridine and 1.4 mL (4.4 mmols) of tetraisopropyldisiloxane dichloride. The mixture was stirred for 4 hours and then evaporated to dryness *in vacuo*. The oily residue was dissolved in ethyl acetate and washed with cold 0.1 N HCl, saturated $NaHCO_3$ and saturated sodium chloride. The organic layer was dried ($MgSO_4$), filtered and evaporated to dryness. The pink coloured foam was crystallized from ACN and afforded 0.6 g of the desired compound. From the mother liquor, an additional crop (0.3 g) was obtained after silica gel chromatography. The product eluted with 98:2 DCM/MeOH. Total yield 47%. UV (MeOH) max. : 265 nm (9600). ^{1}H-NMR (DMSO-d_6, 200 MHZ) δ (ppm) : 7.20 (1H, s, H-2), 6.75 (2H, m, $CONH_2$), 5.82 (2H, m, NH_2), 5.60 (1H, d, OH-2'), 5.52 (1H, d, H-1'), 4.28 (1H, dd, H-3'), 4.15-3.85 (4H, m, H-2', H-4', H-5',5"), 1.00 (2H, m, iPr). TLC (90:10 DCM/MeOH) Rf : 0.45.

2'-O-Phenoxythiocarbonyl-3',5'-O-tetraisopropyldisiloxanyl-5-amino-1-(β-D-ribofuranosyl) imidazole-4-carboxamide.

To 750 mg (1.5 mmols) of dried 3',5'-O-TPDS-AICA riboside was added 25 mL of anhydrous ACN and 915 mg (7.5 mmols) of N,N-dimethylaminopyridine. To the mixture was added 0.27 mL (1.6 mmols) of phenoxytiocarbonyl chloride and the mixture was stirred at room temperature for 2-3 hrs. Solvent was evaporated and the residue was treated as described above. The resulting product was sufficiently pure to be used directly in the reduction step. UV (MeOH) max : 261 nm. TLC (90:10 DCM/MeOH) Rf : 0.65.

5-amino-1-(β-D-2'-deoxyribofuranosyl)imidazole-4-carboxamide.

The total product obtained above (2'-O-PTC-3',5'-TPDS-AICA riboside) was dissolved in 20 mL of dry toluene and 32 mg (0.2 mmols) of AIBN and 0.65 mL (2.5 mmols) of tributyltin hydride were added. The mixture was degassed with oxygen-free nitrogen for 20 min and then heated at 75°C for 3 hrs. Then the reaction was monitored by TLC and additional 0.26 mL of tributyltin hydride were added. After 5 hrs of magnetic stirring, the mixture was cooled at room temperature, 3 mL (3 mmols) of 1M solution of tetrabutylammonium fluoride in THF were added and the solution stirred for 15 minutes. Solvent was evaporated and the residue was partitioned between ethylic ether and water. The aqueous phase was concentrated and applied to a Dowex 1x2 (OH^-) column. Elution was done with a 0.25 M solution of triethylammonium bicarbonate pH 8.0. After evaporation of the desired fractions, the residue was dissolved in water and lyophilized. This procedure was repeated four times to remove residual triethylammonium bicarbonate. The residue was purified through a reversed phase column (Lobar B310-25, LiChroprep RP-18, 40-63 μm) eluted with 0.01 M triethylammonium bicarbonate. The chromatography was monitored by measuring the UV absorption at 260 nm, and the fractions containing the desired product were combined and evaporated to a foam. Yield 50 mg (15%). UV (MeOH) max : 267 nm. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ (ppm) : 7.32 (1H, s, H-2); 6.68 (m, $CONH_2$); 5.91 (3H, m, NH_2, H-1'); 5.27 (m, OH-3'); 5.12 (m, OH-5'); 4.30 (1H, m, H-3'); 3.75 (1H, m, H-4'); 3.52 (2H, m, H-5',5"); 2.39 (1H, m, H-2'); 2.12 (1H, m, H-2"). EM (FAB+): 242 (M+); 265 (M+Na).

3',5'-O-tetraisopropyldisiloxanyl-2-azainosine.

3.7 g (13.3 mmols) of dried 2-azainosine in 225 mL of anhydrous DMF were reacted with 4.5 mL (14.3 mmols) of tetraisopropyldisiloxane dichloride and 15 mL of dry pyridine. The reaction mixture was treated as described above for the preparation of 3',5'-O-TPDS-AICA riboside. Yield 5.7 g (11.2 mmol, 74%). TLC (10% MeOH/DCM) Rf = 0.55. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ (ppm) : 15.12 (1H, NH); 8.42 (1H, s, H-8); 6.03 (1H, d, J=0.9 Hz, H-1'); 5.70 (1H, d, OH-2'); 4.65 (2H, m, H-3', H-2'), 4.05 (3H, m, H-4', H-5'), 1.0 (24H, m, 4 isopropyl).

2'-O-Phenoxythiocarbonyl-3',5'-O-tetraisopropyldisiloxanyl-2-azainosine.

The product obtained above (11.2 mmols) was dissolved in 200 mL of anhydrous ACN and 6.8 g (56 mmol) of N,N-dimethylaminopyridine were added to the solution and, after, 1.75 mL (12.6 mmols) of phenoxythiocarbonyl chloride. After 3 hrs of magnetic stirring, the mixture was treated as described above in the preparation of 2'-O-TPC-3',5'-TPDS-AICA riboside. The product obtained was purified on a silica gel column eluted with a solution from 0% to 5% of MeOH in DCM. Yield 4.5 g (63%). TLC (5% MeOH/ DCM) Rf = 0.4. U.V. (MeOH) max. 242, 291 nm. I.R. ($CHCl_3$, cm^{-1}) : 2970, 2890, 1725, 1290, 1200, 1050. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ (ppm) : 15.29 (1H, NH); 8.61 (1H, s, H-8); 7.49 (2H, t, Ar.); 7.33 (1H, t, Ar.); 7.15 (2H, d, Ar.); 6.56 (1H, d, H-1'); 6.43 (1H, dd, $J_{1',2'}$= 1Hz, $J_{2',3'}$ = 5.4 Hz, H-2'); 5.29 (1H, dd, $J_{2',3'}$= 5.4Hz, $J_{3',4'}$= 8.6 Hz, H-3'); 4.05 (3H, m, H-4', H-5'); 1.0 (24H, m, 4 isopropyls).

2'-O-Phenoxythiocarbonyl-3',5'-O-tetraisopropyldisiloxanyl-1-N-(N',N'-

diphenylcarbamoyl)-2-azainosine.

3 g (4.6 mmols) of 2'-O-TPS-3',5'-O-TPDS-2-azainosine were dried by evaporation with anhydrous pyridine. The residue was dissolved with 60 mL of anhydrous pyridine and 2.3 g (10 mmols) of diphenylcarbamoyl chloride were added together with 1.2 mL of diisopropylethylamine. After 1 hr of magnetic stirring, 0.25 mL of MeOH was added to the red coloured solution. The solvent was evaporated and the residue was partitioned between ethyl acetate and water. The organic phase was washed with 0.1N HCl, saturated sodium bicarbonate, saturated NaCl and evaporated to dryness. The product obtained was applied to a silica gel column eluted with DCM. The desired product was eluted with a 0.5% solution of MeOH in DCM. Yield 3.1 g (85%). TLC (2% MeOH/ DCM) Rf = 0.60.

1-N-(N',N'-Diphenylcarbamoyl)-2-aza-2'-deoxyinosine.

3 g (3.6 mmols) of the product obtained above were reacted with 2 mL (7.5 mmols) of tributyltin hydride and 50 mg of AIBN as described above for the preparation of 2'-deoxy AICA. The reaction mixture was stirred for 6 hrs at 75°C. After cooling, the solvent was evaporated and the residue was purified on a silica gel column eluted with a 0.5% solution of MeOH in DCM, obtaining 1.5 g (2.2 mmol) of an homogeneous product of lower Rf (Rf = 0.35, 2% MeOH/ DCM).

2The product obtained was dissolved in 20 mL of anhydrous THF and deprotected with 4 mL of a 1M solution of tetrabutylammonium fluoride in THF. After stirring for 15 min, the solvent was evaporated and the oily residue was partitioned between water and ethyl acetate. The organic layer was washed with 0.1 M HCl, saturated sodium bicarbonate and saturated NaCl. The solvent was evaporated and the product was purified on a silica gel column. The desired compound was eluted with a 4% solution of MeOH in DCM. Yield : 0.4 g (0.9 mmol, 25%). TLC (10% MeOH/ DCM) Rf = 0.50. U.V. (MeOH) max. = 295 nm. I.R. (KBr, cm^{-1}) : 3600-3300, 1750, 1600, 1500, 1350. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ (ppm) : 8.70 (1H, s, H-8); 7.18-7.52 (10H, m, Ar.); 6.43 (1H, t, H-1'); 5.35 (1H, d, 3'-OH); 4.90 (1H, t, 5'-OH); 4.36 (1H, m, H-3'); 3.86 (1H, m, H-4'); 3.52 (2H, m, H-5'); 2.62 (1H, m, H-2'); 2.39 (1H, m, H-2"). ^{13}C-NMR (CD_3OD, 50 MHz) δ (ppm) : 154.6; 153.2; 145.9; 145.5; 143.79; 142.73; 131.67; 131.44; 130.68; 129.86; 128.76; 128.06; 90.73; 88.16; 72.90; 63.59; 43.00. E.M. (FAB+) : 471 (M+Na).

5'-O-Dimethoxytrityl-1-N-(N',N'-diphenylcarbamoyl)-2-aza-2'-deoxyinosine.

0.3 g of 1-N-(N',N'-diphenylcarbamoyl)-2-aza-2'-deoxyinosine (0.6 mmol) were dried by coevaporation with anhydrous pyridine (2 x 10 mL). The residue was dissolved with 7 mL of anhydrous pyridine and 0.25 g (0.7 mmol) of 4,4'-dimethoxytrityl chloride were added. After 16 hrs of magnetic stirring, 1 mL of methanol were added and the solvents were evaporated. Toluene was added and evaporated in order to eliminate the residual pyridine. The residue was dissolved in DCM and the solution was washed with saturated sodium bicarbonate and water. The organic layer was concentrated to dryness and the resulting oil was applied to a silica gel column eluted with a solution containing from 0% to 2% of MeOH in DCM. Fractions containing the desired product were pooled and evaporated to dryness. The oily product was dissolved with the minimal amount of DCM and precipitated from a mixture of ethyl ether / hexane (1:1), obtaining 350 mg of a white solid (50% yield). TLC (10% MeOH/ DCM) Rf = 0.60.

5'-O-Dimethoxytrityl-1-N-(N',N'-diphenylcarbamoyl)-2-aza-2'-deoxyinosine, 3'-O-2-cyanoethyl-N,N-diisopropylaminophosphoramidite.

300 mg (0.4 mmol) of the product obtained above were dried by coevaporation with anhydrous acetonitrile. The residue was dissolved in 2 mL of anhydrous DCM under a dry argon atmosphere and 0.225 mL of triethylamine were added. After complete dissolution, 0.142 mL (1.5 mmol) of 2-cyanoethyl-N,N-diisopropylaminochlorophosphine were added. The solution was stirred for 30 minutes and then 0.2 mL of methanol were added. The resulting mixture as

diluted with a 5% solution of triethylamine in ethyl acetate and, then, washed with a saturated solution of sodium bicarbonate and a saturated solution of NaCl. The solvent was evaporated and the residue was purified on a silica gel column eluted with a 5% solution of triethylamine in a mixture DCM : ethyl acetate (1:1). The oily product was dissolved in the minimal amount of dichloromethane and the solution was added dropwise to a cold solution of hexane with stirring. The white precipitate formed was separated by centrifugation and dried *in vacuo*. Yield 250 mg (70%) of the title compound. TLC (ethyl acetate/ DCM/ triethylamine 45:45:10) Rf = 0.4. ^{31}P-NMR ($CDCl_3$, 81 MHz) δ (ppm) : 149.3; 149.1.

Isolation of the imidazole-triazene derivative produced during the ammonia treatment of 1-N-(N',N'-diphenylcarbamoyl)-2'deoxy-2-azainosine.

20 mg (44.6 µmol) of 1-N-(N',N'-diphenylcarbamoyl-2'-deoxy-2-azainosine were treated with conc. ammonia at room temperature for 1 hour. 5 mL of water were added and the solution was concentrated to dryness. The residue was dissolved in 1 mL MeOH/ water and was purified by semi-preparative HPLC. Solvent A: water; solvent B: ACN/ water (1:1). A 20 min linear gradient from 10% to 70%B was used. Flow rate : 3 mL/ min. Column : Hypersyl ODS C-18, 10 µm, 250x 7 mm. Yield : 2.3 mg (4.9 µmol, 11%) of a yellow product characterized as 5-(N',N'-diphenylcarbamoyl)triazene-2'-deoxyribofuranosyl imidazole-4-carboxamide. U.V. (MeOH) max. : 228, 350 nm. ^{1}H-NMR (CD_3OD, 200 MHz) δ (ppm) : 8.38 (1H, s, H-8); 7.65-7.35 (10H, m, Ar.); 6.50 (1H, t, H-1'); 4.52 (1H, dd, H-3'); 4.05 (1H, m, H-4'); 3.85 (2H, m, H-5'); 2.75 (1H, m, H-2'); 2.4 (1H, m, H-2"). E.M. (FAB+) : 466 (M+ 1); 488 (M+ Na).

Oligonucleotide synthesis.

Oligonucleotides were synthesized on an Applied Biosystems automatic DNA synthesizer on a 1 µmol scale using the protocols recommended by the supplier. Standard 2-cyanoethyl phosphoramidite and controlled-pore glass supports were used (12). The efficiency of the coupling reactions were 98% by measuring DMT optical density at 500 nm. Cleavage from the resin together with deblocking of the base and phosphate protecting groups was achieved with a conc. ammonia treatment at 40 °C for 3 days. DMT-oligonucleotides were purified by HPLC (5) and using OPC^R cartridges (Applied Biosystems).

1,2,3-Benzotriazen-4(3H)-one.

A solution of anthranylamine (13.6 gr, 0.1 mols) in 8N HCl (50 mL) was cooled down to ice-bath temperature and 66 mL of a 3M solution of sodium nitrite were added slowly. After 1 hour of magnetic stirring, the solution was warmed to room temperature and neutralized with a saturated NaOH solution. The product precipitated was collected and recrystallized from methanol. Yield : 11.35 g (77%). m.p. 215-217 °C. TLC (10% MeOH/ DCM) Rf = 0.68. ^{1}H-NMR (DMSO-d_6, 60 MHz) δ (ppm) : 14.7 (s, 1H, NH); 7.6-8.1 (m, 4H, Ar). ^{13}C-NMR (DMSO-d_6, 50 MHz) δ (ppm) : 155.6; 144.1; 135.5; 132.5; 127.8; 124.2; 120.2.

3-N-(N',N'-Diphenylcarbamoyl)-1,2,3-benzotriazen-4-one.

5 gr of 1,2,3-benzotriazen-4(3H)-one (34 mmols) were dried by coevaporation with anhydrous pyridine. The residue was dissolved in 250 mL of pyridine and 15 gr (68 mmols) of N,N-diphenylcarbamoyl chloride and 8.8 mL of N-ethyldiisopropylamine were added. After 1 hr of magnetic stirring, 1.5 mL of methanol were added and the reaction mixture was concentrated to dryness. The residue was dissolved in DCM and the solution was washed with 0.1N HCl, a saturated solution of sodium bicarbonate and, finally, with a saturated solution of NaCl. The organic phase was dried with anhydrous $MgSO_4$ and concentrated to dryness. The residue was washed with hexane and after with methanol obtaining a white solid that was crystallized from ethanol. Yield : 8.8 gr (76%). m.p. 194-196 °C. TLC (2% MeOH/ DCM) Rf = 0.76. IR (KBr, cm^{-1}) : 1710, 1750. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ (ppm) : 8.22 (1H, dd, H-7); 8.07 (1H, dd, H-10); 7.90 (1H, td, H-9), 7.74 (1H, td, H-8); 7.0-7.5 (10H, m, Ar).

^{13}C-NMR ($CDCl_3$, 50 MHz) δ (ppm) : 153.7; 151.3; 142.9; 136.8; 134.4; 130.0; 125.0; 129.8-126.4; 119.2.

Isolation of the triazene benzamide derivative produced during the ammonia treatment of 3-N-(N',N'-diphenylcarbamoyl)-1,2,3-benzotriazen-4-one.

4 g (12 mmol) of 3-N-(N',N'-diphenylcarbamoyl)-1,2,3-benzotriazen-4-one were treated with 50 mL of conc. ammonia and 50 mL of methanol at 50-60°C for 24 hours. The resulting solution was concentrated to dryness and the residue was dissolved with the minimal amount of DCM and precipitated by adding hexane. Yield : 2.4 g of a yellow solid (6.6 mmol, 57%) characterized as 2-N-(N',N'-diphenylcarbamoyl)triazene benzamide. m.p. 163-167°C. TLC (5% MeOH/DCM) Rf= 0.47). U.V. (EtOH) max. : 355 nm. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ (ppm) : 12.50 (NH); 7.1-8.1 (m, Ar.). E.M. (FAB+) : 360 (M+1); 382 (M+ Na).

Crystal structure of 3-N-(N',N'-diphenylcarbamoyl)-1,2,3-benzotriazen-4-one.

Crystals for X-Ray diffraction were obtained by slow evaporation from a ethanol solution.

Crystal data. $C_{20}H_{14}N_4O_2$. Fw = 342.36, triclinic, a = 12.320(3), b = 9.812(2), c = 8.776(2) Å, α = 104.14(3), β = 99.31(2), γ = 118.40(2)° , V = 855.5(6)Å^3, space group $P1$, Dx = 1.329 g cm^{-3}, Z = 2, $F(000)$ = 365.0, λ (Mo Kα) = 0.71069 Å, μ(Mo Kα) = 0.97 cm^{-1}. 298 °K.

A tabular crystal (0.2 x 0.2 x 0.1 mm) was selected and mounted on a Phillips PW-1100 four-circle diffractometer. Unit-cell parameters were determined from automatic centring of 25 reflections ($4 \leq \theta \leq 12°$) and refined by least-squares method. Intensities were collected with graphite monochromatized Mo Kα radiation, using the ω-scan technique, scan width 0.8°, scan speed 0.03 ° s^{-1}. 1492 reflections were measured in the range $2 \leq \theta \leq 25°$; 889 of which were assumed as observed applying the condition $I \geq 2.5\ \sigma(I)$. Three reflections were measured every two hours as orientation and intensity control, significant intensity decay was not observed. Lorentz-polarization but no absorption corrections were made.

The structure was solved by direct methods, using the SHELXS computer program (17) and refined by full-matrix least-squares method, using SHELX76 computer program (18). The function minimized was $\Sigma\ w\ |\ |Fo| - |Fc|\ |^2$, where $w = (\sigma^2(Fo) + 0.0046\ |Fo|^2)^{-1}$, f, f' and f" were taken from International Tables of X-Ray Crystallography (19). The position of H atoms was computed and refined with an overall isotropic temperature factor, using a riding model, while were anisotropically refined the remaining atoms. The final R factor was 0.068 (wR = 0.074) for all observed reflections. Number of refined parameters = 236. Max. shift/ e.s.d. = 0.4 in U_{11} of C(21); Max. and min. peaks in final difference synthesis were 0.1 and -0.2 eÅ^{-3}, respectively.

Table 1 : Final atomic coordinates (x10000) OF $C_{20}H_{14}N_4O_2$
($B_{eq} = 8\ \pi^2/3\ \Sigma\ U_{ij}\ a_i^*\ a_j^*\ a_i.a_j$)

	X/A	Y/B	Z/C	B_{eq}
N(1)	4365(9)	2079(12)	3931(11)	7.22(56)
N(2)	5061(8)	1788(10)	4793(9)	4.77(44)
N(3)	6422(8)	2903(10)	5243(8)	4.38(43)
C(4)	7069(12)	4281(15)	4931(12)	5.06(59)
O(4)	8193(7)	5211(9)	5519(10)	7.10(44)
C(5)	6639(13)	5791(14)	3231(15)	7.11(73)
C(6)	5751(20)	5965(17)	2282(15)	8.26(98)
C(7)	4428(18)	4948(22)	1861(14)	8.75(101)
C(8)	3973(12)	3603(17)	2424(14)	7.01(71)
C(9)	4918(13)	3453(13)	3393(12)	5.05(60)
C(10)	6188(11)	4538(14)	3777(12)	5.21(61)
C(11)	7166(10)	2505(12)	6316(14)	4.95(59)
O(11)	7129(7)	2756(9)	7751(9)	7.02(46)
N(12)	7854(7)	1954(9)	5627(9)	4.35(43)
C(13)	7653(9)	1462(11)	3817(11)	3.77(49)

C(14)	8716(10)	2381(11)	3348(13)	5.01(59)
C(15)	8557(12)	1979(14)	1712(17)	6.54(73)
C(16)	7370(14)	735(15)	500(14)	6.55(73)
C(17)	6345(11)	-140(13)	1041(12)	5.29(56)
C(18)	6466(10)	230(11)	2701(11)	4.42(48)
C(19)	8808(9)	1820(12)	6630(11)	4.11(48)
C(20)	9797(10)	3189(13)	7960(12)	5.71(57)
C(21)	10730(10)	3056(16)	8931(14)	6.84(64)
C(22)	10649(12)	1559(18)	8507(16)	6.98(78)
C(23)	9648(12)	176(15)	7147(15)	6.72(70)
C(24)	8708(9)	297(13)	6212(13)	5.57(58)

Table 2 : Bond lenghts and angles for $C_{20}H_{14}N_4O_2$

N(2) ---N(3)	1.41 (1)	N(12)---C(11)	1.35 (1)
C(4) ---N(3)	1.33 (1)	C(13)---N(12)	1.48 (1)
C(11)---N(3)	1.46 (1)	C(19)---N(12)	1.43 (1)
N(1) ---N(2)	1.23 (1)	C(14)---C(13)	1.39 (1)
C(9) ---N(1)	1.43 (1)	C(18)---C(13)	1.35 (1)
C(8) ---C(9)	1.42 (1)	C(15)---C(14)	1.35 (1)
C(10)---C(9)	1.33 (1)	C(16)---C(15)	1.39 (1)
C(7) ---C(8)	1.42 (2)	C(17)---C(16)	1.39 (1)
C(6) ---C(7)	1.37 (2)	C(18)---C(17)	1.38 (1)
C(5) ---C(6)	1.37 (2)	C(20)---C(19)	1.37 (1)
C(10)---C(5)	1.33 (1)	C(24)---C(19)	1.39 (1)
C(4) ---C(10)	1.52 (1)	C(21)---C(20)	1.39 (1)
O(4) ---C(4)	1.16 (1)	C(22)---C(21)	1.37 (1)
O(11)---C(11)	1.24 (1)	C(23)---C(22)	1.39 (1)
		C(24)---C(23)	1.38 (1)

C(4) -N(3) -N(2)	129.0 (8)	N(12)-C(11)-O(11)	127.7 (9)
C(11)-N(3) -N(2)	112.6 (8)	C(13)-N(12)-C(11)	121.8 (8)
C(11)-N(3) -C(4)	118.2 (8)	C(19)-N(12)-C(11)	120.6 (8)
N(1) -N(2) -N(3)	116.3 (8)	C(19)-N(12)-C(13)	117.5 (8)
C(9) -N(1) -N(2)	121.3 (9)	C(14)-C(13)-N(12)	116.6 (9)
C(8) -C(9) -N(1)	113.5 (12)	C(18)-C(13)-N(12)	120.6 (9)
C(10)-C(9) -N(1)	124.9 (11)	C(18)-C(13)-C(14)	122.7 (9)
C(10)-C(9) -C(8)	121.5 (10)	C(15)-C(14)-C(13)	117.9 (10)
C(7) -C(8) -C(9)	117.6 (12)	C(16)-C(15)-C(14)	122.5 (11)
C(6) -C(7) -C(8)	116.3 (13)	C(17)-C(16)-C(15)	117.0 (10)
C(5) -C(6) -C(7)	124.6 (13)	C(18)-C(17)-C(16)	122.4 (10)
C(10)-C(5) -C(6)	118.0 (12)	C(17)-C(18)-C(13)	117.4 (9)
C(5) -C(10)-C(9)	121.9 (11)	C(20)-C(19)-N(12)	119.5 (9)
C(4) -C(10)-C(9)	115.0 (11)	C(24)-C(19)-N(12)	118.8 (8)
C(4) -C(10)-C(5)	123.0 (12)	C(24)-C(19)-C(20)	121.8 (9)
C(10)-C(4) -N(3)	113.1 (10)	C(21)-C(20)-C(19)	119.4 (10)
O(4) -C(4) -N(3)	123.2 (10)	C(22)-C(21)-C(20)	119.2 (11)
O(4) -C(4) -C(10)	123.7 (12)	C(23)-C(22)-C(21)	121.2 (11)
O(11)-C(11)-N(3)	117.0 (10)	C(24)-C(23)-C(22)	119.6 (11)

Decomposition studies on N,N-diphenylcarbamoyl imidazole-triazene derivatives.

a) UV experiments with the triazene imidazole nucleoside derivative.

Approx. 0.1 mg of 5-(N',N'-diphenylcarbamoyl)triazene-2'-deoxyribofuranosyl imidazole-4-carboxamide were dissolved in 2.5 mL of methanol. The solution located in a UV spectrophotometer and heated at 75°C with and without the UV lamp on. At different times, the UV spectrum was recorded (see figure 6). The reaction was stopped when the UV spectrum show no variation (7 hrs with UV irradiation, 16 hrs with no UV irradiation). At this time, the solution was cooled to room temperature and the products were analyzed by analytical HPLC. Column Nucleosil-120, C-18, 5µm, 250 x 4 mm.

Flow rate : 1 mL/ min. Solvent A : 10 mM triethylammonium acetate. Solvent B : ACN/ water (1:1). A 5 min linear gradient from 5% to 15% B followed by a 10 min linear gradient from 15% to 80% B. Retention times : dAICA 5.2 min; starting material (triazene imidazole nucleoside) 17.5 min.

A similar experiment with UV irradiation but without heat was also performed and very little changes on the UV spectrum of the triazene derivative were observed even after 24 hours.

b) UV experiments with the oligonucleotide containing the triazene derivative. Isolation of the oligonucleotide containing dAICA.

Approx. 3 absorvance units at 260 nm of the oligonucleotide containing the triazene derivative were dissolved with 2 mL of water and 0.5 mL of methanol. The solution was located in the UV spectrophotometer and heated at 75°C with the UV lamp on. The reaction was monitored by analytical HPLC. Same conditions as above except that a 20 min linear gradient from 5% to 50% B was used. After 6 hrs of treatment, the solution was cooled to room temperature and the new product formed was purified by semi-preparative HPLC. Column Hypersyl ODS, C-18, 10µm, 250 x 7 mm. Flow rate : 3 mL/ min. A 20 min linear gradient from 5% to 35% B. The presence of dAICA in the oligonucleotide was confirmed by HPLC analysis of the enzymatic digestion of the oligonucleotide.

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