Base Pairing of Cytosine Analogues with Adenine and Guanine in Oligonucleotide Duplexes: Evidence for Exchange between Watson–Crick and Wobble Base Pairs using ^{1}H NMR Spectroscopy

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^{1}H NMR spectra of oligodeoxynucleotide duplexes incorporating N^4-methoxycytosine and a related analogue paired with adenine and guanine demonstrate that Watson–Crick and wobble base pairs are in slow exchange on the chemical shift timescale.

In seeking pyrimidine base analogues for oligonucleotide probe or primer construction which would form base pairs of comparable stability with both adenine (A) and guanine (G), N^4-methoxycytosine (M) and its bicyclic analogue (P) have been studied.[1–3] The tautomers and possible geometric conformers of the two bases are shown in Fig. 1. UV melting studies,[2] using heptadecamer duplexes, have shown that P forms more stable base pairs than M with both purine bases. A·P and G·P base pairs were of comparable stability to each other and to the natural G·C and A·T pairs. The G·M base pair, too, had approximately equal stability to the A·M pair. Although less stable than the normal base pairs, they were considerably more stable than the mismatches G·T and A·C. Only in the case of the G·M pair has any structural study been undertaken. A crystallographic investigation of the self-complementary duplex d(CGCGMG)$_2$ showed that the base pair had the wobble configuration.[4]

Two series of self-complementary duplexes, designated 8mer-APy and 8mer-GPy, of the general form d(CGAATPyCG)$_2$ and d(CGGATPyCG)$_2$, respectively (where Py = T,C,M or P), have been synthesised and studied using ^{1}H NMR spectroscopy. Variable temperature studies have been used to probe duplex stability by monitoring temperature-dependent line-broadening and chemical shift changes of base protons over the range 273 to 340 K. In a cooperative melting transition, the rate of change of chemical shift is at a maximum at the melting temperature (T_m), when the concentrations of duplex and single strands are equal. The following T_m values were obtained: 8mer-AT (325 K), 8mer-AP (324 K), 8mer-AM (305 K), 8mer-AC (no duplex formed), 8mer-GC (343 K), 8mer-GP (315 K), 8mer-GM (297 K) and 8mer-GT (306 K). It appears, therefore that the preferential binding of P with A is of sufficient magnitude to be easily observed in the shorter oligonucleotide duplexes reported here, but was less marked in the more stable 17-mer duplexes used in the previous studies.[1,2]

The nature and integrity of the base pairing interactions taking place in solution are demonstrated by the detection of slowly exchanging imino proton resonances in 80% H_2O solutions. It is well established that imino protons involved in

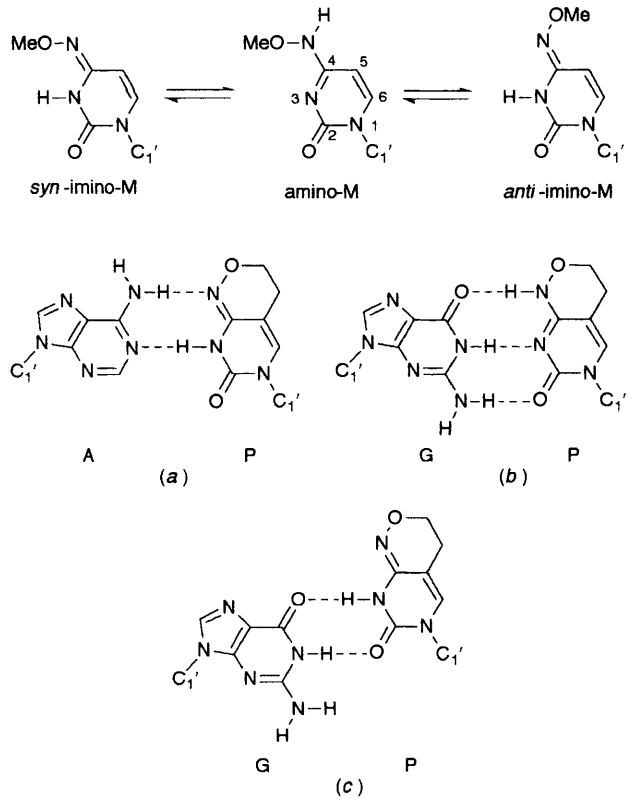

Fig. 1 Structures of the pyrimidine analogues N^4-methoxycytosine (M) and 6*H*,8*H*-3,4-dihydropyrimido[4,5-*c*][1,2]oxazin-7-one (P), showing the possible tautomeric and geometrical isomers. P is shown in the following base pairs: (*a*) Watson–Crick A·P; (*b*) Watson–Crick G·P; (*c*) wobble G·P.

hydrogen bonds to ring nitrogen atoms (as in a Watson–Crick base pair) show large downfield shifts, appearing in the δ 12–14 range, whereas imino protons hydrogen bonded to carbonyl oxygen atoms (as in a G·T wobble base pair) appear in the δ 10–12 chemical shift range.[5] Chemical shifts are therefore a reliable diagnostic of the base pairing scheme prevalent in solution. By virtue of the number and position of the peaks, the spectrum of 8mer-GC [Fig. 2(*a*)] indicates Watson–Crick base pairing alone, whereas the 8mer-GT spectrum [Fig. 2(*b*)] confirms the presence of a wobble base pair.

Imino proton spectra for 8mer-AP and -AM are indicative of Watson–Crick base pairing, with imino protons lying to low field (δ 12–14). In contrast, 8mer-GP and -GM have signals characteristic of the Watson–Crick base pairing scheme and in addition resonances in the predicted chemical shift range for wobble base pairs (Fig. 2). We provide direct evidence that G·P base pairs undergo slow chemical exchange between the Watson–Crick and wobble forms through the use of saturation transfer difference spectra. As the NOEs observed within the duplexes are of the order of 10%, a larger intensity change indicates that chemical exchange mechanisms may be occurring. In the water-suppressed spectrum of 8mer-GP, irradiation of the small peak at δ 10.6 leads, in addition to a series of NOEs, to an intensity change at δ 12.9 well beyond the NOE range (Fig. 3). This observation provides striking evidence for chemical exchange between Watson–Crick and wobble base pairs. The spectrum of 8mer-GM [Fig. 2(*d*)] is also indicative of slow exchange between Watson–Crick and wobble forms of base pair, although no saturation transfer evidence is available for this duplex. In this spectrum, the imino proton resonances are all of similar intensity, indicating that the two forms of the base pair are of roughly comparable stability. The presence of at least 4 peaks in the δ 9–12 region is comparable with more than one form of the wobble base pair, but not proven as such.

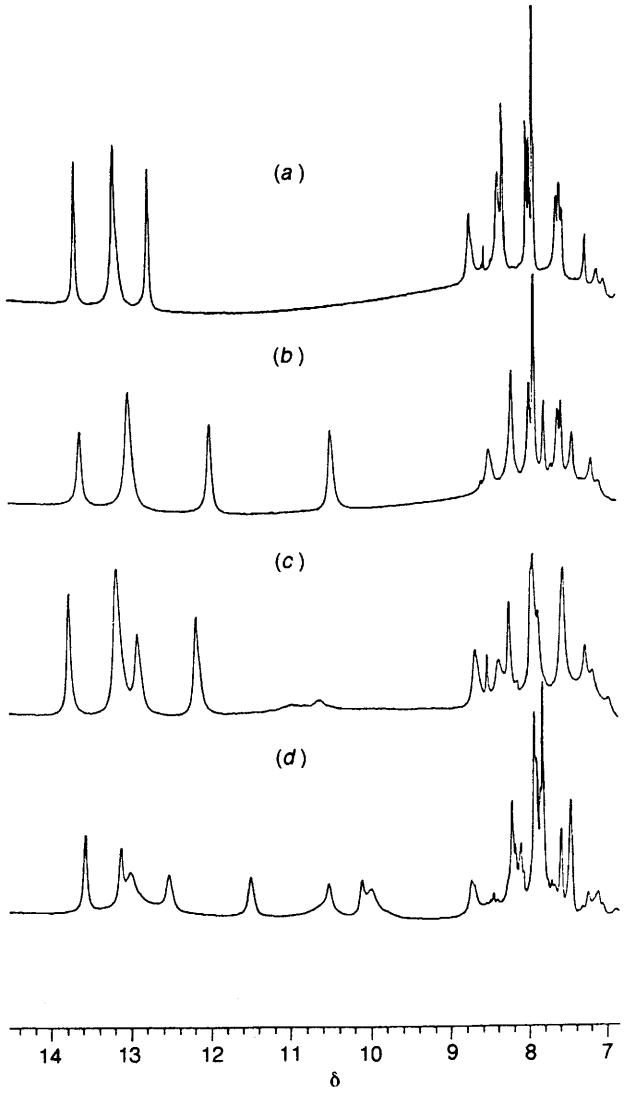

Fig. 2 Imino proton NMR spectra of: (*a*) 8mer-GC; (*b*) 8mer-GT; (*c*) 8mer-GP; (*d*) 8mer-GM, using a 1-3′-3-1′ binomial water suppression sequence.[9] Spectra were recorded in H_2O–D_2O (4:1) buffer (1 mol dm^{-3} NaCl, 50 mmol dm^{-3} phosphate, 1 mmol dm^{-3} ethylenediaminetetraacetic acid) at pH 7.0 and 10 °C.

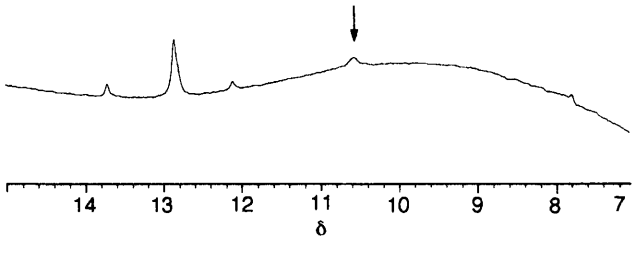

Fig. 3 Saturation transfer difference NMR spectrum of 8mer-GP with irradiation at δ 10.6, showing a chemical exchange effect at δ 12.9

It is possible that this is due to the two orientations of the methoxy group in the imino tautomer of the M base.

Assignment of the imino resonances for 8mer-GC and -GT was confirmed by variable temperature studies, NOE difference spectroscopy and comparison with previous studies.[5] It was also possible to assign the imino proton spectrum of 8mer-GP using these methods and by analogy with the spectra recorded for 8mer-GC and -GT (data not shown).

Previous studies, both in aqueous solution and in the crystalline state,[6–8] have revealed that the N^4-methoxycytidine nucleoside and the free base demonstrate a preference for the

imino tautomer and adopt the *syn* isomeric form. This fact may explain the increased stability of the wobble form of the G·M base pair, in which this stabilised conformation can be adopted. Interestingly, in the crystalline state, the wobble structure alone was observed.[4] P cannot take up the *syn* conformation. This factor must account for the fact that in the G·P base pair the Watson–Crick form is highly populated, utilising the less stable amino tautomer.

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