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Françoise Debart, Albert Meyer, J.J. Vasseur, Bernard Rayner. Anomeric inversion (from  $\beta$  to  $\alpha$ ) in methylphosphonate oligonucleosides enhances their affinity for DNA and RNA. *Nucleic Acids Research*, 1998, 26 (20), pp.4551 - 4556. 10.1093/nar/26.20.4551 . hal-03008434

**HAL Id: hal-03008434**

**<https://hal.science/hal-03008434>**

Submitted on 16 Nov 2020

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# Anomeric inversion (from $\beta$ to $\alpha$ ) in methylphosphonate oligonucleosides enhances their affinity for DNA and RNA

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Received July 24, 1998; Revised and Accepted September 7, 1998

## ABSTRACT

Here we report that the poor binding of methylphosphonate oligodeoxynucleosides (MP-ODNs) to their nucleic acid targets can be improved by additional inversion of the anomeric configuration (from  $\beta$  to  $\alpha$ ) in the sugar moieties to give a new class of analogs, MP  $\alpha$ -oligonucleosides. MP  $\alpha$ -dT<sub>12</sub> and MP 5'  $\alpha$ -d(TCTTAA-CCCACA) 3' were synthesized and their ability to form hybrids with complementary single stranded (ss)DNA and ssRNA, as well as with double stranded (ds)DNA, was evaluated. The thermal stability of hybrids formed with MP  $\alpha$ -analogs was compared with the affinity of phosphodiester (PO) and phosphorothioate (PS)  $\beta$ - and  $\alpha$ -oligomers for their targets. Non-ionic MP  $\alpha$ -oligonucleosides bound to their complementary DNA and RNA strands more tightly than their homologues with natural  $\beta$ -anomeric configuration did. With DNA target, MP  $\alpha$ -oligomers formed duplexes more stable than the corresponding natural PO  $\beta$ -oligomer did. MP  $\alpha$ -heteropolymer hybridized to RNA target better than PS  $\beta$ -oligonucleotide did but the hybrid was less stable ( $\Delta T_m -0.5^\circ\text{C}$  per mod.) than the hybrid formed with the natural PO  $\beta$ -oligomer. Only MP  $\alpha$ -dT<sub>12</sub> bound to dsDNA target at low salt concentration (0.1 M NaCl).

## INTRODUCTION

Synthetic oligonucleotides (ODNs) are compounds of major interest in the development of new therapeutic agents. These compounds can be rationally designed to specifically interfere with gene expression via antisense or antigene mode of action (1–4). Various structural modifications have been introduced to improve their binding properties to complementary target nucleic acids and their resistance to nuclease-mediated hydrolysis (5,6). Some typical modifications have included alteration of the internucleoside phosphodiester links through substitution of one or two of the four oxygen atoms linked to the phosphorus atom by another atom or group, or replacement of the phosphodiester function by another chemical group leading to oligonucleotide analogs containing dephospho internucleoside links (7). Oligo-

nucleoside methylphosphonates (MP-ODNs) belong to the first class of modified ODNs and were among the first analogs studied (8). These non-ionic analogs are resistant towards nucleases and they have been used in several *in vitro* and *in vivo* systems including herpes simplex virus infected cells and mice (9–11).

The major problem associated with the replacement of one non-bridging oxygen atom by any substituent is the appearance of a new chiral center at the phosphorus. Indeed, physicochemical properties of MP-ODNs are strongly affected by the absolute configuration of the methylphosphonate links (12) and the non-stereoselectivity of standard methods of ODN synthesis leads to a mixture of diastereoisomers. Only a few of them would possess the properties supporting the desired biological effects. In particular, although MP-ODNs hybridize to complementary single stranded (ss)DNAs better than their phosphorothioate counterparts, they exhibit much weaker affinity for RNAs (13). Furthermore, no triple helix was formed, even at high salt concentration, when any normal strand in (dA)<sub>19</sub> + 2 (dT)<sub>19</sub> or (dU)<sub>19</sub> was replaced by an MP strand (14). As illustrated in the literature, the originally expected increase in complex stability due to the reduction of charge repulsion when substituting non-ionic MP links for normal phosphodiester ones is overcome by adverse effects under broadly physiological conditions (15). The destabilization of duplexes formed between an MP-ODN and a complementary natural oligonucleotide arises mainly from the unfavorable steric interactions of the P-methyl group. This effect is more pronounced when the P-methyl group is pseudoaxial (Sp configuration) relative to the double helix axis than when it is pseudoequatorial (Rp configuration) (16). Despite considerable effort which has been expended to the synthesis of chirally pure MP-ODNs, the solid-phase synthesis of all Rp MP-ODNs long enough for antisense applications still requires further developments (15,17). However, Rp chirally enriched MP-ODNs with alternating Rp/Rp + Sp internucleoside links were synthesized by sequential coupling of Rp dinucleoside methylphosphonates and were shown to bind RNA with significantly higher affinity than non-enriched MP-ODNs (18). Alternatively, a similar effect was observed upon replacement of deoxyribonucleosides with 2'-O-methylribonucleosides in MP-ODNs (19), and relies on the high affinity of oligo 2'-O-alkylribonucleotides for complementary RNA target molecules (20). This last example is representative of

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how two chemical modifications, one located in the sugar moiety and the second in the internucleoside link, may be combined to improve the effectiveness of antisense oligonucleotides.

Several years ago, we introduced nuclease-resistant  $\alpha$ -anomeric oligodeoxynucleotides ( $\alpha$ -ODNs) constituted of unnatural  $\alpha$ -anomeric nucleotide units (21). These analogs form stable duplexes with both complementary DNA and RNA and the two strands in these duplexes are oriented in a parallel arrangement (22,23). Unexpectedly,  $\alpha$ -oligodeoxynucleoside phosphorothioates (PS  $\alpha$ -ODNs) hybridize to RNA better than their  $\beta$ -anomeric counterparts (PS  $\beta$ -ODNs) (24,25). The observed increase was modest ( $\Delta T_m$  0.1–0.4°C per modified nucleotide), but subsequently was found to be higher upon replacement of phosphodiester links by non-ionic phosphoramidate links (26). These results prompted us to extend the study of this particular behavior to  $\alpha$ -ODNs bearing methylphosphonate links ( $\alpha$ -MP-ODNs). In this paper, we report on the synthesis of these new analogs and we describe their interactions with ssDNA and ssRNA as well as with double stranded (ds)DNA.

## MATERIALS AND METHODS

Protected deoxy-2'- $\beta$ -ribonucleoside-3'-*O*-(*N,N*-diisopropyl)cianoethylphosphoramidites were purchased from Perseptive Biosystems GmbH and deoxy-2'- $\beta$ -ribonucleoside-3'-*O*-(*N,N*-diisopropyl)methylphosphonamidites were obtained from Glen Research (Sterling, VA). Deoxy-2'- $\alpha$ -ribonucleoside-3'-*O*-(*N,N*-diisopropyl)cianoethylphosphoramidites ( $\alpha$ -dT,  $\alpha$ -dC<sup>Bz</sup> and  $\alpha$ -dA<sup>Bz</sup>) were prepared according to published procedures (27). Except as noted, chemicals were reagent grade or better and used without further purification. Silica gel TLC was carried out on Kieselgel 60 F<sub>254</sub> plates (Merck), and compounds were visualized by UV shadowing. Silica gel 60 (230–240 mesh Merck) was used for flash column chromatography. FAB mass spectra were recorded on a JEOL DX300 spectrometer operating with a JMA-DA 5000 mass data system in positive ion mode; MALDI-TOF mass spectra of MP  $\alpha$ -oligonucleosides were obtained on a Bruker BIFLEX instrument (Bremen, Germany). The <sup>1</sup>H spectra were recorded with a Bruker DRX 400 spectrometer, and chemical shifts were measured relative to CHD<sub>2</sub>CN fixed at 1.94 p.p.m. The signals are described as: s, singlet; d, doublet; t, triplet; m, multiplet. The <sup>31</sup>P-NMR spectra were recorded with a Bruker AC 250 spectrometer, and chemical shifts were measured relative to 85% H<sub>3</sub>PO<sub>4</sub> as external reference. Reversed phase (RP)-HPLC was performed on a Waters 600E system equipped with a Model 990 photodiode array detector and using an EC Nucleosil 5 $\mu$  C<sub>18</sub> column (150  $\times$  4.6 mm, Macherey-Nagel) or a Lichrospher 5 $\mu$  C<sub>18</sub> EC column (150  $\times$  4.6 mm) for analytical purposes (flow rate 1.0 ml/min) and a Waters Delta Pak 15 $\mu$  C<sub>18</sub> 100 Å column (300  $\times$  7.8 mm) for preparative work (flow rate 2.0 ml/min). Elution was performed with linear gradients of acetonitrile in 50 mM triethylammonium acetate (TEAAC) pH 7.

### $\beta$ - and $\alpha$ -oligonucleoside phosphodiester 1, 2, 7, 9, 10 and 15

Poly rA and poly dA were purchased from Pharmacia Biotech. The RNA target oligomer r-(AGAAUUGGGUGU) **8** was a gift from ISIS Pharmaceuticals (Carlsbad, CA). The DNA target oligomers d-(AGAATTGGGTGT) **7** and d-(GCT<sub>12</sub>CGT<sub>4</sub>CGA<sub>12</sub>GC) **15**, the complementary phosphodiester  $\beta$ -D-(ACAC-CCAATTCT) **9** and  $\alpha$ -D-(TCTTAACCCACA) **10**, the  $\beta$ - and

$\alpha$ -dodecathymidylate phosphodiester **1** and **2** were prepared on a 1  $\mu$ mol scale with an Applied Biosystems Inc. Model 381A DNA synthesizer. The protected oligomers were cleaved from the support and deprotected upon treatment with concentrated ammonium hydroxide for 5 h at 55°C. The oligomers were purified by preparative RP-HPLC using a linear gradient of 5–50% acetonitrile in 50 mM TEAAC.

### Synthesis of protected deoxy-2'- $\alpha$ -ribonucleoside-3'-methylphosphonamidites

5'-*O*-dimethoxytrityl- $\alpha$ -2'-deoxythymidine and 5'-*O*-dimethoxytrityl-*N*-6-benzoyl- $\alpha$ -2'-deoxyadenosine were prepared as described previously (27). *N*-4-isobutyryl- $\alpha$ -2'-deoxycytidine was prepared from  $\alpha$ -2'-deoxycytidine with isobutyric anhydride (28). Diisopropylethylamine and diisopropylamine were both distilled from calcium hydride and anhydrous methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>) was distilled from phosphorus pentoxide. 5'-*O*-dimethoxytrityl-(base protected)- $\alpha$ -2'-deoxynucleosides were phosphitylated with (*N,N*-diisopropylamino)methylchlorophosphine prepared just prior to use following a published procedure (19). The methylphosphonamidite  $\alpha$ -nucleosides were isolated as diastereoisomeric mixtures.

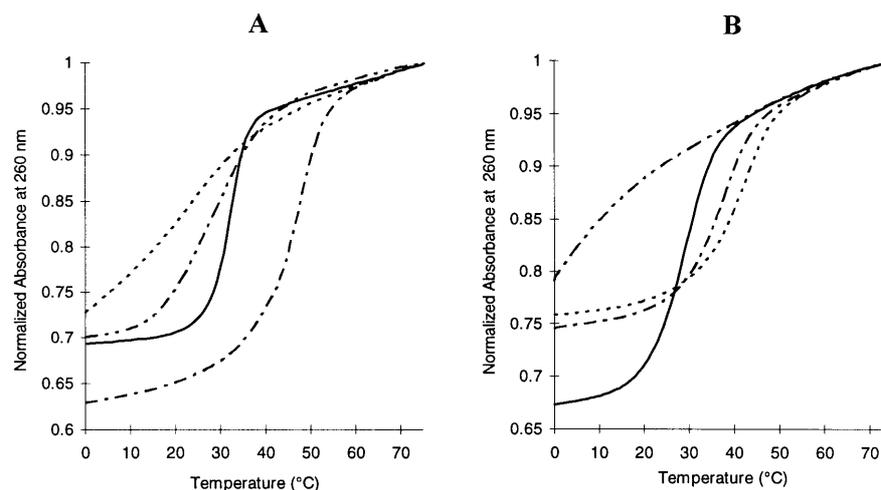
*O*-[5'-*O*-(4,4'-dimethoxytrityl)- $\alpha$ -thymidin-3'-yl]-*N*-(diisopropyl)methylphosphonamidite. Yield 81%. <sup>1</sup>H-NMR (CD<sub>3</sub>CN)  $\delta$  8.87 (broad s, 1H, NH), 7.63 and 7.57 (2s, 1H, H6), 7.47–7.24 (m, 9H, trityl), 6.92–6.88 (m, 4H, trityl H), 6.28 (m, 1H, H1'), 4.48 and 4.42 (2m, 1H, H4'), 4.32 (m, 1H, H3'), 3.79 (s, 6H, 2 OCH<sub>3</sub>), 3.49 (m, 2H, CH isopropyl), 3.18 and 3.06 (2m, 2H, H5' and H5''), 2.67 (m, 1H, H2'), 2.13 and 2.00 (2m, 1H, H2'), 1.86 (s, 3H, CH<sub>3</sub> thymine), 1.10–1.07 (m, 15H, CH<sub>3</sub> isopropyl and P-CH<sub>3</sub>). <sup>31</sup>P-NMR (CD<sub>3</sub>CN)  $\delta$  121.45 and 120.26. FAB mass spectrum (negative mode, polyethyleneglycol 500): 688 [M-H]<sup>-</sup>.

*O*-[5'-*O*-(4,4'-dimethoxytrityl)-4-*N*-isobutyryl- $\alpha$ -2'-deoxycytidin-3'-yl]-*N*-(diisopropyl)methylphosphonamidite. Yield 54%. <sup>1</sup>H-NMR (CD<sub>3</sub>CN)  $\delta$  8.69 (broad s, 1H, NH), 8.00 (d, 1H, H6), 7.49–7.24 (m, 10H, trityl and H5), 6.92–6.88 (m, 4H, trityl), 6.15 (m, 1H, H1'), 4.59 and 4.50 (2m, 1H, H4'), 4.29 (m, 1H, H3'), 3.79 (s, 6H, 2 OCH<sub>3</sub>), 3.47 and 3.36 (2m, 2H, CH isopropyl), 3.20 and 3.10 (2m, 2H, H5' and H5''), 2.65 (m, 2H, H2' and CH isobutyryl), 2.36 and 2.11 (2m, 1H, H2''), 1.25–0.9 (m, 21H, CH<sub>3</sub> isobutyryl, CH<sub>3</sub> isopropyl and P-CH<sub>3</sub>). <sup>31</sup>P-NMR (CD<sub>3</sub>CN)  $\delta$  122.27 and 119.96. FAB mass spectrum (negative mode, polyethyleneglycol 500): 743 [M-H]<sup>-</sup>.

*O*-[5'-*O*-(4,4'-dimethoxytrityl)-6-*N*-benzoyl- $\alpha$ -2'-deoxyadenosin-3'-yl]-*N*-(diisopropyl)methylphosphonamidite. Yield 69%. <sup>1</sup>H-NMR (CD<sub>3</sub>CN)  $\delta$  9.35 (broad s, 1H, NH), 8.71–8.46 (m, 2H, H2 and H8), 8.04 (m, 2H, benzoyl), 7.69–7.24 (m, 12H, trityl and benzoyl), 6.93–6.89 (m, 4H, trityl), 6.64 (m, 1H, H1'), 4.52–4.43 (m, 2H, H3' and H4'), 3.80 (s, 6H, 2 OCH<sub>3</sub>), 3.46 (m, 2H, CH isopropyl), 3.30 and 3.13 (2m, 2H, H5' and H5''), 2.90 (m, 1H, H2'), 2.73 and 2.51 (2m, 1H, H2''), 0.98–1.16 (m, 15H, CH<sub>3</sub> isopropyl and P-CH<sub>3</sub>). <sup>31</sup>P-NMR (CD<sub>3</sub>CN)  $\delta$  122.20 and 120.95. FAB mass spectrum (negative mode, thioglycerol): 801 [M-H]<sup>-</sup>.

### $\beta$ - and $\alpha$ -oligonucleoside methylphosphonates 5, 6, 13 and 14

$\beta$ - and  $\alpha$ -oligonucleoside methylphosphonates **5**, **13** and **6**, **14**, respectively, were prepared according to published procedures (29) on 1  $\mu$ mol scale with an Applied Biosystems Inc. Model



**Figure 1.** Melting curves of the duplexes formed with modified dodecathymidylate analogs and (A) poly dA, (B) poly rA. (—), PO  $\beta$ -dT<sub>12</sub> 1; (···), PO  $\alpha$ -dT<sub>12</sub> 2; (— · — · — · —), MP  $\beta$ -dT<sub>12</sub> 5; (— · — · — · —), MP  $\alpha$ -dT<sub>12</sub> 6. Experimental conditions as in Table 1.

381A DNA synthesizer using either  $\beta$ -methylphosphonamidite synthons or  $\alpha$ -methylphosphonamidite synthons. To facilitate the oligonucleotide purification, the final trityl group was kept on the oligonucleotide at the end of the synthesis. The oligomers were deprotected by treatment with ethylenediamine/ethanol (1/1, v/v) for 6 h as described by Lin *et al.* (30). The oligomers were purified by OPEC™ cartridge (Clontech) (30). The  $\beta$ - and  $\alpha$ -dodecathymidine methylphosphonates **5** and **6** were obtained without further purification whereas the  $\beta$ - and  $\alpha$ -heteropolymers **13** and **14** were purified by preparative HPLC using a 30 min linear gradient of 5–40% CH<sub>3</sub>CN in 50 mM TEAAC.

MP  $\alpha$ -oligonucleosides **6** and **14** were characterized by MALDI-TOF mass spectrometry. The samples were dissolved in a solution of 2% trifluoroacetic acid in water and the matrix was  $\alpha$ -cyanohydroxycinnamic acid. MS (negative ion mode MALDI-TOF) **6**  $m/z$  calculated 3566.7, found 3565.6; **14**  $m/z$  calculated 3527.7, found 3528.2.

### Melting experiments

Concentration of each separated oligonucleotide was determined spectrophotometrically at 260 nm and at 80°C assuming that the molar extinction coefficient of each  $\alpha$ - or  $\beta$ -oligomer is the sum of the molar extinction coefficients of the constitutive deoxynucleotides. Optical measurements were performed on a UVI-KON 931 spectrophotometer (Kontron). The temperature of the cell was controlled through a HUBER PD415 temperature programmer connected to a refrigerated ethyleneglycol–water bath (HUBER Ministat). Cuvettes were 1 cm pathlength quartz cells. The cell compartment was continuously flushed with dry nitrogen when the temperature was below room temperature. Prior to the experiments, the oligonucleotides were mixed together each at a final concentration of 3 or 5  $\mu$ M in 100 mM sodium chloride and 10 mM sodium cacodylate (pH 7), and allowed to incubate at 90°C for 30 min. During the melting and hybridization experiments the heating rate was 0.5°C/min. Digitized absorbance and temperature values were stored in a computer for subsequent plotting and analysis.  $T_m$  values were

determined from the maxima of the first derivative plots of absorbance versus temperature.

## RESULTS AND DISCUSSION

### Preparation of methylphosphonate oligonucleosides **5**, **6**, **13**, **14**

MP  $\beta$ - and  $\alpha$ -dodecathymidine **5** and **6**, and MP  $\beta$ - and  $\alpha$ -heteropolymers **13** and **14** were prepared on solid support using the appropriately protected nucleoside methylphosphonamidite synthons and following the procedure of Hogrefe *et al.* (29). The heteropolymer sequence was complementary to the splice acceptor site of mRNA (AGAAUUGGGUGU) coding for HIV-1 *tat* protein. A decrease in the synthesis yield of the  $\beta$ - and  $\alpha$ -heteropolymers **13** and **14** was observed (coupling yields averaged 93% per cycle) compared with the synthesis yield of the  $\beta$ - and  $\alpha$ -dodecathymidine oligomers **5** and **6** (98% per cycle). All the oligomers were deprotected and purified following a dimethoxytrityl-on method in combination with the OPEC cartridges (30). Due to the low coupling efficiency, the purity of oligonucleotides **13** and **14** was not satisfactory after the OPEC cartridge purification, so they were further purified by RP-HPLC.

### Interactions of homothymidine dT<sub>12</sub> analogs with poly dA and poly rA

Interactions between dodecathymidine analogs with poly dA and poly rA were investigated by measuring the absorbance at 260 and 284 nm versus temperature of 1:1 mixtures of the oligomer and the target molecule (60  $\mu$ M nucleotide concentration). At 260 nm, each mixture exhibited a single transition (Fig. 1) and no differences were observed between thermal association and dissociation curves. Melting temperatures were determined at the inflexion point of the sigmoidal curves (Table 1).

**Table 1.** Thermal stability of duplexes formed by various dodecathymidine dT<sub>12</sub> oligonucleotide analogs with poly dA and poly rA

Oligomers	Anomeric configuration Internucleotidic linkages		Nucleic acid target			
			Poly dA		Poly rA	
			T <sub>m</sub> (°C)	ΔT <sub>m</sub> (°C)	T <sub>m</sub> (°C)	ΔT <sub>m</sub> (°C)
1	beta	phosphodiester	31.5	—	29.0	—
2	alpha	phosphodiester	22.5	-9.0	41.8	+12.8
3	beta	phosphorothioate	14.2	-17.3	8.0	-21.0
4	alpha	phosphorothioate	10.5	-21.0	27.8	-1.2
5	beta	methylphosphonate	28.1	-3.4	<0	—
6	alpha	methylphosphonate	46.9	+15.4	37.2	+8.2

Experiments were carried out at 60 μM nucleotide concentration for each strand in a buffer containing 10 mM sodium cacodylate, pH 7, 100 mM sodium chloride.

Among all the studied analogs, we noticed that only the non-ionic MP α-ODN **6** formed with poly dA a duplex more stable than the natural PO β-ODN **1** (ΔT<sub>m</sub>+15.4°C). Regarding each backbone-modification, the anomeric inversion from β to α induced a decrease of affinity of ionic PO **2** (-9.0°C) and PS **4** (-3.7°C) oligonucleosides whereas an enhancement of binding of non-ionic MP (+18.8°C) oligomers was observed. Furthermore, the transition for MP α-ODN **6** was sharper than the transition for MP β-ODN **5** (Fig. 1A). No hyperchromic change at 284 nm was noticed during melting experiments, ruling out the possible formation of triple helices (31,32).

With poly rA target, the combination of α-anomeric configuration with internucleosidic linkages PO and MP in homopolymers stabilized hybrids in comparison with the natural duplex with PO β-ODN **1**. Whatever the internucleosidic linkage was, duplexes formed between α-ODNs and RNA target were more stable than duplexes formed with β-homologues. This difference between α- and β-ODNs was more pronounced with MP oligomers (more than +37.2°C) than with ionic PO (+12.8°C) and PS (+19.8°C) oligomers. Note that the non-ionic MP β-ODN **5** was not able to form hybrid with the RNA target in these experimental conditions.

The data reported here related to MP α-ODNs corroborated the data obtained previously with NH<sub>2</sub>-phosphoramidate α-ODNs, another class of non-ionic oligonucleotides (26). Extension of this study with MP α-homopolymers was to examine the behavior of MP heteropolymers with their complementary DNA and RNA targets.

### Interactions of heteropolymer analogs with complementary DNA and RNA targets

It is well known that PO and PS α-ODNs hybridize to their nucleic acid targets with a parallel orientation (23,33). Here we have checked if MP α-dodecamer 5'-TCTTAACCCACA-3' **14** bound to their complementary strand in parallel or antiparallel orientation. For this, we synthesized two MP α-ODNs 5'-ACACCCAATTCT-3' and 5'-TCTTAACCCACA-3' **14** both complementary to the splice acceptor site of mRNA coding for HIV-1 *tat* protein but in reverse orientation. Only **14** hybridized to its targets which demonstrated that MP α-oligonucleosides also hybridize to their targets in parallel orientation.

As shown in Table 2, among all the modified oligomers, MP α-ODN **14** formed a hybrid with the DNA target that was more

stable than that from the corresponding natural PO β-ODN **9** (ΔT<sub>m</sub>+2.6°C). The more destabilizing analog was PS β-ODN **11** (ΔT<sub>m</sub>-10.5°C).

Regarding the RNA target, all the modifications of the backbone combined or not to the anomeric inversion affected the thermal stability of duplexes. The MP β-ODN **13** formed the less stable hybrid with RNA target (ΔT<sub>m</sub>-18.3°C).

**Table 2.** Thermal stability of duplexes formed by various backbone-modified β- and α-heteropolymers with DNA and RNA targets

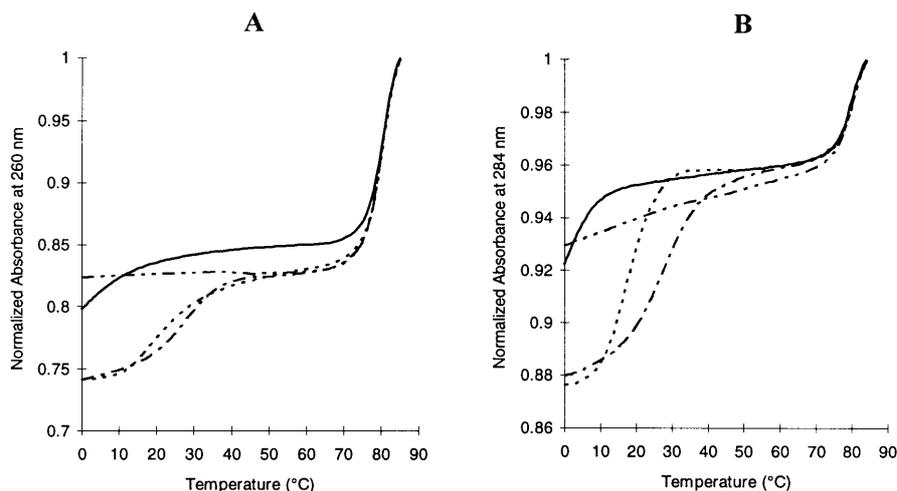
Oligomers	Anomeric configuration Internucleotidic linkages		Nucleic acid target			
			DNA <b>7</b>		RNA <b>8</b>	
			T <sub>m</sub> (°C)	ΔT <sub>m</sub> (°C)	T <sub>m</sub> (°C)	ΔT <sub>m</sub> (°C)
9	beta	phosphodiester	45.4	—	44.6	—
10	alpha	phosphodiester	43.4	-2.0	42.0	-2.6
11	beta	phosphorothioate	34.9	-10.5	35.0	-9.6
12	alpha	phosphorothioate	36.8	-8.6	38.6	-6.0
13	beta	methylphosphonate	40.0	-5.4	26.3	-18.3
14	alpha	methylphosphonate	48.0	+2.6	38.8	-5.8

DNA and RNA targets: *tat* HIV-1 sequence (position 5357–5368 of genomic RNA): **7**: 5'-d(AGAATTGGGTGT)-3' and **8**: 5'-r(AGAAUUGGGUGU)-3'. Complementary oligonucleotide sequence: β-configuration 5'-d(ACACCCAATTCT)-3' and α-configuration 5'-d(TCTTAACCCACA)-3'. Experiments were carried out at 3 μM oligomer strand concentration in a buffer containing 10 mM sodium cacodylate, pH 7, 100 mM sodium chloride.

Whatever the target was, the anomeric inversion from β to α induced a slight destabilization of phosphodiester duplexes (ΔT<sub>m</sub>-2°C versus DNA and -2.6°C versus RNA), whereas duplexes formed with phosphate-modified α-ODNs were more stable than duplexes formed with their β-homologues. This increase of affinity of PS α-ODNs for their targets was reported previously in the literature with other examples (25). The stabilization obtained in changing the anomeric configuration from β to α was more pronounced for non-ionic MP oligonucleosides (+8°C with DNA target and +12.5°C with RNA target) than with ionic PS oligomers (+1.9°C with DNA target and +3.6°C with RNA target).

Concerning backbone modification, the change of PO in PS linkage decreased the stability of duplexes with DNA and RNA targets whatever the anomeric configuration was. Nevertheless, this decrease was more pronounced with β-ODNs **9** and **11** (ΔT<sub>m</sub>-10.5°C versus DNA and -9.6°C versus RNA) than with α-oligonucleotides **10** and **12** (-6.6°C versus DNA and -3.4°C versus RNA). On the contrary, the replacement of PO linkage by MP non-ionic linkage enhanced the affinity of α-ODN **14** for its DNA target (+4.6°C). When MP α-oligonucleoside **14** was hybridized to its RNA target, a slight decrease of affinity (ΔT<sub>m</sub>/mod. -0.53°C) was observed.

As already observed (13), with RNA target MP β-ODN **13** formed a duplex less stable (ΔT<sub>m</sub>-18.3°C) than that formed by PS β-ODN **11** (ΔT<sub>m</sub>-9.6°C), whereas versus DNA target MP β-ODN **13** hybridized better (ΔT<sub>m</sub>-5.4°C) than PS β-ODN **11** (ΔT<sub>m</sub>-10.5°C). This data indicated an appreciable distortion of the structure of the MP β-ODN-RNA duplex and this distortion was weaker with DNA target. Concerning α-oligonucleotides, MP modification stabilized the duplex formed with DNA target



**Figure 2.** Melting curves of the triplexes formed with modified dodecathymidylate analogs and the target d(GCT<sub>12</sub>CGT<sub>4</sub>CGA<sub>12</sub>GC) **15**, detected at (A) 260 nm and (B) 284 nm. (—), PS α-dT<sub>12</sub> **4**; (···), PO α-dT<sub>12</sub> **2**; (-·-·-·-), MP β-dT<sub>12</sub> **5**; (- - - - -), MP α-dT<sub>12</sub> **6**. Experiments were carried out at 3 μM oligomer strand concentration in a buffer containing 10 mM sodium cacodylate, pH 7, 1 M NaCl.

(+4.6°C compared with **10**) whereas PS modification destabilized the duplex (-6.6°C compared with **10**). Versus RNA target, MP α-oligonucleoside **14** ( $\Delta T_m$  -5.8°C) was as destabilizing as PS α-oligomer **12** ( $\Delta T_m$  -6°C).

Non-ionic MP ODNs with α-anomeric configuration bound to their complementary nucleic acid targets more tightly than their homologues with natural β-anomeric configuration did. This difference of behavior between MP α- and β-ODNs was more pronounced with RNA than with DNA target. The anomeric inversion restored the affinity of backbone-modified oligonucleotides for their single-stranded targets. These results prompted us to extend our investigations on the ability of non-ionic methylphosphonate α-oligonucleosides to form triple helices with dsDNA targets.

### Interactions of homothymidine dT<sub>12</sub> analogs with double-stranded target d(GCT<sub>12</sub>CGT<sub>4</sub>CGA<sub>12</sub>GC)

The ability of backbone-modified MP α-dT<sub>12</sub> oligonucleoside **6** to form triple helix with a complementary dsDNA target was evaluated using thermal denaturation experiments. The target was d(GCT<sub>12</sub>CGT<sub>4</sub>CGA<sub>12</sub>GC) **15**, which forms a self-complementary structure (hairpin) of high stability ( $T_m$  67.5°C in 0.1 M NaCl and 80°C in 1 M NaCl). Melting curves were recorded at two different salt concentrations (0.1 and 1 M NaCl) and  $T_m$  data are reported in Table 3.

It is noteworthy that none of the β-dT<sub>12</sub> oligonucleotides analogs, PO **1**, PS **3** and non-ionic MP **5**, formed a stable complex with the target **15** under the conditions of the melting experiment, even at high salt concentration (1 M NaCl). This result is consistent with the well-known limited stability of triple helices containing only T·A·T triplets (32) when the third strand is a backbone-modified β-oligonucleotide (14,34).

On the contrary, the ionic PO and PS α-dT<sub>12</sub> oligonucleotides **2** and **4** formed a triplex with **15** at high salt concentration (1 M NaCl) with  $T_m$  values of 17.5 and 3°C, respectively, but were not able to hybridize to their target at low salt concentration (0.1 M NaCl). Of special interest is that the non-ionic MP α-dT<sub>12</sub> **6**

hybridized to their double-stranded target **15** more tightly than the ionic α-analogs **2** and **4** whatever the salt concentration was (0.1 or 1 M NaCl). When the experiment was followed at 260 nm, the melting curve for an equimolar mixture of the non-ionic α-dT<sub>12</sub> **6** and the hairpin **15** showed a transition ( $T_m$  25°C at 0.1 M NaCl concentration) for dissociation of the analog from the duplex segment **15** and another transition ( $T_m$  67.5°C) for denaturation of the hairpin **15**.

**Table 3.** Thermal stability of triplexes formed by various dodecathymidine dT<sub>12</sub> α-oligonucleotide analogs with the hairpin d(GCT<sub>12</sub>CGT<sub>4</sub>CGA<sub>12</sub>GC) **15**

Oligomers	Anomeric configuration		$T_m$ (°C)	
	Internucleotidic linkages		0.1 M NaCl	1 M NaCl
<b>2</b>	alpha	phosphodiester	—	17.5
<b>4</b>	alpha	phosphorothioate	—	3.0
<b>6</b>	alpha	methylphosphonate	25	29

Experiments were carried out at 3 μM oligomer strand concentration in a buffer containing 10 mM sodium cacodylate, pH 7, 0.1 M NaCl or 1 M NaCl. No transition was observed with the corresponding β-analogs.

When melting experiments were carried out at 284 nm,  $T_m$  values were identical to  $T_m$  values obtained on the melting curves recorded at 260 nm (Fig. 2). However, a strong hyperchromic change for the lower temperature transition was observed at 284 nm in agreement with the formation of dT·dA·dT triplets (31). It should be noted that the lower transition was sharp in the case of MP α-dT<sub>12</sub> **6**.

Generally, in the case of pyrimidines-containing oligonucleotides it is known that triplex formation is a much slower process than duplex formation and long incubation times of the third strand with the duplex are required to allow their formation (35). The slow rate of triple helix formation could be a limiting factor for use in regulating gene transcription or replication since the oligonucleotide would have to compete with proteins for binding to DNA (36). Surprisingly, here, no significant differences were

observed between thermal dissociation and association curves (heating and cooling rate of 0.5°C/min) of non-ionic MP  $\alpha$ -ODN **6** with their target **15**, which suggests a relatively fast formation of triplex.

The stability of the complex formed with non-ionic MP  $\alpha$ -oligo **6** was slightly enhanced (+4°C) as the salt concentration increased. At high ionic strength, the enhancement was more pronounced for ionic  $\alpha$ -oligonucleotides. However, the triplexes formed with these ionic ODNs were less stable than with MP  $\alpha$ -oligonucleoside **6**. The finding that an increase of the ionic strength raised the  $T_m$  value of the PO  $\alpha$ -dT<sub>12</sub> more than the  $T_m$  of MP  $\alpha$ -oligomer is consistent with the greater charge density of the triplex formed with ionic PO  $\alpha$ -oligo compared with the triplex formed with non-ionic MP  $\alpha$ -ODN. Although the present results do not allow us to state the orientation of the third strand in triplexes formed between MP  $\alpha$ -dT<sub>12</sub> and its dsDNA target, they provide evidence that thermal stability of triple helices is highly dependent on the anomeric configuration of the oligonucleotide analog.

In conclusion, we have demonstrated that the inversion of the anomeric configuration in the sugar moieties in non-ionic methylphosphonate oligonucleosides restored their affinity for ssDNA and ssRNA targets as well as for dsDNA target. The enhancement of the affinity with ssDNA and ssRNA induced by the combination of two structural modifications was previously observed with non-ionic PNH<sub>2</sub> oligonucleosides (**26**) and at a lower extent with ionic PS-ODNs (**25**), and this finding seems to be a general phenomenon for backbone-modified oligonucleotides. We are currently trying to explain it by circular dichroism, NMR and molecular modeling studies. With respect to potential applications, the high binding of MP  $\alpha$ -oligonucleosides to dsDNA is promising for the development of antigene oligonucleotides as therapeutically useful drugs.

## ACKNOWLEDGEMENT

We thank the Agence Nationale de Recherche sur le SIDA (ANRS, France) for financial support.

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