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Tautomerism, acid-base properties and conformation of methylated analogues of the promutagenic N^4-hydroxycytosine

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Abstract

UV and NMR spectroscopy were employed to study the tautomerism, acid-base properties and conformation of the exocyclic N^4–OH group in 1-methyl-N^4-hydroxycytosine (1-mOH^{4}C), and its methyl derivatives, viz. the fixed *imino* forms (1,3-m^{2}OH^{4}C and 1,3,5-m^{3}OH^{4}C), the fixed *amino* form (1,N^4-m^{2}OH^{4}C), and analogues sterically constrained to the form *syn* (1,5-m^{2}OH^{4}C) or *anti* (1,3-m^{2}OH^{4}C) with respect to the ring N(3). Relative to 1,N^4-m^{2}OH^{4}C, UV spectroscopy showed that the other analogues were predominantly *imino* and that all analogues formed a structurally common cation in acid medium, with results pointing to ~90% population of the *imino* species for 1-mOH^{4}C and 1,5-m^{2}OH^{4}C, further supported by NMR spectroscopy. Both exhibited two sequential dissociations in alkaline medium, the first due to N^4–OH, followed by the N(3)–H. ^{1}H and ^{13}C NMR spectroscopy showed 1-mOH^{4}C in the conformation *syn*. With 1,3,5-m^{3}OH^{4}C, an 'overcrowded' planar molecule with steric constraints to both the *syn* and *anti* conformations, a *syn–anti* equilibrium is observed, with a preference of ~75% for the *anti* rotamer, independently of the polarity of the medium. Exchange between the rotamers is slow on the NMR time-scale, with a minimal barrier to exchange exceeding 100 kJ/mol. In low-polar media, the analogues associate as dimers via O^4–H···O^2 or O^4–H···N^4 hydrogen bonds, with association constants at ambient temperature of 4.6 (1,3-m^{2}OH^{4}C), 12.8 (*anti* 1,3,5-m^{3}OH^{4}C), 36 (1,5-m^{2}OH^{4}C), 109 (*syn* 1,3,5-m^{3}OH^{4}C) M^{-1}. Implications of the overall findings to the promutagenic activities of OH^{4}C and OMe^{4}C are examined.

Keywords: Hydroxylamine mutagenesis; Methylated analogues of N^4-hydroxycytosine; Tautomerism; Ionic forms; Conformational equilibrium; Autoassociation

Abbreviations: Cyt, cytosine; Ura, uracil; Gua, guanine; Ade, adenine; OH^{4}C, N^4-hydroxycytosine; OMe^{4}C, N^4-methoxycytosine; 1-mOH^{4}C, 1-methyl-N^4-hydroxycytosine; 1-mOMe^{4}C, 1-methyl-N^4-methoxycytosine; 1,3-m^{2}OH^{4}C, 1,3-dimethyl-N^4-hydroxycytosine; 1,5-m^{2}OH^{4}C, 1,5-dimethyl-N^4-hydroxycytosine; 1,5-m^{2}OMe^{4}C, 1,5-dimethyl-N^4-methoxycytosine; 1,N^4-m^{2}OH^{4}C, 1,N^4-dimethyl-N^4-hydroxycytosine; 1,3,5-m^{3}OH^{4}C, 1,3,5-trimethyl-N^4-hydroxycytosine; 3-mOH^{4}Cyd, 3-methyl-N^4-hydroxycytidine; 3-mOMe^{4}Cyd, 3-methyl-N^4-methoxycytidine; OMe^{6}Ado, N^6-methoxyadenosine; TMS, tetramethylsilane; TSP, 2,2,3,3-tetradeuter-3-(trimethylsilyl)-propionic acid (sodium salt); DMSO-d_6, deuterated dimethylsulfoxide; C^{2}HCl$_3$, deuterated chloroform

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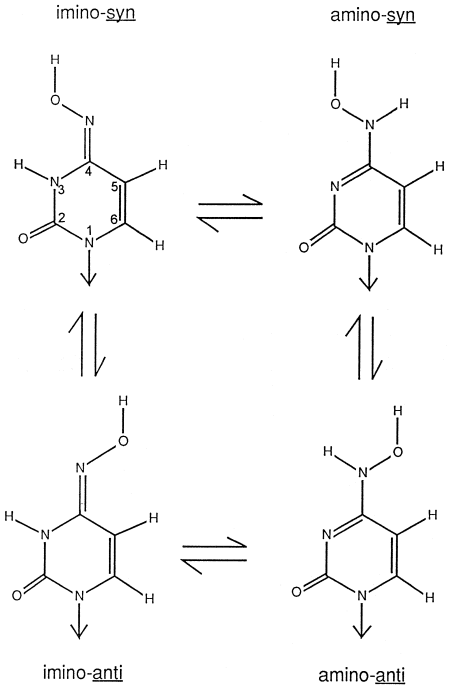
1. Introduction

The promutagen N^4-hydroxycytosine (OH^4C, Scheme 1) [1,2] is a product of the reaction of the known mutagen hydroxylamine (NH_2OH) with cytosine and its nucleotides, and with cytosine residues in RNA and DNA [3]. The promutagenic activity of OH^4C stems from its ability to base pair like cytosine or uracil, the latter leading to a $C \rightarrow U(T)$ transition [1–10]. This dual functionality of OH^4C is due in part to its existence as an equilibrium mixture of *amino* and *imino* tautomers (Scheme 1), with the latter predominant [11–17]. In addition, the exocyclic N^4–OH group may exist as an equilibrium mixture of rotamers, *syn* and *anti*, relative to the ring N(3) (Scheme 1). Both tautomeric and conformational equilibria play a key role in base pairing with potentially complementary bases [18], and also by inhibition of thymidylate synthase by OH^4–dCMP, where the *syn* conformer leads to a 10^4-fold diminution of inhibitory potency [19].

N^4-methoxycytosine (OMe^4C), the product of reaction of methoxyamine (NH_2OMe) with cytosine, is also a promutagen. A spectroscopic study of self-complementary oligonucleotide duplexes showed both Watson–Crick and 'wobble' base pairing of OMe^4C with Gua, with OMe^4C in the *imino* form and the N^4–OMe in the *syn* and *anti* conformations, respectively [9]. The preferred *syn* conformation had a destabilizing effect on an octomer duplex with an OMe^4C–Ade base pair, in equilibrium between Watson–Crick and 'wobble' configurations at low temperatures, with an *anti*-oriented methoxy group and a melting transition accompanied by isomerization to the *syn* conformation [10]. A similar destabilizing effect has been noted for other self-complementary oligonucleotide duplexes [20,21], a crystalline Z-DNA oligomer [22], and monomeric *imino* OMe^4C pairing with Ade in solution [15].



Scheme 1. Amino–imino tautomeric equilibrium and *syn–anti* rotamer equilibrium of the exocyclic N^4–OH group relative to the ring N(3), in N^4-hydroxycytosine (OH^4C).

For the rotameric free analog of OH^4C, *imino* 1-mOH^4C, the exocyclic group is in the conformation *syn* [23]. For the *imino* 1,5-m^2OH^4C (and 1,5-m^2OMe^4C), the 5-methyl substituent sterically confers a preference for the *syn* rotamer, additionally stabilized by interaction of the exocyclic O^4 with the ring N(3)–H, in the solid state [24] and in solution [11–15], as for the corresponding N^6-methoxyadenosine [25]. It may be anticipated that, in the fixed *imino* 1,3-m^2OH^4C, the methyl group at N(3) would sterically lead to preference of the rotamer *anti*. Furthermore, theoretical calculations [26] point to existence of an unusually high barrier to exchange between the two rotamers of OH^4C. This prompted us to compare the solution conformations of 1,3-m^2OH^4C and 1,5-m^2OH^4C with that of 1,3,5-m^3OH^4C, with steric constraints at both N(3) and C(5). Interestingly, the latter proved to be a planar 'overcrowded' molecule, like 1,N^4,N^4,5-tetramethylcytosine [27], uniquely in the conformation *anti* in the solid state [28]. Earlier studies [13–15,28–30] have also been extended to an examination of the acid-base properties of OH^4C and its methylated analogues, as well as their potential to form autoassociates in apolar media.

2. Materials and methods

2.1. Materials

1,N^4-dimethyl-N^4-hydroxycytosine hydrochloride and 1-methyl, 1,3-dimethyl, 1,5-dimethyl and 1,3,5-

trimethyl derivatives of N^4-hydroxycytosine were synthesized as described [11,31]. All compounds were dried under vacuum over P_2O_5 and characterized by melting point determinations. Their purity and structures were confirmed by chromatography, pH-dependent UV absorption spectra, ^1H and ^{13}C NMR spectroscopy.

Deuterated chloroform (C^2HCl_3, $\geq$ 99.8% mol ^2H), deuterated dimethylsulfoxide (DMSO-d_6, $\geq$ 99.8% mol ^2H), deuterated water (^2H_2O, $\geq$ 99.95% mol ^2H), TMS and TSP were obtained from Merck (Darmstadt, Germany). Sodium hydroxide, orthophosphoric acid and acetic acid were from OCh (Lublin, Poland). Boric acid was from POCh (Gliwice, Poland), and hydrochloric acid was from Aldrich (Milwaukee, USA). Buffers were made using MilliQ water. All other chemicals, reagents and materials were of the highest, spectral-grade quality commercially available.

2.2. UV spectroscopy

UV spectra were run on a Kontron (Rotkreuz, Switzerland) Uvikon 930 UV–VIS instrument, using 10-mm pathlength quartz cuvettes in a cell holder thermostated at 22°C. Spectrophotometric titrations at several different wavelengths made use of 30–200 mM Britton–Robinson buffers in the pH range 2–12, and HCl and NaOH outside this range. Measurements of the pH of HCl and NaOH solutions took account of activity coefficients as a function of concentration and temperature [32]. Changes in ionic strength of the Britton–Robinson buffers were without effect on the spectra. Monitoring of pH made use of a Jenway (Dunmow, UK) pH-meter to an accuracy of 0.02 pH units and 0.05°C.

UV-irradiation of OH^4C derivatives was conducted with a Philips mercury resonance lamp (254 nm), short wavelength radiation being filtered out with an acetic acid filter. Radiation intensity was determined by actinometry, from the quantum yield for photohydration of uridine [33]. Solution concentrations were 50 μM, and irradiations were performed in buffered medium (pH 6.5), in water and in 1 N HCl.

2.3. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded on Varian Gemini 200 MHz and Varian UNITYplus 500 MHz spectrometers, relative to internal TMS in C^2HCl_3 and DMSO, and internal TSP in ^2H_2O, to an accuracy of $\pm$0.001 and $\pm$0.03 ppm for proton and carbon chemical shifts, respectively. The concentrations were determined with 10% accuracy and the temperature stabilized within $\pm$0.1°C. Identification of the ^1H resonances of the *anti* and *syn* rotamers of 1,3,5-m^3OH^4C (Scheme 1) was based on the ^1H NMR spectra of 1,3-m^2OH^4C and 1,5-m^2OH^4C, the sterically preferred *anti* and *syn* rotamers, respectively. Relative populations of the rotameric forms were determined from the integral intensities of the H6 and/or C(5)–CH_3 protons of the *anti* and *syn* rotamers of 1,3,5-m^3OH^4C to an accuracy of $\pm$2%. Assuming these are in thermodynamic equilibrium, the difference in free enthalpy, $\Delta G°$, between the energy minima at temperature T is as follows:

$$\Delta G° = RT \ln\left(\frac{P(anti)}{P(syn)}\right) \quad (1)$$

The concentration-dependence of the chemical shifts of the N^4–OH proton in 1,3-m^2OH^4C, 1,5-m^2OH^4C and the *anti* and *syn* rotamers of 1,3,5-m^3OH^4C, permitted calculations of autoassociation constants. Assuming formation only of dimers, the apparent constants K are as follows (where C_{xx} is the concentration of dimer, and C_x the monomer):

$$K = \frac{C_{xx}}{(C_x)^2} \quad (2)$$

The total molar concentration of a compound (for 1,3-m^2OH^4C and 1,5-m^2OH^4C) or of the conformational form (for the *syn* and *anti* rotamers of 1,3,5-m^3OH^4C), expressed as C_{x0}, is:

$$C_{x0} = 2C_{xx} + C_x \quad (3)$$

For the model involving one hydrogen bond per dimer, the measured value, δ_m, of the chemical shift of the N^4–OH proton is:

$$\delta_m^{1HB} = \frac{(C_x + C_{xx}) \cdot \delta_x + C_{xx} \cdot \delta_{xx}}{C_{x0}} \quad (4)$$

For the second model, where both O^4–H protons of a dimer are hydrogen bonded, we have:

$$\delta_m^{2HB} = \frac{C_x \cdot \delta_x + 2C_{xx} \cdot \delta_{xx}}{C_{x0}} \quad (5)$$

where δ_x is the chemical shift of the free proton, and δ_{xx} is the chemical shift of the hydrogen-bonded proton. From Eqs. (2)–(5) we obtain the dependence of δ_m on C_{x0}:

$$\delta_m^{1HB}(C_{x0}) = \frac{1}{8KC_{x0}}\left(1 - \sqrt{1 + 8KC_{x0}}\right) \times (\delta_{xx} - \delta_x) + \frac{\delta_x + \delta_{xx}}{2} \quad (6)$$

and

$$\delta_m^{2HB}(C_{x0}) = \frac{1}{4KC_{x0}}\left(1 - \sqrt{1 + 8KC_{x0}}\right) \times (\delta_{xx} - \delta_x) + \delta_{xx} \quad (7)$$

Fitting of Eqs. (4) and (7) to the experimental points demonstrated that, in fact, the complexes formed are indeed dimers and not higher order structures (see Section 3.3, below).

All numerical analyses were made on an IBM PC 486 computer, using nonlinear regression analysis. The reduced χ_R^2, residuals and/or relative residuals were used to test the quality of the fits.

3. Results

3.1. Tautomerism and acid-base properties of methylated OH⁴C analogues

Fig. 1. exhibits the UV spectra of 1-mOH^{4}C and its methylated derivatives at acid, neutral and alkaline pH. As shown below, all of these undergo protonation at pH < 5, and dissociation at pH > 8. The spectra at pH 6.5 therefore represent the neutral forms. Allowing for the bathochromic effects of methylation, it will be noted that the spectra of the neutral forms of 1-mOH^{4}C and 1,5-m^{2}OH^{4}C are similar to those of the fixed *imino* forms 1,3-m^{2}OH^{4}C and 1,3,5-m^{3}OH^{4}C, whereas they all differ appreciably from the fixed *amino* form 1,N^4-m^{2}OH^{4}C. It follows that 1-mOH^{4}C and 1,5-m^{2}OH^{4}C are predominantly, if not exclusively, in the *imino* form.

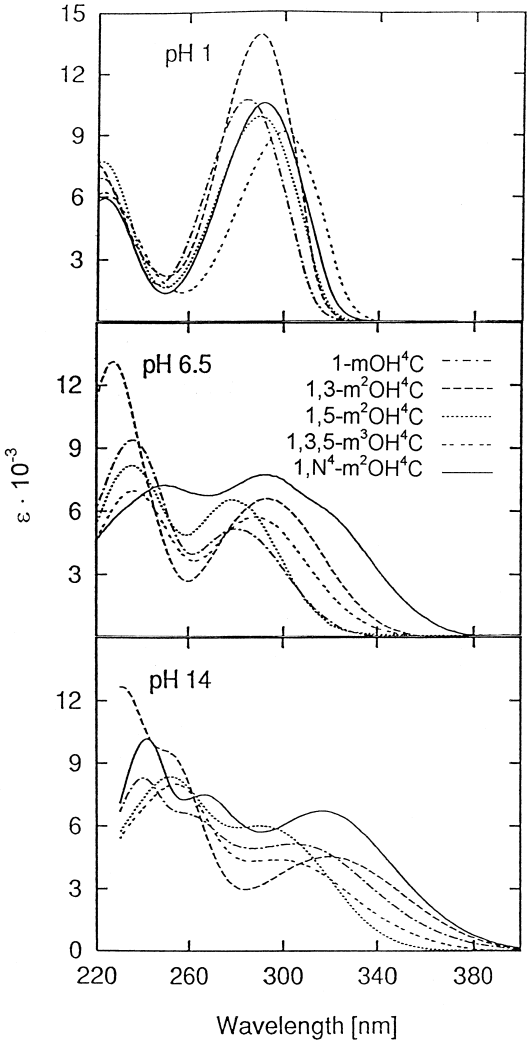


Fig. 1. UV absorption spectra of cationic (top), neutral (middle) and anionic (bottom) forms of 1,N^4-m^{2}OH^{4}C, 1,3-m^{2}OH^{4}C, 1,3,5-m^{3}OH^{4}C, 1,5-m^{2}OH^{4}C and 1-mOH^{4}C in aqueous medium at pH values indicated.

Spectrophotometric titration below pH 6.5 demonstrated that all compounds undergo protonation and exist as cations at pH 1 (Fig. 1). The pK_a values for protonation at N(3) or N^4 are listed in Table 1. Allowing for effects of methylation, the striking similarity of all spectra point to formation of a structurally common cation, with distribution of charge between N^4 and N(3). This permits applica-

Table 1
Protonation (pK_a) and dissociation (pK_b) constants (± 0.05) for methylated analogs of OH^4C and OMe^4C

Analogue	pK_a	pK_b	Dissociation site
1-mOH^4C	3.02	9.0[a,b]	O^4–H
		11.1	N(3)–H
1-mOMe^4C	2.0[c]	11.65[c]	N(3)–H
1,5-m^2OH^4C	2.89	8.75[a]	O^4–H
		11.83	N(3)–H
1,5-m^2OMe^4C	2.05[c]	12.1[c]	N(3)–H
1,3-m^2OH^4C	3.02	12.22	O^4–H
1,3,5-m^3OH^4C	3.43	12.4[b]	O^4–H
1,N^4-m^2OH^4C	3.85	9.01	O^4–H

[a] Values previously reported [17], 10.4 for 1-mOH^4C and 11.1 for 1,5-m^2OH^4C, overlooked existence of two functional dissociating groups.
[b] Accuracy ± 0.1.
[c] From Ref. [17].

tion of the basicity method [34] to calculate the *amino–imino* tautomeric equilibrium from the pK_a values of the fixed *amino* (1,N^4-m^2OH^4C) and *imino* (1,3-m^2OH^4C) forms, leading to a $K_T = 0.15 \pm 0.03$, hence a predominant population ($\sim 90\%$) of the *imino* species, as earlier postulated for OH^4C by Brown et al. [11,12] and as deduced above from the UV spectra of the neutral forms (Fig. 1).

The spectra of the same compounds in strongly alkaline medium (Fig. 1) testify to formation of the anionic forms, previously characterized as monoanions [17]. However, if 1-mOH^4C and 1,5-m^2OH^4C are also predominantly in the *imino* form in alkaline medium, we should expect two functional dissociating groups, the O^4–H and the N(3)–H (or the O^4–H and the N^4–H for the *amino* species). And, in fact, closer examination by spectral titration placed in evidence two dissociating groups, partially overlapping (Fig. 2, Table 1). With N(3) or N^4 methylated (1,3-m^2OH^4C, 1,3,5-m^3OH^4C or 1,N^4-m^2OH^4C, respectively) with no proton on N(3) and N^4, only one dissociating group is observed.

The pK_b values for anion formation are listed in Table 1. For 1-mOH^4C these are 8.96 and 11.07, and for 1-mOMe^4C, where only N(3)–H may dissociate, it is 11.65. For 1,5-m^2OH^4C, the corresponding values are 8.75 and 11.83, and for 1,5-m^2OMe^4C it is 12.10. It follows that, for 1-mOH^4C and 1,5-m^2OH^4C, it is the O^4–H which dissociates first, followed by the N(3)–H. Even assuming 1-mOH^4C and 1,5-m^2OH^4C to be in the *amino* form in alkaline medium, the first dissociating group is still the N^4–OH, and not the N^4–H, since $pK_b = 9.01$ for 1,N^4-m^2OH^4C, and 11.65 for 1-mOMe^4C (Table 1).

It is of interest that the values of pK_b for formation of the monoanions of 1-mOH^4C and 1,5-m^2OH^4C are similar to that for the fixed *amino* 1,N^4-m^2OH^4C, but much lower than the pK_b for the fixed *imino* species 1,3-m^2OH^4C and 1,3,5-m^3OH^4C, where the methyl substituent at N(3) sterically confers a preference for the *anti* rotamer of the exocyclic N^4–OH (see Section 3.2). The foregoing is subject to two possible interpretations: (a) dissociation of the O^4–H proton in 1-mOH^4C and 1,5-m^2OH^4C may depend on the tautomeric equilibrium and proceeds for the minor *amino* tautomer ($\sim 10\%$ population, see above) at the same pH as for the fixed *amino* 1,N^4-m^2OH^4C; (b) the pK_b may be

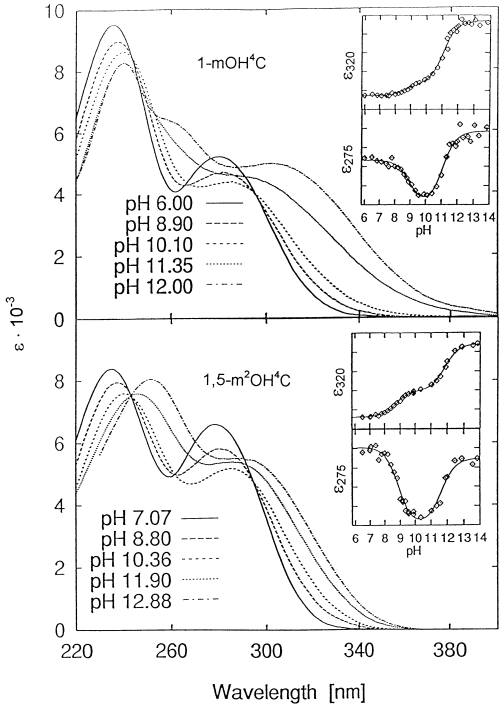


Fig. 2. UV absorption spectra in aqueous medium showing dissociation of the N^4–OH and the N(3)–H in 1-mOH^4C (top) and 1,5-m^2OH^4C (bottom). Note the isosbestic points at 243, 267, 295 nm (upper frame), and 243, 261, 295 nm (lower frame). Inserts show the pH-dependence of the molar extinction coefficients at 275 and 320 nm.

Table 2
Proton chemical shifts (ppm) vs. internal TMS (in C^2HCl_3, DMSO-d_6) or TSP (in ^2H_2O), proton–proton coupling constants (Hz) and average populations (%) of the *anti* and *syn* rotamers of 0.02 M methylated analogs of OH^4C in various solvents, at 17°C[a]

Protons and coupling constants	C^2HCl_3					DMSO-d_6		^2H_2O	
	1,3-m^2OH^4C	1,5-m^2OH^4C	1-mOH^4C	1,3,5-m^3OH^4C		1,3,5-m^3OH^4C		1,3,5-m^3OH^4C	
	anti	*syn*	*syn*	*anti* 77%	*syn* 23%	*anti* 76%	*syn* 24%	*anti* 74%	*syn* 26%
O^4–H[b]	7.64[c]	6.37[c]	6.14[c]	6.664	7.435	9.584	10.093	–	–
H(6)	6.200	6.346	5.527	6.195	6.257	6.596	6.622	6.593	6.60[c]
N(1)–CH_3	3.235	3.215	3.232	3.202	3.191	3.059	3.064	3.163	3.181
N(3)–CH_3	3.280	–	–	3.208	3.633	3.081	3.425	3.197	3.489
C(5)–CH_3	–	1.793	–	2.271	1.768	2.158	1.694	2.230	1.784
N(3)–H	–	8.14[c]	8.13[c]	–	–	–	–	–	–
H(5)	6.619	–	6.504	–	–	–	–	–	–
J(6,5-CH_3)	–	1.3	–	1.28	1.28	1.12	1.3	1.25	1.4
J(5,6)	8.1	–	8.0	–	–	–	–	–	–

Carbon chemical shifts (ppm ±0.03) of 0.02 M methylated analogs of OH^4C vs. internal TMS in chloroform, at 17°C

Carbons	1-mOH^4C	1,3,5-m^3OH^4C	
	syn	*anti*	*syn*
C(2)	149.81	151.10[d]	–
C(4)	145.31	149.38[d]	–
C(5)	97.52	104.72	107.61
C(6)	136.04	134.21	130.73
N(1)–CH_3	35.28	35.94	35.52
N(3)–CH_3	–	30.03	29.64
C(5)–CH_3	–	18.98	14.27

[a] Chemical shifts ±0.001 ppm, coupling constants ±0.1 Hz, rotamer populations ±2%.
[b] OH^4 chemical shifts strongly dependent on degree of autoassociation.
[c] Lower accuracy (±0.01 ppm) due to signal broadening.
[d] Identification uncertain, because of low signal intensities.

dependent on electrostatic interaction of N^4–OH with the lone pair of N(3) in 1,N^4-m^{2}OH^{4}C, and the N(3)–H with the lone pairs of O^4 in 1-mOH^{4}C and 1,5-m^{2}OH^{4}C. Such interaction could conceivably weaken the O^4–H bond and facilitate dissociation at pH ~ 9. It should be noted that this is not possible for the N(3)-substituted analogues, reflected by the fact that their pK_b values are three units higher (Table 1). Resolution of this problem may be feasible by analyses of ^{1}H NMR spectra in strongly alkaline media to determine to what extent, if any, anion formation affects rotamer populations under these conditions.

3.2. Conformation of the exocyclic N^4-hydroxy group of 1,3-m^{2}OH^{4}C, 1,5-m^{2}OH^{4}C and 1,3,5-m^{3}OH^{4}C

The exocyclic N^4–OH in 1-mOH^{4}C, where there is no steric hindrance to its rotation, is in the conformation *syn* in the solid state [23]. Methylation at C(5) constrains the conformation to *syn* both in solution and in the solid state [13,24]. Similarly, methylation at the ring N(3) constrains the conformation to *anti* in solution, as may be seen from a comparison of proton chemical shifts in Table 2[14]. Furthermore, comparison of proton chemical shifts of 1-mOH^{4}C and its two fixed tautomeric forms, in particular H(6) and N(3)–H, lead to the conclusion that 1-mOH^{4}C in solution exhibits a marked preference for, or is exclusively in, the conformation *syn*, as proposed elsewhere [14,15].

In the 'overcrowded' molecule 1,3,5-m^{3}OH^{4}C, where the two methyl groups at N(3) and C(5) confer steric hindrance to adoption of either conformation, one observes at 17°C two sets of signals for all protons in C^{2}HCl$_3$, DMSO-d_6 and ^{2}H$_2$O, as well as for the carbon signals in C^{2}HCl$_3$ solution (Table 2 and Fig. 3). From a comparison of the chemical shifts of H(6) and the methyl protons with those of the fixed rotamers *syn* and *anti* (Table 2), it follows that 1,3,5-m^{3}OH^{4}C exists in solution in a *syn–anti* dynamic equilibrium, with about 75% in the form *anti*, only the latter of which exists in the crystal [28]. Furthermore, the populations of the two conformers in solution are independent of the polarity of the solvent (Table 2).

It was, however, noted that attainment of a dynamic equilibrium is a slow time-dependent process. Immediately following dissolution of the compound in DMSO at room temperature, the population of the

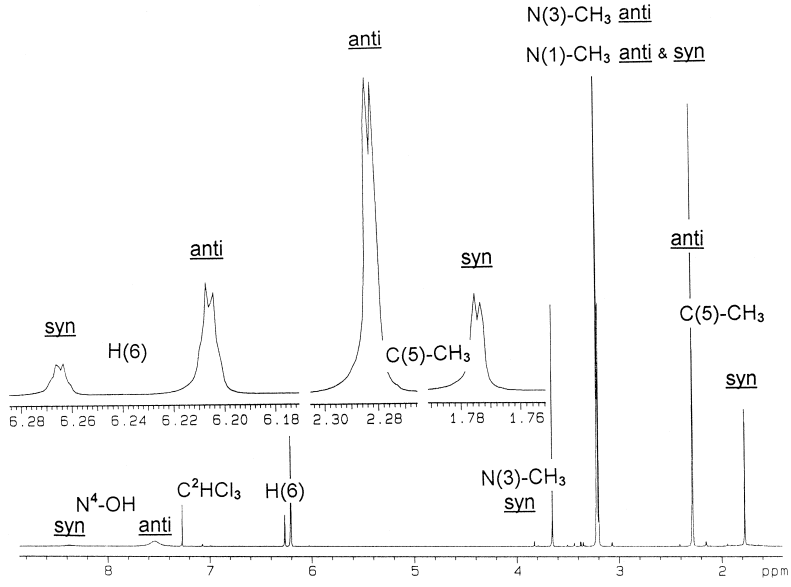


Fig. 3. ^{1}H NMR spectrum of 1,3,5-m^{3}OH^{4}C in chloroform. Inserts show the extended region containing signals of protons: H(6) (ppm ~ 6) and (5)-CH$_3$ (ppm ~ 2). The higher signal for each pair corresponds to the *anti* rotamer and the lower to the *syn* rotamer. Coupling between H(6) and (5)-CH$_3$ can be seen.

anti rotamer was about 90%, and this decreased to 76% after several hours. And, with an increase in temperature, there was a weak, but systematic, reversible and reproducible increase of the population *syn*. The spectra did not, on the other hand, display any temperature-dependent broadening of signals in the range up to 100°C. Assuming that broadening of signals is below the accuracy of our measurements (0.2 Hz), the Eyring equation permits evaluation of the lower limit of the barrier to rotation between the two conformers as about 100 kJ/M. This is consistent with ab initio quantum mechanical calculations by the SCF method, corrected for electron correlation effects by the MBPT(2) method which predict a value of 180 kJ/M [26].

It is also of interest that, for 1,3,5-m^{3}OH^{4}C in DMSO, an increase in temperature to 65–100°C led to splitting of the signals for both the *syn* and *anti* N^4–OH and H(6) protons into doublets. This was accompanied by broadening of the remaining signals in the spectrum (not shown). The foregoing testifies to an additional equilibrium observable on the NMR time-scale, and is being subjected to further investigation.

It had earlier been reported that UV-irradiation of 3-mOH^{4}Cyd and 3-mOMe^{4}Cyd resulted in a shift of the *syn–anti* equilibrium in the direction *syn* [35,36], based on analysis of irradiation-induced changes in UV absorption spectra. We have examined the effects of such irradiation on all five OH^{4}C analogues embraced in the present study, and found that the changes in absorption spectra are readily interpreted in terms of hydration of the 5,6 bond, or formation of cyclobutane photodimers [33]. We also found no evidence for irradiation-induced cleavage of the N^4–OH bond [37], which should have resulted in a 5–8-nm smaller blue-shift of the absorption spectra, than observed.

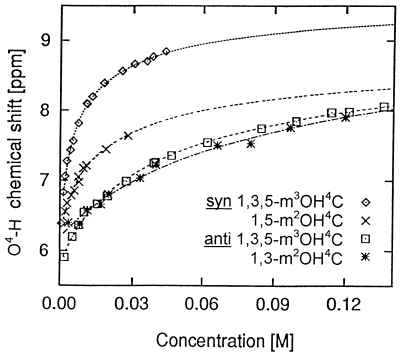
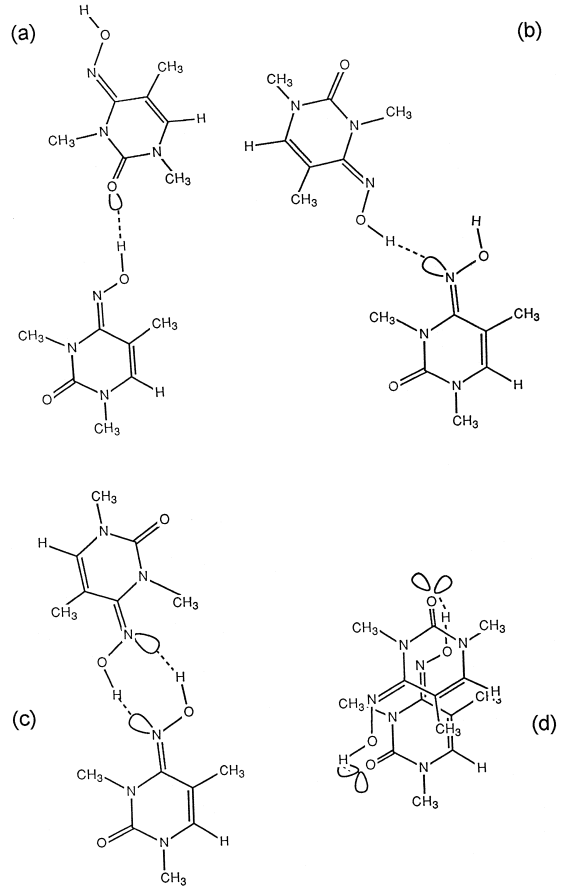


Fig. 4. Concentration-dependence of the ^{1}H chemical shifts of the exocyclic N^4–OH group in 1,5-m^{2}OH^{4}C, 1,3-m^{2}OH^{4}C, and *syn* and *anti* rotamers of 1,3,5-m^{3}OH^{4}C in chloroform solution at 17°C. Lines denote the numerical-fitted theoretical curves with parameters given in Table 3.



Scheme 2. Proposed structures of hydrogen-bonded autoassociates of 1,3,5-m^{3}OH^{4}C with one (a–b) and with two (c–d) hydrogen bonds shown by dotted lines. Analogous structures prevail for *syn* rotamers.

3.3. Autoassociation of 1,3-m^{2}OH^{4}C, 1,5-m^{2}OH^{4}C and 1,3,5-m^{3}OH^{4}C

The chemical shifts of H^4 in 1,3,5-m^{3}OH^{4}C (equilibrium mixture of *syn* and *anti*), 1,3-m^{2}OH^{4}C (*anti*) and 1,5-m^{2}OH^{4}C (*syn*) in the low-polar sol-

vent chloroform exhibit a sigmoidal dependence on concentration (Fig. 4). This testifies to formation of autoassociates via one (Scheme 2a,b) or two (Scheme 2c,d) hydrogen bonds involving O(2), N^4 and O^4 as acceptors, and N^4–OH as donor.

Bearing in mind the experimentally observed dependence of the *syn* and *anti* populations of 1,3,5-m^3OH^4C on its concentration in chloroform (0.0025–0.18 M, see Fig. 4), theoretical curves describing dimer formation were fitted to the experimental points and found to exhibit good correlation (Fig. 4, Table 3). No such correlation was observed when higher-order associates were taken into consideration. However, the difference between the values for one and two hydrogen bonds was too small to distinguish between these two cases, because the proton involved in hydrogen bonding gave signals in the range 10–14 ppm, so that both values (Table 3) are within the expected limits. A plausible argument in favor of formation of linear associates via one hydrogen bond (O^4–H · · · N^4 or O^4–H · · · O2) is forthcoming from the fact that, for stacked or linear associates with two hydrogen bonds (O^4–H · · · N^4, see Scheme 2c,d), these bonds would not be linear. Both crystallographic data [28] and theoretical calculations by the DFT method (G. Bakalarski et al., in preparation) show that formation of two such symmetrical hydrogen bonds (O^4–H · · · N^4) would require each to deviate from linearity by 40°, whereas deviations from linearity for normal hydrogen bonds do not exceed 25° [38].

Autoassociation of the minor conformer *syn* of 1,3,5-m^3OH^4C is much stronger than that of the major conformer *anti*, with an association constant an order of magnitude higher (Table 3). Simultaneously, the observed concentration-dependent increase of the population *syn* from 21% to 25% (Fig. 5) is induced by progressive dimerization. It follows that minimalization of the energy involved in formation of the hydrogen bond with the conformer *syn* is comparable to, or greater than, the difference in free enthalpy between the two conformers. Associates of 1,3,5-m^3OH^4C in DMSO were not detectable (data not shown), presumably due to competing hydrogen bonding between O^4–H and this solvent.

Table 3
Parameters for autoassociation of methylated analogs of OH^4C at 17°C in chloroform, derived from concentration-dependent changes of N^4–OH chemical shifts, taken from Fig. 4

Parameter	1,3-m^2OH4C	1,5-m^2OH^4C	1,3,5-3m^3OH^4C	
	anti	*syn*	*anti*	*syn*
K	4.6 ± 1.6	36 ± 13	12.8 ± 1.3	109 ± 10
δ_x	6.19 ± 0.07	6.20 ± 0.11	5.78 ± 0.04	6.05 ± 0.05
δ_{xx} (1HB)[a]	14.7 ± 1.2	12.0 ± 0.5	13.5 ± 0.2	13.69 ± 0.08
δ_{xx} (2HB)[a]	10.5 ± 0.6	9.1 ± 0.3	9.64 ± 0.10	9.87 ± 0.06

[a] See Eqs. (6) and (7).

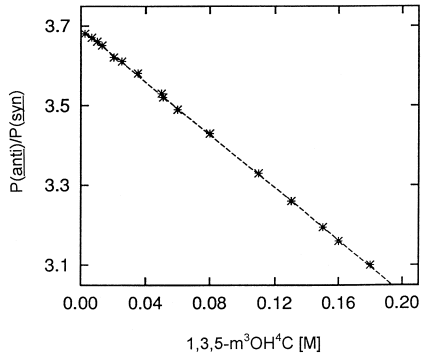


Fig. 5. Concentration-dependence of *syn* ↔ *anti* equilibrium of 1,3,5-m^3OH^4C in chloroform solution at 17°C, $P(anti)/P(syn)$ indicates proportion of populations of both rotamers.

Autoassociation of 1,3,5-m^3OH^4C is accompanied by chemical shifts of protons other than the O^4–H, indicative of interactions between aromatic rings. Although these shifts are small, they are reproducible. Fig. 6 exhibits the differences in response of these protons in chloroform solution at total concentrations below and above 0.06 M. At the higher concentrations, shielding of all protons by ring currents increases with concentration. By contrast, with increasing concentration in the range 0.0025–0.06 M, stacking either decreases (*syn* (3)-CH_3, *anti* (5)-CH_3, *syn* and *anti* H(6)), or is unaltered (*anti* (3)-CH_3, *syn* (5)-CH_3, *syn* and *anti* (1)-CH_3). The monotonic decrease in chemical shifts of all methyl protons with an increase in concentration, or decrease in temperature, was noted also in DMSO, despite absence of formation of hydrogen-bonded associates. The foregoing testifies to cooperativity of hydrogen bonding and interaction between the rings on formation of associates of 1,3,5-m^3OH^4C in chloroform. The relative orientations of the molecules are dependent on concentration and the dielectric con-

stant of the medium. The absence of increased stacking with an increase in concentration clearly excludes formation of 'classical' stacked associates. More likely is an orientation 'edge-to-face', as for π-electron coupled systems in non-polar media [39], e.g. such a geometry is preferentially exhibited in Phe–Phe interactions in the hydrophobic environment of a protein core.

Analogous measurements in chloroform solution were conducted with 1,3-m^{2}OH^{4}C and 1,5-m^{2}OH^{4}C in the concentration ranges 0.0035–0.12 M and 0.00086–0.028 M, respectively. The resulting calculated association constants are in accord with the fact that, for 1,3,5-m^{3}OH^{4}C, the conformation *syn* constrains H^4 to hydrogen bond with an association constant an order of magnitude greater than that of the *anti* conformation (see Table 3). It should be noted that the association constant determined here by NMR for 1,5-m^{2}OH^{4}C is in accord with that reported by IR spectroscopy [13] for linear associates of the same compound via O^4–H $\cdots$ O^2 bonding. The thermodynamic parameters determined by IR spectroscopy were $\Delta H = -2.8$ kcal/mol and $\Delta S = -2.7$ cal/mol K, corresponding to an association constant $K = 33 \pm 5$ M^{-1} at 290 K.

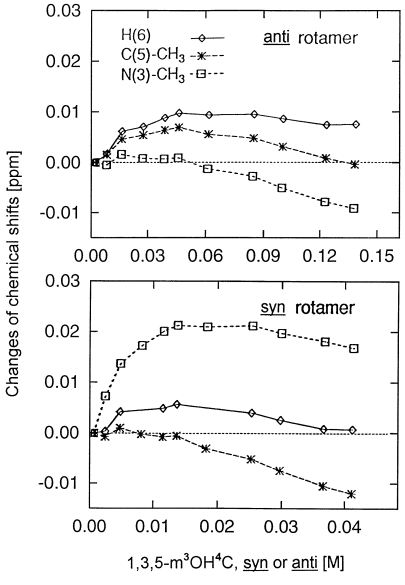


Fig. 6. Dependence of ^{1}H chemical shifts of the C–H protons on the concentrations of the *anti* (top) and *syn* (bottom) rotamers of 1,3,5-m^{3}OH^{4}C in chloroform at 17°C.

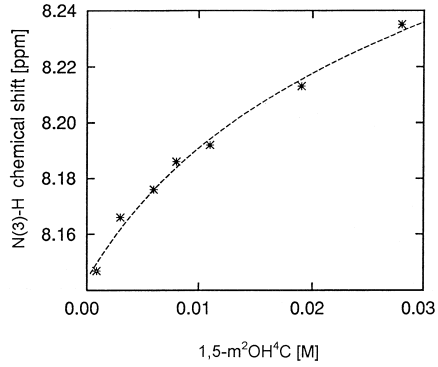


Fig. 7. Concentration-dependence of ^{1}H chemical shift of the N(3)–H proton in 1,5-m^{2}OH^{4}C in chloroform at 17°C.

The signals of H^4 engaged in hydrogen bonding in associates of 1,5-m^{2}OH^{4}C are located upfield relative to those of H^4 involved in autoassociates of *syn* 1,3,5-m^{3}OH^{4}C; whereas the signals of the free protons are identical for both compounds. This is most likely due to shielding of H^4 in the former compound by ring currents of the bases forming a dimer with spatial orientation other than in *syn* 1,3,5-m^{3}OH^{4}C. The minor changes in location of the N(3)–H signal in 1,5-m^{2}OH^{4}C as a function of concentration (Fig. 7) suggest that it is not a good donor for typical intermolecular hydrogen bonding, in line with earlier observations that N(3)–H is involved in intramolecular interaction with O^4 [13,23,24] not only in the crystal, but also in solution. This is, however, not a typical intramolecular hydrogen bond, inasmuch as its sigmoidal dependence on concentration is due to competing interactions with an external acceptor.

4. Discussion

Most studies on physico-chemical aspects of hydroxylamine and methoxyamine mutagenesis have concentrated on the latter, because OMe^{4}C monomer derivatives are more soluble in both aqueous and non-aqueous solvents, and the absence of the N^4–OH simplifies acid-base, as well as spectroscopic, properties.

It is of interest to first note some differences in their behavior in enzymatic systems, e.g. OH^{4}C nucleoside triphosphate simulates both UTP and CTP

in transcription by RNA polymerase and Qβ replicase, and in synthetic templates containing OH^4C residues, e.g. poly(C) +8% OH^4C residues, these also simulate both Ura and Cyt [6]. Under analogous conditions OMe^4C in a template appears to simulate only Ura, with incorporation of ATP [7], e.g. with a template of poly(U) +40% OMe^4C residues, the resulting transcript is poly(A) [8].

In line with the foregoing, an NMR study in aqueous medium of an oligonucleotide duplex containing an OMe^4C opposite an Ado residue demonstrated Watson–Crick base pairing with OMe^4C in the *imino* form and the OMe^4 group *anti* to the ring N(3) [9,10]. But, in crystals of a Z-form oligonucleotide duplex containing an OMe^4C-G pair, with OMe^4C also in the *imino* form, only 'wobble' base pairing was observed with the OMe^4 group *syn* to N(3) [22]. In striking contrast, a similar B-duplex in aqueous medium exhibited a temperature-dependent equilibrium of both Watson–Crick and 'wobble' base pairs, the former involving *amino* OMe^4C with OMe^4C *anti* to N(3), the latter *imino* OMe^4C with OMe^4C *syn* to N(3) [20,21]. It follows that both tautomeric forms exist simultaneously within DNA base pairs, the equilibrium being dependent on the base in the opposing strand (and probably also on adjacent bases in each strand).

Highly relevant to the foregoing is the demonstration that the tautomeric equilibrium of the corresponding N^6-methoxyadenosine (the product of the reaction of adenosine with methoxyamine) is shifted in solution at the monomer level in the presence of uracil, which base pairs with the *amino* form; and the presence of cytosine, which base pairs with the *imino* form of OMe^6Ado in various planar configurations [25]. It has been also shown that OMe^6Ado in oligodeoxyribonucleotides paired both with TTP and with dCTP, creating quite stable B-like right handed DNA [40].

It is clear that more reliant are the results obtained from studies in solution, adequately illustrated here by the demonstration that 1,3,5-m^3OH^4C, with the O^4–H group uniquely *anti* in the crystal [28], exhibits a very marked *syn–anti* equilibrium in solution, despite existence of steric hindrance to both conformers. It follows that the N(3)-methyl in 1,3-m^2OH^4C, while sterically constraining the N^4–OH from adopting the *anti* conformation, does not exclude existence of the *syn* rotamer. Similarly, in 1,5-m^2OH^4C, the *syn* rotamer is preferred, but not to exclusion of the *anti* rotamer.

The latter point is particularly relevant to reported potent mutagenicity of hydroxylamine vs. the T-even bacteriophages [41]. The DNA of these phages contains, in place of cytosine, 5-hydroxymethylcytosine and/or glucosylated 5-hydroxymethylcytosine, both of which were shown to react readily with hydroxylamine [42]. The products of reaction of such residues with hydroxylamine would be expected to exhibit a predominant population of the *syn* rotamer, which would lead largely to 'wobble' base pairing.

An additional fact, relevant to the base pairing ability of *imino* OH^4C, in contrast to OMe^4C, is the possibility of involvement of the N^4–OH in such pairing, depending in part on whether it is in the *syn* or *anti* conformation. The structures of autoassociates of 1,3,5-m^3OH^4C involving the O^4–H proton as a donor, the N^4 and O(2) atoms as acceptors, and neglecting O^4 (Scheme 2) well agree with the observations in the crystal [28], where the N^4 and O(2) atoms create hydrogen bonds with water molecules but the O^4 atom does not.

Consideration must also be given to the relatively low pK for dissociation of the exocyclic hydroxyl of the *imino* form, so that it exists in detectable proportions at physiological pH.

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