Nucleic Acids Research, 1999, Vol. 27, No. 9 **1991–1998**

Theoretical calculations, synthesis and base pairing properties of oligonucleotides containing 8-amino-2′-deoxyadenosine

Ramón Güimil García, Elisenda Ferrer, Maria J. Macías, Ramon Eritja* and Modesto Orozco[1]

European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany and [1]Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

Received January 21, 1999; Revised and Accepted March 8, 1999

ABSTRACT

Theoretical calculations on double and triple helices containing 8-amino-2′-deoxyadenosine were made to analyze the possible differences in base pairing properties between 8-aminoadenine and adenine. These calculations indicate a strong preferential stabilization of the triplex over the duplex when adenine is replaced by 8-aminoadenine. In addition, a protected phosphoramidite derivative of 8-amino-2′-deoxyadenosine was prepared for the introduction of 8-aminoadenine into synthetic oligonucleotides using the phosphite-triester approach. DNA triple helical structures are normally observed at acidic pH. However, when oligonucleotides carrying 8-aminoadenine are used, very stable triple helical structures can be observed even at neutral pH. Biological applications of triple helices could benefit from the use of 8-aminoadenine derivatives.

INTRODUCTION

Several years ago, oligonucleotides that could bind on homopurine–homopyrimidine sequences of double-stranded DNA by forming triple helices were described. The formation of nucleic acid triple helices opens the way to designing sequence-specific DNA-binding molecules which may have therapeutic and diagnostic uses such as antigene therapy (1,2). Due to these potential uses of triple helices, a lot of effort has been put into the design and preparation of modified oligonucleotides in order to enhance triple helix stability (1,2). One of the most successful modifications was to replace natural bases with some modified bases such as 5-methylcytidine (3,4), 5-bromouracil (5,6), 5-aminouracil (7), N^4-spermine-5-methylcytidine (8) or 5-methyl-2,6(1*H*,3*H*)-pyrimidinedione (9). Due to the lack of structural information, all chemical modifications were made after screening a large number of derivatives.

A detailed picture of the DNA triplex in solution, including the specific pattern of hydration which may be used for the design of new bases with triplex stabilization properties, was recently obtained by molecular dynamics simulations (10). Average triplex structures in Shields *et al*. (10) displayed the existence of a small groove [the minor-Major (mM) groove] in the surroundings of the C8 of adenines. Such a narrow groove has a defined pattern of hydration and a region of large electronegative molecular interaction potential (see fig. 3 in ref. 10). These structural features suggest that a notable increase in the stability of the triplex can be achieved by introducing a polar group near C8, which will replace highly ordered water molecules in the mM groove. The stabilization should increase if the group displays a positive electrostatic potential so that it can interact with the negative potential of the DNA in the mM groove. Analysis of a d(A·T·T) triad suggests that the introduction of an amino group at C8 might lead to the formation of an additional A·T (Hoogsteen) hydrogen bond (H-bond), which should add extra stabilization to the triple helix (Fig. 1). A similar model was suggested by Kumar *et al*. (11) and, recently, a preliminary study on the stability of triple helices containing 8-aminoadenine has been reported (12). In order to test this hypothesis, quantum mechanics, molecular dynamics and thermodynamic integration techniques were used in the analysis of a triplex containing 8-aminoadenine (**M**). In addition, oligodeoxynucleotides carrying 8-aminoadenine were prepared and their triple helix binding properties were determined. Substitution of A by 8-aminoadenine increases the stability of the triple helix, as predicted in our theoretical calculations.

MATERIALS AND METHODS

Molecular modeling

The Insight-II computer package (13) was used for all the modeling. The Gaussian 94 (14) program was used for quantum mechanical (QM) calculations. Molecular dynamics (MD) and MD-thermodynamic integration (TI) calculations were made using AMBER5.0 (15). All calculations used the ORIGIN-2000 parallel supercomputer of the Centre de Supercomputació de Catalunya, as well as work stations in our laboratory.

*To whom correspondence should be addressed. Tel: +49 6221 387210; Fax: +49 6221 387306; Email: eritja@embl-heidelberg.de

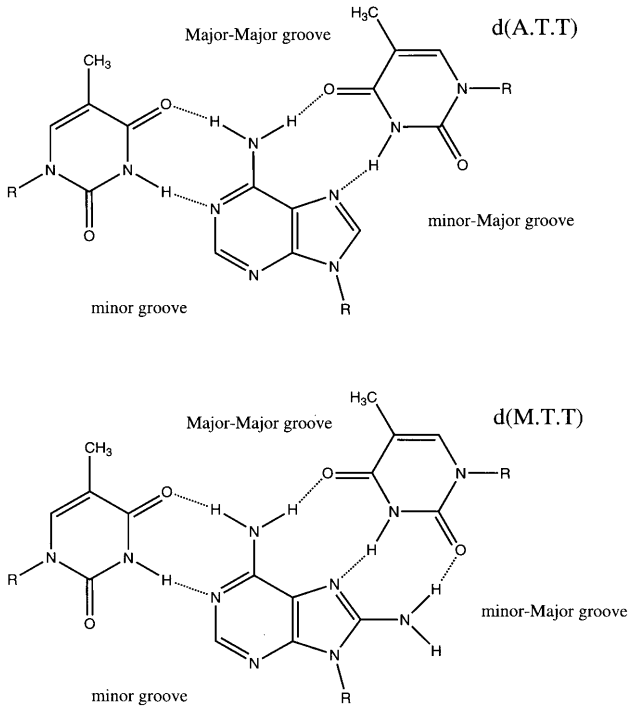


Figure 1. Schematic representation of A·T·T and **M**·T·T triads. The grooves generated in the structure of a triplex DNA are indicated following Shields *et al.* (10).

Theoretical calculations

QM calculations. The first stage in the theoretical study was to determine the suitability of the H-bond pattern shown in Figure 1 and the contribution of the third H-bond to the stability of **M**-T (Hoogsteen pairing). This was explored using QM calculations. In particular, we determined the binding energies of the A-T (Hoogsteen) and **M**-T (Hoogsteen) dimers at the B3LYP/6-31G(d) level (16). The selection of this DFT level of calculation follows previous studies which demonstrated its accuracy in the determination of H-bonding energies in related systems (17). In all cases, geometries of dimers and monomers were fully optimized at the same level and BSSE errors were corrected using the counterpoise method (18).

MD and free energy calculations. Free energy calculations evaluated whether the presence of an amino group at position 8 of adenine induces greater stabilization in the triple helix than in the parent duplex. Calculations used TI coupled with MD simulations.

A standard B-DNA d(A·T)$_{10}$ duplex and a DNA triplex of sequence d(A·T·T)$_{10}$ were generated as follows. The duplex structure was constructed using Arnott's standard fibber parameters (19), as implemented in Insight-II (13). For the triplex structure a MD-averaged structure of the d(A·T·T)$_{10}$ triplex was used as template (10). In both cases the fifth adenine was substituted by 8-aminoadenine using Insight-II, while the rest of the bases remained unchanged in the model.

Duplex and triplex structures were immersed in a water bath and, in order to obtain electroneutral systems, Na^+ ions were added (10). The duplex system contains 3211 water molecules and 18 Na^+ ions while the triplex system has 4207 water molecules and 27 Na^+ ions, for a total of 10 177 and 13 607 atoms, respectively. 5000 cycles of minimization were applied to both systems prior to the heating and relaxation process during 130 ps. The heating and relaxation process consisted of seven stages: (i) 10 ps of MD (T = 100 K, P = 1 atm) with restraints of 100 kcal/mol Å^2 in DNA and sodium; (ii) 10 ps of MD (T = 100 K, P = 1 atm) with restraints of 100 kcal/mol Å^2 in DNA; (iii) 10 ps of MD (T = 300 K, P = 1 atm) with restraints of 100 kcal/mol Å^2 in DNA; (iv) 25 ps of MD (T = 300 K, P = 1 atm) with restraints of 50 kcal/mol Å^2 in DNA; (v) 25 ps of MD (T = 300 K, P = 1 atm) with restraints of 25 kcal/mol Å^2 in DNA; (vi) 25 ps of MD (T = 300 K, P = 1 atm) with restraints of 10 kcal/mol Å^2 in DNA; (vii) 25 ps of MD (T = 300 K, P = 1 atm) with restraints of 5 kcal/mol Å^2 in DNA. After these stages the system was equilibrated [the starting model for triplex DNA had already been equilibrated for 1 ns (see ref. 10), so 100 ps of free MD were considered enough to equilibrate the system fully] through 100 (triplex) and 300 (duplex) ps of non-restrained MD at constant pressure (1 atm) and temperature (298°C).

Equilibrated duplex and triplex structures were the starting point for two MD-TI studies (20). In MD-TI calculations the free energy change relating to the **M**→A mutation in a given environment is computed as the reversible work necessary to mutate an 8-aminoadenine molecule into an A molecule. The mutation coordinate is then defined as the mixing parameter (λ) which determines the force field parameters defining the mutated residue: $V_\lambda = \lambda V_{\mathbf{M}} + (1 + \lambda)V_A$. These calculations give us a first approximation to the relative stability of the triplex arising from the substitution of a central adenine by 8-aminoadenine under physiological conditions. Simulations were always performed in the **M**→A direction using 21 windows. Each window consisted of 10 ps of equilibration and 10 ps of averaging for a total of 420 ps in each mutation study. Not only inter-group contributions to the free energy difference, but also the non-bonded intra-group contributions and the bond PMF corrections (21) were considered.

All simulations (equilibration MD and MD-TI) used periodic boundary conditions and the particle-mesh Ewal approach (22) to introduce long-range electrostatic effects. All bond lengths were frozen using SHAKE (23), which allows the use of a 2 fs time step for integration. In all cases the DNA structures were represented using AMBER-95 force field parameters (24,25), while water was represented by means of the well-known TIP3P model (24,25). Atomic changes for 8-aminoadenine were determined (data available on request) using the standard RESP strategy (26).

Chemicals

Protected phosphoramidites and DNA synthesis reagents were purchased from Perkin Elmer-Applied Biosystems (USA). Nucleosides were purchased from Pharma-Waldorf (Germany). Anhydrous solvents were obtained from SDS (France). Phosphodiesterase and alkaline phosphatase were obtained from Boehringer Mannheim (Germany). The rest of the chemicals were from Fluka (Switzerland) and Aldrich (USA) and were used without further purification. 8-Amino-2′-deoxyadenosine (**1**) was prepared following previously described protocols (27,28).

8-Amino-2′-deoxy-N^2,N^8-bis(dimethylaminomethyliden) adenosine (2)

8-Amino-2′-deoxyadenosine (27,28) (**1**) (3.25 g, 12.2 mmol) was suspended in 200 ml of methanol and 8.2 ml of *N*,*N*-dimethylformamide dimethylacetal (61 mmol) were added. The mixture was stirred overnight at room temperature. The reaction mixture was concentrated to dryness and the residue was dissolved in 10% ethanol (EtOH) in dichloromethane (DCM) and purified on a silica gel column eluted with 15% EtOH in DCM. Yield 3.52 g (9.35 mmol, 76%). TLC (10% EtOH in DCM) R_f 0.29. UV (H_2O) λ_{max} 267, 334 nm. ^{1}H-NMR (DMSO-d_6) δ_H: 8.81 (1H, s), 8.66 (1H, s), 8.17 (1H, s), 6.57 (1H, t), 5.55 (1H, m), 5.25 (2H, m), 4.46 (1H, m), 3.86 (1H, m), 3.5–3.6 (2H, m), 3.2–3.05 (13H, 3s and 1m), 2.05 (1H, m). ^{13}C-NMR (DMSO-d_6) δ_C: 157.6, 157.1, 156.0, 155.6, 151.6, 148.4, 124.7, 87.8, 82.9, 71.7, 62.6, 40.4 (under solvent peak), 37.1, 34.5, 34.4. Analysis of $C_{16}H_{24}N_8O_3.½H_2O$: predicted, C 49.86, H 6.54, N 29.07; found, C 49.52, H 6.57, N 28.88.

8-Amino-2′-deoxy-5′-*O*-dimethoxytrityl-N^2,N^8-bis(dimethylaminomethyliden) adenosine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite (4)

Compound **2** (1.0 g, 2.65 mmol) was dissolved in 50 ml of dry pyridine and 1.08 g of dimethoxytrityl chloride (3.18 mmol) were added. The mixture was stirred for 2 h at room temperature. To the reaction mixture 1 ml of methanol was added and the solution was concentrated to dryness. The residue was dissolved in DCM and washed with 5% $NaCO_3H$ and saturated NaCl aqueous solution. The organic phase was dried with anhydrous Na_2SO_4 and concentrated to dryness. The residue was purified by chromatography on silica gel. The column was packed with silica gel using a 1% triethylamine solution in DCM. The product was eluted with a 0–4% methanol gradient in DCM. Compound **3** was obtained as a white foam (1.5 g, 2.21 mmol, 83%). TLC (5% EtOH/DCM) R_f 0.6.^{1}H-NMR (Cl_3CD) δ_H: 8.71 (1H, s), 8.68 (1H, s), 8.14 (1H, s), 7.5–7.1 (9H, m), 6.8–6.6 (5H, m), 4.85 (1H, m), 4.18 (1H, m), 3.69 (6H, s), 3.4 (2H, m), 3.16, 3.07, 3.02, 2.93 (13H, 4 s and 1m), 2.2 (1H, m). ^{13}C-NMR (Cl_3CD) δ_C: 158.1, 157.5, 156.8, 156.7, 155.3, 152.1, 149.2, 144.8, 136.0, 129.9, 128.0, 127.4, 126.4, 124.8, 112.8, 85.9, 85.3, 82.4, 73.2, 64.4, 55.0, 40.8, 40.6, 36.4, 34.8, 34.6.

Compound **3** (1.16 g, 1.7 mmol) and diisopropylethylamine (0.9 ml, 5.1 mmol) were dissolved in 20 ml of dry acetonitrile. The solution was cooled with an ice bath and 0.57 ml of 2-cyanoethoxy-*N*,*N*-diisopropylamino-chlorophosphine (2.55 mmol) were added. After 1 h of magnetic stirring at room temperature, the mixture was concentrated to dryness. The residue was dissolved in DCM and washed with 5% $NaCO_3H$ aqueous solution. The organic phase was dried with anhydrous Na_2SO_4 and concentrated to dryness. The residue was purified on silica gel. The column was packed using 1% triethylamine solution in DCM/hexane 9:1 and the product was eluted with DCM/hexane 9:1. Compound **4** was obtained as a white foam. Yield 1.4 g (1.59 mmol, 93%). TLC (DCM/hexane 9:1) R_f 0.8. ^{1}H-NMR as described in Kawai *et al.* (12). ^{31}P-NMR (Cl_3CD) δ_P: 144.96. Analysis of $C_{46}H_{59}N_{10}O_6P$: predicted, C 62.86, H 6.77, N 15.93; found, C 62.58, H 6.80, N 15.64.

Deprotection studies

Aliquots of dinucleotide supports containing 8-amino-deoxyadenosine (5′-**M**T-3′) were treated with concentrated ammonia either at room temperature or at 55°C. At different intervals, the ammonia solutions were filtered, concentrated to dryness and analyzed by HPLC. HPLC conditions are described below. A 30 min linear gradient from 0 to 60% B was used. Elution at 260 nm was followed.

Dimer **M**T. HPLC: retention time 17.2 min; UV_{max} 269 nm. Mass spectra found 570.4 (expected for $C_{20}H_{27}N_8O_{10}P$: 570.4).

Dimer **M**T containing a N^6,N^8-bis-dimethylaminomethyliden (dmf) group. HPLC: retention time 18.8 min. UV_{max} 270, 305 nm. Mass spectra found 625.3 (expected for $C_{23}H_{32}N_9O_{10}P$: 625.5).

Dimer **M**T containing two dmf groups. HPLC: retention time: 20 min. UV_{max} 266, 335 nm. Mass spectra found 679.3 (expected for $C_{26}H_{37}N_{10}O_{10}P$: 680.5).

Oligonucleotide synthesis

Oligonucleotides were prepared on an automatic DNA synthesizer using standard 2-cyanoethyl phosphoramidites and the modified phosphoramidite **4**. Complementary pentadecamers and undecamers containing natural bases were also prepared using commercially available chemicals and following standard protocols. After the assembly of the sequences, the oligonucleotide supports were treated with 32% aqueous ammonia at 55°C for 16 h. Ammonia solutions were concentrated to dryness and the products were purified by reverse phase HPLC. Dimer A and pentamer B were prepared without the last DMT group (DMT-off protocol) on 1 µmol scale. The remaining oligonucleotides were synthesized on 0.2 µmol scale and with the last DMT group at the 5′-end (DMT-on protocol) to help reverse phase purification. All purified products presented a major peak which was collected and analyzed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis of the nucleosides (HPLC conditions B). The retention times of nucleosides obtained after enzyme digestion were: dC, 4.6 min; dG, 8.4 min; T, 9.2 min; dA, 17.6 min; 8-amino-2′-deoxyadenosine, 21.2 min. Mass spectra: sequence B, found 1501.8 (expected for $C_{49}H_{63}N_{21}O_{27}P_4$ 1501.9); sequence C, found 4579 (expected for $C_{146}H_{185}N_{59}O_{86}P_{14}$ 4575.5). Yield (OD units at 260 nm after HPLC purification, 0.2 µmol): sequence B, 6.3 OD; sequence C, 10.4 OD; sequence D, 16.8 OD; sequence E, 16.9 OD; sequence F, 13.5 OD; sequence G, 9.6 OD.

HPLC conditions

HPLC solutions were as follows: solvent A, 5% ACN in 100 mM triethylammonium acetate (pH 6.5); solvent B, 70% ACN in 100 mM triethylammonium acetate (pH 6.5). For analytical runs the following conditions were used: column, Nucleosil 120C_{18}, 250 × 4 mm; flow rate, 1 ml/min; condition A, a 40 min linear gradient from 0 to 75% B; condition B, a 20 min linear gradient from 0 to 20% B. For preparative runs the following conditions were used: column, PRP-1 (Hamilton), 250 × 10 mm; flow rate, 3 ml/min; 30 min linear gradient from 10 to 80% B (DMT-on) or a 30 min linear gradient from 0 to 50% B (DMT-off)

Melting experiments

Melting experiments of pentadecamer duplexes were carried out by mixing equimolar amounts of two pentadecamer strands

dissolved in a solution containing 0.15 M NaCl, 0.05 N Tris–HCl buffer, pH 7.5. Duplexes were annealed by slow cooling from 80 to 4°C. UV absorption spectra and melting curves (absorbance versus temperature) were recorded in 1 cm pathlength cells using a Varian Cary 13 spectrophotometer having a temperature controller with a programmed temperature increase of 0.5°C/min. Melts were run on duplex concentrations of 4 μM at 260 nm.

Melting experiments with triple helix were performed as follows. Solutions of equimolar amounts of the hairpin oligonucleotide (h_{26}) and the 11mer (s_{11}) were mixed in the appropriate buffer. The solutions were heated to 80°C, allowed to cool slowly to room temperature and then samples were kept in the refrigerator overnight. UV absorption spectra and melting experiments (absorbance versus temperature) were recorded in 1 cm pathlength cells using a spectrophotometer having a temperature controller with a programmed temperature increase of 0.5°C/min. Melts were run on duplex concentrations of 4 μM at 270 nm.

RESULTS AND DISCUSSION

QM calculations

The first stage in the theoretical study was to determine the suitability of the H-bond pattern shown in Figure 1 and the contribution of the third H-bond to the stability of **M**-T (Hoogsteen pairing). This was explored using QM calculations. In particular, we determined the binding energies of the A-T (Hoogsteen) and **M**-T (Hoogsteen) dimers at the B3LYP/6-31G(d) level (16). Results in Figure 2 demonstrate the stability of the two dimers and of the expected H-bonds. For instance, for the **M**·T (Hoogsteen) pairing the calculated H-bond distances are 2.95, 2.89 and 2.95 Å for the N6–O4, N7–N3 and N8–O2 H-bonds. These distances are similar to those found for the A·T (Hoogsteen) dimer, which are 2.98 (N6–O4) and 2.85 Å (N7–N3), and are consistent with those expected for strong H-bond interactions.

The stabilization energy determined for the A·T Hoogsteen pairing is –12.7 kcal/mol (after distortion and BSSE corrections) and a value of –17.9 kcal/mol is reached for the **M**·T Hoogsteen pairing (after distortion and BSSE corrections). The extra H-bond present in the **M**·T (Hoogsteen) dimer is very strong and it seems to contribute to the stability of the purine–pyrimidine (Hoogsteen) dimer. Remarkably, the difference in stability between the A·T (Hoogsteen) and **M**·T (Hoogsteen) dimers (–5.2 kcal/mol from Fig. 2) is similar to the average energy × H-bonds in the A·T (Hoogsteen) and **M**-T (Hoogsteen) dimers (~6 kcal/mol). This result demonstrates that the formation of the third H-bond leads neither to unfavorable contacts nor to distortions of dimer geometries.

M·T (Hoogsteen) binding energy determined from B3LYP/6-31G(d) calculations cannot be compared directly with experimental data to verify the correctness of the theoretical estimate. However, an early study of heterodimerization of adenine and thymine in the gas phase suggests (19) an enthalpy of dimerization of –13 kcal/mol, which is consistent with our theoretical estimation of –12.7 kcal/mol and gives additional support to the B3LYP/6-31G(d) calculations presented in this paper.

B3LYP/6-31G(d) calculations were also performed to investigate the role of the extra 8-amino group in the Watson–Crick binding of adenine and thymine. Geometry optimization (data not shown) is not able to find any significant change in dimer geometry. Thus, for the Watson–Crick A:T dimer, the H-bonds are 2.95 Å for the N6–O4 and 2.88 Å for the N1–N3 bond. Almost the same values (2.96 and 2.87 Å) are found for the **M**·T Watson–Crick dimer.

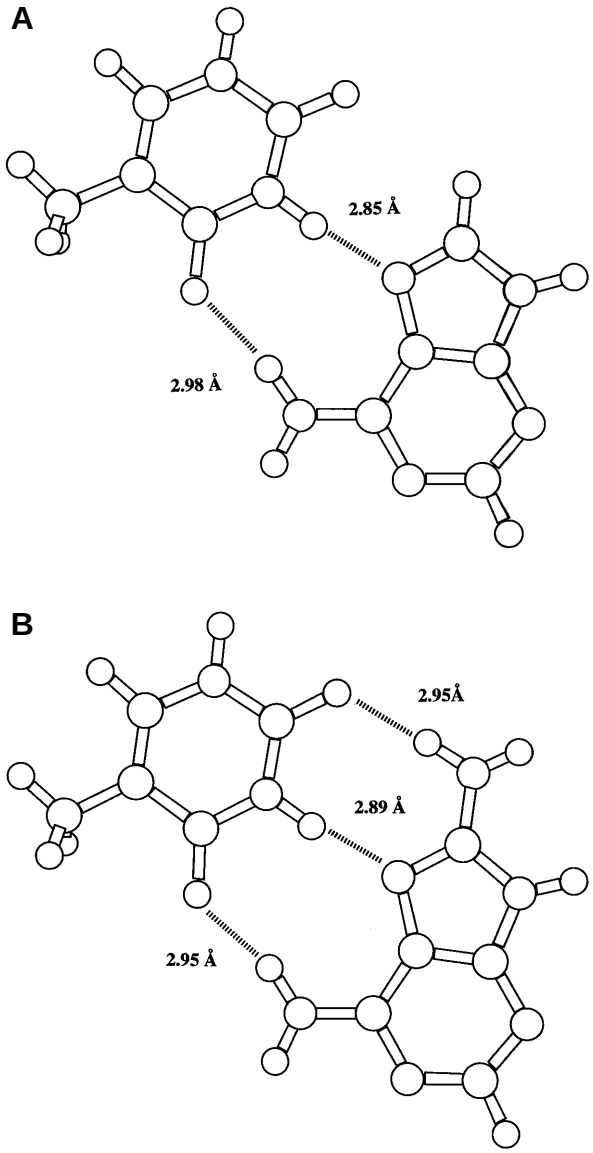


Figure 2. B3LYP/6-31G(d) optimized geometries for Hoogsteen A·T and **M**·T pairs in the vacuum.

Dimerization energy for the Watson–Crick pairing of A·T is –12.1 kcal/mol, which compares with –12.7 kcal/mol found for the most stable Hoogsteen pairing. The Watson–Crick pairing of **M**·T has a stabilization energy of –11.7 kcal/mol, clearly less than the Hoogsteen binding of **M**·T (–17.9 kcal/mol), reflecting the fact that the Hoogsteen binding is stabilized for **M**·T by one more H-bond than the Watson–Crick binding. Comparison of **M**·T and A·T Watson–Crick pairing reveals that the presence of the amino group at position 8 leads to a slight decrease (0.4 kcal/mol) in the stability of the pairing, due probably to a decrease in the acidity of the purine N6 group.

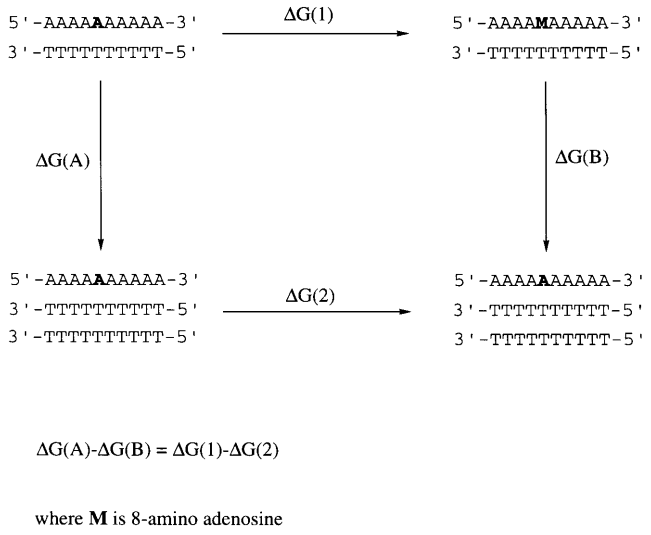


Figure 3. Schematic representation of the thermodynamic cycle used to compute the stabilization of the triplex due to the replacement of an adenine by 8-aminoadenine. Note that the magnitude in a physical sense is $\Delta G(A) - \Delta G(B)$, while the magnitude computed from simulations is $\Delta G(1) - \Delta G(2)$.

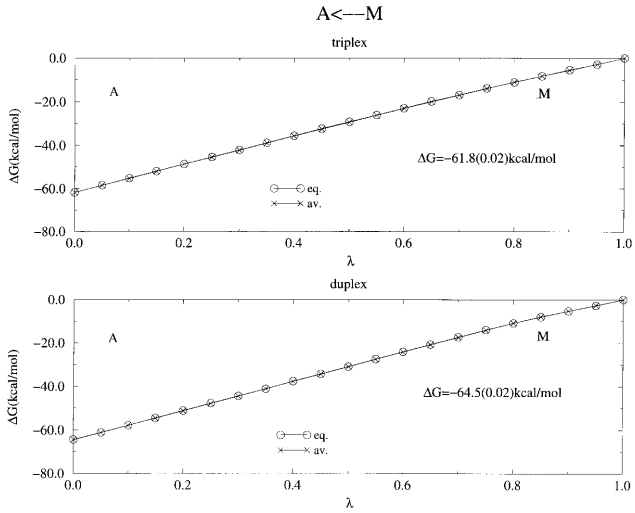


Figure 4. Free energy profile for the mutation from 8-aminoadenine ($\lambda = 1$) to adenine ($\lambda = 0$) in the triplex and duplex systems.

MD and MD-TI calculations

Quantum mechanical calculations suggest that the replacement of A by **M** can lead to the formation of an extra H-bond which stabilizes *in vacuo* the purine·pyrimidine Hoogsteen H-bond. However, 8-aminoadenine behavior in solution cannot be completely described with this method because water greatly hinders the formation of H-bonds. For a more direct estimate, MD-TI calculations were performed. MD-TI calculations can provide a direct estimate of the influence of the replacement of an A by **M** on triplex stability, by computing the differences in the reversible work necessary to replace an **M** by A in a duplex and in a triplex DNA (Fig. 3). Calculations were performed on a standard B-DNA $d(A{\cdot}T)_{10}$ duplex and a DNA triplex of sequence $d(A{\cdot}T{\cdot}T)_{10}$.

Change of H8 by an amino group does not lead to any significant difference in duplex or triplex geometry. The Watson–Crick and Hoogsteen H-bonds were preserved during all the simulations. The average **M**·T (WC) and **M**·T (Hoogsteen) H-bond distances found in the MD are close to the optimum values determined from gas phase calculations. The amino group is placed in the mM groove of the triplex and seems well hydrated.

Mutations were done in the 8-aminoadenine ($\lambda = 1$) to adenine ($\lambda = 0$) direction. The free energy profiles for the duplex and triplex were smooth, without apparent discontinuity. When the free energy values were computed with the first 10 ps of each window (equilibration part) or with the last 10 ps (averaging part), almost the same free energy profiles were obtained (to <0.1 kcal/mol), confirming the suitability of the sampling protocol used to compute the free energy profile (Fig. 4). There is always concern in TI or FEP calculations about the dependence of free energy differences on the length of the simulation. The perturbation studied here is moderate and implies only small changes in geometry, so 420 ps of trajectory seems reasonable. Test calculations were performed in which the 'free energy' difference after seven windows of **M**→A mutation in the duplex were determined for shorter (equivalent to 210 ps) and longer (equivalent to 1050 ps) runs. The results obtained were 14.2 and 14.8 kcal/mol, respectively, which compare very well with the estimate of 14.1 kcal/mol for the 420 ps trajectory. It seems then that the free energy values reported here converge reasonably. The numbers obtained in the mutations are large due to the introduction of intra-group contributions. Note that such contributions are the same in the duplex and triplex simulations and, accordingly, they should have little effect on the determination of the triplex-inducing effect of the 8-amino group.

Comparison of free profiles in the duplex and triplex simulations clearly demonstrates that the change from **M** is 2.7 kcal/mol more favorable in a duplex DNA than in a triplex DNA (Figs 4 and 5), which indicates that the presence of the **M** leads to a preferential stabilization of the triplex. In fact, the free energy difference of 2.7 kcal/mol detected here indicates a strong preferential stabilization of the triplex over the duplex as a result of the replacement of a single adenine by **M** in close accord with previous gas phase calculations. Note that the stabilization due to the amino group at position 8 is smaller in DNA in solution than the values obtained for an isolated base pair in the gas phase, confirming the well-known water interference with H-bond formation. However, when these systems are compared to other H-bonding systems (see for instance ref. 17) the effect of water interference on the **M**·T Hoogsteen H-bond is a lot less than usual, which suggests that the formation of the **M**·T Hoogsteen dimer is reasonable. In summary, we believe that the roughly 3 kcal/mol stabilization in the triplex due to the existence of a single 8-aminoadenine molecule is related not only to the existence of an extra H-bond, but also to the ability of the amino group to mimic a water molecule in the mM groove of triplex DNA (10).

Moreover, our molecular dynamic simulation results suggest that the change of a single A residue to an 8-aminoadenine in $d(A)_{10}$ does not introduce dramatic changes in the structural properties of the helix. The 8-amino group does not induce any change of conformation in the glycosidic bond and the amino group interacts nicely with water and the ribose moiety (O4′ and O5′). These results suggest that the A→**M** mutation is conservative in terms of DNA duplex structure. These results confirm QM

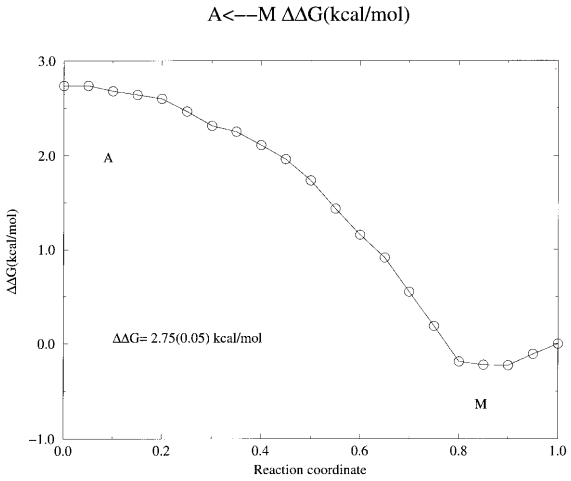


Figure 5. Differential (triplex–duplex) free energy profile for the mutation of 8-aminoadenine (λ = 1) into adenine (λ = 0).

calculations noted above, which demonstrate the similar stability of Watson–Crick A·T and **M**·T pairings.

In a similar manner, the introduction of the 8-amino group into a step of the $d(A{\cdot}T{\cdot}T)_{10}$ triplex does not lead to significant changes in the structure of the triplex. MD simulations during 500 ps allowed us to define an average structure (for details see ref. 10) which is very similar to that obtained for the pure $d(A{\cdot}T{\cdot}T)_{10}$ triplex. As in the duplex, the extra amino group seems reasonably solvated and establishes several H-bonding interactions with the ribose moiety and, to a lesser extent, with the phosphate group. In summary, MD suggests that a single A→**M** mutation in a poly(A) tract does not lead to dramatic alterations in the structure of the triplex.

Preparation of the phosphoramidite derivative and deprotection studies

The synthesis of the phosphoramidite derivative of **M** is illustrated in Figure 6. 8-Amino-2′-deoxyadenosine (**1**) was prepared by bromination of dA followed by azide displacement and catalytic hydrogenation of the resulting 8-azido-deoxyadenosine using previously described protocols (27,28). Reaction of **1** with dimethylaminoformamide dimethylacetal gave protected nucleoside **2** in excellent yield. The dmf nucleoside **2** was stable to silica gel purification. Subsequent dimethoxytritylation and phosphitylation gave the desired 8-aminoadenine building block in good yield. The same building block has been described elsewhere (12), but the route described here is shorter and yields are higher.

The stability of the dmf groups to ammonia was analyzed on the dimer 5′-**M**T-3′. The dimer was assembled on controlled pore glass using standard phosphoramidite protocols. Aliquots of dinucleotide supports were treated with concentrated ammonia at room temperature and at 55°C. HPLC analysis of the resulting products followed by mass spectrometry analysis of the products showed complete deprotection at 55°C in <3 h. At room temperature only one dmf group was removed rapidly and the dimer containing a single dmf group was slowly converted to the unprotected dimer. Other minor products due to exchange of the dmf groups to acetyl groups during the capping reaction were also

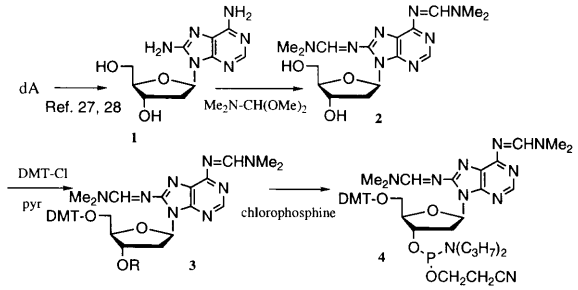


Figure 6. Preparation of 8-amino-2′-deoxyadenosine phosphoramidite.

observed. All these intermediate compounds were not detected when deprotection was performed at 55°C. Therefore, for the rest of the sequences containing **M**, all deprotection reactions were carried out overnight at 55°C.

Oligonucleotide synthesis

Oligonucleotide sequences containing **M** ranging from 5 to 26 bases (Table 1) were prepared using phosphoramidite chemistry on an automatic DNA synthesizer. Coupling efficiency of 8-aminoadenine phosphoramidite **4** was similar to the commercially available phosphoramidites. After standard deprotection with concentrated ammonia, the products were purified by reverse phase HPLC using the DMT-on and DMT-off protocols. In all cases a major peak was obtained and collected. The purified oligonucleotides were analyzed by mass spectrometry (electrospray and MALDI-TOF) and by enzyme digestion followed by HPLC analysis of the resulting nucleosides. In all cases the purified oligonucleotides were obtained in good yield and had the correct nucleoside composition and the expected mass.

Table 1. Oligonucleotide sequences containing 8-amino-2′-deoxyadenosine (**M**) prepared in this research

		Sequence (5′→3′)
A		**M**T
B		AG**M**CT
C		GCAATGGA**M**CCTCTA
D	$h_{26}1$	GAAGG**M**GGAGATTTTTCTCCTCCTTC
E	$h_{26}2$	GAAGG**M**GG**M**GATTTTTCTCCTCCTTC
F	$h_{26}3$	GA**M**GG**M**GG**M**GATTTTTCTCCTCCTTC
G	$h_{26}4$	G**MM**GG**M**GG**M**G**M**TTTTTCTCCTCCTTC

Base pairing properties

The hybridization properties of 8-aminoadenine were measured spectrophotometrically on a duplex formed by two complementary pentadecamers in which **M** is paired with the four natural bases (Table 2). 8-Aminoadenine forms the strongest base pairs with T as expected. This base pair is slightly less stable (1.6°C) than the natural A·T. Compared with adenine, 8-aminoadenine base pairs are between 1.6 and 6.4°C less stable than the A·T base pair, while the same base pairs with A were from 0 to 9.6°C less stable

than the A·T base pair. There is a tendency to level the melting temperatures of mismatches that comes from a small decrease in the stability of the **M**·T (1.6°C compared to A·T) base pair and an increase in the **M**·C (3°C compared to A·C) and **M**·A (2.9°C compared to A·A) base pairs. This tendency to similar melting temperatures for the different mismatches may assist in the design of degenerate primers or probes. This possibility was investigated with primers containing multiple 8-aminoadenine mismatches for PCR reactions. Unfortunately, no special properties were found in the oligonucleotides carrying **M** as against the same primers carrying A (data not shown), so precluding its use as a universal base.

Table 2. Melting temperatures (°C) of duplexes containing 8-amino-2′-deoxyadenosine (**M**) base pairs (0.15 M NaCl, 0.05 N Tris–HCl buffer, pH 7.5)

	Y[a]		
X[a]	**M**	A	G
A	52.6	49.7	54.2
C	54.5	51.5	60.9
T	57.7	59.3	51.3
G	54.9	54.8	56.8

[a]5′-TAGAGG**X**TCCATTGC-3′, 3′-ATCTCC**Y**AGGTAACG-5′

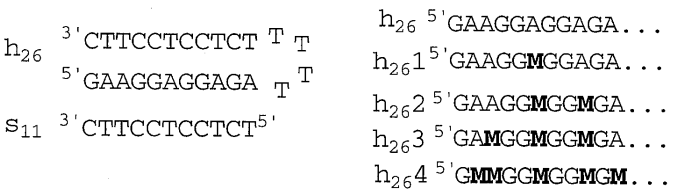
QM calculations suggest that the Watson–Crick pairing of **M**·T is slightly less favorable than A·T. This and the large desolvation penalty upon binding of 8-aminoadenine justify that duplexes containing **M** are less stable than those with A. Preliminary NMR and circular dichroism analysis of duplexes containing **M**·T base pairs show no major changes in the duplex structure on the introduction of 8-aminoadenine (data not shown). In summary, neither experimental nor theoretical results suggest that changes of adenine to **M** cause any dramatic effect on duplex structure.

Triple helix stabilization properties of **M** were investigated using a triple helix model formed by a self-complementary hairpin of 26 bases (h_{26}) and an all-pyrimidine single-strand oligonucleotide (s_{11}) described previously (3). Sequential replacement of A by **M** in triple helix results in a stabilization of 5–8°C per substitution in the range from pH 5.5 to 7.0, except when the number of **M** substitutions goes from three to five residues (Table 3). In any case, the observation of triple helical structures at neutral pH becomes possible only when the **M**-modified base is used. The pH dependence of triplex stability is due to the presence of G·C·C^+ triads. These results corroborate those of Kawai *et al.* (12) obtained on a three-strand triple helix. The degree of stabilization and its additive character are consistent with the existence of an extra hydrogen bond between the 8-amino group of 8-aminoadenine and the 2-keto group of T (Fig. 1) and with theoretical calculations reported above both in the gas phase and in aqueous solution.

CONCLUSIONS

In summary, we predicted by theoretical calculations that the introduction of an amino group at position 8 of adenine would broadly stabilize triple helix formation, due to the combined effect of the gain in one Hoogsteen purine–pyrimidine H-bond and the ability of the amino group to be integrated into the ‘spine of hydration’ located in the mM groove of the triplex structure. Oligonucleotides carrying **M** residues were synthesized using protected phosphoramidite **4**. As we predicted, these oligonucleotides form very stable triple helices. As a matter of fact, the degree of stabilization is one of the highest reported for a non-natural base analog and it could be further increased by adding other reported stabilizing molecules such as intercalating compounds (1,2). The high degree of stabilization obtained by this simple substitution is especially relevant to the development of new applications based on triple helix formation such as structural studies, DNA-based diagnostic tools and antigene therapy (1,2). However, it should not be overlooked that applications using DNA from natural sources such as control of gene expression (1) may not benefit from the use of 8-aminoadenine. Since the modified purine is on the target strand, the direct applicability of this modification in certain applications may be limited. One possible way to overcome this problem may be the preparation of oligonucleotides containing the purine Watson–Crick strand (with 8-aminoadenine) linked with the pyrimidine Hoogsteen strand. If the polarity of the strands is correct, these oligonucleotides may bind the pyrimidine Watson–Crick strand and leave the natural purine Watson–Crick strand unpaired due to the exceptionally high stability of 8-aminoadenine triplexes. Experiments along these lines are currently being undertaken.

Table 3. Melting temperatures[a] (°C) for the triplex h_{26}:s_{11} containing 8-amino-2′-deoxyadenosine (**M**)



Sequence	**M**[b]	pH 5.5	pH 6.0	pH 6.5	pH 7.0
h_{26}:s_{11}	0	40	~20	–	–
h_{26}1:s_{11}	1	48.3	30.9	24	–
h_{26}2:s_{11}	2	56	41.8	32.4	22.2
h_{26}3:s_{11}	3	65.2	48.7	39.6	26.9
h_{26}4:s_{11}	5	68.3	51.9	43.5	32.2

[a]1 M NaCl, 100 mM sodium phosphate/citric acid buffer.
[b]Number of substitutions A→**M**. The duplex T_m values of h_{26} occurred between 82 and 75°C.

Our results demonstrate the use of ‘state-of-the-art’ quantum mechanics, molecular dynamics and thermodynamic integration techniques in the analysis and design of polynucleotidic sequences. Overall, our results suggest that introduction of amino groups at position 8 of purines may lead to new products with interesting triple helix stabilization properties. Just recently, oligonucleotides carrying 8-aminoguanine have also been shown to form a very stable triple helix (29), which indicates that, indeed, replacement at position 8 of purines by amino groups is key to triple helix stabilization.

See supplementary material available in NAR Online.

ACKNOWLEDGEMENTS

We thank M. Wiersma for valuable suggestions. We also thank Dr Matthias Mann and his group for mass spectroscopic measurements. This research was partially supported by the Centre de Supercomputació de Catalunya (CESCA, Molecular Recognition Project) and the Spanish Dirección General de Investigación Científica y Técnica (DGICYT, PB96-1005).

REFERENCES

1 Thuong,N.T. and Hélène,C. (1993) *Angew. Chem. Int. Ed. Engl*., **32**, 666–690.
2 Soyfer,V.N. and Potaman,V.N. (1996) *Triple Helical Nucleic Acids*. Springer-Verlag, New York, NY.
3 Xodo,L.E., Manzini,G., Quadrifolio,F., van der Marel,G.A. and van Boom,J.H. (1991) *Nucleic Acids Res*., **19**, 5625–5631.
4 Sun,S., François,J.C., Montenay-Garestier,T., Saison-Behmoaras,T., Roig,V., Thuong,N.T. and Hélène,C. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 9198–9202.
5 Povsic,T.J. and Dervan,P.B. (1989) *J. Am. Chem. Soc*., **111**, 3059–3061.
6 Ferrer,E., Fàbrega,C., Güimil García,R., Azorín,F. and Eritja,R. (1996) *Nucl. Nucl*., **15**, 907–921.
7 Rana,V.S., Barawkar,D.A. and Ganesh,K.N. (1996) *J. Org. Chem*., **61**, 3578–3579.
8 Barawkar,D.A., Rajeev,K.G., Kumar,V.A. and Ganesh,K.N. (1996) *Nucleic Acids Res*., **24**, 1229–1237.
9 Xiang,G., Soussou,W. and McLaughlin,L.W. (1994) *J. Am. Chem. Soc*., **116**, 11155–11156.
10 Shields,G.C., Laughton,C.A. and Orozco,M. (1997) *J. Am. Chem. Soc*., **119**, 7463–7469.
11 Kumar,R.K., Gunjal,A.D. and Ganesh,K.N. (1994) *Biochem. Biophys. Res. Commun*., **204**, 788–793.
12 Kawai,K., Saito,I. and Sugiyama,H. (1998) *Tetrahedron Lett*., **39**, 5221–5224.
13 Biosym (1994) *Insight-II Computer Program*. Biosym, San Diego, CA.
14 Frisch,M.J., Trucks,G.W., Schegel,H.B., Gill,P.M.W., Johnson,B.G., Robb,M.A., Cheeseman,J.R., Keith,T.A., Petersson,G.A., Montgomery,G.A. *et al.* (1995) *Gaussian-94* (*Rev. D.3)*. Gaussian Inc., Pittsburg, PA.
15 Pearlman,D.A., Case,D.A., Caldwell,J.W., Ross,W.S., Cheatham,T.E., DeBolt,S., Fergurson,D., Seibel,G. and Kollman,P.A. (1995) *Comp. Phys. Commun*., **91**, 1–41.
16 Lee,C., Yang,W. and Parr,R.G. (1988) *Phys Rev. B*, **37**, 785–802.
17 Colominas,C., Teixidó,J., Cemelí,J., Luque,F.J. and Orozco,M. (1998) *Phys. Chem. B*., **102**, 2269–2276.
18 Boys,S.F. and Bernadi,F. (1970) *Mol. Phys*., **19**, 553–574.
19 Arnott,S. and Hukins,D.W.L. (1972) *Biochem. Biophys. Res. Commun*., **47**, 1504–1510.
20 Kirkwood,J.J. (1935) *J. Chem. Soc*., **3**, 300–308.
21 Pearlman,D.A. and Kolman,P.A. (1991) *J. Chem. Phys*., **94**, 4532–4552.
22 Essmann,U., Perera,L., Berkowitz,M.L., Darden,T., Lee,H. and Pedersen,L.G. (1995) *J. Chem. Phys*., **103**, 8577–8593.
23 Rykaert,J.P., Ciccote,G. and Berendsen,J.C. (1977) *J. Comp. Phys*., **23**, 327–342.
24 Cornell,W.D., Cieplack,P., Bayly,C.I., Gould,I.R., Merz,K., Ferguson,D.M., Spellmeyer,D.C., Fox,T., Caldwell,J.W. and Kollman,P.A. (1995) *J. Am. Chem. Soc*., **117**, 5179–5197.
25 Jorgensen,W.L., Chandresekhar,J., Madura,J., Impey,R.W. and Klein,M.L. (1983) *J. Chem. Phys*., **79**, 926–935.
26 Bayly,C.I., Cieplak,P., Cornell,W.D. and Kollman,P.A. (1983) *J. Phys. Chem*., **97**, 10269–10292.
27 Holmes,R.E. and Robins,R.K. (1965) *J. Am. Chem. Soc*., **87**, 1772–1776.
28 Long,R.A., Robins,R.K. and Townsend,L.B. (1967) *J. Org. Chem*., **32**, 2751–2756.
29 Güimil-García,R., Bachi,A., Eritja,R., Luque,F.J. and Orozco,M. (1998) *Bioorg. Med. Chem. Lett*., **8**, 3011–3016.