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Synthesis and Hybridization Properties of Modified Oligodeoxynucleotides Carrying Non-Natural Bases

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ABSTRACT

The impact of the presence of non-natural bases on the properties of oligodeoxynucleotides has been studied. First, oligodeoxynucleotides carrying 2'-deoxyzebularine were prepared and the stability of duplexes carrying this analogue was determined by DNA melting experiments. Melting temperatures and thermodynamic data showed the preference of 2'-deoxyzebularine for 2'-deoxyguanosine, which behaves as a 2'-deoxycytidine analogue forming a less stable base pair due to the absence of the amino group at position 4. Moreover, the duplex-hairpin equilibrium of a self-complementary oligodeoxynucleotide carrying several natural and non-natural bases including 2'-deoxyzebularine as a central mispair, was studied. Depending of the base present in the middle of the sequence it is possible to affect the stability of the bimolecular duplex modulating the duplex-hairpin equilibrium. Magnesium ions were shown to stabilize preferentially the bimolecular duplex form. The results indicate the importance of the modifications and the role of cations in shifting the structural equilibrium.

Introduction.- Oligonucleotides containing non-natural bases are important tools as model compounds in structural studies involving DNA, RNA and nucleic acid-binding proteins. In particular, oligonucleotides containing 2'-deoxyzebularine are of special interest due to the important biological properties of this compound. 2'-Deoxyzebularine, (dZ, 1-β-D-deoxyribofuranosyl-1,2-dihydropyrimidin-2-one, Scheme

1) is an inhibitor of DNA methyltransferases and may have activity against cancer [1-4]. This nucleoside is similar to 2'-deoxycytidine but it lacks the exocyclic amino group at position 4. In order to inhibit DNA methyltransferases this nucleoside must be incorporated to DNA at the GpC methylation sites. Recently it has been shown that 2'-deoxyzebularine triphosphate (dZTP) is incorporated by DNA polymerases opposite to guanine (G) and when Z is at the template it directs dGTP incorporation [5]. In spite of the absence of the amino group, 2'-deoxyzebularine behaved similar to 2'-deoxycytidine pairing with guanine [5-7]. In this report we describe the melting behaviour of DNA duplexes carrying the 1, 2-dihydropyrimidin-2-one (Z) residue opposite to all four natural bases.

In addition, we describe the melting behaviour of a self-complementary oligodeoxynucleotide having a central mispair. This type of oligodeoxynucleotides exists in duplex, hairpin and single-stranded form. Several natural and modified bases including 2'-deoxyzebularine were introduced in the middle of the sequence generating a central mismatch that is able to destabilize the duplex form without affecting the stability of the hairpin form. The nature of the compounds present at the central position as well as the presence of magnesium ions were shown to modulate the equilibrium between duplex and hairpin forms.

Results and Discussion.- *1. Synthesis of oligodeoxynucleotides*. Oligodeoxynucleotides were prepared using solid-phase 2-cyanoethylphosphoramidite chemistry. The required synthon to incorporate 2'-deoxyzebularine into oligodeoxynucleotides was prepared starting from the corresponding nucleoside that was synthesized as described [8]. Previous investigations have shown that 1,2-dihydropyrimidin-2-one nucleosides are labile both to acids and bases [9] but the DMT

group may be used for the protection of the 5'-end [7]. Reaction of 2'-deoxyzebularine with DMT chloride in pyridine gave the desired DMT-nucleoside that was reacted with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphine to yield the desired phosphoramidite [7]. Solid phase synthesis using this phosphoramidite revealed a step-coupling yield similar to standard phosphoramidites. Ammonia treatment was performed at 55ºC for 30 min to minimize decomposition of the 1,2-dihydropyrimidin-2-one residue. For this reason, the dimethylformamidine group was selected for the protection of the 2'-deoxyguanosine. The resulting oligodeoxynucleotides were purified by HPLC and gave the expected molecular weight by mass spectrometry.

2. Melting experiments with pentadecamer duplexes. Duplexes having 1,2-dihydropyrimidin-2-one (Z) opposite to the four natural bases were analyzed using the pentadecamer sequence (5'- d(GCA ATG GAX CCT CTA)-3' / 3'-d(CGT TAC CTY GGA GAT)-5', X = C, Z; Y= A, G, C, T, Z;). Melting temperatures (T_m) and thermodynamic parameters are shown in Table 1. Cytosine base pairs were included as reference. As expected, in all cases the most stable base pair was formed between the guanine and cytosine (ΔG = -13.4 Kcal/mol). The most stable base pair of 1, 2-dihydropyrimidin-2-one (Z) is formed with guanine (ΔG = -10.3 Kcal/mol). The relative stability of the Z base pairs is Z•G > Z•A = Z•T = Z•Z (ΔG = -8.0 Kcal/mol) > Z•C (ΔG = -7.5 Kcal/mol). On the other hand, the C•A mispair (ΔG = -9.6 Kcal/mol) is clearly the most stable of the C mispairs (ΔG = -8.0-8.4 Kcal/mol) in accordance with the literature [10, 11].

Recently, the design and synthesis of a new class of metal-binding nucleobases have been described [12, 13]. Most of these new analogues are derived from pyridine bases and carry carbonyl groups that can stabilize DNA duplexes in the presence of

metal ions by forming specific ion-mediated base pairs [12, 13]. The analogy of these compounds to Z prompted us to measure melting curves of the duplex carrying the Z•Z mispair in presence of metal cations. Unfortunately, no differences were observed with or without the presence of Fe^{2+} , Cu^{2+} or Ni^{2+} cations, thus precluding the formation of an ion-mediated base pair involving Z•Z mispair.

2. *Melting experiments with self-complementary oligodeoxynucleotides.* In addition to the previous oligodeoxynucleotides we prepared modified *Dickerson-Drew* dodecamers [14, 15] with an additional base in the central position (5'-d(CGCGAAXTTCGCG)-3' X= C, Z). The idea was to study intermolecular duplexes with a central C•C or Z•Z mispair. Melting curves of the tridecamers carrying C or Z at central positions were very different from the melting curve of the parent *Dickerson-Drew* dodecamer (Figure 1). The self-complementary dodecamer had a transition with a melting temperature of 44.0 ºC while the tridecamers carrying C or Z at central positions had a wider transition with a Tm of 70.6 and 69.2 ºC respectively (Table 2). The melting behavior of the tridecamers was attributed to the formation of a hairpin with the C or Z bases occupying an extrahelical position in the loop (Scheme 2). The *Dickerson-Drew* dodecamer, as all self-complementary oligonucleotides, can form both a unimolecular hairpin and a bimolecular duplex [15-18]. Low salt and low oligomer concentration favors the formation of the hairpin [15-18]. In our case, it is expected that the introduction of a central mismatch will decrease the stability of the bimolecular duplex without affecting the stability of the hairpin, thus favoring the formation of hairpin relative to the bimolecular duplex.

In order to study if this distinct behavior is caused by the extrahelical disposition of the base at the central position, regardless of the chemical nature of the base, several

tridecamers were prepared (Table 2). This included oligodeoxynucleotide sequences having one of the four natural bases (A, C, G, T), some base analogues such as Z, 2-aminopurine (P), apurinic site model compounds such as ribitol (R), and 1,3-propandiol (D), an intercalating compound (acridine (I)), as well as a few tetradecamers carrying AA and CC sequence at the central position.

All tridecamers and tetradecamers had the transition at around 70 ºC observed for the tridecamers containing C and Z (Table 2). In addition, when A and G were at the central position we observed a second transition at low temperature assigned to the bimolecular duplex.

The native polyacrylamide gel electrophoresis (PAGE) was used to analyze the secondary structure of the oligodeoxynucleotides carrying the modification at the central position. Since the interconversion of the hairpin and the duplex is slow at low temperature, the identification of the species was performed by gel electrophoresis at 7ºC. Figure 2 shows the secondary structures of the modified tridecamers and tetradecamers in 40 mM Tris, 2 mM EDTA, 20 mM acetic acid, and 12.5 mM magnesium acetate (TAE-Mg^{2+}). Tridecamers containing A and G at the central position run as a single spot with low mobility (between 20-25 bases) corresponding to the bimolecular duplex. The tridecamer containing T was mainly a single spot with low mobility (between 20-25 bases) but a faster spot is observed. This new spot has mobility similar to the 10 bases marker and corresponds to the unimolecular hairpin. Oligodeoxynucleotides containing P, C, Z, AA and R have increasing amounts of the hairpin (Figure 2, Table 2). Finally, oligodeoxynucleotides containing D, I, and CC are mainly in hairpin form (Figure 2, Table 2). It is important to notice that the oligodeoxynucleotide carrying 2'-deoxyzebularine behaves similarly to the

oligodeoxynucleotide carrying the ribitol apurinic site (50:50 duplex/ hairpin ratio) indicating a low stability for the duplex with the Z•Z mispair.

Melting experiments were also performed in TAE-Mg^{2+} buffer used in the PAGE experiment (Figure 3). This buffer has similar ion strength than the previously used NaCl buffer but contains magnesium and Tris. Under these conditions, the bimolecular duplex is stabilized and it is possible to observe the duplex to hairpin transition in most of the modified oligodeoxynucleotides except for oligodeoxynucleotides carrying AA, CC mispairs (Figure 3, Table 2). This confirms the assignment of the hairpin as the fast running spot and the duplex as the slow running spot. Little changes were observed in the melting temperatures of the hairpin to random coil transitions in the Mg^{2+} buffer (Table 2).

These results indicate that the presence of purines (G, A and P) at the central position stabilize the bimolecular duplex with the oligodeoxynucleotide carrying G being so stable than the melting curve of the duplex to hairpin transition in Mg^{2+} overlaps with the hairpin to random coil transition. This is in agreement with fact that purine-purine mismatches are more stable than pyrimidine-pyrimidine mismatches [10, 11]. Following in order of stability, duplexes carrying pyrimidine mismatches (T, C and Z) were found to be less stable. Amongst these, tridecamers carrying T and C are more stable than duplexes carrying Z, indicating again a low stability for the duplex with the Z•Z mispair. A clear difference is observed with the two apurinic site model compounds. Ribitol (R) with a closed furanose ring can form a bimolecular duplex at low temperature (Tm_1 = 35.5 ºC) in the presence of Mg^{2+}, while the open 1,3-propandiol (D) exists mostly as a hairpin although a small duplex-to-hairpin transition is observed (Tm_1 = 24.3 ºC). A similar behavior was also observed with the tetradecamers carrying AA and CC. A bimolecular duplex was observed at low temperature in the

presence of Mg^{2+} with the tetradecamer carrying AA and no duplex was observed for the tetradecamer carrying CC; no duplex-to-hairpin transition was observed in any of these oligodeoxynucleotides. It is worth mentioning that PAGE experiment with the tetradecamer carrying AA shows the presence of two minor components running close to 40 bases marker and between 50-60 bases marker. We believe that these components are trimolecular (three-way junction [19]) and the tetramolecular (Holliday junction [20]) species that may be stabilized by adenine stacking. Unfortunately, these compounds are formed in small amounts and no further characterization was possible. Finally the presence of a bigger group such as an acridine (I) favors hairpin formation although a duplex-to-hairpin transition was also observed (Tm_1 = 25.4 ºC).

Conclusions.

Oligodeoxynucleotides carrying the DNA methylase inhibitor 2'-deoxyzebularine (dZ) were prepared in order to measure the stability of duplexes carrying Z in front of the four natural bases. Melting temperatures and thermodynamic data show the preference of Z for guanine behaving as a cytosine analogue with a less stable base pair due to the absence of the amino group at position 4.

Additionally, the duplex-hairpin equilibrium of self-complementary oligodeoxynucleotides carrying different moieties, from nucleobases to apurinic sites, plus an intercalating acridine at the central position, was studied. It was observed that the nature of the molecule present in the middle of the sequence has a strong influence on the stability of the bimolecular duplex modulating the duplex-hairpin equilibrium. In this system, we observed that duplexes carrying the Z•Z mispair were less stable than duplexes carrying the C•C mispair, being similar to the duplex formed by the abasic

(ribitol) derivative. The absence of the amino group at position 4 is likely to be the reason of this destabilization.

Magnesium cations were shown to stabilize preferentially the bimolecular duplex form. The results demonstrate the structural flexibility of palindromic sequences and show the importance of the modifications at the central position as well as the presence of cations in modulating the duplex-hairpin structural equilibrium. These results are relevant for understanding the structural characteristics of palindromic sequences.

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Experimental Part

General. Phosphoramidites and ancillary reagents used during oligonucleotide synthesis were from *Applied Biosystems* (*PE Biosystems Hispania S.A.,* Spain). The rest of the chemicals were purchased from *Aldrich, Sigma* or *Fluka* (*Sigma-Aldrich Química S.A.,* Spain). 2'-Deoxyzebularine was prepared as described [8].

Oligodeoxynucleotide synthesis. Oligodeoxynucleotides were prepared using an Applied Biosystems 3400 DNA synthesizer. The corresponding dZ [6, 7] and acridine

[21] phosphoramidites were synthesised as reported. The phosphoramidites of 2-aminopurine, ribitol, 1,3-propandiol were from commercial sources (*Glen Research, USA*). For the addition of dZ and acridine phosphoramidites we used a longer coupling time (300 sec). The standard bases were added using the standard LV200 synthesis cycle. Syntheses were performed without the removal of the last DMT group. For the synthesis of oligodeoxynucleotides carrying Z, 2'-deoxyguanosine was protected with the dimethylformamidine group. Ammonia treatment was performed at 55ºC for 30 min to preserve the integrity of Z. Oligodeoxynucleotides were purified using reversed-phase HPLC (DMT on). HPLC solutions are as follows. Solvent A: 5% ACN in 100 mM triethylammonium acetate (pH 6.5) and solvent B: 70% ACN in 100 mM triethylammonium acetate pH 6.5. Columns: Nucleosil 120C18 (10 μm), 200 x 10 mm. Flow rate: 3 ml/min. Conditions A: 20 min linear gradient from 15-80% B (DMT on). The DMT was removed with 80% acetic acid and the acetic acid was removed by extraction with ethyl ether. The resulting products were desalted with Sephadex G-25 (NAP-10 column). Mass spectrometry of modified tridecamers: G: found 3968.2, expected for $C_{126}H_{159}N_{51}O_{76}P_{12}$ 3975.1; T: found 3941.1, expected for $C_{126}H_{160}N_{48}O_{77}P_{12}$ 3950.1; Z: found 3926.6, expected for $C_{125}H_{158}N_{48}O_{76}P_{12}$ 3920.1; C: found 3932.3, expected for $C_{125}H_{159}N_{49}O_{76}P_{12}$ 3935.1; A: found 3955.4, expected for $C_{126}H_{159}N_{51}O_{75}P_{12}$ 3959.1; I: found 4017.6, expected for $C_{134}H_{164}N_{48}O_{75}P_{12}$ 4018.2; R: found 3824.1, expected for $C_{121}H_{156}N_{46}O_{75}P_{12}$ 3826.0; D: found 3824.9 ($M+K^+$), expected for $C_{119}H_{154}N_{46}O_{74}P_{12}$ 3784.0, P: found 3951.1, expected for $C_{126}H_{159}N_{51}O_{75}P_{12}$ 3959.1. Modified tetradecamers: AA: found 4281.0, expected for $C_{136}H_{179}N_{56}O_{80}P_{13}$ 4280.3; CC: found 4234.9, expected for $C_{134}H_{171}N_{52}O_{82}P_{13}$ 4224.2. Pentadecamer sequences: 5'- GCAATGGAZCCTCTA-3': found 4524.6, expected for

$C_{145}H_{183}N_{55}O_{87}P_{14}$ 4521.4; 3'-CGTTACCTZGGAGAT-5': found 4556.6, expected for $C_{146}H_{184}N_{54}O_{89}P_{14}$ 4552.4.

Melting experiments. Melting experiments were performed as follows. Solutions of equimolar amounts of oligodeoxynucleotides (5'-d(GCAATGGAXCCTCTA)-3', X = C, Z) and the complementary strand (5'-d(TAGAGGYTCCATTGC)-3', Y = Z, A, C, G, T) were mixed in 50 mM NaCl, 10 mM sodium phosphate buffer pH 7.0. The DNA concentration was determined by UV absorbance measurements (260 nm) at 90ºC, using for the DNA coil state the following extinction coefficients: 7500, 8500, 12500, and 15000 M^{-1} cm^{-1} for C, T, G, and A respectively. The solutions were heated to 90 ºC, allowed to cool slowly to room temperature, and stored at 4ºC until UV was measured. UV absorption spectra and melting experiments (absorbance vs temperature) were recorded in 1 cm path-length cells using a spectrophotometer, with a temperature controller and a programmed temperature increase rate of 1 ºC/min. Melts were run by duplicate using a strand concentration of 7-8 μM at 260 nm.

Melting curves were analyzed by computer-fitting the denaturation data, using Meltwin 3.5 software. On the basis of multiple experiments, the uncertainty in Tm values was estimated at +/- 0.7 ºC.

Melting experiments on the self-complementary oligodeoxynucleotides derived from the *Dikerson-Drew* dodecamer were run in a similar way at a concentration of 4-5 μM. In these case melting temperatures were estimated by the maximum of the first derivative. Due to the presence of two close transitions we could not use the computer-fitting program. In the melting curve of the tridecamer carrying G at central position in TAE-Mg buffer both transitions overlap. Melting temperature of the hairpin-to-coil (Tm_2) transition was measured at 282 nm where the duplex-to-hairpin transition was not

observed [16]. On the basis of multiple experiments, the uncertainty in Tm values was estimated at +/- 1.5 ºC.

Polyacrylamide gel electrophoresis (PAGE). Samples (0.5 O.D. units, 15 μg) were dissolved in 40 mM Tris, 2 mM EDTA sodium, 20 mM acetic acid, 12.5 mM magnesium acetate (TAE-Mg^{2+}), cooled to 4ºC and loaded on the native 20% polyacrylamide gel (acrylamide:bisacrylamide = 19:1) termostabilized at 7ºC. The running buffer was TAE-Mg. After the electrophoresis, oligodeoxynucleotides were stained by STAINS-ALL (Sigma) using a solution of 20 mg of the dye in formamide (100 ml). After 15 min, the dye solution was removed and the gel was washed with water. After bleaching the background with IR light, the remaining blue stains were visualized and photographed.

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LEGENDS

Scheme 1. 2'-Deoxyzebularine (dZ) and 2'-Deoxycytidine (dC).

Scheme 2. The three possible conformations of the self-complementary tridecamer sequence 5'-d(CGCGAAXTTCGCG)-3'.

Scheme 3. Structures of modifications introduced at the central position of the *Dickerson-Drew* dodecamer. D= 1,3-propanediol, I: acridine, P = 2-aminopurine, R= ribitol, Z= 1, 2-dihydropyrimidin-2-one

Figure 1. *Melting curves showing the absorption at 260 nm of Dickerson-Drew dodecamer and Z- and C-modified tridecamer.*

Figure 2. *Native polyacrylamide gel electrophoresis (PAGE) at 7ºC for modified oligodeoxynucleotides prepared in 40 mM Tris, 2 mM EDTA sodium, 20 mM acetic acid, 12.5 mM magnesium acetate (TAE-Mg^{2+}).* M: marker having 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 bases. D= 1,3-propanediol, I: acridine, P = 2-aminopurine, R= ribitol, Z= 1, 2-dihydropyrimidin-2-one.

Figure 3. *Melting curves showing the absorption at 260 nm of X-modified tridecamer in A) 10 mM sodium phosphate, 50 mM NaCl and B) 40 mM Tris, 2 mM EDTA sodium, 20 mM acetic acid, 12.5 mM magnesium acetate (TAE-Mg^{2+}) buffers.*

Table 1. Thermodynamic Parameters of Double-Stranded DNA to Single-Stranded DNA Transition. Duplex sequence: 5'- d(GCAATGGAXCCTCTA)-3' / 3'-d(CGTTACCTYGGAGAT)-5', X = C, Z; Y= A, G, C, T, Z;). Conditions: 50 mM NaCl, 10 mM sodium phosphate buffer pH 7.0.

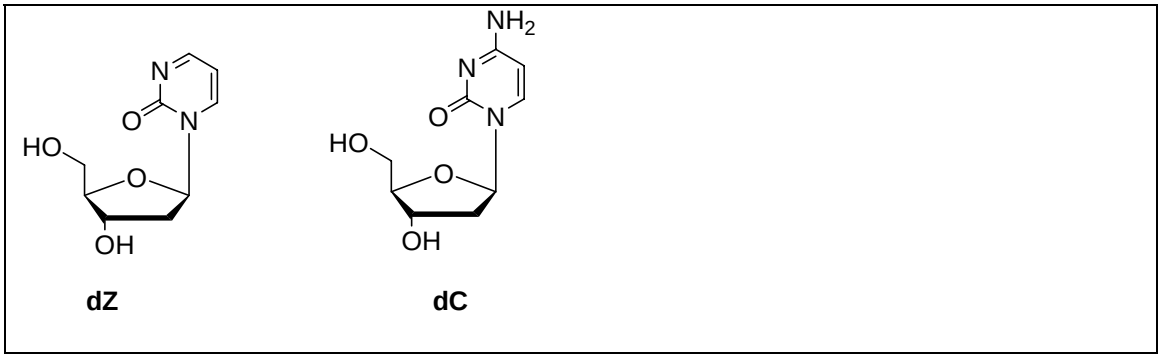
basepair	ΔH (kcal/mol)	ΔS (cal/K.mol)	ΔG (kcal/mol)	Tm (ºC)
C-A	-96.5	-280.3	-9.6	41.5
C-C	-85.1	-248.6	-8.0	36.5
C-G	-100.3	-280.5	-13.4	54.0
C-T	-80.6	-233	-8.2	37.3
C-Z	-104.8	-310.9	-8.4	38.2
Z-A	-72.3	-207.5	-8.0	36.0
Z-C	-78.2	-228.2	-7.5	34.2
Z-G	-79.3	-222.6	-10.3	45.6
Z-T	-89.4	-262.3	-8.0	36.5
Z-Z	-98.6	-292.1	-8.0	36.9

Table 2. Melting Temperatures and Duplex-Hairpin Ratio Estimated by PAGE of Self-Complementary Oligodeoxynucleotides Derived from the Dickerson-Drew Dodecamer.

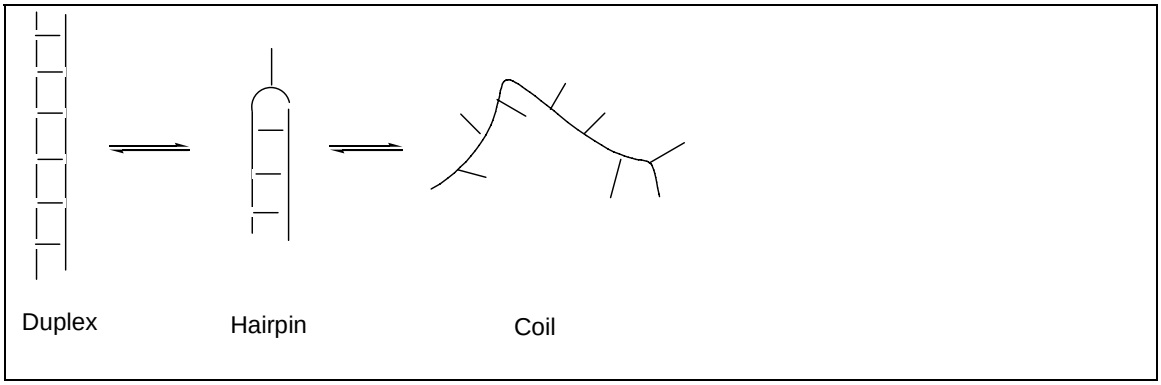
Sequence[a])	Tm_1 (ºC)[b])[c])	Tm_2 (ºC)[b])[d])	% duplex[e])	% hairpin[e])	Tm_1 (ºC)[c])[f])	Tm_2 (ºC)[d])[f])
CGCGAATTCGCG	44.0	--	n.d.	n.d.	n.d.	n.d.
CGCGAA**C**TTCGCG	--	70.6	60	40	33.6	70.0
CGCGAA**Z**TTCGCG	--	69.2	50	50	27.0	71.9
CGCGAA**A**TTCGCG	36.3	70.1	100	0	40.8	70.2
CGCGAA**G**TTCGCG	39.5	70.3	100	0	>42[g])	68.1[g])
CGCGAA**T**TTCGCG	--	71.3	90	10	40.4	71.6
CGCGAA**P**TTCGCG	--	70.3	80	20	37.7	70.2
CGCGAA**R**TTCGCG	--	72.2	50	50	35.5	70.1
CGCGAA**D**TTCGCG	--	72.3	0	100	24.3	72.5
CGCGAA**I**TTCGCG	--	71.4	10	90	25.4	73.0
CGCGAA**AA**TTCGCG	--	72.7	50	50	--	71.8
CGCGAA**CC**TTCGCG	--	72.4	0	100	--	70.4

[a]) D= 1,3-propanediol, I: acridine, P = 2-aminopurine, R= ribitol, Z= 1, 2-dihydropyrimidin-2-one.
[b]) conditions: 50 mM NaCl, 10 mM sodium phosphate buffer pH 7.0
[c]) melting temperature of duplex-to-coil or duplex-to-hairpin transition
[d]) melting temperature of hairpin-to-coil transition
[e]) percentage of duplex / hairpin by PAGE electrophoresis at 4ºC (TAE-Mg^{2+}).
[f]) conditions: 40 mM Tris, 2 mM EDTA sodium, 20 mM acetic acid, 12.5 mM magnesium acetate (TAE-Mg)
[g]) both transitions are superimposed. Tm_2 was measured at 282 nm where the duplex-to-hairpin transition is not observed [16].

Scheme 1



Scheme 2



Scheme 3

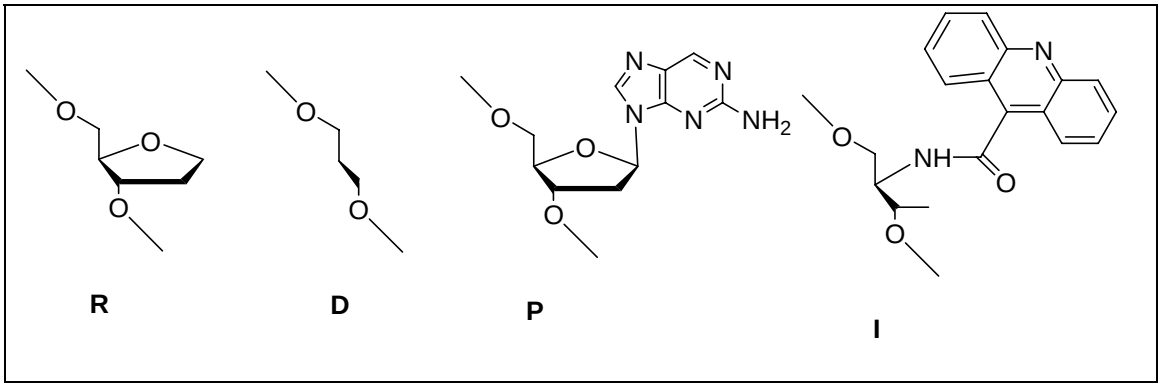


Figure 1

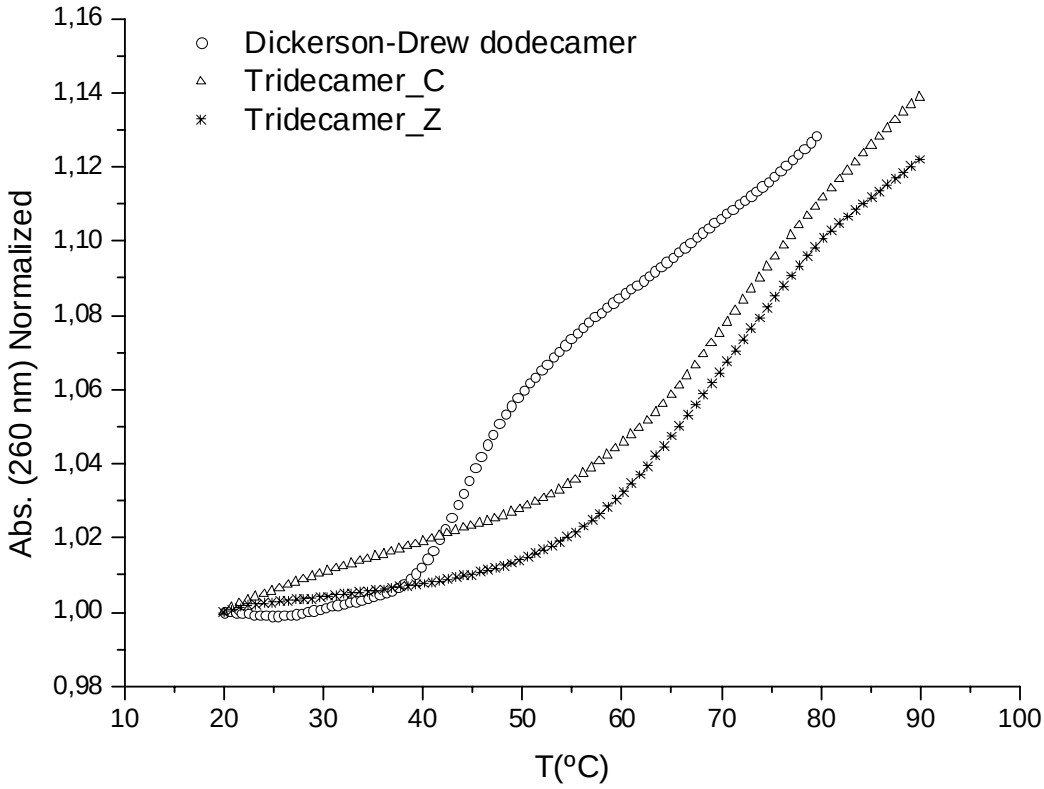

Dickerson-Drew dodecamer
Tridecamer_C
Tridecamer_Z
Abs. (260 nm) Normalized
T(ºC)
1,16
1,14
1,12
1,10
1,08
1,06
1,04
1,02
1,00
0,98
10
20
30
40
50
60
70
80
90
100

Figure 2

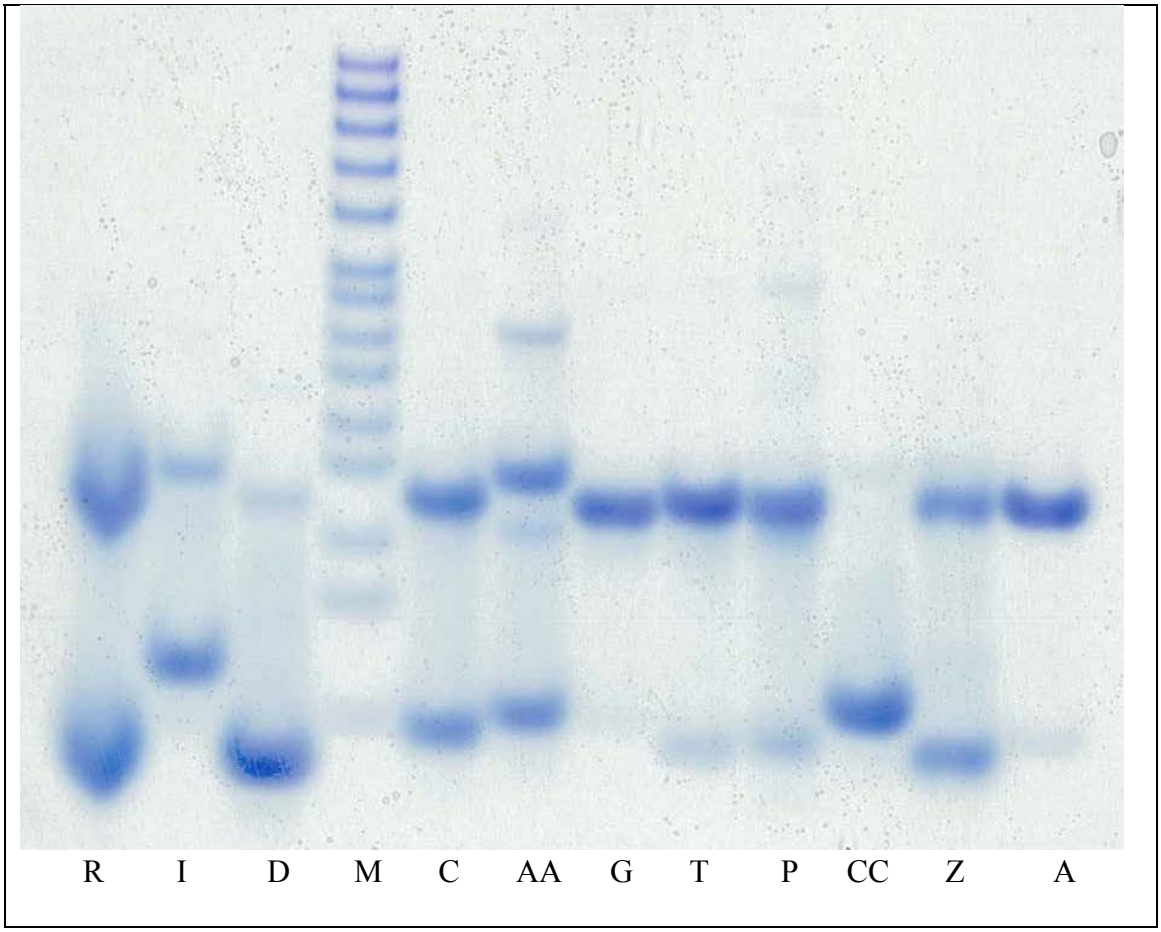
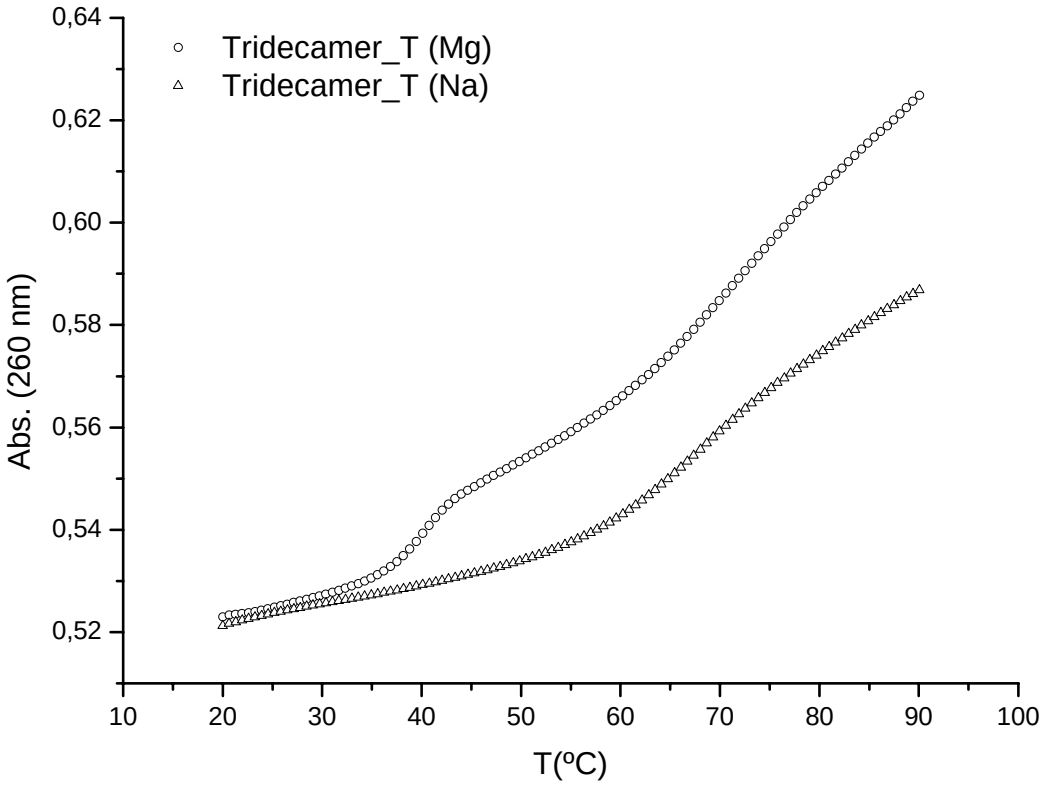


Figure 3


Tridecamer_T (Mg)
Tridecamer_T (Na)
Abs. (260 nm)
0,64
0,62
0,60
0,58
0,56
0,54
0,52
10
20
30
40
50
60
70
80
90
100
T(ºC)