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Base pairing of anhydrohexitol nucleosides with 2,6-diaminopurine, 5-methylcytosine and uracil as base moiety

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ABSTRACT

Hexitol nucleic acids (HNAs) with modified bases (5-methylcytosine, 2,6-diaminopurine or uracil) were synthesized. The introduction of the 5-methylcytosine base demonstrates that *N*-benzoylated 5-methylcytosyl-hexitol occurs as the imino tautomer. The base pairing systems (G:C^{Me}, U:D, T:D and U:A) obey Watson–Crick rules. Substituting hT for hU, hC^{Me} for hC and hD for hA generally leads to increased duplex stability. In a single case, replacement of hC by hC^{Me} did not result in duplex stabilization. This sequence-specific effect could be explained by the geometry of the model duplex used for carrying out the thermal stability study. Generally, polypurine HNA sequences give more stable duplexes with their RNA complement than polypyrimidine HNA sequences. This observation supports the hypothesis that, besides changes in stacking pattern, the difference in conformational stress between purine and pyrimidine nucleosides may contribute to duplex stability. Introduction of hC^{Me} and hD in HNA sequences further increases the potential of HNA to function as a steric blocking agent.

INTRODUCTION

Duplex stability of dsDNA and dsRNA can be increased by modifications of the carbohydrate moiety, the base moiety or the internucleoside linkage. Hexitol nucleic acid (HNA) is an example of how sugar modifications may influence duplex stability in a beneficial way (1–3). The two most studied base modifications, leading to an increase in hybridization strength, are the replacement of cytosine by 5-methylcytosine and the replacement of adenine by 2-aminoadenine (or diaminopurine) (4). Introduction of a methyl group in the 5 position of uracil and cytosine bases increases hydrophobic interactions between the 5-methylpyrimidines and neighbouring bases, generally resulting in more stable complexes (5–7). It has been observed, however, that the 5-methyl group may sometimes reduce the cooperativity of the duplex melting process (5) and may induce a conformational transition of oligonucleotides in solution (5–8). Three Watson–Crick hydrogen bonds can be formed between the diaminopurine base and a regular uracil (thymine) base and this is the basis for the expected and observed higher stability of duplexes when adenine is replaced by 2,6-diaminopurine (D). However, such replacement may lead as well to duplex stabilization as to destabilization or may end up in no effect at all on thermal stability (9–12), mainly depending on the influence of the 2-amino group on the geometry of hydrogen bonding, hydration of the minor groove, helical conformational transitions, groove width and base stacking pattern.

HNAs hybridize sequence-selectively and very strongly with natural nucleic acids. Until now, only two Watson–Crick base pairing schemes have been evaluated, i.e. G-C and A-T base pairs. To further investigate the influence of base modifications on HNA-containing complexes, we incorporated uracil (U), 5-methylcytosine (C^{Me}) and 2,6-diaminopurine (D) modified hexitol nucleosides into regular HNA sequences. The influence on the thermal stability of HNA:DNA, HNA:RNA and dsHNA hybrids was evaluated. This study led to the conclusion that, even with these modified and unnatural bases, HNA obeys the general Watson–Crick pairing rules and that replacement of D for A and C^{Me} for C may further increase the potential of HNA to function as a steric blocker.

MATERIALS AND METHODS

Synthesis of protected hexitol nucleosides with modified bases

All experiments were carried out using instrumentations and manipulations as described previously (2,3,13). $[BH_2]^+$ stands for protonated base. ^{1}H NMR and ^{13}C NMR data of all compounds are available as supplementary material.

*1,5-Anhydro-4,6-O-benzylidene-2,3-dideoxy-2-(uracil-1-yl)-*D*-arabino-hexitol (**2**).* A suspension of 2.24 g (20 mmol) of uracil and 152 mg of LiH in DMF (150 ml) was heated at 120°C for 1 h, after which a solution of 3.9 g (10 mmol) of 1,5-anhydro-4,6-*O*-benzylidene-3-deoxy-2-*O*-(*p*-toluenesulfonyl)-D-ribo-hexitol (13) (**1**) in DMF (20 ml) was added. The reaction mixture was stirred for 24 h at 120°C, H_2O (1 ml) was added and the mixture was evaporated at reduced pressure. The residue was diluted with brine (300 ml) and extracted with EtOAc (3 times). The combined organic layer was dried and evaporated. The title compound was

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obtained in 57% yield (3.5 g, 10.6 mmol) after column chromatographic purification (EtOAc/hexane 80:20).
LSIMS (THGLY): *m/z* 331 $[MH]^+$, 113 $[BH_2]^+$.
Elemental analysis. Calculated for $C_{17}H_{18}N_2O_5$: C, 61.81; H, 5.49; N, 8.48. Found: C, 62.24; H, 5.62; N, 8.64.

1,5-Anhydro-2,3-dideoxy-2-(uracil-1-yl)-D-arabino-hexitol ***(3)***. A solution of 2.20 g (6.67 mmol) of 1,5-anhydro-4,6-*O*-benzylidene-2,3-dideoxy-2-(uracil-1-yl)-D-arabino-hexitol in 100 ml of 80% aqueous HOAc was stirred overnight at room temperature and for an additional 2 h at 60°C. Acetic acid was evaporated and the residual oil was purified by column chromatography (CH_2Cl_2/MeOH 90:10). The title compound was obtained in 75% yield (1.21 g, 5 mmol).
LSIMS (THGLY): *m/z* 243 $[MH]^+$, 113 $[BH_2]^+$.
Elemental analysis. Calculated for $C_{10}H_{14}N_2O_5$: C, 49.58; H, 5.83; N, 11.56. Found: C, 49.57; H, 5.82; N, 11.47.

1,5-Anhydro-2,3-dideoxy-2-(uracil-1-yl)-6-O-monomethoxytrityl-D-arabino-hexitol ***(4)***. A solution of 2.0 g (8.3 mmol) of 1,5-anhydro-2,3-dideoxy-2-(uracil-1-yl)-D-arabino-hexitol and 3.95 g (12.8 mmol) of monomethoxytrityl chloride in 40 ml of pyridine was stirred overnight at room temperature. The reaction mixture was diluted with 200 ml of saturated $NaHCO_3$ solution and extracted three times with CH_2Cl_2. The organic layer was dried, evaporated, co-evaporated with toluene and purified by column chromatography (CH_2Cl_2/MeOH 98:2). Yield 3.34 g (6.5 mmol, 81%)
LSIMS (THGLY/NaOAc): *m/z* 559 $[M+2Na-H]^+$.
Elemental analysis. Calculated for $C_{30}H_{30}N_2O_6 \cdot H_2O$: C, 67.64; H, 6.06; N, 5.26. Found: C, 67.56; H, 5.87; N, 5.22.

1,5-Anhydro-2,3-dideoxy-2-(5-methylcytosin-1-yl)-D-arabino-hexitol ***(7)***. A mixture of 1.7 g (5 mmol) of 1,5-anhydro-4,6-*O*-benzylidene-2,3-dideoxy-2-(thymin-1-yl)-D-arabino-hexitol (1,2), $POCl_3$ (1 ml) and 1,2,4-triazole (2.96 g) in 120 ml of pyridine was stirred at room temperature for 4 h. The reaction mixture was cooled to 0°C and ammonia gas was bubbled through the mixture for 10 min. The reaction mixture was further stirred for 10 min at room temperature, evaporated and co-evaporated with toluene (3 times). The principal reaction product was isolated by silica gel chromatography (CH_2Cl_2/MeOH 95.5) giving 1.3 g (3.8 mmol, 80% yield) of an oil. The compound was identified after removal of the benzylidene protecting group. Hereto, the oil was dissolved in 80% HOAc (100 ml) and heated for 5 h at 80°C. The mixture was evaporated, co-evaporated with toluene (3 times) and purified by column chromatography (CH_2Cl_2/MeOH 90:10, followed by 80:20). The title compound was obtained in 60% yield (600 mg, 2.35 mmol).
LSIMS (THGLY): *m/z* 256 $[MH]^+$.
Elemental analysis. Calculated for $C_{11}H_{17}N_3O_4$: C, 51.76; H, 6.71; N, 16.46. Found: C, 51.52; H, 6.59; N, 16.27.

1,5-Anhydro-2-(N^4-benzoyl-5-methylcytosin-1-yl)-2,3-dideoxy-6-O-monomethoxytrityl-D-arabino-hexitol ***(9)***. To a solution of 600 mg (2.3 mmol) of 1,5-anhydro-2-(5-methylcytosin-1-yl)-2,3-dideoxy-D-arabino-hexitol in pyridine (20 ml) was added 2.17 ml (16.1 mmol) of chlorotrimethylsilane. The reaction mixture was stirred for 15 min at room temperature and 0.75 ml (6.5 mmol) of benzoyl chloride was added. After stirring for 3 h at room temperature, the reaction mixture was cooled in an ice bath, water (1 ml) was added, stirring was continued for 5 min and concentrated ammonia (2 ml) was added. Stirring was continued for 30 min at 0°C after which the reaction mixture was evaporated and co-evaporated with EtOH and toluene. The resulting oil was dissolved in pyridine (20 ml) and 772 mg (2.5 mmol) of monomethoxytrityl chloride was added. After keeping the solution for 16 h at room temperature, it was evaporated after addition of solid sodium bicarbonate. The resulting oil was diluted with CH_2Cl_2 and washed with H_2O, saturated $NaHCO_3$ solution and H_2O. The organic layer was evaporated and purified by column chromatography (hexane/EtOAc 50:50). Yield 390 mg (0.62 mmol, 27%).
LSIMS (THGLY/NaOAc): *m/z* 654 $[M+Na]^+$.
Elemental analysis. Calculated for $C_{38}H_{37}N_3O_6$: C, 72.24; H, 5.91; N, 6.65. Found: C, 72.02; H, 6.04; N, 6.69.

1,5-Anhydro-4,6-O-benzylidene-2,3-dideoxy-2-(2-amino-6-chloropurin-9-yl)-D-arabino-hexitol ***(12)*** *and 1,5-anhydro-4,6-O-benzylidene-2,3-dideoxy-2-(2-N-phosphinimine-6-chloropurin-9-yl)-D-arabino-hexitol* ***(13)***. A suspension of 2-amino-6-chloropurine (3.59 g, 21.19 mmol) in dry dioxane (200 ml) was heated to reflux for 30 min. After cooling to room temperature, the suspension was treated with triphenylphosphine (7.78 g, 29.65 mmol) and 1,5-anhydro-4,6-*O*-benzylidene-3-deoxy-D-glucitol **11** (14) (2.00 g, 8.47 mmol) in dry dioxane (60 ml). After 5 min a solution of diethyl azodicarboxylate (4.67 ml, 29.65 mmol) in dry THF (45 ml) was added dropwise over 90 min and the mixture was stirred at room temperature for 30 h. The reaction mixture was concentrated and purified by silica gel column chromatography using a gradient of 0–100% CH_2Cl_2 in *n*-hexane followed by 10–100% EtOAc in CH_2Cl_2 to afford a mixture of compounds **12** and **13** contaminated with triphenylphosphine oxide. Analytical samples of **12** and **13** were obtained after further purification of 200 mg of this mixture by chromatotron (1 mm silica gel layer; elution at 3 ml/min, 50 ml fractions of EtOAc/CH_2Cl_2 0:100, 10:90, 20:80, 30:70, 40:60 and 50:50).
Compound **12** LSIMS (THGLY/NBA): *m/z* 388 $[MH]^+$.
Compound **13** LSIMS (THGLY/NBA): *m/z* 648 $[MH]^+$, 430 $[BH_2]^+$.

1,5-Anhydro-4,6-O-benzylidene-2,3-dideoxy-2-(2,6-diaminopurin-9-yl)-D-arabino-hexitol ***(14)***. The mixture of compounds **12** and **13** previously obtained was placed in a Parr pressure reactor and treated with a solution (300 ml) of methanol saturated with ammonia at 100°C for 24 h. After concentration, purification of the residue by silica gel column chromatography (0–7% MeOH in CH_2Cl_2) led to compound **14** (2.29 g, 73% from **11**).
UV (MeOH): λ_{max} = 258 nm (ε 11 500), 283 nm (ε 13 300).
LSIMS (THGLY/NaOAc): *m/z* 391 $[M+Na]^+$, 369 $[MH]^+$.

1,5-Anhydro-4,6-O-benzylidene-2,3-dideoxy-2-[2,6-di-(N-benzoylamino)purin-9-yl]-D-arabino-hexitol ***(15)***. A solution of compound **14** (2.25 g, 6.11 mmol) in dry pyridine (70 ml) was treated by benzoyl chloride (2.84 ml, 24.44 mmol) and stirred overnight. Water (10 ml) was added and after 20 min, the mixture was treated with a solution of concentrated ammonia (15 ml) in pyridine (100 ml) for 45 min. The solvents were removed *in vacuo*, the residue was co-evaporated with toluene and methanol and then purified by silica gel column chromatography (0–5% MeOH in CH_2Cl_2) to give pure compound **15** (3.23 g, 92%).
UV (MeOH): λ_{max} = 235 nm (ε 25 000), 257 nm (ε 28 100), 297 (ε 16 000).
LSIMS (THGLY/NBA): *m/z* 577 $[MH]^+$.
HRMS: calculated for $C_{32}H_{28}N_6O_5$+H, 577.21994; found, 577.21660.

*1,5-Anhydro-2,3-dideoxy-2-[2,6-di-(N-benzoylamino)purin-9-yl]-D-arabino-hexitol (**16**)*. The benzylidene moiety of compound **15** (3.10 g, 5.37 mmol) was cleaved with 80% acetic acid (140 ml) at 60°C for 6 h. The reaction mixture was concentrated to dryness, the oily residue was co-evaporated with toluene and methanol and then purified by silica gel column chromatography (0–8% MeOH in CH_2Cl_2) to afford compound **16** (1.67 g, 63%).
UV (MeOH): λ_{max} = 257 nm (ε 11 400), 297 (ε 6300).
LSIMS (THGLY/NBA): *m/z* 489 $[MH]^+$, 359 $[BH_2]^+$.

*1,5-Anhydro-2,3-dideoxy-2-[2,6-di-(N-benzoylamino)purin-9-yl]-6-O-dimethoxytrityl-D-arabino-hexitol (**17**)*. To a solution of diol **16** (1.60 g, 3.27 mmol) in dry pyridine (40 ml) cooled at 0°C, was added dropwise a solution of dimethoxytrityl chloride (1.55 g, 4.59 mmol) in dry dichloromethane (13 ml) over 2 h. The solution was stirred at 0°C for 30 min, then warmed to room temperature. After 17 h, the reaction was quenched with methanol (5 ml) and the solvents were removed *in vacuo*. The residual oil was dissolved in dichloromethane and washed successively with a saturated solution of $NaHCO_3$, brine and water. The organic layer was dried over Na_2SO_4, evaporated to dryness and co-evaporated with toluene and methanol. The crude material was purified by flash silica gel column chromatography [10–100% CH_2Cl_2 in *n*-hexane + 1% triethylamine (TEA) then 0–5% MeOH in CH_2Cl_2 + 1% TEA] to give pure compound **17** (1.64 g, 63%).
UV (MeOH): λ_{max} = 243 nm (ε 36 100), 257 nm (ε 32 700), 276 nm (ε 21 500), 300 nm (ε 17 600).
LSIMS (THGLY): *m/z* 791 $[MH]^+$, 359 $[BH_2]^+$.
HRMS (THGLY/NaOAc): calculated for $C_{46}H_{42}N_6O_7$+Na, 813.30127; found, 813.30470.

Preparation of the amidite building blocks

About 1 mmol of the modified nucleoside was treated with dry *N,N*-diisopropylethylamine (3 equiv.) and 2-cyanoethyl-*N,N*-diisopropylchloro-phosphoramidite (1.5 equiv.) in dry dichloromethane (10 ml) and stirred at room temperature for 30 min. The reaction was quenched by addition of water (3 ml) and stirred for 15 min. The mixture was washed with 5% sodium bicarbonate solution (30 ml) and saturated NaCl solution (3 × 30 ml), dried and evaporated. Column chromatography with *n*-hexane/acetone/triethylamine as eluent afforded the amidite. The product thus obtained was dissolved in dry dichloromethane (2 ml) and precipitated by dropwise addition to cold (–70°C) *n*-hexane (100 ml). The product was isolated, washed with *n*-hexane, dried and used as such for DNA synthesis. Yields, starting quantity, R_f values, mass analysis and ^{31}P NMR data are given in Table 1.

Table 1. Phosphoramidite analysis

	Starting alcohol (mmol)	Yield (%)	R_f value[a]	MS[b]	^{31}P NMR[c]
5	1.55	85	0.33	715.5 $(M+H)^+$	149.16/148.57
10	0.42	82	0.63	854.4 $(M+Na)^+$	149.29/148.55
18	0.44	90	0.48	1013.4 $(M+Na)^+$	149.57/148.62

[a]Ratios are given for the system *n*-hexane/acetone/TEA (49:49:2).
[b]LSIMS (positive mode, thioglycerol, NaOAc; NPOE for **5**).
[c]85% H_3PO_4 in water as external reference.

Solid-phase oligonucleotide synthesis

Oligonucleotide synthesis was performed on an ABI 392 A DNA synthesiser (Applied Biosystems) by using the phosphoramidite approach. The standard DNA assembly protocol was used, except for a 3 min coupling time using 0.12 M of the incoming amidite. The oligomers were deprotected and cleaved from the solid support by treatment with concentrated aqueous ammonia (55°C, 16 h). After gel filtration on a NAP-10® column (Sephadex G25-DNA grade; Pharmacia) with water as eluent, purification was achieved on a Mono-Q® HR 10/10 anion exchange column (Pharmacia) with the following gradient system (A = 10 mM NaOH, pH 12.0, 0.1 M NaCl; B = 10 mM NaOH, pH 12.0, 0.9 M NaCl; gradient used depended on the oligo; flow rate 2 ml min^{-1}). The low pressure liquid chromatography system consisted of a Merck-Hitachi L 6200 A intelligent pump, a Mono Q®-HR 10/10 column (Pharmacia), a Uvicord SII 2138 UV detector (Pharmacia LKB) and a recorder. The product-containing fraction was desalted on a NAP-10® column and lyophilized.

Synthesis of the diaminopurine-containing oligos was essentially the same as for other oligonucleotides, except that deprotection was done for 1 h at 70°C with a 1:1 mixture of concentrated ammonia and 40% aqueous methylamine (15).

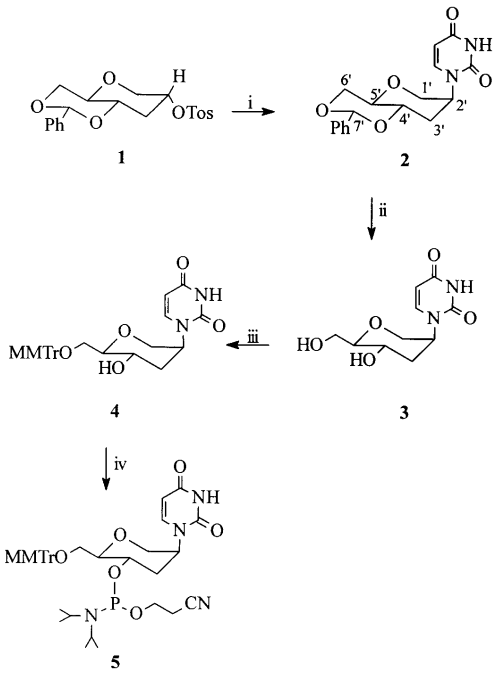
Melting temperatures

Oligomers were dissolved in 0.1 M NaCl, 0.02 M potassium phosphate, pH 7.5, 0.1 mM EDTA. The concentration was determined by measuring the absorbance at 260 nm at 80°C and assuming the hexitol nucleoside analogues to have the same extinction coefficients in the denatured state as the natural nucleosides. The following extinction coefficients were used: dA and hA, ε = 15 000: dT and hT, ε = 8500; dG and hG, ε = 12 500; dC and hC, ε = 7500; U and hU, ε = 10 000; hD, ε = 7400; hC^{Me}, ε = 6300. The concentration in all experiments was 4 µM for each strand unless otherwise stated. Melting curves were determined with a Uvikon 940 spectrophotometer. Cuvettes were maintained at constant temperature by means of water circulation through the cuvette holder. The temperature of the solution was measured with a thermistor directly immersed in the cuvette. Temperature control and data acquisition were done automatically with an IBM-compatible computer. The samples were heated at a rate of 0.2°C min^{-1} and no difference was observed between heating and cooling melting curves unless stated. Melting temperatures were determined by plotting the first derivative of the absorbance versus temperature curve.

RESULTS AND DISCUSSION

Synthesis of the protected uracil nucleoside (hU) followed classical procedures (Scheme 1). Reaction of the 2-*O*-tosylate of 1,5-anhydro-4,6-*O*-benzylidene-3-deoxy-D-ribo-hexitol (13) (**1**) with the lithium salt of uracil, followed by deprotection of the benzylidene group yielded hU (**3**). The primary hydroxyl group of **3** was protected with a monomethoxytrityl group (**4**) and the secondary hydroxyl group was esterified to obtain the phosphoramidite, which is the hU building block **5** for oligonucleotide synthesis.

The 5-methylcytosine building block could be obtained starting from the previously described thymine congener **6** (13,14) by a well-known procedure (16; Scheme 2). Removal of the benzylidene protecting group in acidic medium gave hC^{Me} (**7**). When the



Scheme 1. Synthesis of the hU building block. (i) Uracil, LiH, DMF, 120°C, 1 h; (ii) HOAc, 60°C, 2 h; (iii) MMTrCl, pyr, room temperature, 16 h; (iv) $(iPr)_2N(CE)PCl$, $(iPr)_2NEt_2$, CH_2Cl_2.

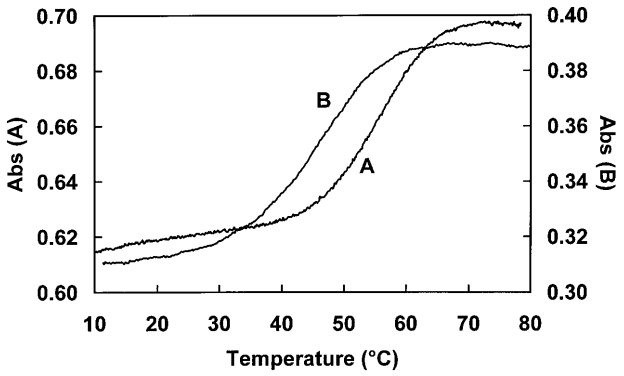
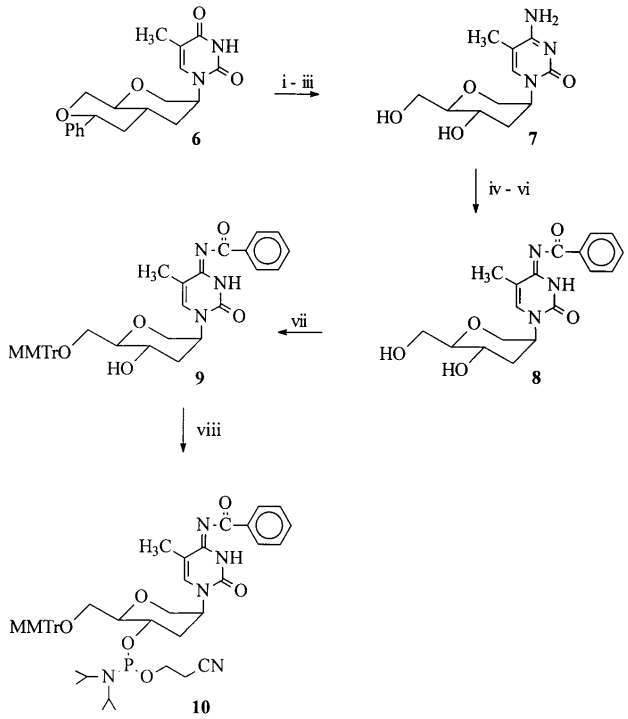


Figure 1. Melting profile of (**A**) the duplex formed between 6′-h(GC^{Me}GTA-GC^{Me}G)-4′ and 5′-r(CGCUACGC)-3′ (in 0.1 M NaCl, at 260 nm) and (**B**) the duplex formed between 6′-h(DGGDGD)-4′ and 5′-d(TCTCCT)-3′ (in 1 M NaCl, at 260 nm), showing the somewhat broader melting profile of the latter duplex.

5-methylcytosine base was protected with a benzoyl group, according to the procedure of Jones (17), compound **8** was obtained, which was converted to **9**. It was clearly shown by ^{13}C NMR and X-ray studies that the obtained compounds **8** and **9** are not the expected benzoylated amino tautomer (18) but the more labile imino tautomer. Finally, compound **9** was converted to the protected phosphoramidite building block for incorporation studies.

Synthesis of the diaminopurine nucleoside **14** albeit in low total yield (19%) has already been described (14). Therefore, the initial procedure was modified using the Mitsunobu alkylation procedure (Scheme 3). Reaction of 2-amino-6-chloropurine with 1,5-anhydro-4,6-*O*-benzylidene-3-deoxy-D-glucitol **11** (14) in the presence of



Scheme 2. Synthesis of the hC^{Me} building block. (i) $POCl_3$, triazole, pyr, room temperature, 4 h; (ii) NH_3, 20 min; (iii) HOAc, 5 h, 80°C; (iv) $ClSiMe_3$, pyr, 15 min, room temperature; (v) BzCl, pyr, 3 h, room temperature; (vi) H_2O and NH_3, 30 min, 0°C; (vii) MMTrCl, pyr, 16 h, room temperature; (viii) $(iPr)_2N(CE)PCl$, $(iPr)_2NEt$, CH_2Cl_2.

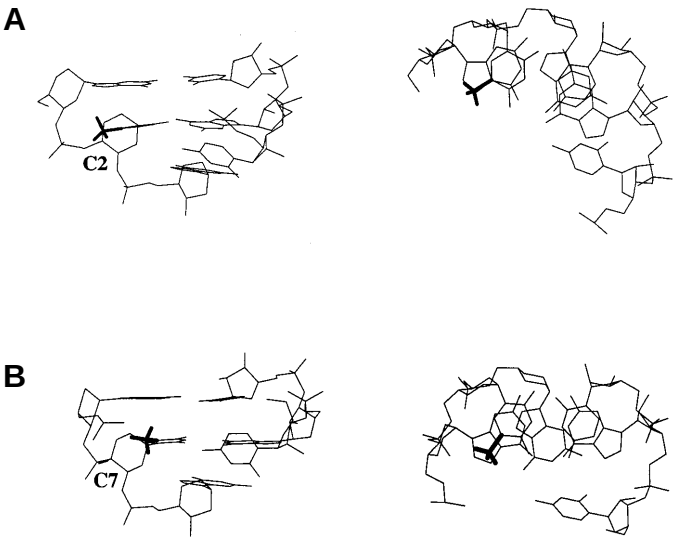
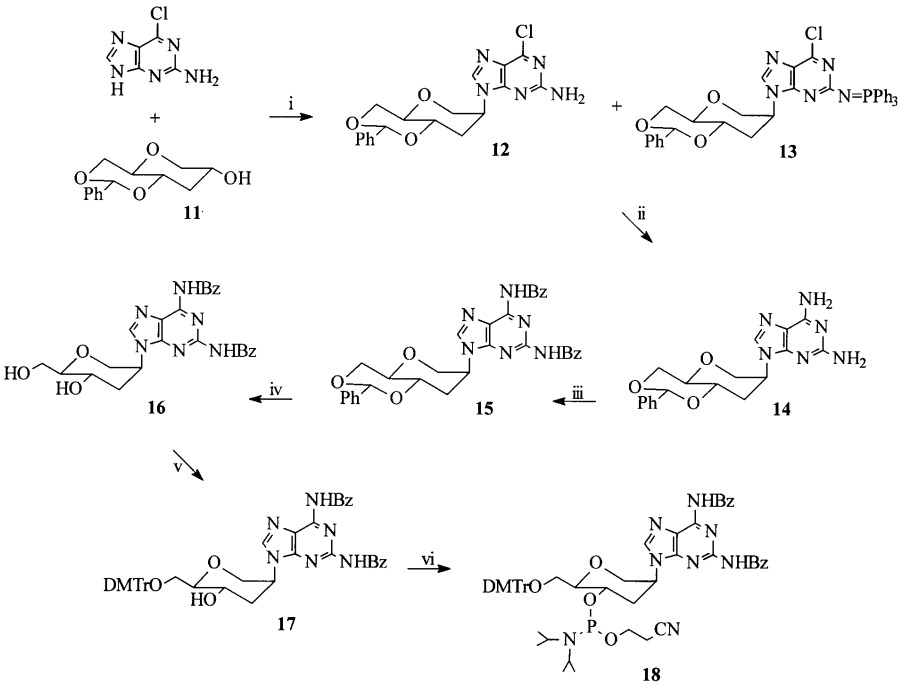


Figure 2. Positioning of the methyl group of hC^{Me} in the sequences 6′-h(GC^{Me}GTAGCG)-4′ and 6′-h(GCGTAGC^{Me}G)-4′, respectively.

triphenylphosphine and DEAD yielded a mixture of **12** and the N^2-phosphinimine **13**. Both compounds give rise to the 2,6-diaminopurine hexitol nucleoside **14** by treatment with ammonia in methanol at elevated temperature. Compound **14** was obtained in 73% yield from **11**. The base moiety of **14** was protected as its dibenzoyl derivative in 92% yield by a slightly modified procedure as described by Strobel *et al.* (19). After



Scheme 3. Synthesis of the hD building block. (i) PPh_3, DEAD/THF, dioxane; (ii) NH_3/MeOH, 100°C; (iii) BzCl/pyr; (iv) AcOH, 80%, 60°C; (v) DMTrCl/pyr; (vi) $(iPr)_2N(CE)PCl$, $(iPr)_2NEt$, CH_2Cl_2.

hydrolysis of the benzylidene moiety in acidic medium, the primary hydroxyl group of **16** was protected by dimethoxytritylation to give **17**. The phosphoramidite **18** was obtained from **17** in the usual way. The building blocks **5**, **10** and **18** were used to synthesize the HNA sequences depicted in Tables 2 and 3. As can be seen in the formulae in the reaction schemes, all newly prepared hexitol nucleosides showed the arabino configuration and thus axially oriented conformation of the base moiety. This followed from chemical evidence in the reactions used and was in all cases very clearly confirmed in the ^{1}H NMR spectra by the absence of characteristic large diaxial *J*-coupling constants for the hydrogen at the 2′ position, thus pointing to an equatorial orientation of this hydrogen.

The influence of base modifications was first evaluated in a mixed polypurine–polypyrimidine duplex, wherein adenine was replaced by diaminopurine in the polypurine strand, or cytosine by 5-methylcytosine and thymine by uracil in the polypyrimidine strand. The hybridization studies were carried out on 6mers and, in contrast to HNA-containing complexes, associations of natural nucleic acids of this length are very unstable, except for the dsRNA duplex. The stability of the duplex associations generally decreases in the order dsHNA > HNA:RNA > RNA:RNA > HNA:DNA. The T_m of the HNA:DNA duplex **26**:**19** is 21.5°C in 0.1 M NaCl (31°C in 1.0 M NaCl), while the stability increases to 45°C (in 0.1 M NaCl) for HNA:RNA (**26**:**20**) and 50°C (in 0.1 M NaCl) for dsHNA (**26**:**21**). The corresponding dsDNA sequence (**24**:**19**) shows a T_m of 10°C at 1.0 M NaCl. When substituting hT for hU in the HNA oligopyrimidine sequence, the T_m of the dsHNA duplex further increases from 50 to 54°C (in 0.1 M NaCl). When substituting hC^{Me} for hC in the same sequence, likewise, the duplex stability increases from 50 to 57°C. Introduction of a methyl group in the 5 position of pyrimidine bases has indeed a beneficial effect on duplex stability of HNA, in analogy with DNA oligos. This is most likely due to increased stacking interactions. At first sight, the effect of C/C^{Me} substitution is somewhat more pronounced than that of U/T replacement. The thymine bases, however, are situated at both ends of the oligomer while the methylcytosine bases are located in the centre of the oligonucleotide so that the influence of the replacement of U by T and C by C^{Me} on the thermal stability of the duplex may be equivalent. The increase in duplex stability per modification is ~2°C. The influence of replacing hA by hD (with hD standing for the hexitol nucleoside with a diaminopurine base moiety) is clearly visible by comparing the T_m of the HNA polypurine strands hybridized with DNA and RNA. After substituting hD for hA, the T_m increases from 31 to 46.5°C (in 1 M NaCl) for the HNA:DNA duplex (**27**:**19**). For the HNA:RNA duplex (**27**:**20**), an increase from 45 to 54.5°C (in 0.1 M NaCl) was observed. In 1.0 M NaCl, the T_m of the D-containing HNA:RNA duplex further increases to 65.5°C, confirming the previously observed RNA selectivity of HNA (1–3). In all cases described here, three diaminopurine bases were introduced. The ΔT_m/mod is +5°C for the HNA:DNA duplex and +3°C for the HNA:RNA duplex. This effect may be largely attributed to the increase in the number of hydrogen bondings. The beneficial effect of substituting D for A is also seen in dsHNA. The T_m values of the dsHNA duplex with U/C, T/C, U/C^{Me} in the polypyrimidine strand and A/G in the polypurine strand (**26**) are 50, 54 and 57°C (0.1 M NaCl), respectively. They increase to ~63, 70 and 71°C (0.1 M NaCl), respectively, when using a D/G polypurine strand (**27**). The ΔT_m/mod ranges between +4 and +5°C. The large duplex stabilization when combining $G:C^{Me}$ and D:U base pairing within a dsHNA complex can be observed by comparing the T_m of the complex **23**:**27** and the T_m of the corresponding dsRNA duplex **20**:**25** (with the usual C:G and U:A base pairs). Values of 71.5 and 43°C, respectively, are noticed in 0.1 M NaCl, meaning an increase in T_m of +28.5°C.

Table 2. Melting temperatures (°C) of DNA, RNA and HNA polypurine sequences with their DNA, RNA and HNA polypyrimidine complement

	3′-d(TCCTCT)-5′ **19**	3′-r(UCCUCU)-5′ **20**	4′-h(**U**CC**U**C**U**)-6′ **21**	4′-h(TCCTCT)-6′ **22**	4′-h(U**C^{Me}C^{Me}**U**C^{Me}**U)-6′ **23**
5′-d(AGGAGA)-3′ **24**	10[a]	13[a]	no T_m[a]	no T_m[a]	no T_m[a]
5′-r(AGGAGA)-3′ **25**	31.5[a]	43[a]	30[b,c]; 40[a,c]	39[b,c]; 48[a,c]	38[b,c]; 50[a,c]
6′-h(AGGAGA)-4′ **26**	21.5[b]; 31[a]	45[b]	50[b,c]; 61[a,c]	54[b,c]	57[b,c]
6′-h(**D**GG**D**G**D**)-4′ **27**	46.5[a,c]	54.5[b,c]; 65.5[a,c]	63.7[b]	70.3[b]	71.5[b]

The stability of the following base pairs are compared: U-A, T-A, U-D, T-D, C:G, G:C^{Me} (d means DNA sequence, r stands for RNA sequence, h represents an HNA sequence).
[a]1 M NaCl.
[b]0.1 M NaCl.
[c]Broad peak.

Table 3. Melting temperature (°C) of HNA–RNA octamer duplexes after incorporation of 5-methylcytosine and 2,6-diaminopurine bases in the HNA strand

	5′-r(CGCUACGC)-3′
6′-h(GCGTAGCG)-4′	54
6′-h(GCG**U**AGCG)-4′	52
6′-h(G**C^{Me}**GTAGCG)-4′	54
6′-h(GCGT**D**GCG)-4′	60
6′-h(G**C^{Me}**GT**D**GCG)-4′	60.5
6′-h(GCGTAG**C^{Me}**G)-4′	55.3
6′-h(G**C^{Me}**GTAG**C^{Me}**G)-4′	55.8

However, care should be taken when comparing the hybridization potential of different modifications. When the hexitol polypurine sequence **26** is used for the evaluation, a clear-cut order in stability of the different duplexes is seen with HNA:DNA < HNA:RNA < HNA:HNA (Table 2, line 3). The same order of stability is noticed using the hexitol pyrimidine sequences **21**, **22** or **23** (Table 2, columns 3–5, respectively). Using the pyrimidine RNA sequence **20** (column 2), the order of affinity reads as RNA:DNA < RNA:RNA < RNA:HNA. With the purine RNA sequence **25** (line 2), however, the dsRNA interaction becomes stronger than the corresponding RNA:HNA interaction (43 versus 40 °C in 1 M NaCl). Comparing both HNA:RNA duplexes (**20**:**26** and **21**:**25**) one notices a 15 °C preference in 0.1 M NaCl, having the purine strand as the hexitol sequence versus the counterpart with the pyrimidine strand as the hexitol oligonucleotide. We believe the conformation of the HNA sequences to be governed by the constrained hexitol moiety. Molecular modelling studies indicated the hexitol purines with an axial base moiety to be energetically more stable than hexitol pyrimidines in the same conformation (Y.Maurinsh, H.Rosemeyer, R.Esnouf, J.Wang, G.Ceulemans, E.Lescrinier, C.Hendrix, R.Busson, F.Seela, A.Van Aerschot and P.Herdewijn, *Chem. Eur. J.*, submitted for publication). This axial orientation of the base moiety in hexitol monomers is necessary to allow base pairing with natural nucleic acids. The pyrimidine-containing hexitol oligomer is expected to form thermally less stable duplexes compared with the purine-containing oligomer (compare line 3 with column 3). Indeed, with the hexitol pyrimidine sequences **21**, **22** or **23**, no duplex formation could be noticed with the DNA complement, where the HNA:DNA duplex **26**:**19** gave a T_m of 31 °C in 1 M NaCl. The same conclusions hold when comparing the duplexes with the RNA complement. Striking as well, is the difference in stability noticed for both DNA:RNA complexes **19**:**25** and **24**:**20**, with a clear preference for the former with the purine sequence as the RNA oligonucleotide (31.5 versus 13 °C in 1 M NaCl).

It should be mentioned, however, that the melting profile of the aforementioned duplexes is not always uniform. This may confirm the previous observation that the introduction of modified bases (*in casu* 5-methylcytosine and 2,6-diaminopurine) may influence the cooperativity of the melting process. The melting curves of duplexes indicated with [c] are broader than the other melting profiles (Fig. 1). The reason for this non-uniform melting is not clear. A possible explanation might be the occurence of polymorphism (parallel and anti-parallel associations) or the formation of associations of a higher order. Therefore, some of these measurements were repeated using a somewhat longer mixed sequence (octamer). Hereby, we focused on complexes between HNA and RNA (as the latter are the prime targets for antisense oligonucleotides) and on the effect of the U, C^{Me} and D substitutions. In this case more uniform melting profiles were obtained. The incorporation of one diaminopurine base gave an increase in T_m of 6 °C. The stabilizing effect of introducing a methyl group in the 5 position of the uracil base is observed by a 2 °C increase in T_m (Table 3). However, when the C^{Me} hexitol nucleoside is incorporated at the 2 position, no effect on duplex stability is observed, neither on the normal HNA:RNA duplex nor on the D-containing HNA:RNA duplex. At first sight, this was somewhat surprising and must be attributed to a sequence-selective effect. Indeed, in order to have a beneficial effect on stacking, the newly introduced methyl group (of C^{Me}) should be able to get involved in hydrophobic interactions with the neighbouring guanine base. When evaluating an NMR model of this HNA:RNA sequence (E.Lescrinier, J.Schraml, R.Busson, H.Heus, C.Hilbers and P.Herdewijn, in preparation), strong interstrand G-G stacking dominates the conformation of the ends of the duplex. This means that the methyl of the 5-methylcytosine base is situated out of the plane of the neighbouring guanine base so that it cannot contribute to the duplex stability, explaining the experimental observation (Fig. 2A). Therefore, we introduced a 5-methylcytosine base at the penultimate position where it should have the opportunity to interact with the five-membered ring moiety of the guanine neighbour (Fig. 2B). In this case a higher T_m was observed. Introduction of a second C^{Me} group further increased T_m marginally.

Finally, we also evaluated the specificity of the C^{Me}-G and D-U pairing system within HNA:RNA duplexes. The same sequence [6′-h(GCGTAGCG)-4′] was used to substitute D for A and C^{Me}

for C. The melting curves of HNA:RNA duplexes with D-A, D-G, C-C^{Me}, A-C^{Me} and U-C^{Me} mismatches show irregular profiles with several transitions and low thermal stabilities (between 30 and 35°C). Only the D-C mismatch gives a higher T_m value, but still 8°C lower than the T_m of the corresponding regular duplex. The large destabilization of the mismatch duplexes demonstrates the consistency of the Watson–Crick base pairing system within HNA-containing complexes.

CONCLUSION

The increased stability of HNA:RNA, as compared with dsDNA, dsRNA and RNA:DNA complexes, is attributed to the conformational pre-organization of ssHNA in an A-type helical shape. In contrast to RNA and DNA, HNA has a six-membered carbohydrate moiety. Increase in duplex stability can also be obtained by base modifications (i.e. by increasing stacking interactions and/or by increasing the strength of the hydrogen bonding network). By substituting hT for hU, hC^{Me} for hC and hD for hA, we demonstrate that both stabilizing factors (i.e. sugar and base modification) work additively and this finding increases the potentiality of HNA to function as a steric blocker in an antisense strategy. The experiments, likewise, demonstrate the sequence-dependent differences in stabilization effects of modified bases within HNA sequences. Finally, the contribution of purine and pyrimidine nucleosides to the stability of oligonucleotide duplexes is dependent on the structure of the phosphorylated sugar from which the backbone is constructed (i.e. HNA:RNA or RNA:HNA of identical sequence).

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REFERENCES

1 Van Aerschot,A., Verheggen,I., Hendrix,C. and Herdewijn P. (1995) *Angew. Chem. Int. Edn Engl.*, **34**, 1338–1339.
2 Hendrix,C., Rosemeyer,H., De Bouvere,B., Van Aerschot,A., Seela,F. and Herdewijn,P. (1997) *Chem. Eur. J.*, **3**, 1513–1520.
3 Hendrix,C., Rosemeyer,H., Verheggen,I., Seela,F., Van Aerschot,A. and Herdewijn,P. (1997) *Chem. Eur. J.*, **3**, 110–120.
4 Luyten,I. and Herdewijn,P. (1998) *Eur. J. Med. Chem.*, **33**, 515–576.
5 Uesugi,S., Miyashiro,H., Tomita,K.-i. and Ikehara,M. (1986) *Chem. Pharm. Bull.*, **34**, 51–60.
6 Ono,A. and Ueda,T. (1987) *Nucleic Acids Res.*, **15**, 219–232.
7 Walker,G.T. (1988) *Nucleic Acids Res.*, **16**, 3091–3099.
8 Richardson,F.C., Boucheron,J.A., Skopek,T.R. and Swenberg,J.A. (1989) *J. Biol. Chem.*, **264**, 838–841.
9 Brennan,C.A. and Gumport,R.I. (1985) *Nucleic Acids Res.*, **13**, 8665–8684.
10 Chollet,A., Chopllet-Domerius,A. and Kawashima,E.H. (1986) *Chem. Scripta*, **26**, 37–40.
11 Cheong,C., Tinoco,I.,Jr and Chollet A. (1988) *Nucleic Acids Res.*, **16**, 5115–5122.
12 Gryaznov,S. and Schultz,R.G. (1994) *Tetrahedron Lett.*, **35**, 2489–2492.
13 De Bouvere,B., Kerremans,L., Rozenski,J., Janssen,G., Van Aerschot,A., Claes,P., Busson,R. and Herdewijn,P. (1997) *Liebigs Ann./Recueil*, 1413–1461.
14 Verheggen,I., Van Aerschot,A., Toppet,S., Snoeck,R., Janssen,G., Balzarini,J., De Clercq,E. and Herdewijn P. (1993) *J. Med. Chem.*, **36**, 2033–2040.
15 Reddy,M.P., Hann,N.B. and Farooqui,F. (1994) *Tetrahedron Lett.*, **35**, 4311–4314.
16 Sung,W.L. (1998) *J. Chem. Soc. Chem. Commun.*, **1**, 1089.
17 Ti,G.S., Gaffney,B.L. and Jones R.A. (1982) *J. Am. Chem. Soc.*, **104**, 1316.
18 Busson,R., Kerremans,L., Van Aerschot,A., Peeters,M., Blaton,N. and Herdewijn P. (1999) *Nucleosides Nucleotides*, in press.
19 Strobel,S.A., Cech,T.R., Usman,N. and Beigelman. L. (1994) *Biochemistry*, **33**, 13824–13835.