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Synthesis and Properties of Oligodeoxynucleotides Carrying 2-Aminopurine

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Abstract: The use of benzoyl, isobutyryl and dimethylaminomethylidene groups for the protection of the exocyclic amino function of 2-aminopurine during oligonucleotide synthesis has been investigated. Best results in the synthesis were obtained with the monomers of 2-aminopurine protected with the isobutyryl group.

Keywords: Amino protection, 2-aminopurine, DNA, mutagenesis, oligonucleotide, solid-phase synthesis.

INTRODUCTION

Oligonucleotides carrying non-natural bases are important tools for the determination of the molecular basis of mutagenesis. Furthermore, their special properties are used for structural determination of nucleic acids and proteins that bind nucleic acids [1].

2-Aminopurine (P, Fig. **1**), an isomer of adenine in which the exocyclic amino group is located at position 2 instead of position 6, can form two hydrogen-bonded base pairs with thymine which are similar to the Watson-Crick A: T base pair [2, 3]. In the 2-aminopurine: thymine base pair, one of the hydrogen bonds occurs in the minor groove instead of in the major groove. Several groups have taken advantage of this difference to characterize DNA-protein interactions [4, 5] and DNA bending [6, 7]. Due to the fluorescent properties of 2-aminopurine, this nucleobase is a useful local probe to study enzymes that interact with DNA, such as DNA polymerases [8-11], helicases [12] and DNA methylases [13, 14] as well as in DNA structural studies [15, 16]. 2-Aminopurine can also form base pairs with adenine [17] and cytosine [2, 15, 18, 19] but not with guanine [20]. The formation of 2-aminopurine: cytosine base pair results in transition mutations during DNA replication [21, 22]. NMR studies showed that 2-aminopurine: cytosine base pair adopts a wobble geometry at neutral and high pH [18, 19].

The preparation of oligonucleotides carrying 2-aminopurine was first described by Eritja *et al.* [20]. In this method, 2-aminopurine-2'-deoxyribonucleotide was prepared enzymatically from the free base [23], and the amino function was protected with the isobutyryl (ibu) group by analogy with 2'-deoxyguanosine (dG) [20]. Afterwards, the synthesis of 2-aminopurine phosphoramidite protected with the benzoyl (Bz) group was described starting from dG [24]. In this method, dG was protected with the Bz group, converted to the 6-hydrazino derivative which was reduced with silver (I) oxide [24]. This method is easily scaled-up and detailed protocols have been published [25, 26]. The Bz group was also selected for the protection of the 2-amino function during the synthesis of oligoribonucleotides containing 2-aminopurine [27]. The synthesis of the ibu derivative of 2-aminopurine-2'-deoxyriboside has been described by reduction of 6-chloroguanosine [28] and thioguanosine [29]. In addition, oligodeoxynucleoside methylphosphonates containing 2-aminopurine have been prepared using the base-labile phenoxyacetyl (PAC) group for the protection of the amino function of 2-aminopurine [30].

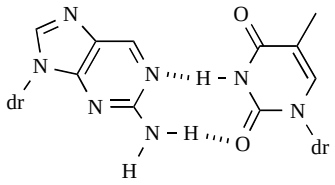

Fig. (1). Scheme of the hydrogen-bonding between 2-aminopurine and thymine.

A major concern for these authors was the high stability to ammonia of the Bz-protected derivative of 2-aminopurine, produced following the McLaughlin's protocol [28, 30]. In this sense, the preparation of the PAC-protected derivative of 2-aminopurine solved this problem, however implied a long synthetic route in which the Bz group is first introduced and then removed to allow the introduction of the PAC protecting group [30]. Finally, the preparation of oligonucleotides containing ^{15}N-2-aminopurine has been described using 2-fluoropurine-2'-deoxynucleoside as convertible nucleoside [31]. However, this method is difficult to scale-up due to the enzymatic preparation of the 2-fluoropurine derivative.

In this paper, we describe the preparation of the isobutyryl derivative of 2-aminopurine-2'-deoxyriboside using McLaughlin's synthetic route [24] and we compare the use of the Bz, ibu and dimethylaminomethylidene (dmf) protecting groups for the amino function of 2-aminopurine.

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1874-0952/11

RESULTS AND DISCUSSION

Synthesis of N^2-Protected Derivatives of 2-Aminopurine-2'-Deoxyriboside

Starting from dG, N^2-Bz-2-aminopurine-2'-deoxyriboside was prepared as previously described [24-26]. The key step in this method was the treatment of a guanine derivative which has the hydrazino group at position 6 with silver oxide. The preparation of the 6-hydrazino derivative was performed by the activation of position 6 with the 2,4,6-triisopropylbenzenesulfonyl (TPS) group, followed by nucleophilic displacement with hydrazine. During these reactions, the 3'- and 5'-hydroxyl groups and the 2-amino group were protected with the Bz group. After the silver oxide reaction 3'-, 5'-benzoate esters were selectively hydrolyzed giving the *N*-2-benzoylated derivative.

The N^2-Bz derivative was reported to be too stable for oligonucleotide synthesis by several authors [28, 30]. For this reason, and using a similar reaction scheme, N^2-ibu-2-aminopurine-2'-deoxyriboside was prepared (Fig. **2**). All reactions went to completion as described for the Bz derivative, giving a major product that was isolated in good yields. NMR data of intermediates was in agreement with the proposed structures. The isobutyryl-protected intermediates were stable under the reaction conditions.

A similar scheme was applied to commercially available 5'-O-dimethoxytrityl (DMT)-N^2-ibu-dG. In this case, the starting material was carrying two of the groups needed for oligonucleotide synthesis: the DMT and the ibu groups. During the activation of position 6 with TPS-Cl, the free 3'-hydroxyl group was protected with the trimethylsilyl group. Unfortunately, the presence of the bulky and labile DMT group at the 5' position slowed down the reaction, and the products were recovered in low yields. Moreover, the ibu group was lost during the treatment with silver oxide, due to the long time needed for this reaction and, 5'-O-DMT-2-aminopurine deoxyriboside (5, Fig. **3**) was isolated instead of the desired 2-aminopurine derivative carrying the DMT and the ibu groups.

Compound 5 was the starting material for the preparation of the 2-aminopurine derivative carrying the dmf group. This group has been reported as a protecting group of 2-amino function of dG in oligonucleotide synthesis, because it is

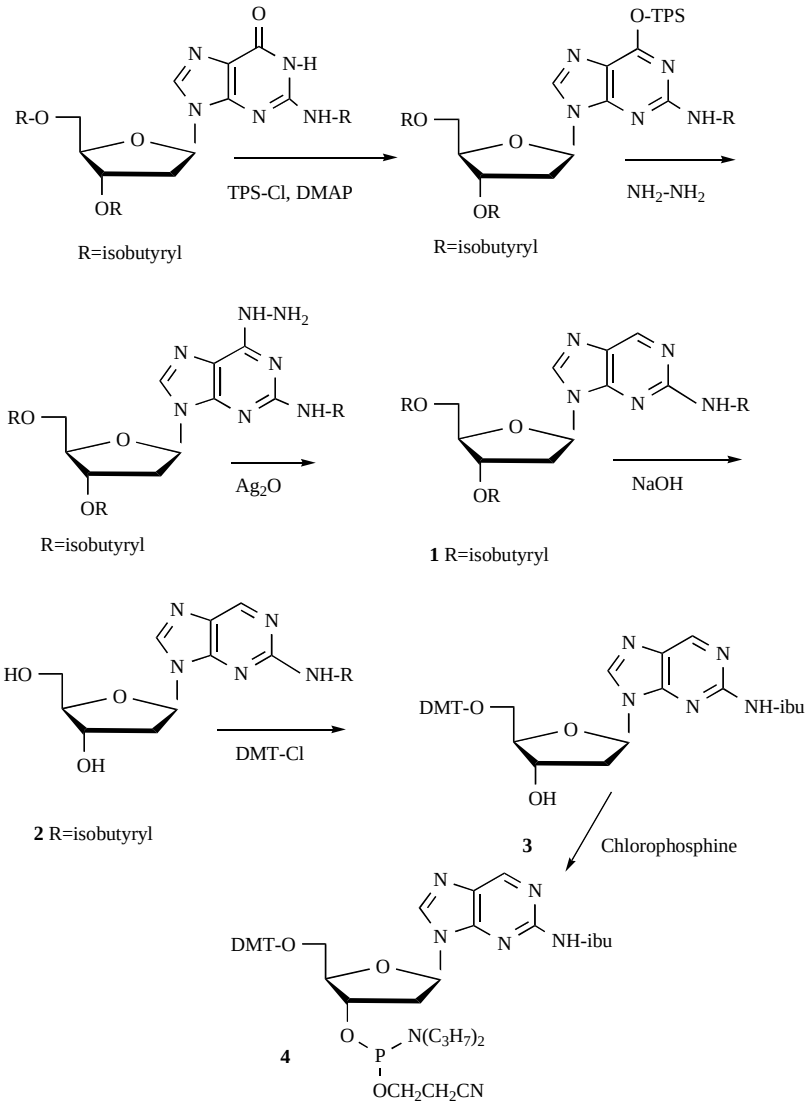

Fig. (2). Synthetic scheme used for the preparation of isobutyryl protected 2-aminopurine phosphoramidite.

more labile than the ibu group [32, 33]. Recently, the phosphoramidite derivative of 2-aminopurine carrying the dmf group has become commercially available. Treatment of compound 5 with dimethylformamide dimethyl acetal yielded compound 6, that was treated with succinic anhydride. The resulting hemisuccinate was coupled to controlled pore glass functionalized with amino groups.

Depurination and Stability Studies

Protected (Bz, ibu) 2-aminopurine 2'-deoxyriboside derivatives carrying the DMT group at 5' position were succinylated and attached to controlled pore glass supports following standard protocols [34]. Stability of the different protecting groups to ammonia was studied by treatment of the different nucleoside supports with concentrated ammonia in order to simulate oligonucleotide synthesis conditions. Prior to ammonia treatment the supports were treated with a 3% trichloroacetic acid (TCA) solution to eliminate the 5'-DMT group. Thereafter, the supports were treated with concentrated ammonia solution at room temperature for one hour to hydrolyze the succinyl linkage and the supports were filtered out. The solutions were left at room temperature and 55 ºC for one day. The study of the efficiency of the removal of protecting groups was followed by analytical HPLC. The dmf group was found to be the most labile protecting group, followed by the ibu group. Both groups can be removed at room temperature although the dmf group was totally removed in 2 hours, while the ibu group needed 8 hours. At the standard oligonucleotide deprotection conditions (55 ºC, minimum 6 hours) both ibu and dmf groups are completely removed. Finally, the Bz group was the most stable group, although it may be removed at 55 ºC if the deprotection time is extended to 1 or 2 days. However, at room temperature the Bz group was removed very slowly (20% in 24 hr).

Another important parameter in the evaluation of protected nucleosides for oligonucleotide synthesis is the stability to depurination during the removal of the dimethoxytrityl group. Previous studies have indicated that this side reaction may be important with 2-aminopurine derivatives [20]. In order to analyze the extent of the depurination reaction, the dinucleotide containing protected 2-aminopurine 5'-TP-3' was prepared using the supports carrying compounds 3 (ibu) and 5 (dmf). Dinucleotide supports were treated with a 3% TCA solution in dichloromethane during different times (up to 7 days) and then the resulting supports were treated with concentrated ammonia. Reverse-phase HPLC showed the appearance of a side product that was characterized by UV spectrometry, enzyme digestion and mass spectrometry as the depurination product. This product was quantified in relation with the dinucleotide containing 2-aminopurine by reading the UV absorption at 260 nm. The dinucleotide carrying dmf-protected 2-aminopurine was found to be more

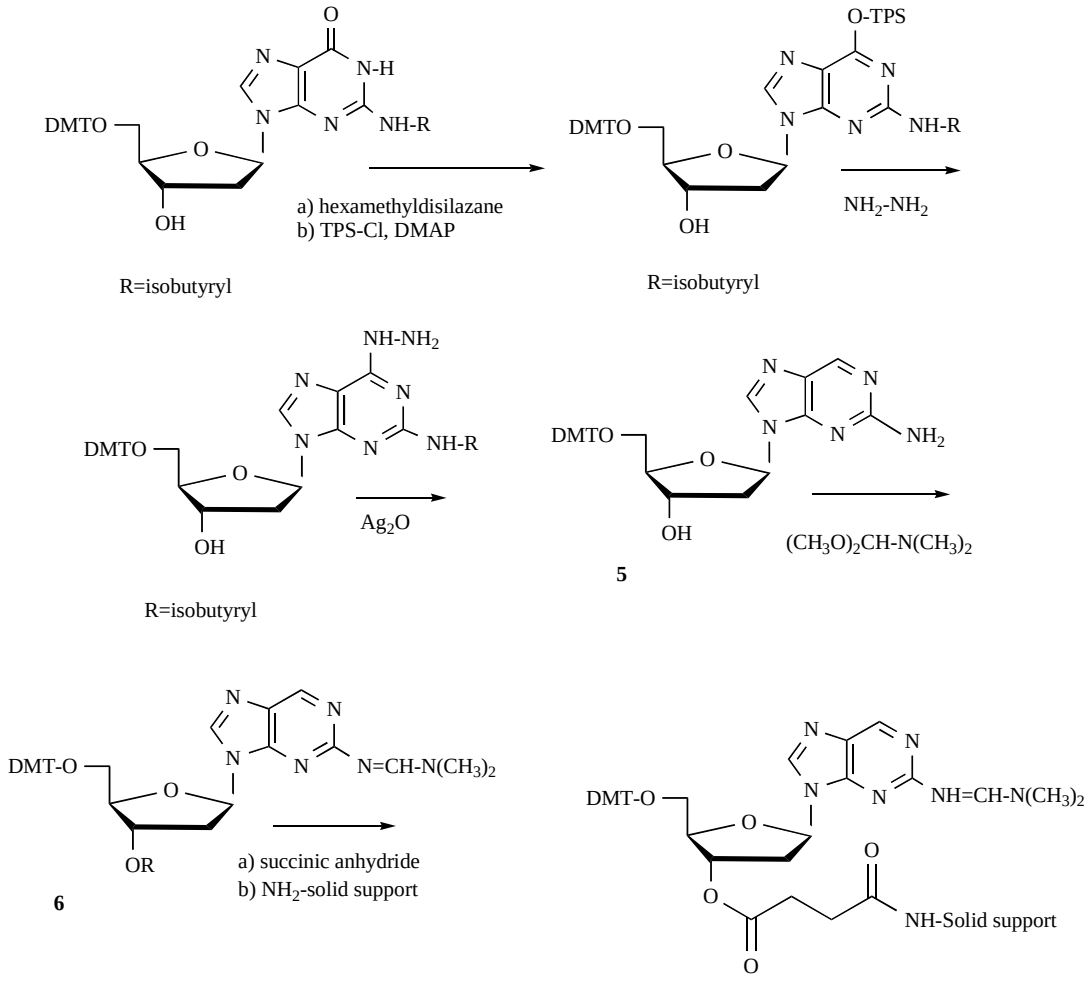

Fig. (3). Synthetic scheme used for the preparation of the solid support carrying dimethylformamidine protected 2-aminopurine.

sensitive to depurination (half-life ≈2 days) than the dinucleotide containing ibu-protected 2-aminopurine (38% depurination after 7 days). These results allowed us to conclude that the dmf group is easier to remove, but, oligonucleotides containing 2-aminopurine with the ibu group are at least 4 times more stable to depurination than oligonucleotides carrying 2-aminopurine with the dmf group.

Oligonucleotide Synthesis

A series of self-complementary dodecamers carrying 2-aminopurine (P) residues P/C: (5'-CGC PAA TTC GCC-3'); P/A: (5'-CGC PAA TTA GCC-3'); P/T: (5'-CGC PAA TTT GCC-3'); and C/P: (5'-CGC CAA TTP GCC-3') were prepared using the ibu-protected 2-aminopurine phosphoramidite derivatives, and the appropriate supports. In all cases, oligonucleotides were obtained in good yields. In addition, the performance of dmf and ibu-protected 2-aminopurine phosphoramidites during oligonucleotide synthesis conditions was analyzed for large-scale syntheses of a short sequence containing 2-aminopurine. Sequence A (5'-CGT AGP GAT GC-3') was prepared twice on 10 μmol-scale. In the first synthesis, ibu-protected 2-aminopurine phosphoramidite (4) was used and in the second synthesis, dmf-protected 2-aminopurine phosphoramidite from commercial sources was used. Coupling efficiencies were similar for both syntheses (99% per step). The last DMT group was left during ammonia deprotection in order to use the hydrophobicity of this group to isolate the full-length sequence from truncated sequences by reversed phase HPLC. After the first HPLC purification, a difference in the amount of full-length oligonucleotide was obtained. Using ibu-protected phosphoramidite, 363 optical density (OD) units at 260 nm (approx. 3.6 μmol) of DMT-oligonucleotide were obtained; while using dmf-protected phosphoramidite, 156 OD units at 260 nm (approx. 1.6 μmol) of the desired sequence were isolated. Moreover, an extra peak after the one of the desired DMT-oligonucleotide was observed only in the HPLC purification coming from the synthesis where dmf-protected 2-aminopurine phosphoramidite was used. These differences could be assigned to 2-aminopurine depurination taking into account the higher sensitivity of the dmf-protected 2-aminopurine derivative to acids described above. Nevertheless, some other side reactions such as premature cleavage of the dmf group or modification of protected 2-aminopurine during capping conditions or iodine oxidation may occur. Overall, the use of the ibu group for the protection of the 2-aminopurine seems advantageous for large scale synthesis.

Thermal denaturation of duplexes, containing self-complementary sequences, were studied in 0.1 M phosphate buffer at different pH. In all cases a clear cooperative curve was observed. Melting temperatures are shown in Table **1**. The most stable duplex contained 2-aminopurine: T base pairs. At acidic pH, duplexes carrying 2-aminopurine: C base pairs are more stable than duplexes carrying 2-aminopurine: A base pairs. At neutral pH, duplexes carrying 2-aminopurine: C and 2-aminopurine: A mismatches have similar stability in agreement with published data [20, 35].

CONCLUSIONS

In summary, we report the preparation of the phosphoramidite derivative of 2-aminopurine 2'-deoxyriboside protected with the ibu group, applying a route previously described for the Bz derivative [24]. This derivative was conveniently deprotected during standard deprotection conditions and is stable to depurination under oligonucleotide synthesis conditions. Alternatively, the dmf group was found to be more labile to ammonia but also more susceptible to depurination. Nevertheless, the extent of depurination of the dmf derivative during normal oligonucleotide synthesis conditions is small and this protecting group is a good alternative for the protection of 2-aminopurine. As described earlier [28, 30], the Bz derivative was found to be very stable to ammonia and for this reason, it is not recommended for normal use, although it could be used if the deprotection is performed during an extended period of time.

Table 1. Melting Temperatures (Tm, ºC) of Duplexes Containing 2-aminopurine in 0.1 M Sodium Phosphate Buffer. Self-Complemantary Sequence: 5'-CGC XAA TTY GCC-3'

Sequence (X/Y)	pH 5.0	pH 6.0	pH 7.0
B (P/T)	49	52.7	53.4
C (P/A)	20.5	22.5	25.5
D (P/C)	24.5	28	25
E (C/P)	24.5	28.5	27

EXPERIMENTAL SECTION

Abbreviations used: ACN: acetonitrile, AcOEt: ethyl acetate, Ar: aromatic, CPG: controlled pore glass, DCM: dichloromethane, DMAP: *N*,*N*-dimethylaminopyridine, DMF: *N*,*N*-dimethylformamide, DMT: dimethoxytrityl, LCAA: long chain amino alkyl, Me : methyl, MeOH: methanol, P: 9-(β-D-2'-deoxyfuranosyl)-2-aminopurine, THF: tetrahydrofurane.

All ^{1}H-, ^{31}P- and ^{13}C-NMR spectra were recorded on a Brucker AM 250 spectrometer. Chemical shifts are reported in parts per million (δ) relative to the internal standard. ^{13}C-NMR spectral data is reported below with broad-band proton noise decoupling; however, assignments were always made with the aid of the off-resonance data.

DMT-N^2-Bz-2-aminopurine-2'-deoxyriboside was prepared from 2'-deoxyguanosine using a multi-step synthesis scheme published previously [24-26]. 5'-O-DMT-N^2-ibu-2'-deoxyguanosine was purchased from ChemGenes (USA). Chemicals for oligonucleotide synthesis were purchased from Perkin Elmer-Applied Biosystems. Anhydrous solvents were obtained from Merck (Germany) and SDS (France). The rest of the chemicals were from Aldrich (USA) and Fluka (Switzeland) and they were used directly without further purification.

General Methods and Standard Work-Up

All reactions were carried out in oven-dried glassware, under a nitrogen or argon atmosphere, unless specified otherwise. Starting materials were dried by evaporation with the dry solvent that will be used for the reaction. Once the reaction was completed, solutions were concentrated to dryness and the residues were dissolved in DCM or $CHCl_3$. The re-

sulting organic solutions were washed with 5% aqueous $NaHCO_3$ and saturated aqueous NaCl. The organic phase was dried (Na_2SO_4) and the solvent was removed.

3',5'-*O*-N^2-triisobutyryl-2'-deoxyguanosine

2'-Deoxyguanosine (4 g, 14.9 mmol) was dried by coevaporation with anhydrous pyridine. 50 mL of anhydrous pyridine and 2.35 g of isobutyric anhydride (14.9 mmol) and 0.52 g of DMAP (4.26 mmol) were added to the residue. The mixture was heated at 50 ˚C overnight. The solution was allowed to cool down at room temperature. Then, 30 mL of a 5% aqueous $NaHCO_3$ solution were added and the mixture was stirred for 1 hr at room temperature and concentrated to dryness. The residue was dissolved in $CHCl_3$ and washed following the standard work-up described above. The product was purified by column chromatography (silica gel) eluted with a 0-4% MeOH gradient in $CHCl_3$. Yield: 6.55 g (13.7 mmol, 92%). TLC (5% MeOH in $CHCl_3$) Rf 0.29. ^{1}H-NMR ($CDCl_3$, 500 MHz,): δ 1.14, 1.15, 1.23 (18H, 3s, ibu), 2.4-2.53 (6H, m, ibu), 2.77 (1H, m, H-2'), 2.99 (1H, m, H-2"), 4.32 (2H, m, H-5', 5"), 4.70 (1H, m, H-4'), 5.35 (1H, m, H-3'), 6,19 (1H, t, H-1'), 7.75 (1H, s, H-8), 9.56 (1H, s, NH-1), 12.20 (1H, m, NH-CO). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 18.7, 18.8 Me ibu), 33.7, 33.8 (CH ibu), 33.6 (CH ibu), 36,6 (C-2'), 63.3 (C-5'), 74.2 (C-3'), 82.5 (C-1'), 85.6 (C-4'), 122.34 (C-5), 137.8 (C-8), 147.5 (C-4, C-2), 155.4 (C-6), 176.1 (CONH), 177.5, 179.0 ($COOCH_2$, $COOCH_2$).

3',5'-*O*-N^2-triisobutyryl-9-(β-D-2'-deoxyfuranosyl)-2-aminopurine (1)

3',5'-*O*-N^2-Triisobutyryl-2'-deoxyguanosine was converted to 3',5'-*O*-N^2-triisobutyryl-9-(β-D-2'-deoxyfuranosyl)-2-aminopurine following a similar protocol to the one described for the benzoyl derivative [24-26]: 3',5'-*O*-N^2-triisobutyryl-2'-deoxyguanosine (6.5 g, 13.7 mmol) was reacted with 2,4,6-triisopropylbenzenesulfonyl (TPS) chloride (27.4 mmol), DMAP (6.85 mmol) and diisopropylethylamine (54.2 mmol) in 85 mL of DCM for 2 hours at room temperature. Then, the solution was concentrated to dryness and the desired TPS derivative was isolated from the reaction mixture by silica gel chromatography eluted with a 0-5% methanol gradient in DCM.

Hydrazine hydrate (53.6 mmol) was added to an ice-cooled solution of the TPS derivative in THF and the mixture was left reacting for 2 hours at room temperature. The reaction mixture was concentrated, dissolved in ethyl acetate and washed following the standard work-up protocol. The hydrazino derivative was isolated in 86% yield by silica gel chromatography eluted with a 0-2% MeOH gradient in DCM. Finally, the hydrazino derivative (11.2 mmol) was dissolved in 250 mL of THF and heated until boiling. Ag_2O (101.8 mmol) was added to the solution in 3 portions every 30 min. After completing the addition process, the reaction was refluxed for 2 hours. Then, the reaction mixture was concentrated to dryness before the work-up. The resulting residue was treated with ethyl acetate and 10% NaI aqueous solution. The mixture was filtered through Celite and the organic phase was decanted. The organic phase was washed with 10% NaI followed by 10% sodium thiosulfate and water. The product was purified by silica gel chromatography eluted with a 0-5% MeOH gradient in DCM. Overall yield: 6.07 mmol (44% from starting 3',5'-*O*-N^2-triisobutyryl-2'-deoxyguanosine). TLC (10% MeOH in DCM) Rf 0.4. ^{1}H-NMR: ($CDCl_3$, 500 MHz): δ 1.11, 1.17, 1.25 (18H, 3s, Me ibu), 2.51-2.63 (3H, m, CH ibu, H-2'), 2.99-3.01 (2H, m, CH ibu, H-2"), 4.32-4.44 (3H, m, H-5', 5", H-4'), 5.42 (1H, m, H-3'), 6,42 (1H, m, H-1'), 8.14 (1H, s, H-8), 8.96 (1H, s, H-6), 8.53 (1H, s, NH-1), 12.17 (1H, m, NH-COPh). ^{13}C-NMR ($CDCl_3$, 62 MHz): δ 18.7, 19.1 (Me ibu), 33.7 (CH ibu), 35.9 (CH ibu), 37.2 (C-2'), 63.4 (C-5'), 74.1 (C-3'), 82.8 (C-1'), 84.7 (C-4'), 131.3 (C-5), 142.8 (C-8), 149.7 (C-6), 150.9 (C-4), 152.9 (C-2), 176,2, 176,5, 176,6 (CONH, $COOCH_2$, $COOCH_2$).

N^2-isobutyryl-9-(β-D-2'-deoxyfuranosyl)-2-aminopurine (2)

To a solution of 3',5'-*O*-2-*N*-triisobutyryl-9-(β-D-2'-deoxyfuranosyl)-2-aminopurine 1 (1.8 g, 3.9 mmol) in anhydrous pyridine (45 mL) and MeOH (6 mL) cooled to -20 ˚C, 5.4 mL of a 2 M solution of NaOH were added. The reaction mixture was stirred 30 min at -20 ˚C. After the solution was neutralized with Dowex 50w x 4 (pyridinium salt) until pH= 7. The Dowex was filtered and washed with a solution of H_2O:pyridine:MeOH (3:1:1). The filtrates were evaporated to dryness and the residue was purified on a silica gel column eluted with a 0-10% MeOH gradient in $CHCl_3$. Yield 1.13 g (3.5 mmol, 90%). TLC (20% MeOH in DCM) Rf 0.15. ^{1}H-NMR as described previously [20]. ^{13}C-NMR: ($CDCl_3$, 62 MHz): δ 19.6 (Me ibu), 36.8 (CH ibu), 41.1 (C-2'), 63.0 (C-5'), 72.2 (C-3'), 85.7 (C-1'), 89.3 (C-4'), 132.1 (C-5), 146.0 (C-8), 149.6 (C-6), 152.9 (C-4), 154.1 (C-2), 178.4 (CONH).

5'-O-DMT-N^2-isobutyryl-2-aminopurine-2'-deoxyriboside-3'-O-(N,N-diisopropyl)-2-cyanoethyl phosphoramidite (4)

N^2-Isobutyryl-9-(β-D-2'-deoxyfuranosyl)-2-amino purine 2 (1.2 g, 3.7 mmol) was reacted with DMT-Cl in pyridine as previously described [20].

DMT-N^2-ibu-9-(β-D-2'-deoxyfuranosyl)-2-amino purine (3, 860 mg, 2.66 mmol) was reacted with 0.58 mL (2.66 mmol) of 2-cyanoethoxy-*N*,*N*-diisopropylamino chlorophosphine in the presence of 1.4 mL (10.64 mmol) of diisopropylethylamine as previously described [24-26]. Yield 650 mg (0.8 mmol, 75%). TLC (1% Et_3N in DCM) Rf 0.9. ^{31}P-NMR: ($CDCl_3$, 101 MHz): δ 149.1 and 149.8.

5'-O-Dimethoxytrityl-9-(β-D-2'-deoxyfuranosyl)-2-aminopurine (5)

When the procedure of McLaughlin [24] was performed with 5'-O-dimethoxytrityl-N^2-ibu-2'-deoxyguanosine the ibu group in position 2 was lost during the silver oxidation. 5'-O-DMT- N^2-ibu-2'-deoxyguanosine (2 g, 3.1 mmol) was dissolved in DMF and 0.8 mL of hexamethyldisilazane were added. After 3 hours of magnetic stirring the mixture was concentrated to dryness and residual DMF was eliminated by repeated evaporation with toluene. The resulting oil was dissolved in 30 mL of DCM and reacted with 2,4,6-triisopropylbenzenesulfonyl (TPS) chloride (6.2 mmol), DMAP (1.55 mmol) and diisopropylethylamine (11.8 mmol) for 2 hours at room temperature. The solution was concentrated to dryness and the desired TPS derivative was isolated from the reaction mixture on a silica gel column eluted with a 0-5% MeOH gradient in DCM. Yield 1.64 g (61%). To an ice-cooled solution of the TPS derivative in 20 mL of tetra-

hydrofurane, NH_2-NH_2.$H_2$0 (3.2 mmol) were added and the mixture was left overnight at room temperature. The reaction mixture was concentrated, dissolved in ethyl acetate and washed following the standard work-up protocol. The hydrazino derivative was isolated on a 49% yield by silica gel chromatography eluted with a 0-15% methanol gradient in DCM. Finally, the hydrazino derivative (0.4 g, 0.67 mmol) was treated with Ag_2O (0.81 mmol) and the mixture was refluxed overnight. The reaction mixture was concentrated to dryness before work-up. The residue was treated with ethyl acetate and a 10% NaI aqueous solution. The mixture was filtered through Celite and the organic phase was decanted. The organic phase was washed with 10% NaI followed by 10% sodium thiosulfate and water. The product was purified on silica gel chromatography eluted with a 0-10% MeOH gradient in DCM. Yield: 120 mg (32%).TLC (10% MeOH in DCM) Rf 0.4. ^{1}H-NMR: ($CDCl_3$, 500 MHz): δ 2.5 (1H, m, H-2'), 2.8 (1H, m, H-2"), 3.7 (6H, s, MeO DMT), 4.2 (2H, m, H-5'), 4.7 (1H, m, H-4'), 5.2 (1H, m, H-3'), 6,3 (1H, t, H-1'), 6.7-7.4 (13H, Ar DMT), 8.1 (1H, s, H-6), 8.6 (1H, s, H-8). ^{13}C-NMR ($CDCl_3$, 62 MHz): δ 39.6 (C-2'), 55.1 (MeO, DMT), 63.7 (C-5'), 72.3 (C-3'), 83.7 (C-4'), 86.1 (C-1'), 86.4 (C_q DMT), 113.1 (DMT), 126.6 (DMT), 128.5 (C-5), 129.9 (DMT), 135.5 (DMT), 140.7 (C-8), 144.5 (DMT), 149.6 (C-6), 152.7 (C-4), 158.4 (DMT), 159.6 (C-2).

5'-O-Dimethoxytrityl-N^2-dimethylaminomethyliden-9-(β-D-2'-deoxy-furanosyl)-2-aminopurine (6)

Compound **5** (100 mg, 0.22 mmol) was dissolved in 2 mL of DMF and 0.2 mL (0.24 mmol) of *N,N*-dimethylformamide dimethyl acetal were added. After 48 hours of magnetic stirring, the solution was concentrated to dryness with a yield of 120 mg (91%) of the desired product that was used in the next step without further purification. Attempts of silica gel purification did not yield the desired compound. Lost of protecting groups was observed, instead. TLC (10% MeOH in DCM) Rf 0.4. ^{1}H-NMR ($CDCl_3$, 250 MHz): δ 2.59-3.01 (2H, m, H-2'), 3.13 (3H, d, Me dmf), 3.16 (3H, d, Me dmf), 3.74 (6H, s, MeO DMT), 3.37 (2H, m, H-5'), 4.20 (1H, m, H-4'), 4.64 (1H, m, H-3'), 6.63 (1H, t, H-1'), 6.7-7.4 (13H, m, Ar DMT), 8.0 (1H, s, H-6), 8.6 (1H, s, H-8), 8.8 (1H, m, CH dmf). ^{13}C-NMR ($CDCl_3$, 62 MHz): δ 34.8 (Me dmf), 40.8 (Me dmf), 41.1 (C2'), 54.9 (Me DMT), 63.8 (C-5'), 71.6 (C-3'), 83.0 (C-4'), 85.9 (C-1'), 86.2 (C_q DMT), 112.9 (DMT), 126.6 (DMT), 129.8 (DMT), 130.2 (C-5), 135.4 (DMT), 141.3 (C-8), 144.4 (DMT), 148.9 (C-6), 158.1 (C-2), 158.3 (DMT), 162.6 (CH dmf).

Preparation of DMT-N^2-protected(Bz, ibu, dmf)-9-(β-D-2'-deoxyfuranosyl)-2-aminopurine-3'-O-succinyl-LCAA-CPG supports.

DMT-N^2-protected(Bz [24], ibu, dmf)-9-(β-D-2'-deoxyfuranosyl)-2-aminopurine derivatives (0.15 mmol) were dissolved in 6 mL of dry DCM. To these solutions 23.2 mg of DMAP (0.19 mmol) and 19 mg of succinic anhydride (0.19 mmol) were added. The mixtures were stirred at room temperature overnight. Completeness of the reactions was confirmed by careful TLC analysis. Thereafter, the mixtures were diluted with DCM and washed with 0.1 M de NaH_2PO_4 pH=5. The organic phases were dried and concentrated to dryness. The residues were dissolved in DCM and then precipitated on 50 mL of (1:1) mixture of hexane and ethyl ether. N^2-Bz derivative: Yield 70%. TLC (5% MeOH in $CHCl_3$) Rf 0.35. N^2-ibu derivative: Yield 54%. TLC (5% MeOH in $CHCl_3$) Rf 0.35. N^2-dmf derivative: Yield 64%. TLC (10% MeOH in DCM) Rf 0.1. ^{1}H- and ^{13}C-NMR confirms the addition of the succinyl group.

DMT-N^2-protected-9-(β-D-2'-deoxyfuranosyl)-2-aminopurine 3'-O-succinates were reacted with amino-LCAA-CPG (500 Å, loading 98 μmol/g) supports following a previously described protocol [34]. Nucleoside loadings were as follows: N^2-Bz: 34 μmol/g; N^2-ibu: 52 μmol/g; N^2-dmf: 48 μmol/g.

Oligonucleotide Synthesis and Purification

Sequence A: 5'-CGT AGP GAT GC-3' was prepared on a 10 μmol scale and sequences B (P/C): 5'-CGC PAA TTC GCG-3'; C (P/A): 5'-CGC PAA TTA GCG-3'; D (P/T): 5'-CGC PAA TTT GCG-3'; and E (C/P): 5'-CGC CAA TTP GCG-3' were prepared on a 1 μmol scale using the cycles implemented by the manufacturer. For the addition of the natural bases, commercially available nucleoside phosphoramidites protected with the benzoyl (A, C) and isobutyryl (G) groups were used. For the preparation of sequence A two comparative 10 μmol-scale synthesis were performed using different 2-aminopurine phosphoramidites: the ibu-protected 2-aminopurine phosphoramidite (**4**) and the commercially available dmf-protected 2-aminopurine phosphoramidite. In all cases the last DMT group was left to help purification. Coupling efficiencies were 99%. Sequence: 5'-TP-3' was assembled using 35 mg of the supports of the 2-aminopurine-2'-deoxyriboside containing the ibu and the dmf groups and T 2-cyanoethyl phosphoramidite. Syntheses were performed using an automatic DNA synthesizer (Applied Biosystems Mod.392).

Sequences A-E were deprotected using standard protocols. After deprotection, sequences B-E were purified using oligonucleotide purification cartridges following the instructions of the suppliers. Further purification was achieved by reversed-phase HPLC. HPLC conditions were as follows: Column PRP-1, 10 μm, (305x8 mm), flow rate 2 mL/min, 30 min linear gradient from 5% to 40% ACN over 0.1 M aqueous triethylammonium acetate pH 6.5. Overall yields were: B 41%, C 43%, D 43%, E 38%. Sequence A was purified by reversed-phase HPLC using the DMT on / DMT off protocol [25]. Column PRP-1, 12-22 μm, (250x21.5 mm), flow rate 5 mL/min, DMT on gradient: a 30 min linear gradient from 18% to 60% ACN over 0.1 M aqueous triethylammonium acetate pH 6.5; DMT off gradient: a 30 min linear gradient from 5% to 50% ACN over 0.1 M aqueous triethylammonium acetate pH 6.5. After the first HPLC purification (DMT-on) the amounts of DMT-oligonucleotide obtained were 363 OD (using the ibu group for 2-aminopurine) and 156 OD (using the dmf group for the 2-aminopurine). After the second HPLC (DMT off) 276 OD (ibu) and 111 OD (dmf) were obtained.

The nucleoside composition of the oligonucleotides carrying 2-aminopurine was confirmed by hydrolysis with snake venom phosphodiesterase and alkaline phosphatase followed by HPLC analysis [20]. The oligonucleotide 5'-CGC PAA TTC GCG-3' was further analyzed by mass spectrometry (electrospray): found 3629.6 expected for $C_{116}H_{147}N_{46}O_{69}P_{11}$ 3629.9.

Deprotection Studies for Bz-, ibu- and dmf-protected 2-aminopurine-2'-deoxyriboside

Supports containing 5'-O-DMT-N^2-(Bz-, ibu- or dmf)-protected 2-aminopurine 2'-deoxyriboside were detritylated with 3% TCA in DCM. Aliquots of the different supports (5 mg each) were treated with concentrated ammonia (1 mL) and were left at room temperature and 55˚C for different periods of time. Then, the solutions were concentrated to dryness and analyzed by reverse-phase HPLC.

Depurination Studies

Aliquots of the 5'-TP-3'-succinyl-CPG were treated with 3% TCA solution in DCM at room temperature for different times up to 7 days. The supports were filtered, washed with ACN and dried. The resulting supports were treated with concentrated ammonia at 55˚C for 2 hours and the solutions were concentrated to dryness. The resulting products were analyzed by reverse-phase HPLC (see conditions in oligonucleotide synthesis and purification). The depurination product eluted 0.5 min after the peak corresponding to the dimer containing 2-aminopurine. 5'-TP-3' dinucleotide: UV (water pH 6.5) max 252 (P), 268 (T) and 307 (P) nm; enzyme digestion showed the presence of 2-aminopurine 2'-deoxyriboside and thymidine; mass spectra M= 555.3 (expected for $C_{20}H_{26}N_7O_{10}P$ 555.4). Depurination product. UV (water pH 6.5) max 270 (T) nm, enzyme digestion showed only the presence of thymidine, mass spectra M= 438 (expected for $C_{15}H_{23}N_2O_{11}P$ 438.3).

Melting Studies

Oligonucleotides P/C, P/A, P/T and C/P were dissolved in a solution of 0.1 M sodium phosphate buffer at different pHs. The solutions were heated at 80˚C and allowed to cool slowly to 4˚C. UV absorption spectra and melting experiments (absorbance *vs* temperature) were recorded in 1 cm path-length cells with a spectrophotometer, which has a temperature controller with a programmed temperature increase of 0.5 deg min^{-1}. Thermal denaturation curves were run on duplex concentration of 4 μM at 260 nm.

ACKNOWLEDGEMENTS

This work was supported by the Spanish Ministry of Education (grant CTQ2010-20541) and the Generalitat de Catalunya (2009/SGR/208).

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Received: April 27, 2011 Revised: May 13, 2011 Accepted: May 16, 2011