Hydrogen bonding between adenine and 2,4-difluorotoluene is definitely not present, as shown by concentration-dependent NMR studies

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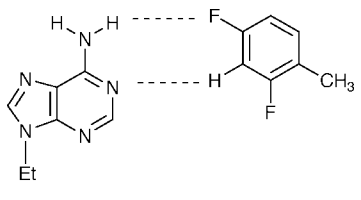
The chloroform-soluble nucleobase derivatives N^9-cyclohexylmethyladenine (A) and N^1-cyclohexylmethylthymine (T) have been synthesized in order to study hydrogen-bonding interactions between A and the thymidine mimic 2,4-difluorotoluene (F) in $CDCl_3$ at high concentrations. Concentration-dependent ^1H NMR experiments show that in the presence of F, A undergoes self-association rather than pairing with F. These results strongly support the assumptions made by Kool with regard to the lack of hydrogen bonding between adenine and 2,4-difluorotoluene.[3]

The commonly accepted view that Watson–Crick hydrogen bonding plays the key role in polymerase fidelity has been recently revised by Kool and coworkers.[1–5] It has been shown that in a synthetic DNA template the nonpolar thymine isostere 2,4-difluorotoluene (F), which is assumed not to form any hydrogen bonds with adenine, successfully encodes the insertion of adenine in DNA replication.[2] Furthermore, polymerase-mediated DNA synthesis using the nucleoside 5′-triphosphate derivative of F (dFTP) led to insertion of F opposite to adenine with a selectivity nearly as high as that observed for thymidine 5′-triphosphate (dTTP).[3]

Hydrogen bonding between F and adenine has been investigated both by denaturation studies of DNA duplexes containing an A/F pair[3] and by titration of 9-ethyladenine (9-EtA) with a dilute solution of F in $CDCl_3$.[6] As both experiments did not indicate hydrogen-bonding interactions between F and adenine, it has been put forward that not Watson–Crick hydrogen bonds, but shape complementarity is the most important criterion of faithful DNA replication.[3]

This hypothesis has been sharply criticized by Evans and Seddon,[7] who stressed the ability of F to form one C–H···N and one C–F···H–N hydrogen bond with 9-EtA (Scheme 1). Their view is supported by NMR titration experiments of F with pyridine and 3-chloropyridine,[7] which are, respectively, stronger and weaker bases than adenine.[8,9] In fact, the experiment, in which a 1 μm 9-EtA solution was titrated with a dilute solution of F in $CDCl_3$,[6] was considered inconclusive because of the poor solubility of 9-EtA in $CDCl_3$.[7]

Apart from this, theoretical studies based on *ab initio* calculations show that hydrogen bonding between adenine and F is rather unlikely, but cannot be definitely excluded.[10–12]



9-EtA **F**

Scheme 1 Hydrogen bonding with a hypothetical A/F pair.

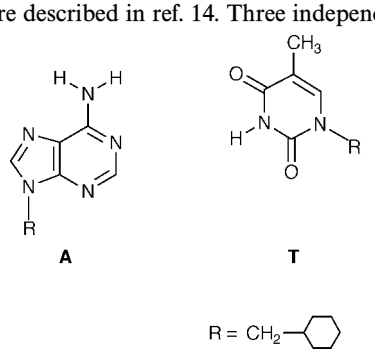
This rather contradictory discussion inspired us to synthesize the highly soluble (up to 100 mM in chloroform) nucleobase derivatives N^9-cyclohexylmethyladenine (A) and N^1-cyclohexylmethylthymine (T)[13] (Scheme 2). These model nucleobases, highly soluble in chloroform, allow a proper study of even weak hydrogen-bonding interactions between A and F in $CDCl_3$, so that the question of whether hydrogen bonding between A and F in $CDCl_3$ is present can be answered unambiguously. The results described below fully support the hypothesis of Kool *et al.*[3]

Experimental

Materials and methods

ESI mass spectra were recorded on a Finnigan MAT TSQ-70 equipped with a custom-made electrospray interface.

^1H and ^{13}C NMR spectra were recorded on a Bruker DPX (300 MHz) instrument and referenced to $CDCl_3$. In the dilution experiments a 1 : 1 mixture of the two components in $CDCl_3$ was diluted stepwise in the concentration range 80–5 mM and each time a ^1H NMR spectrum was recorded. The measurements were carried out at 296 K under an argon atmosphere in deuterated chloroform that had been dried over 4 Å molecular sieves. The added amount of $CDCl_3$ was weighed every time. The concentration-dependent shifts of the NH protons involved in hydrogen bonding were fitted with a non-linear least-squares program after Newton–Gauss, similar to a procedure described in ref. 14. Three independent dilution



A **T**

Scheme 2 Chloroform-soluble adenine and thymine derivatives.

experiments were performed and evaluated for each system. In the cases of the A/A and the A/F systems the final association constants K correspond to the weighted mean of the three individually calculated values obtained from the concentration-dependent ^{1}H NMR shifts of the NH_2 protons of A. In the case of the A/T system the concentration-dependent ^{1}H NMR shifts of the NH_2 of protons and N3-H of A/T could be evaluated for the calculation of K. For each experiment the weighted mean K^* of these two values was calculated. The final stability constant K corresponds to the weighted mean of the three K^* values. All errors correspond to twice the standard deviation.

Synthesis

Thymine and adenine were purchased from Acros and Aldrich, respectively. Bromomethylcyclohexane was from Fluka.

N^1-Cyclohexylmethylthymine was synthesized as follows: 6.076 g (48.2 mmol) thymine and 6.667 g K_2CO_3 (48.2 mmol) were suspended in 150 ml DMSO and stirred for 1 h at room temperature. Bromomethylcyclohexane (2.224 ml, 15.9 mmol) was added and the reaction mixture stirred for 3 h at 70 °C then for 14 h at room temperature. After removal by filtration of KBr, 200 ml water was added to the filtrate and the solution was extracted 3 times with 150 ml dichloromethane. The organic layer was washed with water (3 × 100 ml) and the dichloromethane evaporated. Purification of the crude product by silica gel chromatography (eluent: dichloromethane–methanol, 100 : 0 to 97.5 : 2.5, v : v) afforded pure N^1-cyclohexylmethylthymine. Yield: 1.68 g (47%). Elem. anal. found (calcd.) C: 64.7 (64.8), H: 8.1 (8.1), N: 12.5 (12.6)%. ^{13}C NMR ($CDCl_3$): δ 164.24 (C-4), 151.1 (C-2), 141.0 (C-6), 110.05 (C-5), 54.52 (CH_2N), 37.29, 30.36, 26.14, 25.52 (cyclohexyl), 12.30 (5-CH_3). ESI-MS: m/z 223 [M^+].

N^9-Cyclohexylmethyladenine was synthesized as follows: 10.09 g (74.7 mmol) adenine and 2.15 g (89.6 mmol) NaH were suspended in 300 ml DMF and stirred for 1 h at room temperature. Bromomethylcyclohexane (9.6 ml, 69.3 mmol) was added and the reaction mixture was stirred for 3 h at 70 °C then for 14 h at RT. After removal by filtration of NaBr, 200 ml water was added to the filtrate and the solution extracted with chloroform (3 × 150 ml). The organic layer was washed with water and the crude product purified by silica gel chromatography (eluent: dichloromethane–methanol 100 : 0 to 95 : 5, v : v) to give pure N^9-cyclohexylmethyladenine. Yield: 2.11 g (12.2%). Elem. anal. found (calcd): C: 62.3 (62.4), H: 7.4 (7.4), N: 30.3 (30.4)%. ^{13}C NMR ($CDCl_3$): δ 155.59 (C-6), 152.94 (C-2), 150.29 (C-4), 140.88 (C-8), 119.58 (C-5), 50.0 (CH_2N), 38.174, 30.58, 26.09, 25.46 (cyclohexyl). ESI-MS: m/z 232 [M^+].

Results and discussion

Firstly, hydrogen bonding between A and T and the self-association of A were determined by concentration-dependent NMR studies (Fig. 1 and 2) and compared with reported data to show the reliability of the technique used. Secondly, hydrogen bonding between A and F was investigated (Fig. 2). From the NMR data stability constants for all systems were calculated.

For hydrogen bonding between A and T (Fig. 1) an association constant of $K = 56.5 \pm 9$ M^{-1} was calculated, which is in agreement with earlier results[15] obtained by ^{13}C NMR [$K = 60 \pm 5$ and 73 ± 4 M^{-1} as calculated from the concentration-dependent chemical shift of C-2 (thymine) and C-4 (thymine), respectively]. Similarly, for the self-association of A in $CDCl_3$ a stability constant of $K = 2.2 \pm 0.3$ M^{-1} (Fig. 2) was obtained, which is in good agreement with earlier results[16] ($K = 3.1$ M^{-1}).

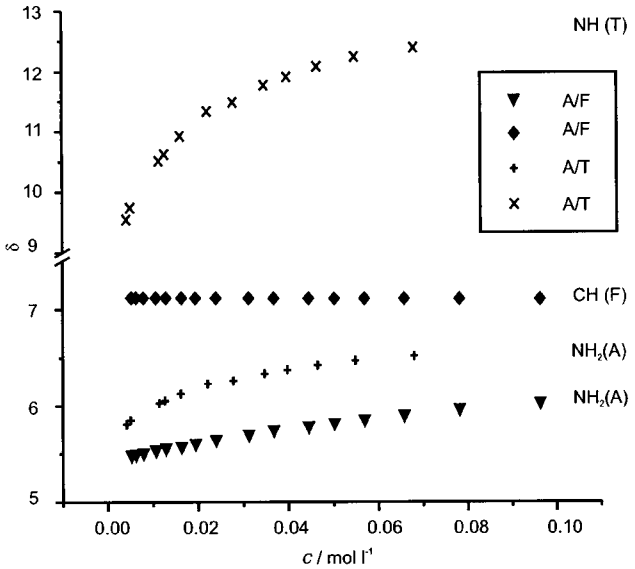


Fig. 1 Concentration-dependent ^{1}H NMR measurements of equimolar A/F and A/T mixtures.

The concentration-dependent ^{1}H NMR measurements of the equimolar A/F mixture show no significant shift except for the NH_2 (A) protons (Fig. 1). From these data an association constant of $K = 2.4 \pm 0.5$ M^{-1} was calculated, which is the same (within the error limits) as the value given above for the self-association of A ($K = 2.2 \pm 0.3$ M^{-1}) obtained from the pure A/A system. The very small downfield shift of the CH (F) resonance at the lower concentrations (Fig. 1) might be explained by decreasing stacking between F molecules, as a dilution experiment of F alone does afford exactly the same curve (data not shown). The concentration dependent shift of the NH_2 (A) protons has thus to be ascribed solely to self-association of A.

We are aware that the proton of chloroform can act as a hydrogen-bond donor, too. However, such H-bonding interactions are usually very weak ($CDCl_3$/DMSO, $K = 2.6$ M^{-1}).[17] If H bonding between the solvent and A was strong, self-association of A should be prevented and hence a concentration dependence of the A-NH_2 resonance as in Fig. 2 should not be observed. This strongly suggests that even weak H-bonding interactions between nucleobases can be detected in this solvent.

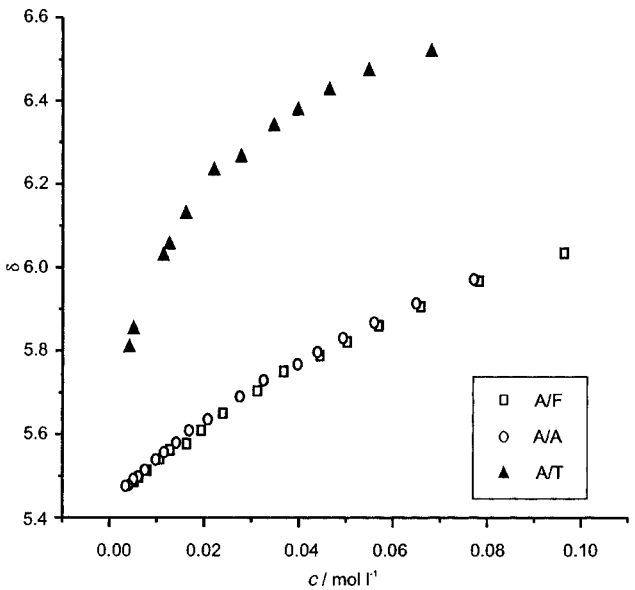


Fig. 2 Concentration-dependent ^{1}H NMR measurements of equimolar A/A, A/F and A/T mixtures [NH_2 (A) resonances].

On the other hand, strong hydrogen-bonding interactions between the proton in $CDCl_3$ and the fluorine substituents in difluorotoluene would significantly affect hydrogen bonding with A. If this interaction was very strong, only A self-pairing would be observed. However, such C–F···H–C interactions are very weak. In fact, an analysis of contacts between fluorine and hydrogen bound to a carbon in the solid state has shown that these contacts cannot be considered as hydrogen bonds due to their low energies.[18] Thus, we believe that in our case the detection of even weak hydrogen bonds between adenine and difluorotoluene would not be hampered by the presence of chloroform.

Conclusions

^{1}H NMR spectroscopy in chloroform does not reveal any hydrogen-bonding interaction between A and F, not even at high concentrations. In fact, we find that in the presence of F, A undergoes self-association rather than pairing with F. In contrast to earlier findings,[7] hydrogen bonding between A and F in chloroform can definitely be ruled out. Our results thus strongly support the assumptions made by Kool and coworkers with regard to the lack of hydrogen bonding between adenine and 2,4-difluorotoluene.[3]

Acknowledgements

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