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Hydrogen bonding in complexes of adenosine and 4-thiouridine: a low-temperature NMR study

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NMR experiments were performed on the association of the two sugar-protected nucleosides 2′-deoxy-4-thiouridine and 2′-deoxyadenosine in solution. Using an aprotic $CDClF_2$–CDF_3 solvent mixture, low-temperature measurements allowed the observation of individual hydrogen-bonded complexes in the slow exchange regime. 2D NOE experiments acquired at 128 K show the preferential formation of a ternary complex with two thiouridine nucleosides simultaneously bound at the Watson–Crick and Hoogsteen site of adenosine at all molar ratios. There is no indication of the 4-thiocarbonyl group involved as proton acceptor in a hydrogen bond to the adenine base to a significant extent. A more downfield chemical shift together with a smaller $^1J(N,H)$ scalar coupling of the Watson–Crick-bound imino proton in specifically 3-^{15}N-labeled thiouridine points to a stronger Watson–Crick than the Hoogsteen hydrogen bond. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; ^{15}N NMR; base pairing; hydrogen bonds; nucleosides

INTRODUCTION

The formation of specific hydrogen bonds constitutes the major determinant for the secondary and tertiary structure of nucleic acids and for the recognition of RNA or DNA targets by antisense or antigene oligonucleotides. Generally, Watson–Crick base pairing provides the most important element for the specific interaction in nucleic acids but, owing to their multiple proton donor and acceptor sites, other base pairing schemes are frequently encountered. Thus, uridine can form various base pairs with adenosine or other nucleobases depending on its use of the 2- or 4-carbonyl oxygen as proton acceptor in a hydrogen bond.

Transfer RNAs of all organisms undergo post-transcriptional modifications that serve to modulate interactions with other nucleic acids such as mRNA or proteins.[1,2] 4-Thiouridine represents the predominant thionucleoside in *Escherichia coli* tRNA and in addition to its modified hydrogen bond acceptor site there is substantial evidence for its function as a cellular sensor for near-UV stress.[3] Upon irradiation, thionucleobases can be selectively photoactivated to give highly reactive species forming internal cross-links with any nucleic acid base. Owing to these photochemical properties, 4-thiouridine modifications have also been extensively incorporated into nucleic acid fragments in the past few years and used as photoaffinity probes of nucleic acid structure.[4–6]

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In order to study nucleobase association without additional base stacking and steric effects of the sugar–phosphate backbone, several NMR studies have been performed in the past on the monomeric bases in aprotic solvents.[7–10] However, little is known about the geometries and strengths of hydrogen bonds in base pairs involving 4-thiouracil. This is mostly due to the fact that the hydrogen-bonded complexes are in fast exchange on the NMR chemical shift time-scale at ambient temperature, preventing the unambiguous characterization of often co-existing associates. Recently, we have employed NMR measurements at very low temperatures to study the various homo- and heteroassociation modes of adenine and uracil nucleosides and some of their analogs.[11–13] With measurements in the liquid state as low as 113 K, individual hydrogen-bonded complexes of nucleobases in slow exchange could be observed and unambiguously characterized in solution for the first time. In these studies no participation of the thiocarbonyl function in a hydrogen bond was observed for the homodimers of 4-thiouridine.[12] We now present NMR measurements on the heteroassociation of *O*-derivatized 2′-deoxyadenosine (A) and 4-thio-substituted 2′-deoxyuridine (s^4U) to explore further the potential alterations in strength and preference of hydrogen bonding upon incorporation of sulfur into the 4-position of the pyrimidine base.

EXPERIMENTAL

[7-^{15}N]-2′-Deoxyadenosine, [3-^{15}N]-2′-deoxy-4-thiouridine and their *O*-acetylated and *O*-silylated derivatives were synthesized as described previously.[12,13] 7-Deaza-2′-deoxyadenosine (2′-deoxytubercidin) was obtained from

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Berry (Ann Arbor, MI, USA) and *O*-silylated according to standard procedures. All nucleosides were purified by HPLC prior to measurements. The deuterated Freon mixture $CDClF_2$–CDF_3 was prepared as described[14] and handled on a vacuum line which was also used for the sample preparation.

NMR experiments were performed on a Bruker AMX500 spectrometer. Temperatures were adjusted by a Eurotherm variable-temperature unit to an accuracy of $\pm 1.0\,^{\circ}$C. ^{1}H chemical shifts in methylene chloride were referenced relative to $CHDCl_2$ ($\delta_H = 5.32$ ppm) and in a Freon mixture relative to $CHClF_2$ ($\delta_H = 7.13$ ppm). For ^{15}N chemical shifts an external reference of $^{15}NH_4Cl$ in 10% HCl was used ($\delta_N = 0$ ppm). *Ab initio* calculations were performed with PC SPARTAN Pro V1.0.5.

RESULTS

The adenine and 4-thiouracil base of the 2′-deoxynucleosides potentially may form four different complexes with two cyclic hydrogen bonds (see Fig. 1). Thus, either the carbonyl oxygen (structures **I** and **II**) or sulfur atom (structures **III** and **IV**) at positions 2 and 4 of the pyrimidine base may serve as proton acceptor in a hydrogen bond to the adenine amino group. In addition, depending on the use of the N-1 or N-7 nitrogen of adenine as proton acceptor, Watson–Crick-like (structures **I** and **III**) and Hoogsteen-like arrangements (structures **II** and **IV**) are formed, respectively.

Generally, the formation of hydrogen bonds can be easily followed in the NMR spectrum by low-field shifts of resonances arising from hydrogen-bonded protons. However, owing to fast exchange of weakly hydrogen-bonded complexes at ambient temperature, ^{1}H chemical shifts are averaged over all associates in solution and fail to yield direct information on the geometry of the various co-existing species. We therefore specifically introduced ^{15}N isotopes at the N-3 and N-7 hydrogen bond donor and acceptor sites of the pyrimidine and purine nucleosides to gain more insight into the predominant association mode of the bases. In Fig. 2 the ^{15}N chemical shift of a 1 : 1 mixture of di-*O*-triisopropylsilyl-protected [7-^{15}N]-2′-deoxyadenosine and di-*O*-acetylated [3-^{15}N]-2′-deoxy-4-thiouridine in methylene chloride is plotted as a function of temperature. As expected for an imino group involved in a hydrogen bond, the thiouridine 3-^{15}N resonance is shifted downfield upon lowering the temperature from 293 to 193 K owing to increasing complex formation. Concomitantly, a pronounced upfield shift of the

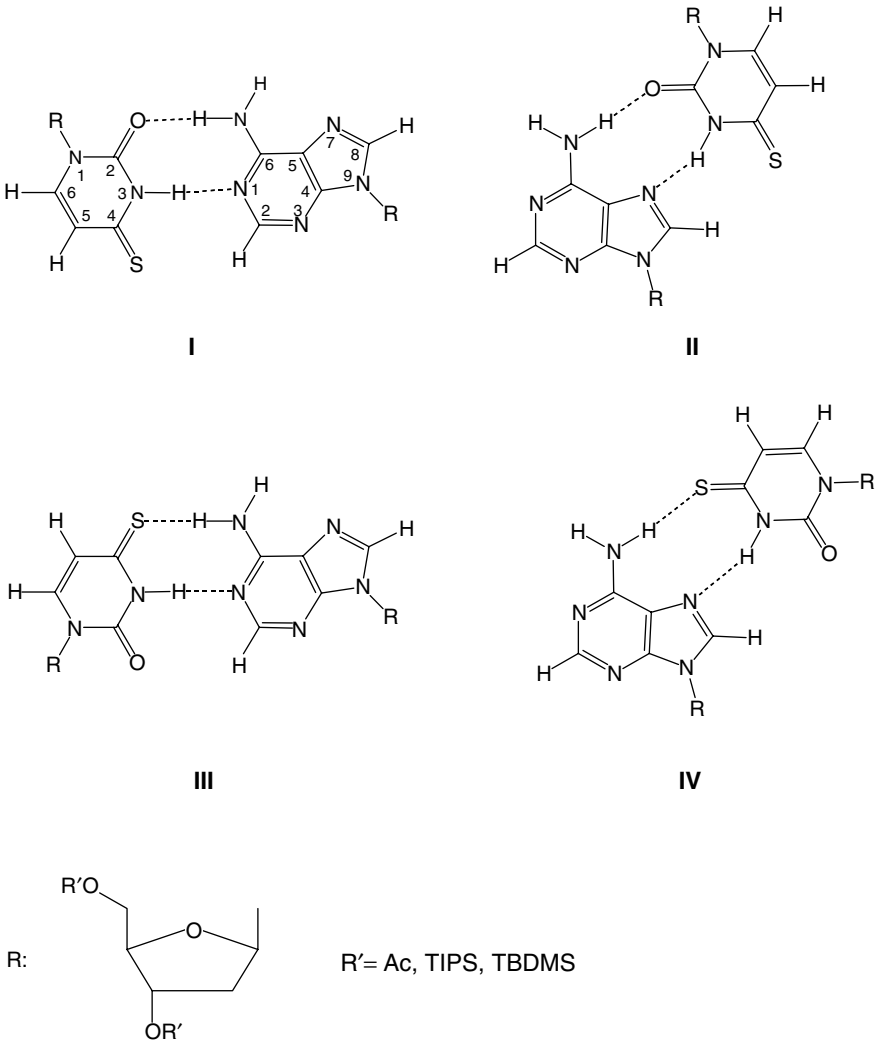

Figure 1. Possible 1 : 1 complexes **I**–**IV** between 4-thiouridine and adenosine derivatives with atom numbering of the pyrimidine and purine heterocyclic ring system indicated for **I**.

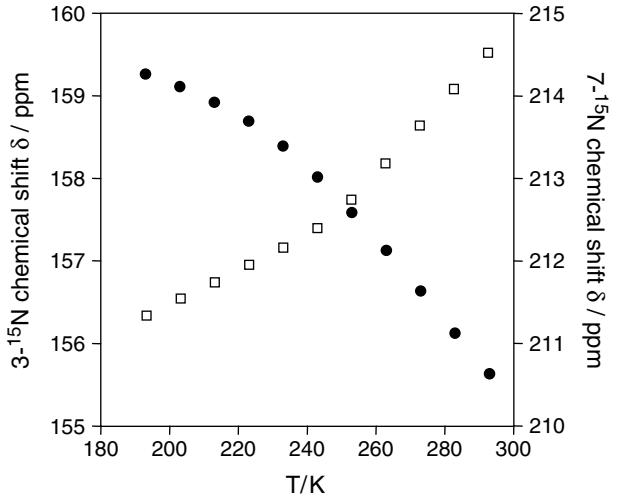

Figure 2. 3-^{15}N (filled circles) and 7-^{15}N (open squares) chemical shift as a function of temperature in a 1 : 1 mixture of 3′,5′-di-*O*-acetyl-[3-^{15}N]-2′-deoxy-4-thiouridine and 3′,5′-di-*O*-triisopropylsilyl-[7-^{15}N]-2′-deoxyadenosine in CD_2Cl_2.

adenosine 7-^{15}N nitrogen points to its participation as acceptor in a hydrogen bond, indicating binding at the adenine Hoogsteen site to a significant extent. Note, however, that these temperature-dependent chemical shifts are not only influenced by the limiting ^{15}N chemical shift of the monomer and the base pair but also the temperature dependence of complex formation, i.e. the enthalpy of association. Correspondingly, a quantitative analysis in terms of populations is largely restricted.

Using methylene chloride as solvent, only one set of averaged NMR signals could be observed at all temperatures. In order to reach the slow hydrogen bond exchange regime, NMR spectra were acquired in a low-melting deuterated Freon solvent. In Fig. 3, s^{4}U imino and A base resonances of a [7-^{15}N]-A +[3-^{15}N]-s^{4}U mixture are plotted as a function of temperature. As expected, the doublet of the s^{4}U ^{15}NH imino signal shifts downfield upon cooling owing to increased formation of hydrogen-bonded complexes. Below the coalescence point at 183 K two individual, slowly exchanging imino resonances H_A and H_B appear in the ^{1}H NMR spectrum. Measurements of signal intensities at 133 K give a ratio H_A : H_B of 1 : 1 at a nucleoside ratio A : s^{4}U of about 1 : 2. Because the imino chemical shift in 4-thiouridine homodimers was found to be at much higher field under corresponding conditions,[12] these downfield-shifted imino resonances must be attributed to heterocomplexes with adenosine.

Unambiguous assignment of the two imino signals to specific complexes is possible through ^{1}H–^{1}H NOE contacts to adenine base protons under slow exchange conditions. From Fig. 1 it becomes apparent, that for a Watson–Crick binding to adenine N-1 NOE cross peaks are expected between the hydrogen-bonded s^{4}U imino and H-2 and amino protons of adenine. On the other hand, in addition to the imino–amino contact there is a close spatial proximity between the s^{4}U imino and the adenine H-8 proton in a Hoogsteen-type base pair with N-7 of adenine serving as

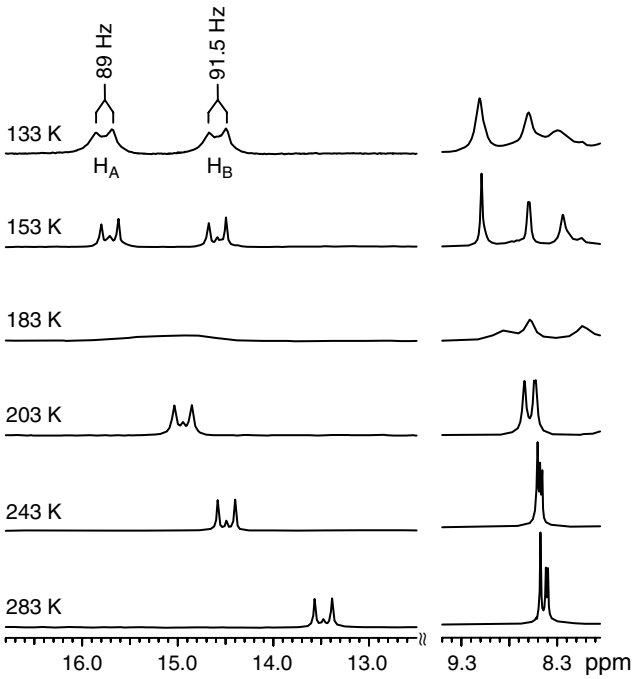

Figure 3. ^{1}H NMR spectra of a mixture of 3′,5′-di-*O*-triisopropylsilyl-[7-^{15}N]-2′-deoxyadenosine and 3′,5′-di-*O*-acetyl-[3-^{15}N]-2′-deoxy-4-thiouridine (1 : 2 molar ratio at 133 K) in a Freon solvent showing the imino (left) and adenine base proton spectral region (right) as a function of temperature.

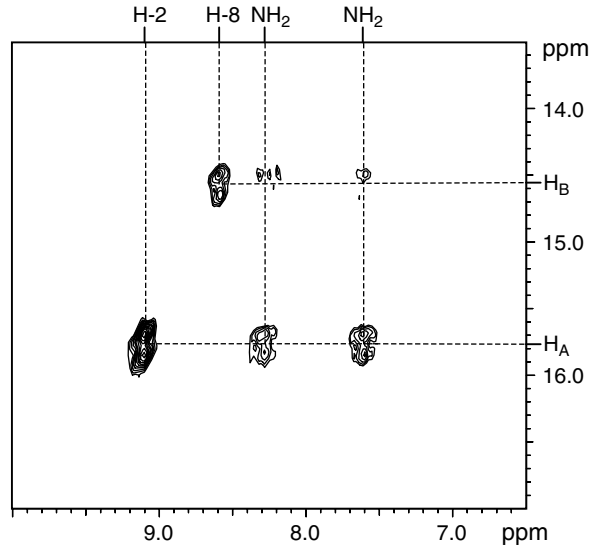

Figure 4. Portion of a 2D NOE spectrum of 3′,5′-di-*O*-triisopropylsilyl)-[7-^{15}N]-2′-deoxyadenosine and 3′,5′-di-*O*-acetyl-[3-^{15}N]-2′-deoxy-4-thiouridine (1 : 2 molar ratio) in a Freon mixture showing cross peaks between imino and base protons. The spectrum was acquired at 128 K with a 60 ms mixing time.

hydrogen bond acceptor. A portion of a 2D NOE spectrum of the specifically ^{15}N-labeled purine–pyrimidine nucleoside mixture with a 1 : 2 molar ratio at 128 K is shown in Fig. 4. At this temperature no exchange cross peaks in the 2D NOE spectrum are observed for the two imino signals H_A and H_B at δ15.78 and 14.57 ppm. However, H_A and H_B exhibit a strong cross peak to signals at δ9.11 and 8.60 ppm,

Table 1. ^{1}H chemical shift δ (ppm) of adenine and 4-thiouracil base protons in A·s^{4}U complexes determined in a Freon mixture at 128 K (molar ratio A : s^{4}U = 1 : 2)[a]

Proton A	δ (ppm)	Proton s^{4}U	δ (ppm)
H-2	9.11	H-5	6.38
H-8	8.60	H-6	7.60
NH_2	7.64	H-3	14.57
	8.30		15.78

[a] Resonances were assigned based on a 2D NOE experiment

respectively. In addition, they are connected by less intense cross peaks to the same two base protons which show a strong mutual NOE contact but no contacts with other adenosine protons. Correspondingly, these are identified as adenine amino protons. Moreover, the resonance at δ8.60 ppm can be unambiguously assigned to H-8 of adenine owing to its ^2J(N,H) scalar coupling of 11 Hz still partly resolved at 153 K. In contrast to this H-8 signal, no NOE connectivities to sugar protons are observed for the resonance at δ9.11 ppm, thus identifying an adenine H-2 in a nucleoside with anti glycosidic torsion angle (not shown). All chemical shifts of adenosine and 4-thiouridine base protons at 128 K are summarized in Table 1.

Obviously, two thiouridine nucleosides simultaneously bind to both the Watson–Crick and Hoogsteen site of adenine forming a single ternary complex at a 1 : 2 molar ratio. As shown in Fig. 5a (top) for an unlabeled sample, an additional high-field shifted resonance at δ12.65 ppm characteristic of thiouridine homodimers is observed together with the Watson–Crick and Hoogsteen bound imino signals when adding more of the pyrimidine nucleoside. However, even for solutions with A concentration $\geqslant$ s^{4}U concentration, the Watson–Crick and Hoogsteen imino resonances of a 1 : 2 complex remain the most intense signals in the NMR spectra indicating predominant heteroassociation to A·(s^{4}U)$_2$ base triples [Fig. 5(a), center and bottom]. Additional imino signals of low intensity can be attributed to small amounts of minor complexes. Residual self-associating purine nucleosides expected for the last two samples become noticeable by an additional set of adenine base proton resonances in the corresponding spectra (not shown). We also studied the association of 3-^{15}N-labeled s^{4}U with 7-deaza-2′-deoxyadenosine. Clearly, substituting N-7 with CH in 7-deaza-A eliminates the Hoogsteen hydrogen bond acceptor site leaving only the possibility of forming a Watson–Crick base pair. In line with our observations on adenosine, only a single Watson–Crick imino resonance split into a doublet with a ^1J(N,H) of 86 Hz at δ15.88 ppm is observed in the ^{1}H NMR spectrum of a 1 : 1 mixture of the 7-deaza analog and 4-thiouridine at 128 K [Fig. 5(b)]. Again, high-field shifted signals of low intensity can be attributed to small amounts of minor species.

DISCUSSION

The results obtained from the one- and two-dimensional NMR experiments described above demonstrate that 1 : 2 A·(s^{4}U)$_2$ complexes with both Watson–Crick and Hoogsteen sites of adenosine occupied by the 4-thiouracil base

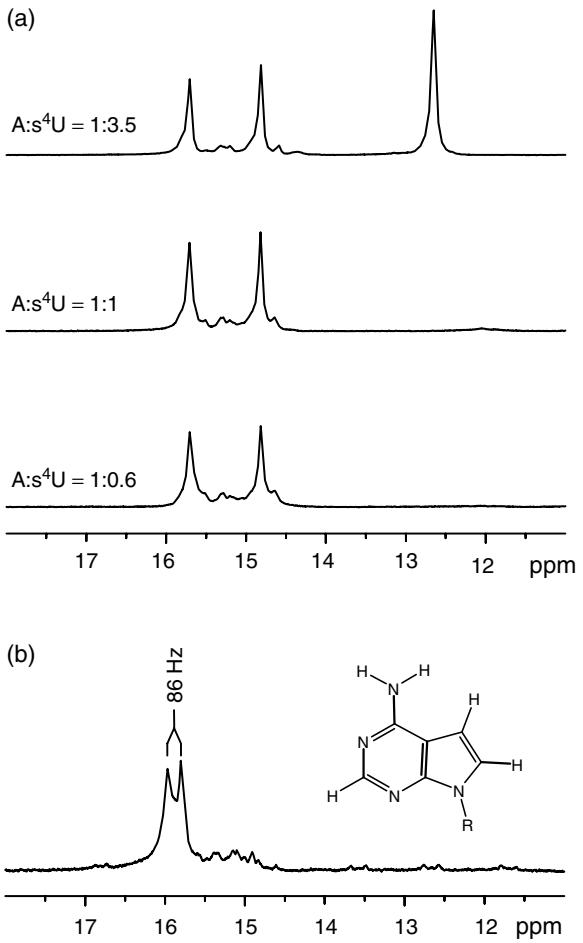

Figure 5. ^{1}H NMR spectra in a Freon mixture at 128 K showing the imino proton spectral region. (a) A mixture of 3′,5′-di-*O*-*t*-butyldimethylsilyl-2′-deoxyadenosine and 3′,5′-di-*O*-acetyl-2'-deoxy-4-thiouridine at different molar ratios. (b) A 1 : 1 mixture of 3′,5′-di-*O*-*t*-butyldimethylsilyl-7-deaza-2′-deoxyadenosine and 3′,5′-di-*O*-acetyl-[3-^{15}N]-2′-deoxy-4-thiouridine. Also shown is the structure of the 7-deaza analog.

are preferentially formed at low temperatures. Obviously, the formation of such ternary complexes co-existing with self-associates of residual pyrimidine or purine nucleosides is thermodynamically favored over the formation of 1 : 1 A·s^{4}U complexes. In contrast, adenosine–uridine mixtures have been found to form predominantly Watson–Crick base pairs. Even with uridine present in large excess, Hoogsteen binding always occurs to a minor extent and competes with the formation of uridine homodimers.[13] The larger tendency of self-association for uridine might be partially attributed to its more negative free enthalpy ΔG of homodimerization when compared with the 4-thio analog.[12] On the other hand, imino proton signals in hetero complexes of the thio derivative are already in slow exchange on the NMR chemical shift time-scale at $T \leq 163$ K and thus at higher temperatures when compared with A·U complexes.[13] This increased kinetic stability may be the result of stronger hydrogen bonds involving the more acidic imino function of 4-thiouridine.

Disregarding additional low-intensity resonances, the low-temperature ^{1}H NMR spectra of A–s^{4}U mixtures

only exhibit a single Watson–Crick and Hoogsteen imino resonance of a ternary hetero complex. This might be attributed to (i) the exclusive participation of either the 2- or 4-position of 4-thiouridine in hydrogen bonding or (ii) to corresponding resonances being isochronous and therefore not resolved. The latter seems less likely given the very different geometries of the hydrogen bonds upon binding to O-2 or S-4 of thiouridine. However, there is no easy way to discriminate by $^1H-^1H$ NOE experiments between complexes **I/II** and **III/IV** with the oxygen at position 2 or the sulfur at position 4 of 4-thiouridine engaged in hydrogen bonding. Generally, thiocarbonyl derivatives have been shown to be stronger bases than the carbonyl analogs in the gas phase and correspondingly high-level *ab initio* calculations have established preferential protonation at the sulfur of 4-thiouracil.[15] In contrast, previous ^{13}C NMR studies in chloroform solution indicated that the 2-carbonyl group of alkylated 4-thiouracil is more often hydrogen bonded than the 4-thiocarbonyl group in complexes with 9-ethyladenine.[16] Recently, low-temperature NMR studies unambiguously showed that the sulfur atom does not participate as proton acceptor in a hydrogen bond for the homodimers of 4-thiouridine in solution.[12] Moreover, association constants between A and s^4U determined at 293 K in chloroform were found to be smaller by a factor of ~ 2 when compared with the corresponding A–U association.[13] Provided that both carbonyls of uridine participate about equally in hydrogen bonding to adenosine, the different association constants may be explained by the statistical disadvantage of forming only base pairs with the 2-carbonyl of 4-thiouridine involved as proton acceptor.

We have also performed geometry optimizations on all four base pairs **I**–**IV** in the gas phase within the Hartree–Fock approximation using the standard 6–31G* basis set. For both Watson–Crick and Hoogsteen geometries base pairs involving NH···S hydrogen bonds are less stable by 3–4 kJ mol^{-1} than base pairs with NH···O hydrogen bonds. Also, distances between the proton and sulfur in (N)H···S(C-4) hydrogen bonds are larger by about 0.6 Å than the (N)H···O(C-2) hydrogen bond length. Because dispersion attraction is neglected within the HF approximation, HF interaction energies are expected to be underestimated and intermolecular distances overestimated. However, the present results are in good agreement with calculations employing high-level *ab initio* methods with inclusion of electron correlation (MP2) on several hydrogen-bonded base pairs containing thiobases.[17] In these studies it was also found that the thio group enhances polarizability as well as the dipole moment of the monomers, thus increasing dispersion attraction and electrostatic interactions upon association.

It is well established that the proton chemical shift and the magnitude of scalar couplings in a hydrogen bond are correlated with its geometry. Thus, gradually shifting the proton from an NH donor to an acceptor atom in a weak hydrogen bond is accompanied by a ^1H downfield shift and a decrease in the $^1J(N,H)$ scalar coupling.[18–21] Correspondingly, observation of a more deshielded Watson–Crick imino proton H_A indicates its participation in a stronger hydrogen bond when compared with the Hoogsteen imino proton H_B. Likewise, $^1J(N,H)$ scalar coupling constants of the 3-^{15}N-labeled thiouridine decrease upon complex formation from 94 Hz for the monomer (determined for dilute solutions at ambient temperatures) to 91.5 ± 0.5 Hz for the Hoogsteen-bound and 89 ± 0.5 Hz for the Watson–Crick-bound pyrimidine base at 133 K. It should be mentioned that in line with its more high-field shifted proton signal, NH···O hydrogen bonds in the homodimer of 4-thiouridine are weaker, exhibiting a $^1J(N,H)$ scalar coupling of 93 Hz at low temperatures. For the 7-deazaadenosine analog, an additional downfield shift and a reduced $^1J(N,H)$ scalar coupling of 86 Hz for the Watson–Crick imino proton is consistent with a more negative charge on the N-1 acceptor atom as derived from electrostatic potential calculations at the HF/6–31G* level of the 1-methylated bases.

CONCLUSIONS

Hydrogen-bonded complexes can be characterized in detail by employing low-temperature NMR techniques in solution. Thus, studies on the association of pyrimidine and purine nucleosides through specific hydrogen bonds have provided new insight into the structure of the associates and the relative strength of hydrogen bonding. The results obtained for the association of adenosine and 4-thiouridine in a Freon mixture demonstrate that a ternary $A\cdot(s^4U)_2$ complex is the most stable species at low temperatures and thermodynamically favored over Watson–Crick or Hoogsteen 1 : 1 $A\cdot s^4U$ base pairs. Only the 2-carbonyl oxygen of the pyrimidine base seems to be involved as proton acceptor in a hydrogen bond to a significant extent. Such an association mode contrasts with the preferred base pairing scheme as found for the uridine nucleoside. Although the thiobase has the same distribution of hydrogen donors and acceptors as the standard uracil base, the sulfur induces significant changes in its interaction. Clearly, this deviating behavior has to be considered not only when studying biological and pharmacological activities of the uracil 4-thio derivative but also when using it as a probe for structural studies.

Acknowledgments

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