

Conformational Analysis by NMR Spectroscopy of 2'-Deoxy-2'-C-Alkyl nucleosides: Building Blocks of New Antisense Fragments

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Abstract. The conformation in solution of new modified nucleosides bearing branched-chain sugars used as building blocks for antisense oligonucleotides is investigated by NMR spectroscopy. Within a classical approach to the conformational study of these molecules, a new methodology is adopted, which is capable of exploring all the possibilities, for a given conformational model, that are compatible with the experimental data. The relationship between conformation of the monomers and hybridization properties of the corresponding oligonucleotides is examined.

1. Introduction

The field of Nucleic Acids and their chemical and biochemical applications, the development of the so called antisense strategy occupies a well-defined place.

Basically, the principle is based on the recognition of complementary sequences through base pair interactions, and the final goal is the inhibition of the expression of a target gene. Therefore, an antisense fragment is a synthetic oligonucleotide carrying a sequence which is complementary (antisense) to a portion of a target m-RNA. This oligonucleotide will hybridize with this RNA molecule and block its translation [1].

This strategy has very important applications not only in molecular biology but also in the development of new therapeutic principles [2].

Some features are characteristic of a suitable antisense fragment. The most important ones are: good up-take by cells, resistance to nucleases, and good hybridization properties.

In order to obtain stable oligonucleotides with regard to the action of nucleases, it is necessary to introduce chemical modifications. These modifications, though, must not

impair the base-pair recognition since this clearly is the basis of the whole mechanism of action of the antisense compound. Whence the interest of a careful analysis of the structural perturbations which are produced by different substituents.

One modification which is widely used is the alkylation at the 2'-hydroxyl of the sugar ring that generates 2'-O-alkyl-oligonucleotides. These fragments are highly resistant to nucleases and have very good hybridization properties [3]. The formation of stable hybrids has been proposed to be due to the preferred C-3'-*endo* conformation adopted by the sugar.

The introduction of an alkyl substituent directly bonded to C-2' has been more recently proposed [4] for the generation of new antisense fragments. The structure of some of their monomeric units is here analyzed. For this purpose we chose two representative pyrimidine nucleosides with the two possible configurations at C-2': (2'S)-2'-deoxy-2'-C-methyluridine (**1**) and (2'R)-2'-deoxy-2'-C-allyluridine (**2**). Additionally, in order to study the influence of the presence of the 3'-hydroxyl group on the conformation preferences of these compounds we studied the 3'-deoxy analogues: (2'S)-2',3'-dideoxy-2'-C-methyluridine (**3**) and (2'R)-2',3'-dideoxy-2'-C-allyluridine (**4**). The chemical structure of these compounds is reported in Fig. 1.

The relative stability of the hybrids formed by oligomers of **1** or **2** with the complementary sequence of RNA is discussed in terms of the conformation of the sugars.

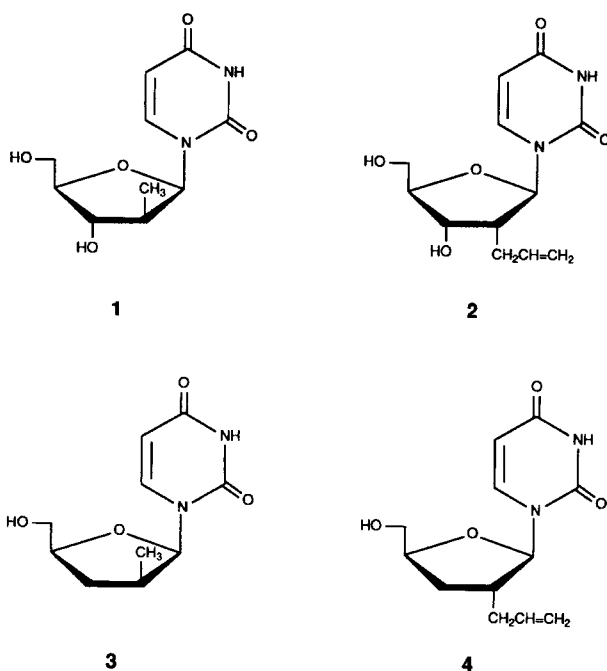


Fig. 1.

Traditionally, the conformational analysis of the sugar ring of nucleosides in solution is tackled by measuring the vicinal ^1H - ^1H J -couplings. The methodology more frequently used in this type of studies was developed by Altona and co-workers [5], combining the concept of pseudorotation [6] for a convenient description of the sugar ring, the use of a generalized Karplus equation [7] for the translation of the J -values into proton-proton torsion angles, and the use of a two-state model for the conformation of the furanose ring. A fitting algorithm is then currently used for generating the solution which best fits the data.

The estimation of each single torsion angle of the furanose ring is affected by several sources of error: a) the approximation of the experimental J -value, b) the calibration of the empirical Karplus equation, c) the possible presence of a significant conformational averaging.

Additionally, the relation between a given torsion angle and the conformational parameters is not known exactly and is likely to introduce another source of inaccuracy.

In spite of all this, the conformational analysis can still be carried out and produce meaningful results since all measurable coupling constants are used together to fit – *via* the estimated torsion angles – few conformational parameters.

The use of a computational program [8] allows one to establish the “best fit” solution to the problem. However, this solution does not reflect the uncertainty that is associated with the measurement and the calculation of each J -coupling value. Therefore, it provides an incomplete piece of information which could often be misleading.

What is necessary and has been done in this paper is expressing the solution as an ensemble of all the possibilities, within a given conformational model, which are capable of reproducing the experimental data within a given degree of confidence.

2. Experimental Methods

The NMR measurements were performed at 500 MHz with a Bruker AMX 500 spectrometer. The coupling constants were obtained, where necessary, after spectra simulation using the routine LAOCOON5. The NOEs were measured by the steady-state method with selective saturations of resolved signals and irradiation times of 40 s.

3. Results

For our compounds the so called pseudorotational model is adopted: if the deviations of heterocyclic five-membered rings from the geometry of cyclopentane are taken to be minor, then we can describe the conformation of the sugar ring in terms of a so called puckering amplitude (Φ_m) and of a phase angle of pseudorotation (P) [6]. This description of the sugar ring is sufficiently accurate for our purposes in that it is not in any respect a major source of error in our analysis.

A further stronger assumption is the so called two-state model: the sugar ring in solution is taken to exist in two well-defined conformations in mutual rapid equilibrium. One conformer is called North (N) (with puckering around $P = 0$) and the other is called South (S) (with puckering around $P = 180$). The problem is eventually reduced to the determination of the five independent parameters P_N , Φ_N , P_S , Φ_S , X_N , where X_N is the molar fraction of the N-conformer, from the observed coupling constants.

The two-state assumption is justified in terms of energy simulation diagrams which show the existence of two energy minima for the sugar ring [9]. Nevertheless it goes without saying that this model represents a deliberate simplification of the expected behaviour in solution of these small molecules, which are likely to be rather flexible around their energetic most favourite conformations. However, it must be admitted that this flexibility does not seem to affect the experimental data very much [5], in that it does not significantly alter the averaged values of the coupling constants. If we now take into account the inherent inaccuracy of this analysis, we may consider the molecular flexibility as a sort of "concealed" variable, which does not affect the data and can therefore be estimated only by other means.

A further point to be risen about the method is the need for a relationship that correlates *exocyclic* proton-proton angles (which we measure through J -values) and the *endocyclic* torsions which are interrelated *via* the parameters P and Φ_m . In the original paper an empirical correlation was established by using a statistical survey of crystal structures which retains its validity for natural nucleic acid constituents [10]. For modified nucleosides one should reconsider this relationship and in this respect molecular mechanics calculations may be helpful.

In practice we found that the definition of a more accurate algorithm for correlating *exo*- and *endo*cyclic angles was on the one hand not easy to achieve and on the other hand unnecessary, the reason being directly related to the major source of error, inherent in the method and considered here below.

The physical basis of the method is represented by the existence of a direct relation between dihedral angles and coupling constants. Such a relation no doubts exists for each specific compound, but it is known only within a limited degree of accuracy. In practice, we assumed that the experimental and calculated J -values are statistically indistinguishable when they differ by less than twice the RMS reported for the generalized Karplus equation used in the present paper [7]. This assumption discriminates between couplings in CHCH and CH₂CH fragments, as it was shown [7] that in the former the couplings can be more accurately calculated. Moreover, when the compound under study presents a conformational equilibrium with $X_N \cong 0.5$, the experimental value to be fitted is almost a mean value between the two conformers. In this case, the errors in the coupling constants for each conformer are likely to compensate to a certain extent. Then we may leave $\sqrt{2} \cdot \text{RMS}$ as our uncertainty for each J -value.

Allowing for our inability to predict the experimental coupling constants with greater accuracy will naturally produce an ensemble of sets of conformational parameters that are all compatible with the data. And this is the fact that turns out to dominate the

whole conformational analysis. We decided to represent this ensemble by giving for each conformational parameter its domain of compatibility with the data. The "best fit" solution will then appear in a general, hopefully more informative, context of plausible solutions.

We set up an algorithm which was then translated into a FORTRAN Program, in order to perform the conformational analysis.

The several tasks that are accomplished by this algorithm are described below. First of all, from a starting model which includes arbitrary initial values of the conformational parameters (P_N , Φ_N , P_S , Φ_S , X_N), the relevant dihedral angles are calculated, whose values are then automatically converted in theoretical coupling constants by the Karplus-Altona equation [7]. Each calculated coupling must not deviate from the experimental value more than what above stated in order to be accepted. The program will then run by changing the conformational parameters until it finds a model which is compatible with all the data. This will constitute the first "feasible" point. Then each conformational parameter will be assigned an interval around the feasible point. Subsequently each variable in turn will be constrained by the program outside the feasible interval in order to search another combination of conformational parameters which could be compatible with the data. After several runs, the program will issue for each variable its corresponding feasible domain, by collecting all the feasible intervals. Clearly we do not attempt at this stage to enlighten the correlation, which certainly exists, among the different conformational variables. The final product of our program will be a sort of feasible conformational space in which our parameters are allowed to sit without violating any experimental data.

In what follows we present the results of the conformational study.

Table 1 summarizes the J -coupling values measured in different solvents and at different temperatures. Table 2 shows the values of a selected set of steady state NOE enhancements for compounds 1 and 2.

For each compound the parameters for the "best fit" solution are reported in Table 3, together with the intervals that enclose the possible values that each conformational parameter is allowed to assume, while retaining the agreement between predicted and measured J -coupling data. The correct view, in our opinion, is to consider the "best fit" solution as merely representative of the sometimes non-narrow family of all the possible (from an experimental standpoint) solutions. This wider perspective is necessary in order to draw structural conclusions with sufficient confidence. Moreover the mere search for the "best fit" can sometimes be blind, in that it may provide an impossible result as judged by the deviation from a specific experimental value. This is the case for compound 3, for which no feasible intervals for the variables could be found.

In order to increase the number of experimental parameters to be fitted, the coupling constants were measured at different temperatures, with the underlying assumption that the geometry of the conformers is not altered, while only their molar fraction can vary. In the case of compounds 1 and 2, the experiments were performed in both MeOD and D₂O.

Table 1. ¹H-¹H *J*-coupling data of compounds 1–4.

Compound	Solvent	Temperature (K)	<i>J</i> -couplings (Hz)				
			1',2'	2',3'	2',3''	3',4'	3'',4'
1	MeOD	260.6	7.6	9.7		8.5	
		274.9	7.6	9.5		8.4	
		299.2	7.6	9.2		8.2	
	D ₂ O	299.2	7.8	8.7		8.7	
		310.0	7.7	8.5		8.5	
		320.0	7.7	8.3		8.3	
2	MeOD	260.6	9.2	5.2		1.0	
		274.9	9.2	5.3		1.1	
		299.2	8.8	5.5		1.5	
	D ₂ O	310.0	8.8	5.3		1.9	
		227.8	7.1	12.6	7.0	11.4	4.8
		266.5	7.2	12.4	7.1	11.9	4.8
3	MeOD	299.2	7.1	12.3	7.1	11.1	4.9
		260.6	5.2	8.0	6.3	6.4	7.3
		274.9	5.2	8.0	6.4	6.8	7.3
4	MeOD	299.2	5.3	8.2	6.5	6.7	7.4

The experimental values seem to indicate that compound **1** presents a slightly different conformation in the two solvents, because of the difference between the values of $J_{2',3'}$. However, in both cases compound **1** exists preponderantly in the N-conformation, with a molar fraction higher than 80 % at all the temperatures. As to the geometry of this C-3' *endo* conformation, the result of the fitting points toward an increased value of P_N in D₂O as compared with the same parameter in MeOD. However, the rather wide range of values of P_N that are compatible with the experimental *J*-couplings in both solvents, prevents any further conclusion.

For compound **2**, it can be concluded that it exists mainly in the S-domain, with molar fraction higher than 80 % at room temperature in both solvents. In this case, the result of the fitting indicates that only the molar fraction changes when changing the

Table 2. Selected steady-state NOE increments.

Compound	NOEs (%) ¹			
	6{1'}	6{2'}	6{3'}	6{CH ₃ }
1	1.13	-0.77	36.17	2.36
2	2.60	10.02	NM ²	—

¹ Notation corresponding to Observed{Saturated}.² Not measured.

Table 3. Least-square results and feasible intervals for compounds 1-4.

Comp.	Solvent	P_N (°)	Φ_N (°)	P_S (°)	Φ_S (°)	Temp. (K)	X_N	RMS (Hz)
1	MeOD	16 (-3-39)	39 (35-45)	162 ¹	35 ¹	260.2	1.00	0.16
						274.9	0.99	
						299.2	0.96	
	D ₂ O	33 (2-50)	43 (31-45)	162 ¹	35 ¹	299.2	0.95	0.07
						310.0	0.92	
						320.0	0.90	
2	MeOD	9 ¹	35 ¹	172 (146-199)	34 (31-44)	260.6	0.00	0.09
						274.9	0.00	
						299.2	0.02	
	D ₂ O	9 ¹	35 ¹	172 (146-199)	34 (31-44)	299.2	0.05	0.15
						310.0	0.07	
							0.00-0.33	
3 ²	MeOD	12	38	162 ¹	35 ¹	227.8	1.00	0.65
						266.5	1.00	
						299.2	1.00	
4	MeOD	20 (1-38)	29 ³ (27-34)	171 (143-192)	31 ³ (27-33)	260.6	0.56	0.38
						274.9	0.58	
							0.52-0.66	
						299.2	0.57	
							0.50-0.64	

¹ Fixed value.² No feasible intervals were found for compound 3; see text.³ Constrained to differ by less than 2°.

solvent. As in the case of 1, however, the uncertainties in the values of P_S and Φ_S associated with their feasible intervals are too high to make this observation sufficiently reliable.

Compound 3 represents a case in which the result of the fitting is not feasible with respect to the error assigned to the J -couplings. This is due to the experimental value for $J_{2',3'}$, which is higher than the highest possible value that can be theoretically reached. We believed that a possible source of error, which is larger in the case of this compound, is the relationship between the pseudo rotational parameters and the ¹H-¹H torsion angles. In fact, compound 3 is an example of 2',3'-dideoxy nucleosides bearing a *cis* methyl to the base and the C-5'. The interaction between these groups

can significantly alter the geometry of the ring, with respect to that found in the solid state for natural nucleosides and nucleotides, which was used for translating the endocyclic angles into the torsion angles between the protons [11]. However, due to the high value in the couplings between H-2'-H-3', and H-3'-H-4', we can safely conclude that this nucleoside populates mainly the N-domain.

The results for (2'R)-2',3'-dideoxy-2'-C-allyluridine (**4**) show that this compound exists in solution as a mixture of N- and S-conformers. The equilibrium shifts slightly towards the S-conformation by increasing the temperature. The conformational parameters are yet again difficult to determine with accuracy. However, compound **4** seems to have a less puckered structure with respect to its 3'-hydroxylated counterpart **2**.

Table 2 shows a set of selected steady-state NOE enhancements at room temperature for compounds **1** and **2** in D₂O. By comparing the values for the NOE between H-6 and H-3' (36.17 %) and between H-6 and H-1' (1.13 %) it can be concluded that the base mainly adopts an *anti* orientation in compound **1**. The same can be concluded for compound **2**, where the NOE between H-6 and H-2' (10.02 %) is significantly higher than the one between H-6 and H-1' (2.60 %).

4. Discussion

According to an overwhelming NMR [5] and X-ray evidence, a pentose ring in nucleosides and nucleotides is generally engaged in a conformational equilibrium described by N- and S-type conformers.

The N-conformations are characterized by a positive value of the torsion angle C-1'-C-2'-C-3'-C-4'; in the S-type conformations this torsion has a negative value. The conformational analysis of a nucleoside involves the description of the averaged geometry of the two types of conformers as well as the assessment of their relative populations in solution.

The inherent difficulty in the conformational description of furanose sugars in solution based only on vicinal ¹H-¹H *J*-couplings was early recognized by Jardetzky [12] and largely by the work of Serianni and co-workers [13-15]. They introduced the analysis of the ¹³C-¹³C and ¹³C-¹H spin coupling in aldoses and erythro-nucleosides enriched with ¹³C in addition to the set of ¹H-¹H coupling constants. The drawbacks of this approach are the need of labelled compounds, which in certain cases can be a severe limitation, and the lack of an accurately parametrized equation for translating the ¹³C-¹H and ¹³C-¹³C coupling constants into dihedral angles.

In the present paper we attempt a revision of the traditional approach based only on ¹H-¹H spin couplings, which involves the consideration of the whole domain for each conformational variable which allows the calculated *J*-couplings to be statistically indistinguishable from the experimental values. The "true" solution can then be regarded as lying within these feasible domains. The wider the latter are, the more uncertainty we have in the experimental determination of a given parameter.

Inspection of Table 3 shows that all that can be safely said about the parameters describing the conformation of nucleosides 1-4 in solution, is that they lie within the normal range found for natural nucleosides and nucleotides. Nevertheless, some further conclusions can be drawn. The feasible intervals for the molar fractions clearly show the preferences of these compounds for the N- or the S-conformation. Sometimes the comparison between related compounds may provide some further insight. One example is the tendency of nucleoside 4 to adopt a less puckered conformation in solution if compared to compound 2.

A more accurate description of the conformation of these compounds in solution, which includes the precise geometry of the conformers and also details about their actual relative distribution around the most favourite positions in solution, is not experimentally accessible by this classical analysis based on proton-proton coupling constants. Even if we could ignore for a moment the inherent sources of inaccuracy of the method, a precise determination of the geometry of the conformers would hardly be meaningful, if we merely consider that it is obtained within the framework of a deliberately oversimplified two-state model.

During the last twenty years, a large number of results about the conformation of natural nucleosides, and of a great number of modified analogues were published. These studies proved that while the geometry of the two conformers in solution is little influenced by the substitution at the 2' or 3' position, the equilibrium can be strongly modified by the nature and orientation of the substituents.

These conclusions were corroborated in the case of our 2'-alkyl derivatives. In fact, the results in the present paper show that although there exists a rather wide range of values for P and Φ_m that are in agreement with the experimental J -couplings, the intervals for these variables lie within the region normally populated by nucleosides and nucleotides in solution, being the composition in the equilibrium altered by the orientation of the alkyl substituent. Thus the "up"-methyl uridine 1 showed a great tendency to adopt the N-conformation, while the "down"-allyl derivative 2 mainly exhibited the S-conformation.

On the other hand, the (2'S)-2',3'-dideoxy-2'-C-methyluridine 3 presents preponderantly the N-conformation in solution, whereas the "down" analogue 4 seems to have no preferences for the S- or N-conformations.

These results can be rationalized by taking into account the steric and stereoelectronic effects that contribute to the stability of the different conformers. These are [3]: a) the anomeric effect, b) the preferred quasi-equatorial orientation of side chains, c) the staggered orientation of rigid substituents, d) the *gauche* effect, and e) a preference for tetrahedral carbon angles in the ring.

In the case of nucleosides and nucleotides, the combined effect of these interactions constrains the geometry of the possible conformers to the N- and S-type. The position of the equilibrium is mostly determined by the relative stabilization or destabilization achieved by these two conformers. The second (b) and fourth (d) effects are predominant, the anomeric effect being very weak as in all the N-glycosides.

Analyzing the two conformations of the 2'-"down"-allyl **2** from this point of view we can see that the N-conformer is destabilized by a repulsive *gauche* effect between the C-4', O-4' and the C of the methyl group and stabilized by the positive anomeric effect, whereas the S-conformer is stabilized by an attractive *gauche* effect between the polar hydroxyl of 3' and the O of the ring. The attractive *gauche* effect seems to determine the preference for the S-conformer. In fact, in the case of the 2',3'-dideoxy analogue **4**, the lack of the 3'-hydroxyl removes the stereoelectronic stabilization of the S-conformer, making the energy of this conformation similar to that of the N-conformer. Additionally, our results seem to indicate that compound **4** is less puckered than its 3'-hydroxylated analogue **2**, resulting in a diminished repulsion in the N-conformer between the C-4', the ring oxygen and the methyl carbon.

For the "up"-isomer **1**, the N-conformer presents neither *gauche* nor 1,3-interactions, being weakly stabilized by the positive anomeric effect. The S-conformer is stabilized by the attractive *gauche* effect between the 3'-OH and the O-4', but largely destabilized by a strong repulsive interaction between the methyl group and the hydroxymethyl substituent of C-4' and by a repulsive *gauche* effect between C-4' and the methyl C. The final effect of these interactions shifts the equilibrium to the N-conformer. Compound **3** shows the same trend, although lacking of the *gauche* attraction in the N-conformer. In this case, the dominant factor that determines the conformation is the destabilization due to the 1,3-diaxial interaction in the S-conformer.

The values of the steady-state NOEs between the 6-base proton and protons 1', 2' and 3' of the sugar suggest a preference for the *anti* orientation of the base. This is in line with the usual observation that pyrimidine nucleosides strongly prefer the *anti* conformation [9]. In the case of the (2'S)-methyl nucleosides **1** and **3**, the sugar is almost fixed in the N-conformation. It was previously observed [16] that isopropiliden-U with the conformation of the sugar fixed in the N-conformation exhibits only the *syn* orientation for the uracil. However, for compounds **1** and **3** the responsible factor for fixing the sugar ring conformation is the 2'-methyl group, which has a *cis*-relationship with the base. The presence of this 2'-"up" substituent could cause a strong interaction with the 2-carbonyl oxygen of the base in the *syn* orientation, thereby raising up its energy.

These structural results, in addition to previous studies performed in our laboratory [4] regarding the thermal stability of homo-oligomers of both **1** and **2** with complementary DNA and RNA, were used to assess the influence of the orientation of the 2'-alkyl group and the conformation of the monomers on the melting points of the hybrids.

In the case of the (2'R) isomers, namely (2'R)-2'-deoxy-2'-C-methyl- and (2'R)-2'-C-allyl-2'-deoxyoligonucleotides no melting points were observed either with the natural oligo dA or with oligo rA. On the contrary, the respective hybrids formed by (2'S)-2'-deoxy-2'-C-methyloligonucleotide showed defined melting transitions [4, 17].

These results seem to support a correlation between the C-3' *endo* conformation of the sugar ring and the hybridization properties of the corresponding oligomer when a bulky 2'-substituent is present. This correlation has been previously suggested [18] in

order to explain the different thermal stabilities of the hybrids formed by RNA and other 2'-modified antisense fragments, such as 2'-fluoro-, and 2'-O-methyloligonucleotides.

The fact that (2'R)-2'-deoxy-2'-C-alkyloligonucleotides do not hybridize could be attributed to steric hindrances of the 2'-substituent in the C-2' *endo* conformation, as it was previously suggested [19] in order to explain the observed puckering of the sugar of RNA.

Our experimental findings about the conformation of these monomers constitute only indirect evidence of the impact that a 2'-substitution exerts on the hybridization properties of the corresponding oligo-nucleotides. In order to further analyze the conformation of these chemically modified nucleic acids fragments we are planning to investigate by NMR the structure of hybrids containing (2'S)-2'-deoxy-2'-C-methyloligonucleotides.

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