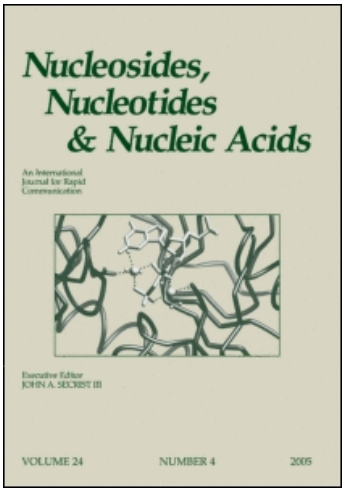


**Nucleosides, Nucleotides and Nucleic Acids**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

# Sugar-Modified Oligonucleotides: Synthesis, Physicochemical and Biological Properties

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**To cite this Article** Imbach, Jean-Louis , Rayner, Bernard and Morvan, François(1989) 'Sugar-Modified Oligonucleotides: Synthesis, Physicochemical and Biological Properties', Nucleosides, Nucleotides and Nucleic Acids, 8: 5, 627 — 648
**To link to this Article: DOI:** 10.1080/07328318908054204
**URL:** http://dx.doi.org/10.1080/07328318908054204

NUCLEOSIDES & NUCLEOTIDES, 8(5&6), 627-648 (1989)

# SUGAR-MODIFIED OLIGONUCLEOTIDES: SYNTHESIS, PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES.

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**ABSTRACT.** A new class of nuclease-resistant oligonucleotides, consisting exclusively of α-anomeric nucleotide units, has been developped. The so- called α-oligodeoxyribonucleotides were shown to strongly hybridize with complementary natural β-strands (DNA or RNA). The two complementary strands of α,β-duplexes adopt a parallel orientation and the double helix exists in aqueous solution in a conformation that belongs to the B family and is 70% right-handed. α-Oligodeoxynucleotides are competitive inhibitors, *in vitro* of HIV and M-MLV reverse transcriptases and *E. Coli* RNase H.

**INTRODUCTION.** Nucleic acids have long been strategic targets for approaches to chemotherapy in view of their roles in replication, transcription and translation.[1] The use of synthetic oligonucleotides, which bind specifically to complementary sequences of nucleic acids (RNA or DNA) through base pairing, is now under extensive investigation. In principle, relatively short oligomers (20 bases or less) can specifically hybridize with DNA or RNA and thus be used for drug design strategies involving targeted interference of genetic expression.

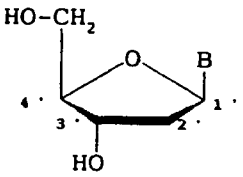
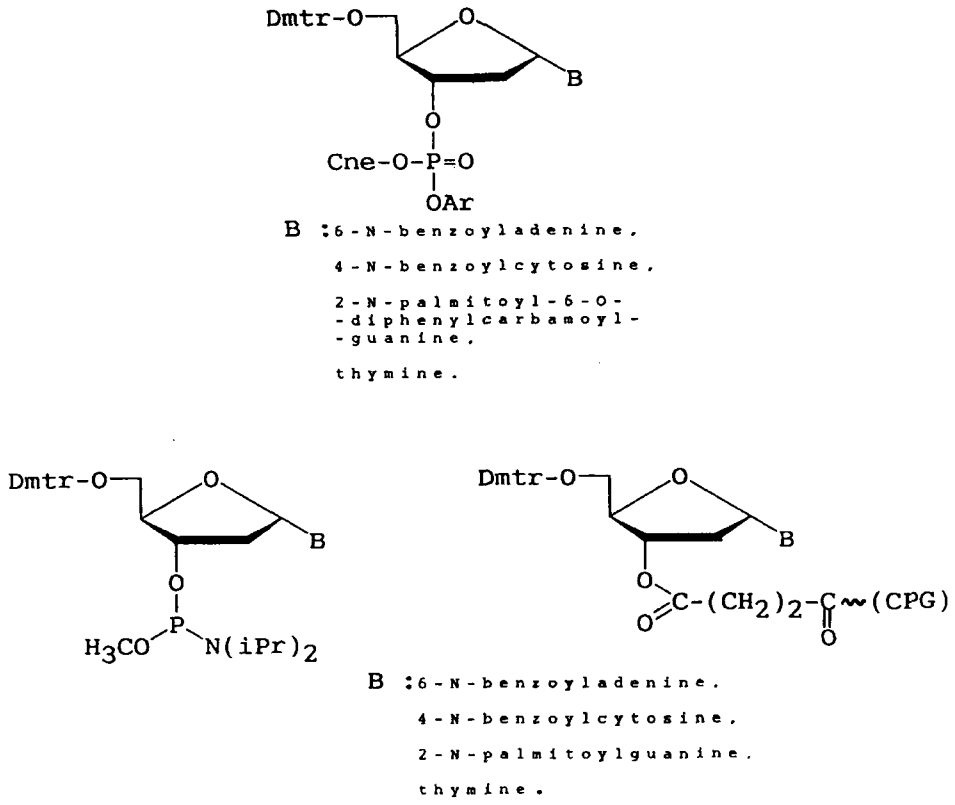



FIGURE 1



However, potential chemotherapeutic applications resulting from sequence-specific hybridization require analogues that are resistant to degradation by various nucleases. Oligonucleotide analogues presenting modifications on the phosphate backbone (i.e. methylphosphonates,[2-5] phosphorothioates[6]) have been introduced and have been shown to present a good resistance to enzyme-mediated depolymerization. However such modifications introduce asymetric linkages and, as the synthesis is not stereocontrolled, lead to a mixture of $2^n$ stereoisomers, where n is the number of these linkages. These phosphate backbone modifications have always been considered using "natural" 2'-deoxy-β-D-ribofuranosyl nucleosides as starting synthons. But another possibility is to consider sugar-modified nucleosides as building blocks and to synthesize the corresponding oligomers with natural phosphodiester linkages.

Let us consider first the structure of a natural 2'-deoxy-β-D-ribofuranosyl nucleoside (Fig. 1).
This molecule presents 3 chiral atoms in 1', 3' and 4' positions. In order to maintain the geometry of the sugar phosphate backbone with a 3',4'-transorientation, configurations of 3' and 4' carbon atoms must be change both together. This modification leads to 2'-deoxy-L-ribonucleosides. Oligomers of L-2'-dU have been shown to be much more resistant to snake venom phosphodiesterase than oligomers of D-2'-dU are, although no evidence was found for binding




Ar, 2-chloro-4-tritylphenyl; Cne, 2-cyanoethyl; Dmtr, 4,4'-dimethoxytrityl; iPr, isopropyl; CPG, Controlled pore glass

FIGURE 2

of a 18-mer of L-2'-dU to poly(dA).[7] Inversion of the 1' carbon atom leads to 2'-deoxy-α-D-ribofuranosyl nucleosides. Only α-oligodeoxyribonucleotides (α-DNA), consisting exclusively of α-anomeric deoxyribonucleotides, will be consider in this paper.

In 1973, U. Sequin,[8] using Dreiding models, considered the possibility of α-oligonucleotides to exhibit a secondary structure similar to that of the natural nucleic acids, featuring base pairing, base stacking and helix formation. This study predicted that an α-strand may form a helix

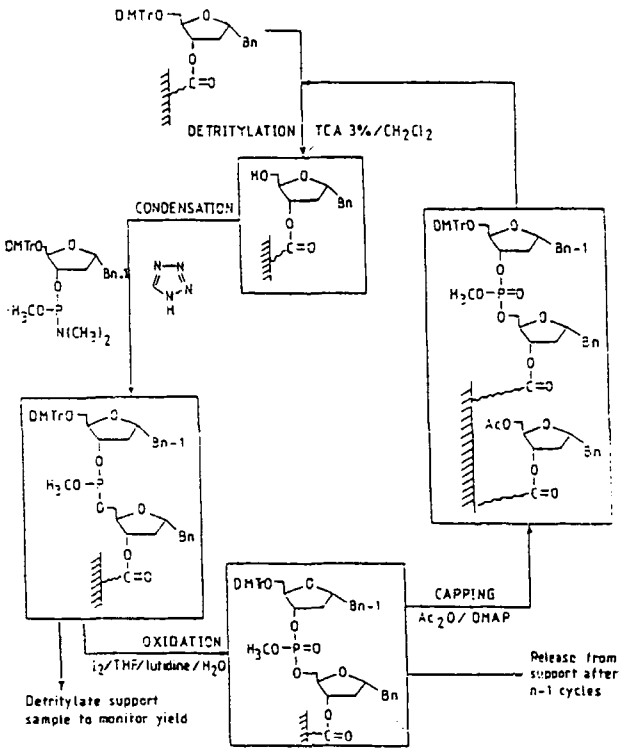



FIGURE 3 - Reaction cycle used during α-oligonucleotide synthesis by the phosphoramidite approach.



duplex with a complementary β- or α-strand by base pairing and the two strands should exhibit parallel and antiparallel polarity respectively. Shortly thereafter, isomeric dithymidine monophosphates bearing one or two α-dT were found to exhibit nuclease-resistance.[9]

The aim of this work, initiated in 1985, was to set up efficient synthesis of α-oligodeoxynucleotides and to study their physical and biological properties.

**SYNTHESIS OF α-OLIGODEOXYNUCLEOTIDES.** The phosphotriester method in solution and the phosphoroamidite method on solid support were applied for the efficient synthesis of α-

oligonucleotides.[10-12] Both methods required the use of unnatural 2'-deoxy-α-ribonucleosides corresponding to the four usual bases: adenine, cytosine, guanine and thymine; the latter beeing the only one commercialy available. They were synthesized by autoanomerisation[13], transglycosylation[13] or glycosylation[14] in moderate yields and were converted into either fully-protected 3'-phosphotriesters or fully-protected 3'-phosphoramidites and α-deoxynucleoside derivatized glass beads (Fig. 3), using standard procedures already described for the β-deoxynucleosides.

The phosphotriester method was applied for the synthesis in relative large amounts of the hexanucleotide α-d(CCTTCC) and the two complementary α-d(CATGCG) and α-d(CGCATG). These three hexanucleotides and their complementary β-strands were used to assess the structural and binding properties of this new class of oligonucleotide analogues, using NMR techniques. Then, in order to evaluate the biological interest of these analogues, an automated solid-phase approach was developped that makes use of α-nucleoside phosphoramidites and allows the fast synthesis of oligomers of defined sequence up to 20 α-nucleotide units in length. Figure 3 depicts the main steps involved in one elongation cycle. The time required for one cycle was 12.5 min (14.5 min for α-dG) and the average coupling yields were higher than 98%. After deprotection and purification by reverse-phase HPLC,purity of α-oligonucleotides (up to 20 bases) was checked by HPLC and gel electrophoresis analysis and was found as good as that of β-oligomers. Furthermore, their primary structure was confirmed after $^{32}$P-labelling and Maxam and Gilbert sequence analysis (Fig. 4).

**ANNEALING PROPERTIES OF NUCLEASE-RESISTANT α-OLIGODEOXYNUCLEOTIDES.** NMR and UV absorption studies[10,15-17] indicated that α-oligodeoxynucleotides exhibit the following features.

(i) They anneal specifically with complementary β-strands. The two strands of the resulting α,β-hybrid adopt a par-

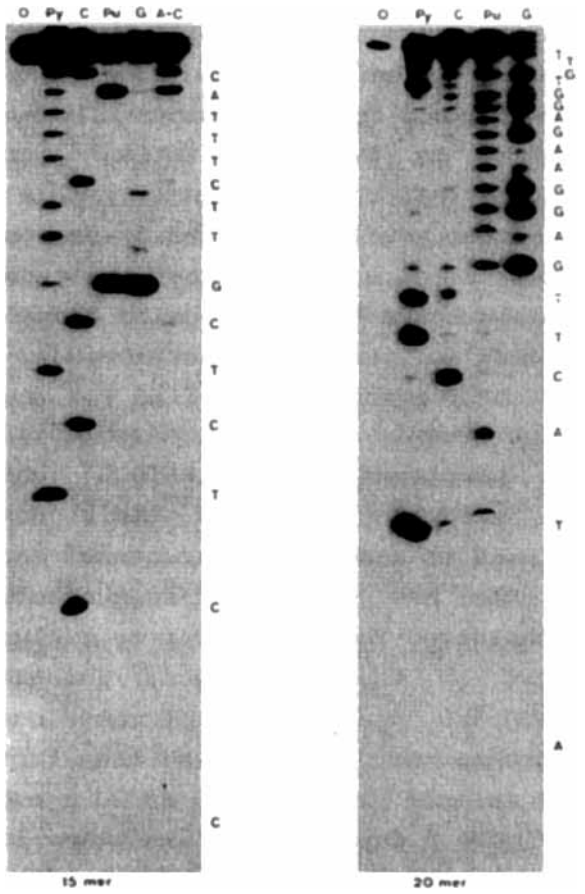



FIGURE 4 - Sequence analysis of two 5'-$^{32}$P-α-oligonucleotides. Lanes O: untreated oligonucleotides.

rallel orientation. Figure 5 shows a computer graphics depiction of the annealed duplex formed from parallel strands of α-d(CpApTpGpCpG) and β-d(GpTpApCpGpC), using the parameters coming from a NMR study.[16] It is worthwhile to mention that this α,β-double helix exists in aqueous solution in a conformation that belongs to the B family and is 70 ± 10% right-handed.

Furthermore, it was shown by Northern blot analysis that two α-oligonucleotides (20 and 18 mer) complementary, in a

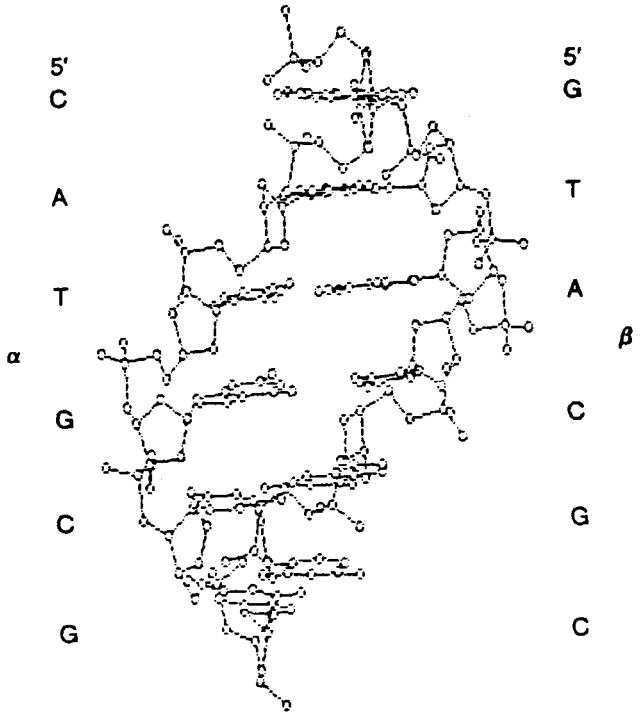



FIGURE 5 - Computer generated stereodiagram of the α,β-duplex, α-d(CATGCG):β-d(CTACGC).



parallel orientation, to the initiation region of two differents mRNA (interferon $\beta_2$ and VSV N protein mRNAs) hybridize specifically with their naturel targets (vide infra). In constrast, an energy minimization study by Sun et al.[18] predicted that the more stable A-type oligo α-(dT)n: Oligo β-(rA)n double helix should have antiparrallel strands. This prediction was confirmed by analysis of the cleavage products induced by an oligo α-$(dT)_8$-phenantroline conjugate on $(rA)_8$ or $(rA)_{10}$ sequence. However the A type of this double helix was not established and it remains to be determined whether the antiparallel orientation observed for the complex α-$(dT)_8$ with β-$(rA)_8$ is a special property due to the formation of adjacent AT base pairs involving, e.g., Hoogsteen or reverse Watson-Crick[19,20] rather than classical Watson-Crick hydrogen bonds.

TABLE 1 - Thermodynamics for single-stranded to double-helix transition for β-CCTTCC:β-GGAAGG, α-CCTTCC:β-GGAAGG and β-CCTTCC:α-GGAAGG. The experimental conditions were cacodylate buffer 10 mM, pH 7.0 and NaCl 1M. Total strand concentration was $10^{-5}$ M. ΔG° represents the free energy at 25°C.

| | ΔH° (Kcal/mol) of duplex | ΔS° (u.e.) | ΔG° (Kcal/mol of duplex) | tm (°C) |
|---|---|---|---|---|
| β-CCTTCC<br>β-GGAAGG | -39.1 ± 3 | -110 ± 10 | -6.3 ± 2 | 20.2 |
| α-CCTTCC<br>β-GGAAGG | -37.5 ± 0.9 | -101 ± 3 | -7.3 ± 1 | 28.1 |
| β-CCTTCC<br>α-GGAAGG | -34.0 ± 0.8 | -94 ± 3 | -6.0 ± 0.9 | 13.8 |



(ii) The α,β-duplex stability is dependent on the base composition and from the data in table 1 it can be deduced that pyrimidine α-nucleosides give more stability than purine α-nucleosides.

(iii) α-oligonucleotide binding to single-stranded β-RNA appears more favorable than to β-DNA.[21] This point may be of great importance for the selective targeting of $mRNA_s$.

Stability of α-oligodeoxynucleotides against hydrolysis by various isolated nucleases (endonuclease or exonucleases) was shown to be one to three orders of magnitude higher then that of the corresponding β-oligomers.[11] The same figure was found in subcellular extracts and culture media. For instance in undiluted fetal bovine serum, β-oligonucleotides were completely degraded within 15 min, whereas one half of an α-oligonucleotide remains intact after 8 h.[22,23]

**BIOLOGICAL PROPERTIES OF α-OLIGODEOXYNUCLEOTIDES.** As we were interested at a first glance on the anti-sense ap-

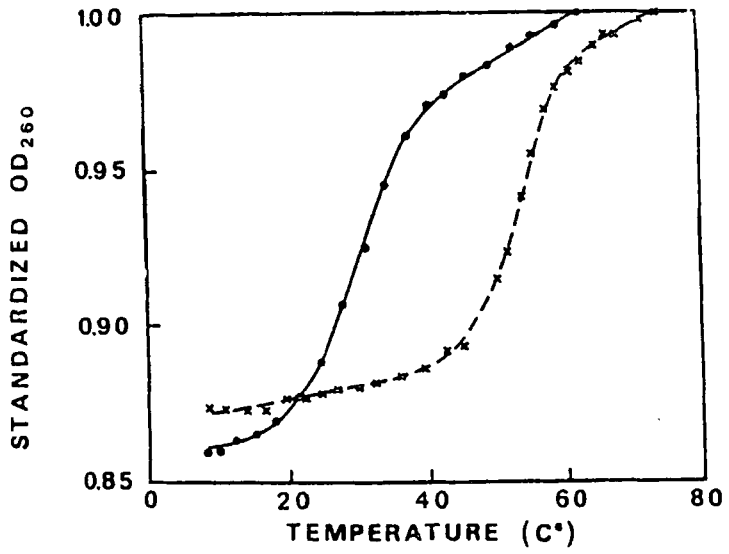



FIGURE 6 - Melting curves of 1:1 mixtures of α- and β-d($G_2T_{12}G_2$) hybridized to $rA_{12}$. Buffer solution was Tris. HCl 20 mM, pH 7.5, $MgCl_2$ 10 mM, KCl 100 mM and DTT 0.1 mM.



proach we focused on RNase H, a nuclease which cleaves the RNA strand of a DNA:RNA duplex, as this enzyme seemed to play a key role in the hybridization arrest process.[24]

In a first *in vitro* experiment we studied the action of *E. Coli* RNase H on a short α-DNA:β-RNA duplex using commercial $rA_{12}$ as a model RNA. Therefore we synthesized the complementary α and β-$dT_{12}$ oligo's with two dG at each extremity to avoid sticky ends.

We showed first we had annealing by measuring the melting temperature of the two duplexes by a spectrophotometric method as shown on the figure 6. The Tm of the β,β-duplex is 27.5° and it raises to 55° for the α,β-hybrid exhibiting a stability enhancement of 27.5°.[24] We then studied the digestion, *in vitro*, of those duplexes by *E. Coli* RNase H after labelling the RNA strand with $^{32}P$.

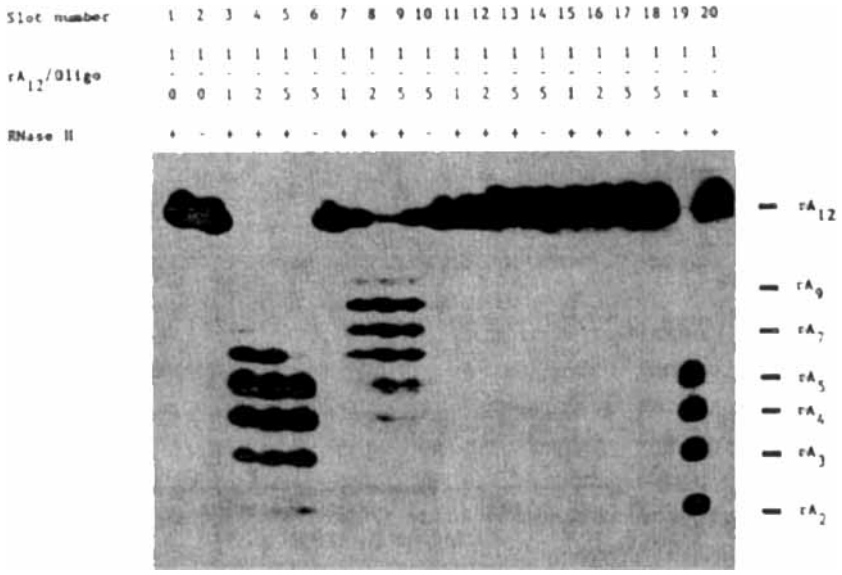



FIGURE 7 - RNase H degradation of 5'-[$^{32}$P]-$rA_{12}$ in presence of α or β-d[$G_2T_{12}G_2$].

The autoradiography of RNase H digestion products (after 4 h of incubation at 20°) of those duplexes is presented on figure 7. As expected this enzyme did not degrade single-stranded $rA_{12}$ alone (lane 1) or in presence of β-poly dC (lane 20). In contrast, the RNase H degraded the β-RNA strand of the β,β-duplex prehybridized (lanes 3-5) or not (lanes 7-9). In the same experimental conditions we did not observe any digestion of the RNA strand in duplexes with α-$dT_{12}$ (lanes 11-18). This *in vitro* experiment shows that RNase H hydrolyses the β-RNA strand hybridized to a complementary β-DNA strand whereas it does not degrade it when duplexed to an α-DNA strand.

Another experiment was designed to investigate the ability of α-DNA to inhibit the translation of mRNA in a cell free system.

IFN $\beta_2$ mRNA 5'.....CCUAUGAACUCCUUCUCCACAAGCGCC...3'

α-probe 5'......ATACTTGAGGAAGAGGTGTT...3'

β-probe 3'......ATACTTGAGGAAGAGGTGTT...5'

FIGURE 8

We use IFN $\beta_2$ mRNA coding for a human 26kD protein and two α- and β-probes (20 mers) complementary to the ribosome binding site were synthesized ( Fig. 8).[25] A Northern blot experiment (Fig. 9) revealed that both probes recognize and hybridize specifically the 26 kD mRNA (lane 1) and do not cross hybridize with VSV mRNAs (lane 2). This point strengthens the fact that there is a parallel hybridization between a mRNA and a complementary α-DNA probe as mentionned before.

This mRNA and the oligo's were then added to a translation system - a rabbit reticulocyte lysate - and after incubation the protein distribution was revealed by fluorography and autoradiography (Fig. 10).

In absence of antisens oligomers the protein was expressed. When the β-probe was added to the medium without RNase H the protein was expressed but not if RNase H was present. Using the α-probe the protein was expressed in presence or in absence of RNase H. This experiment shows that α-oligodeoxynucleotides are unable to inhibit translation, in this reticulocyte lysate, despite they very likely hybridize with their target sequences.

It was then interesting to check if a similar mechanism occurs in intact cells in which the role of RNase H is not yet well known. And we reach here the key problem of the poor penetration of charged oligonucleotides into cells. But as it was shown that poly(L-lysine) (PLL) conjugation of an oligonucleotide could facilitate its internalization, two set of oligo's in α- or β-configuration have been cou-

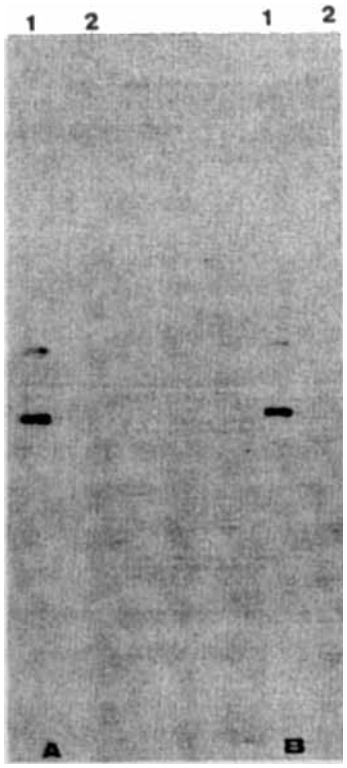



FIGURE 9 - Northern blot analysis with 5'-end labeled α- or β-oligonucleotide probes. "26 kD protein" mRNA (lanes 1) or VSV mRNAs (lanes 2) were analysed on agarose gel in denaturing conditions, transfered on nylon membrane and then hybridized to 5'-end [$^{32}$P] labelled α- or β- probes.

pled to PLL and compared for their antiviral activity against VSV in EL-4T Lymphocyte cell line.

The chemical method we used to link PLL to α- or β--oligodeoxynucleotides is presented on figure 11. A ribonucleoside was first introduced on the solid support and the DNA synthesis was then proceeded as usual with a DNA synthesizer. After removal of the protecting groups and cleavage from the solid support the 3'-terminal ribose moeity was oxidised with sodium periodate and reacted with PLL and sodium borohydride.

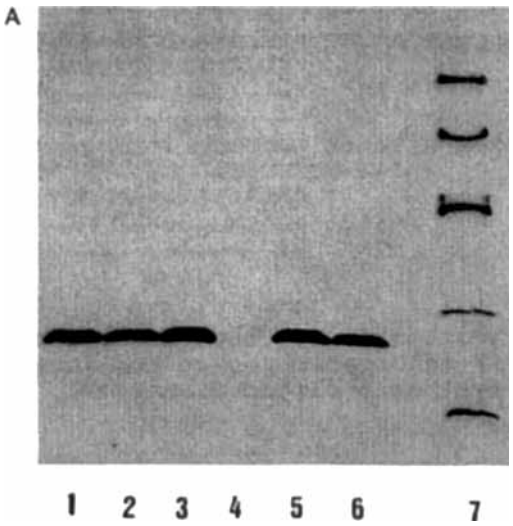



FIGURE 10 - Translation arrest assays mediated by α- or β-oligonucleotides. Lanes 1,2: no oligonucleotide; lanes 3,4: β-oligonucleotide was added; lanes 5,6: α-oligonucleotide was added; lane 7: radiolabelled molecular weight standards.

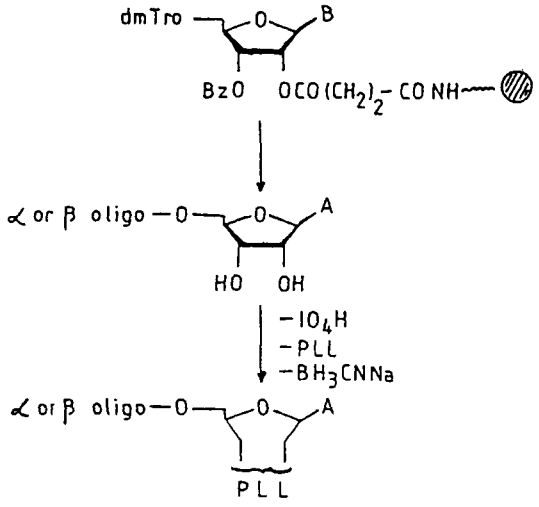



FIGURE 11 - Synthesis of oligonucleotide-PLL conjugates.

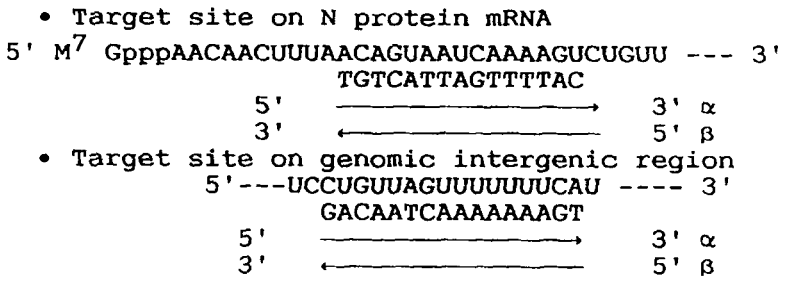



FIGURE 12 - Partial nucleotide sequences of VSV N protein mRNA and VSV intergenic region and location of the complementary synthetic oligonucleotides.



The two sets of oligonucleotide-PLL conjugates thus synthesized (Fig. 12) were targeted against:

- the initiation region of VSV N protein mRNA,
- VSV intergenic regions.

It was further shown (Fig. 13) that both β-oligo-PLL conjugates reduce VSV yield by about 2 log units but none of the α-oligo-PLL conjugates affects significantly VSV multiplication.

Those data support the role played by RNase H as a mediator of short antisense oligo's biological activity in intact cells and could be related to the previously herein described *in vitro* experiments which show that the β-RNA strand of an α-DNA:β-RNA duplex is not degraded by this enzyme. In other words a complementary α-strand protects a β-RNA strand from degradation. Although the exact role of the RNase H in the hybridization arrest of translation is not yet very clear, those data were disapointing at a first sight. But they do not preclude α oligo's further utilisation for example through conjugation with nucleic acid interactive groups such as intercalating agents, free radical generating groups, alkylating agents or photoactivable groups providing thus specific artificial enzymes.

It seemed then interesting to study further this absence of degradation of the RNA strand by RNase H in the

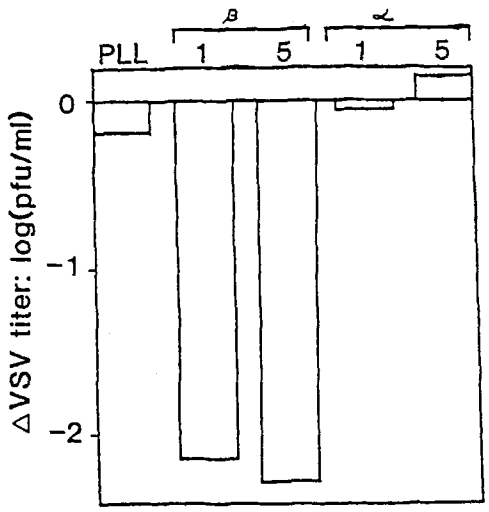



FIGURE 13 -Antiviral activity of α- and β-anomeric oligonucleotide-PLL conjugates. EL 4 cells were incubated with 0.9 μM of the conjugates 30 min. prior to VSV infection. Virus titers are expressed with reference to cells incubated in absence of oligo-PLL conjugates.



α-DNA:β-RNA duplex. For that purpose the degradation of 5'-$^{32}P(rA)_{12}$ either alone or hybridized with α or β-d[$G_2T_{12}G_2$] in rabbit reticulocyte lysate or wheat germ extracts was assayed. Gel electrophoresis analysis (Fig. 14) indicated that $rA_{12}$ was degraded in both lysates (lanes 4,7) by the RNase activity of those biological media. When $rA_{12}$ was hybridized with the complementary α-oligo (lanes 5,8) quite no degradation was observed whereas when it was hybridized with the complementary β-oligo, degradation by the RNase H present was observed under identical conditions (lanes 6,9). This indicates that hybridization with the α-strand protects *in vitro* the β-RNA strand against both aspecific RNase and RNase H activities of the extract.

It seems therefore reasonable to assume that α,β-hybrids associate RNase H and form a stable complex i.e. RNase H is able to bind to α,β-hybrids but cannot degrade them. In orther words one can predict that α,β-hybrids would be com-

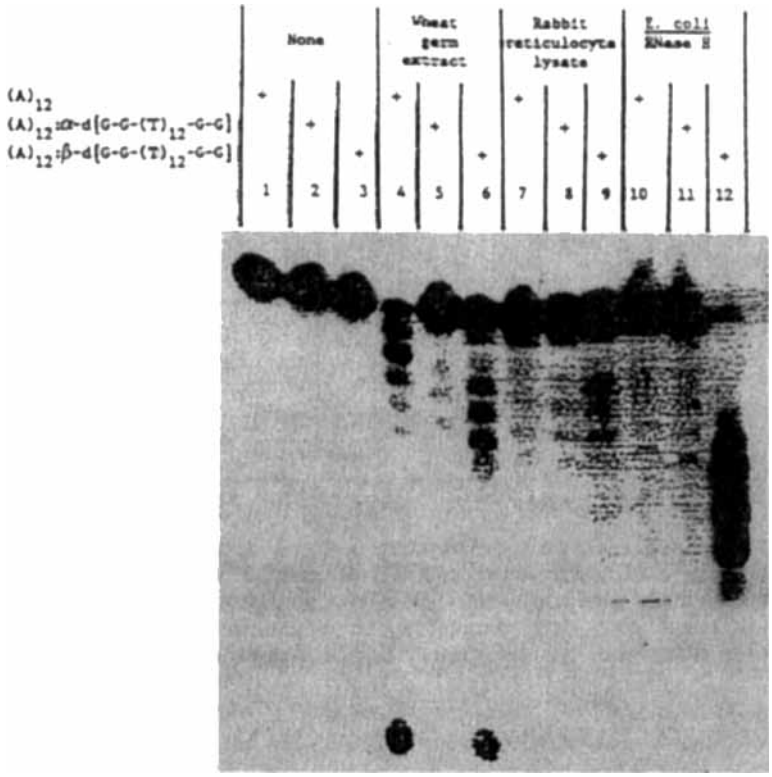



FIGURE 14 - Complementary α-oligonucleotides protects [5'-$^{32}$P] $(rA)_{12}$ against degradation by non-specific RNase H activities [5'-$^{32}$P]$(rA_{12})$ was incubated either alone (lanes 1,4,7,10) or after prehybridization with α-$d(G_2T_{12}G_2)$ (lanes 2,5,8,11) or with β-$d(G_2T_{12}G_2)$ (lanes 3,6,9,12), for 1 h. at 20°C.

petitors of natural DNA/RNA substrates in their binding to the active site of RNase H and hence would inhibit enzyme activity.

In another experiment the degradation of the prehybridized duplex $rA_{12}$:$d(G_2T_{12}G_2)$ by *E. Coli* RNase H was measured (Fig. 15) after about 20 min of incubation at 25°C. The $(rA)_{12}$ strand was quite completely hydrolyzed. But upon addition of the corresponding α,β-duplex, an inhibition of

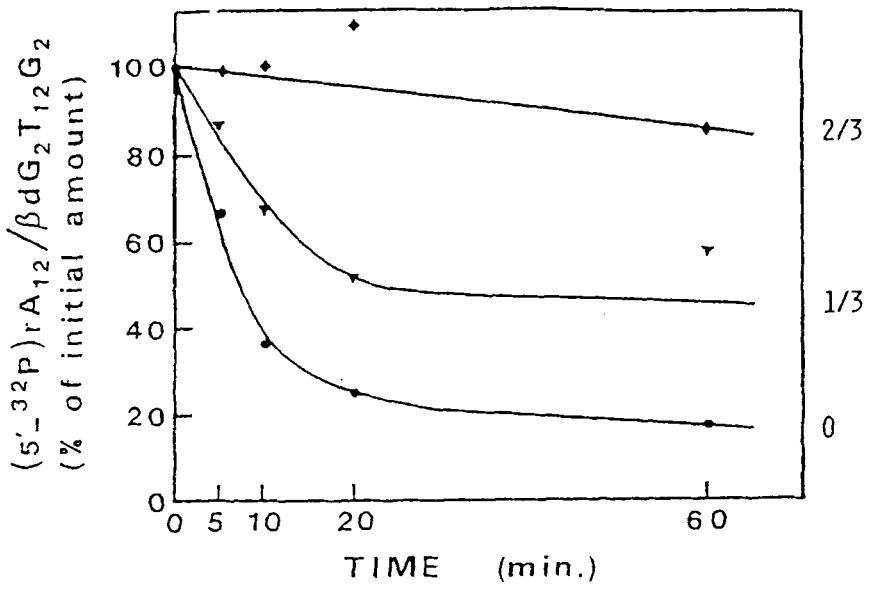



FIGURE 15 - Digestion kinetics of $(rA)_{12}$ : $d(G_2T_{12}G_2)$ by *E. Coli* RNase H in presence of increasing concentration of $(rA)_{12}$ : α-$d(G_2T_{12}G_2)$. Prehybridized $[5'-^{32}P](rA)_{12}$ : $d(G_2T_{12}G_2)$ (94 pM) was incubated in presence on RNase H (2.3 units) with the following concentrations of unlabeled $(rA)_{12}$: α-$d(G_2T_{12}G_2)$ hybrid : (●) 0, (▾) 31 pM and (◆) 61 pM.

the action of RNase H was observed. The most likely mechanism which can account for this inhibition involves the binding of the α,β-duplex to the enzyme.[26]

Another related enzyme which also binds to ds nucleic acid but does not recognize specific sequences is reverse transcriptase (RT), which presents RNase H and RNA dependent DNA polymerase activities. Therefore it seemed interesting to look at the behavior of α-DNA (single stranded or hybridized with a β-strand) in presence of RT.
We first check *in vitro* if an α oligo could act as a primer for the HIV reverse transcriptase.

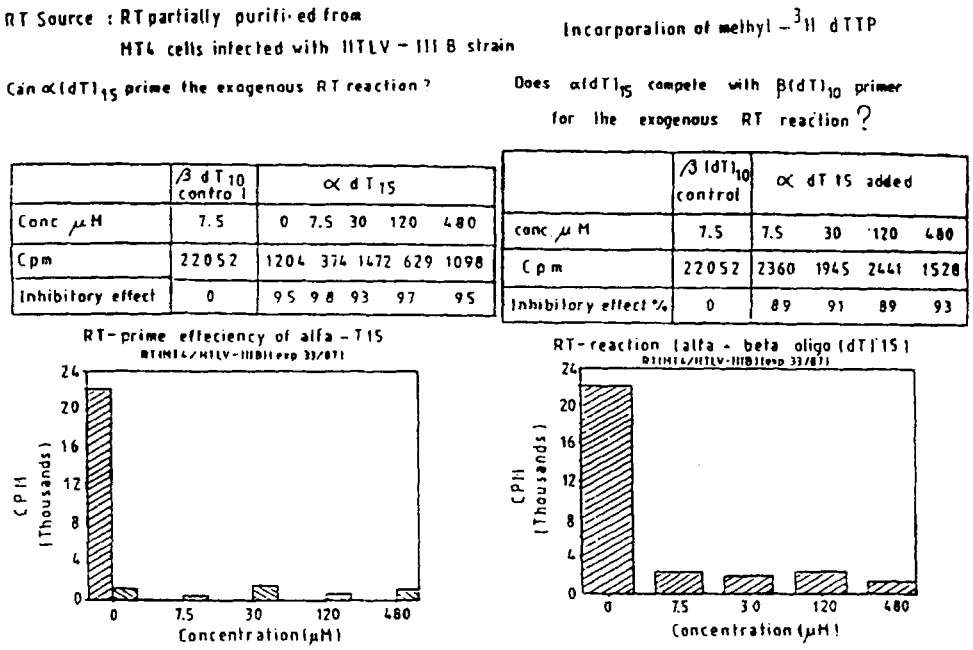



FIGURE 16 - Inhibitory effect of α-$(dT)_{15}$ on HIV reverse transcriptase with exogenous template-primer poly(rA) : oligo $(dT)_{10}$.

In the experiment presented on figure 16 an α-oligomer, α-$dT_{15}$, was first investigated for its priming effect on the poly(rA)-directed RT reaction. In the absence of the normal primer β-$dT_{10}$, there was in incorporation of [methyl-$^3$H]dTTP detectable even at α-dT oligomer concentrations up to 100 μM, suggesting that α-dT oligomers did not function as primers for the RT reaction. However, when these compounds were added to the RT assay mixture in the presence of the β- $dT_{12-18}$, 50% inhibition of the RT reaction was achieved at concentration of about 1 μM with α-$dT_{12}$, α-$dT_{14}$ and α-$dT_{16}$ (Table 2). These observations indicate that α-$dT_n$ oligomers which are complementary to the β-poly(rA) template efficiency block RT reaction provided n > 10.

In another set of experiments we have also shown that α:β

TABLE 2 - Inhibitory effects of various α-oligodeoxynucleotides on HIV associated reverse transcriptase.

| Compound | Priming activity[a] (μM) | ID-50[b] (μM) |
|---|---|---|
| α-$dT$ | - | > 100 |
| α-$dT_2$ | - | > 100 |
| α-$dT_4$ | - | > 100 |
| α-$dT_6$ | - | 45 |
| α-$dT_8$ | > 100 | 12 |
| α-$dT_{10}$ | > 100 | 4.4 |
| α-$dT_{12}$ | > 100 | 2.1 |
| α-$dT_{14}$ | > 100 | 1.4 |
| α-$dT_{16}$ | > 100 | 1.2 |

a Dose required to obtain a 50% incorporation of [methyl-$^3$H]dTTP in the absence of normal primer β-$dT_{12-18}$.

b Dose required to inhibit the HIV-RT reaction by 50% in the presence of 7.5 μM of β-primer β-$dT_{12-18}$.



hybrids inhibit M-MLV reverse transcriptase activity in the same range of importance than an α oligo alone.

We have demonstrated in all these preliminary experiments that a β-RNA strand hybridized in an α/β duplex becomes resistant to non-specific RNases; and that α:β duplexes could be considered as potential inhibitors of specific enzymatic systems which recognize ds nucleic acids. This behavior could be related to the conformational similitude between a natural ds nucleic acid and an α,β-hybrid which, as we have shown, belongs to the B family. And we presume also that the presence of the negative charges on the phosphate backbone, which makes those compounds isoelectric with DNA, is important for such enzymatic interactions. A lot of other very important applications could be envisaged for α-DNA particularly the one related to the fact that α-DNA could form triple helix[27] and hence could interfere with replication.

In conclusion, we have presented preliminary data on α-DNA, the first sugar modified Chimeric Nucleic Acid (CNA) that we recently developped. One can presume that αCNA's could be envisaged as inhibitors of enzymatic systems which recognize, specifically or not, single-stranded or double stranded nucleic acids i.e. their role as potential inhibitors of regulatory enzymes has to be explored. Among all the possible CNA, we presume that sugar modified CNA (and we are not considering only the α) are very promising tools and will become more and more interesting for molecular biologists, enzymologists, as such fake nucleic acids could interfere specifically, or not, with numerous targets of cellular machinery.



**ACKNOWLEDGEMENTS** : This research was supported by Grants from Association pour la Recherche sur le Cancer, and from Programme National de Recherche sur le SIDA (INSERM, CNRS).

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