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SYNTHESIS AND BIO-PHARMACOLOGICAL ACTIVITY OF *ANTISENSE* PHOSPHATIDYL-OLIGONUCLEOTIDES

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Imagination is more important than knowledge….

Albert Einstein

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AcOH	Acetic acid
AIDS	Acquired Immune Deficiency Syndrome
APCI	Atmospheric-Pressure Chemical Ionization
AP-CPG	Aminopropyl-CPG
AS-ODNs	Antisense Oligodeoxynucleotides
BOPCl	Bis(2-oxo-3-oxazolidinyl)-phosphinyl chloride
CMV	Cytomegalovirus
COSY	Correlation Spectroscopy
CPG	Controlled Pore Glass
DBU	1,8-Diazabicycloundec-7-ene
DEPC	Diethylpirocarbonate
DMF	Dimethylformamide
DMT	Dimethoxytrityl
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
ESI	Electrospray Ionization
HDL	High Density Lipoprotein
HOBt	1-Hydroxy-Benzotriazole
HPLC	High Performance Liquid Chromatography
LCAA-CPG	Long chain amino alkyl- CPG
LDL	Low Density Lipoprotein
LNA	Locked Nucleic Acid
LUV	Large Unilamellar Vesicle
MALDI	Matrix Assisted Laser Desorption Ionization

MeOH	Methanol
MF-ODN	Morpholino Oligodeoxynucleotide
mRNA	Messenger Ribonucleic acid
MS	Mass Spectroscopy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMP	1-Methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance
PNA	Peptide Nucleic Acid
PS	Phosphorothioate
RNase H	Ribonuclease H
RP	Reverse Phase
TBAF	Tetrabutylammonium fluoride
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TOF	Time Of Flight
Tm	Melting Temperature
VEGF	Vascular Endothelial Growth Factor

ABSTRACT

Lipid conjugated oligonucleotides are of great interest in the field of antisense oligonucleotides used for functional genomics, gene target validation and therapeutic pourpose. Although various lipid conjugates of oligonucleotides have already been prepared, it was not possible until now to prepare phosphatidyl conjugates owing to an actual difficulty encountered in a direct attachment of the phosphatidyl group to oligonucleotides elongated on the solid phase by standard phosphoramidite chemistry procedures. Now, appropriately exploiting some synthetic opportunities, available in the recent literature for the preparation of oligonucleotides bearing residual base-labile side groups, we designed a synthetic path to obtain 5'-*O*-phosphatidyloligonucleotides.

By applying this synthetic route we prepared some phosphatidyltetradecanucleotides all having the *antisense* sequence against the Vascular Endothelial Growth Factor (VEGF) gene, but differing from each other in their phosphatidyl moiety. This consisting of different fatty acyl residues, such as myristoyl, palmitoyl and stearoyl as well.

The newly synthesized phosphatidyloligonucleotides have been analyzed for their spectroscopic (NMR, MS) and chromatographic (HPLC) properties which confirmed the expected structure. The annealing features of these compounds have been investigated by Differential Scanning Calorimetry analysis .

As a preliminary experiment, the *antisense* effectiveness of 1,2-*O*-dimyristoyl- and 1,2-*O*-dipalmitoyl-*sn*-glycero-3-*O*-phosphoryl-tetradeca-mers has been assessed by observing their effect on the expression

of VEGF at mRNA level in human Neuroblastoma cells. Both the phosphatidyl-tetradecamers were able to inhibit the expression of VEGF mRNA with an effective concentration significantly lower than was found, in parallel experiments, for the corresponding unmodified antisense oligonucleotide.

1. INTRODUCTION

The backbone for all research within the field of biology lies within the "*central dogma*" which describe the basic processes occurring in living cell. Genomic DNA is transcribed into messenger RNA (mRNA) in the cell nucleus. The mRNA, which is then transported in the cytoplasm, codes for the subsequent transcription of protein (Fig. 1).

Many diseases are caused by over-expression of certain genes belonging to the same cell or of recombinant genes derived from viral infections in various cell types. Considering that the complete sequence of the human genome has been known since 2001 [1], the knowledge of the coding sequences of many genes would allow the suppression of the gene of interest by a set of gene manipulation techniques.

Antisense oligodeoxynucleotides (AS-ODNs) are molecules whose sequence is complementary to a codifying region of a gene which and so, they are able to inhibit gene expression. Using antisense DNA fragments to block the expression of selected genes, and thereby assess their function, is a powerful and currently used new tool. Conceptual simplicity, the possibility of rational design, relatively inexpensive cost, and developments in the sequencing of human genome have led recently to consider antisense oligonucleotides as potentially useful therapeutic agents for the treatment of viral infections, cancer or inflammatory diseases.

1.1 Molecular mechanism of antisense oligonucleotides

The regulatory effects of DNA-mediated antisense was discovered in 1978 [2] (Paul Zamenick and Mary Stephenson). The concept underlying

antisense technology is relatively straightforward: the use of a sequence, complementary by virtue of Watson-Crick base-pair hybridization, to a specific mRNA can inhibit its expression and then induce a blockade in the transfer of genetic information from DNA to protein. However, although antisense oligonucleotides are commonly in use both in the laboratory and clinic, this theoretical simplicity belies the many questions concerning the molecular mechanisms of action of these compounds. Oligonucleotides are in theory designed to specifically modulate the transfer of the genetic information to protein, but the mechanisms by which an oligonucleotide can induce a biological effect are subtle and complex. Although some of these mechanisms of inhibition have been characterized [3], rigorous proof for others is still frequently lacking. On the basis of mechanism of action, two classes of antisense oligonucleotide can be discerned:

1) RNase H-dependent oligonucleotides, which induce the degradation of mRNA;
2) Steric-blocker oligonucleotides, which physically prevent or inhibit the progression of splicing or the translational machinery.

The majority of the antisense drugs investigated in the clinic function *via* an RNase H-dependent mechanism. RNase H is a ubiquitous enzyme that hydrolyzes the RNA strand of an RNA/DNA duplex. Oligonucleotide-assisted RNase H-dependent reduction of targeted RNA expression can be quite efficient, reaching 80–95% down-regulation of protein and mRNA expression. Furthermore, in contrast to the steric-blocker oligonucleotides, RNase H-dependent oligonucleotides can inhibit protein expression when targeted to virtually any region of the mRNA.

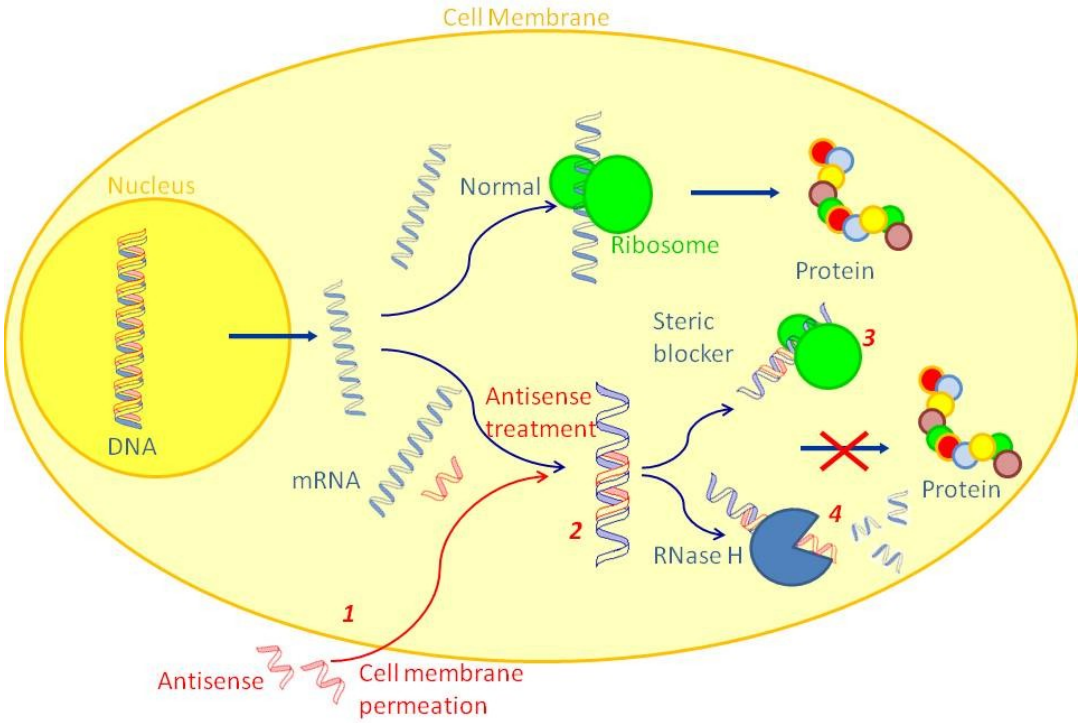


Fig.1

Fig.1 shows the RNase H-dependent antisense mechanism. Single-stranded oligonucleotides are transported across the plasma membrane (step 1). Once in the cytoplasm, single-stranded oligonucleotides bind to their targeted RNA (step 2). The hetero duplex RNA-DNA blocks the translational machinery (step 3), than the protein expression. Further, RNase H recognizes the oligonucleotide/RNA duplex as a substrate and so cleaves the RNA strand releasing the antisense oligonucleotide (step 4). Although the cleavage of the RNA by RNase H is shown to occur in the nucleus, RNase H is also present in the cytosol, allowing for cleavage to occur in that cellular compartment as well.

1.2 Chemical modifications of native structure of ODNs (State of Art)

One of the recurring problems encountered for in vivo application of oligonucleotides is the low permeability of cell membranes to these large polyionic molecules [4]. In order to overcome this problem and to enhance resistance to nuclease degradation of these molecules as well, many chemical modifications of the native structure of ODNs have been attempted, although no one of the proposed solution has been shown to be free from drawbacks. Presented below is a brief report of what until now has been done to improve the practical utility of antisense.

As above cited, in 1978 Paul Zamecnik and Mary Stephenson demonstrated the feasibility of using short antisense oligodeoxynucleotides to block the expression of targeted genes within intact cells [2]. This work provided the first evidence that oligonucleotides, although highly negatively charged, could be transported into cells at some finite rate.

Further, John Goodchild [5] in 1998 described electron microscopy studies using radiolabeled derivatives that provided further evidence for intracellular uptake of oligonucleotides.

Following these studies, it was clear that oligonucleotides can be taken up into mammalian cells and can exert inhibitory effects on selected genes.

Over the past decade, substantial development in antisense technology and manufacturing led to the approval of the first antisense drug Fomivirsen (VitraveneTM), for the treatment of Acquired Immune Deficiency Syndrome (AIDS) related Cytomegalovirus (CMV) retinitis [6].

In the mean time, up to 50 new AS-ODNs have entered phase I/II, and in some cases, phase III trials (Table 1).

All these AS-ODNs have a chemical structure different from that of the relevant native ODNs; most of changes having been made in order to

enhance the ability of these molecules to permeate cell membrane and their resistance to nucleases as well.

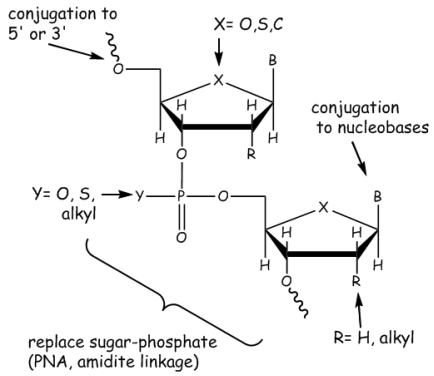


Fig.2

In general, three types of modifications of oligonucleotides (Fig.2) can be distinguished [7]:

- Modification of nucleobases.
- Modification of sugar moieties and phosphate backbones.
- Conjugation to side moieties.

Product	Chemistry	Disease	Administration	Phase
Vitravene™	PS	CMV Retinitis	Intavitreal	On market
Affintak™	PS	Cancer	Parenteral	III
Alicaforsen™	PS	Crohn's Disease	Parenteral	III
ISIS 2302	PS	Topical Psoriasis	Topical	II
ISIS 2302	PS	Ulcerative Colitus	Enema	II
ISIS 2503	PS	Cancer pancreatic	Parenteral	II
ISIS 14803	PS-DNA	Hepatitis C	Parenteral	II
ISIS 104838	Chimeric-PS (2nd generation)	Rheumatoids Arthritis	Parenteral/Oral	II
ISIS 104838	Chimeric-PS (2nd generation)	Psoriasis	Topical	II
OGX-011	N/A	Cancer	Parenteral	I
Genasense™	PS	Cancer	Intravenous	II/III
E2F Decoy	N/A	Artherosclesis	Ex-vivo	II/III
Resten-NG	MF (3rd generation)	Restenosis	Intravenous	III
Heptazyme™	RNA/DNA	Hepatitis C	Intravenous	II
1018-ISS	PS	Hepatitis B	Intravenous	II/III
1018-ISS	PS	Asthma	Intravenous	I/II

***Table 1**: Representatives of AS-ODNs Approved or in Clinical Trials.*

- ***Modifications of nucleobases***

Nucleobases of nucleic acids are the recognition sites for the Watson-Crick base pairing rules and so any ODN modification must maintain these specific hydrogen-bonding interactions. Therefore, the aims of nucleobase modifications are rather limited. These modifications should not greatly affect the sugar conformation of heteroduplex, would provide little nuclease resistance, but should generally support an RNase H cleavage mechanism [8-10]. In conclusion, the modification of nucleobases might not be a fruitful approach [8, 11-13].

- ***Modifications of sugar moieties and phosphate backbones***

Chemical modifications of ODNs have been mainly focused on the phosphodiester backbone and the sugar moiety and, based on variations of these modifications, AS-ODNs are classified into three generations [14].

1) "First Generation" Antisense Oligonucleotides

They are oligodeoxynucleotides with modified phosphate group. Phosphorothioate oligodeoxynucleotides (PS-ODNs) are the major representatives of first generation DNA analogs that are the best known and most widely used AS-ODNs to date. In this class of AS-ODNs, one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by sulfur. The major disadvantage of PS-ODNs, however, is their binding to certain proteins, particularly those that interact with polyanions such as heparin-binding proteins [15-22].

2) *"Second Generation" Antisense Oligonucleotides*

The problems associated with PS-ODNs are to some degree solved in the "second generation" ODNs, as they contain nucleotides with alkyl

modifications at the 2' position of the ribose. 2'-*O*-Methyl and 2'-*O*-methoxyethyl RNAs are the most important members of this class [12, 13].
AS-ODNs made of these building blocks are less toxic than PS-ODNs and have a slightly enhanced affinity towards their complementary RNAs [20,23]. These desirable properties are, however, counterbalanced by the fact that 2'- O-alkyl RNA cannot induce RNase H cleavage of the target RNA [24].

For most AS-ODNs approaches, however, the cleavage of the target RNA by RNase H is desired in order to increase antisense potency. Therefore, 'chimeric strategy' has been developed.
Chimeric ODNs analogs bring together the beneficial properties of the two types of chemistry mentioned so far. In general, they have two segments: one that contains an ODN derivative capable of activating RNase H and another that provides increased binding affinity and fewer side effects.
Accordingly, chimeric ODNs consist of a central stretch of DNA or PS-DNA monomers and modified nucleotides such as 2'-*O*-methyl RNA at each end.

3) "Third Generation" Antisense Oligonucleotides

Another group of DNA and RNA analogs have been more recently developed in which phosphate linkages or riboses units have been modified; in some cases, nucleotides with a completely different chemical moiety substituting the furanose ring have been also synthesized [12]. These novel nucleotides have been subsequently grouped under the term '*third generation AS-ODNs*'. Some examples of modified nucleotides belonging to these vast class are discussed hereinafter.

Peptide Nucleic Acids (PNAs)

In PNAs, the sugar-phosphate backbone is completely replaced with a peptide-based backbone. These DNA analogs have favourable hybridization

properties and high biological stability, but do not elicit target RNA cleavage by Rnase [25-27].

N(3')-O(5') Phosphoroamidates (NPs)

NPs are another example of a modified phosphate backbone, in which the 3'-hydroxyl group of the 2'-deoxyribose ring is replaced by a 3'-amino group. NPs exhibit both a high affinity towards a complementary RNA strand and nuclease resistance [28]. Because phosphoroamidates do not induce RNase H cleavage of the target RNA, they might prove useful for specific applications, where RNA integrity needs to be maintained, like modulation of splicing.

Locked Nucleic Acids (LNAs)

Locked nucleic acid are a ribonucleotide containing a methylene bridge that connects the 2'-oxygen of the ribose with the 4'-carbon [29,30]. Introduction of LNA into an ODN induces a conformational change of the ODN-RNA duplex and prevents RNase H cleavage of the target RNA [31]. Full LNA-ODNs were successfully used *in vivo* to block the translation of the large subunit of RNA polymerase II. These ODNs inhibited tumour growth in a xenograft model with an effective concentration that was five times lower than was found previously for the corresponding PS-DNA.

Morpholino Oligonucleotides (MF-ODNs)

MF-ODNs are nonionic DNA analogs, in which the ribose is replaced by a morpholino moiety and phosphoroamidate bonds. MF-ODNs are promising antisense molecules that possess favourable hybridization, nuclease stability, and toxicity profiles. They do not prevent gene expression by activation of RNase H, rather, they function by translational arrest [32-35].

Conjugated Oligonucleotides

In addition to the previously mentioned modifications, various molecular residues have been attached (conjugated) to ODNs in order to improve their pharmacokinetic properties. Some other potential goals include increasing the solubility, enhancing the lipophilicity, and the ability to bind synthetic cleaver intercalaters [36-40]. Although conjugation of various functionalities (e.g. cholesterol, folic acid, long alkyl chain, bleomycins, etc.) to ODNs has been reported to achieve these objectives, the data supporting some of the claims are limited and generalizations are not possible based on the data presently available [41].

The main objective of conjugating a lipid moiety to an oligonucleotide is to increase the hydrophobic character of the latter and its lipid-solubility, then it would pass across the highly lipophilic cell membrane and into the cytosol. Yet, depending on the lipid's nature these conjugates may have also some other new biological properties.

Cholesterol is a lipid that has been extensively used in the production of these conjugates. The cholesterol tag can be added at the 3' or 5' end of an ODN usually using a C4- to C8-linker or a polyethylene glycol linker. Some cholesterol-ODN conjugates in addition to better transduction have shown improved nuclease resistance and anti-viral activity. Conjugation of antisense phosphorothioate-ODNs with cholesterol has yielded compounds with a higher antisense activity than their unconjugated counterparts but this effect resulted not dependent on the oligonucleotide sequence in some cases [42-50].

In addition to cholesterol and other sterols, additional lipid moieties such as alkyl chains, phospholipid-like units, fatty acids and lipid substituted crown ethers have been also used [51-64]. Like cholesterol-ODN conjugates, ODNs conjugated to alkyl chains larger than 12 carbons would bind to the

serum lipoproteins LDL and HDL to form complexes that are taken up by the proteins' cell receptors facilitating their entry.

Also lipophilic dendrimers have been conjugated to either the 5'- or 3'-ends of antisense ODNs to increase their cellular uptake [65, 66]. Increasing in the size of the conjugate's dendrimer results in a significant decrease in binding activity as shown by a marked drop in melting temperature.

Despite the significant improvement in the targeting efficiency *in vitro* of conjugated ODNs (also called pendant ODNs), only a limited number of *in vivo* experiments has been performed. Nevertheless, the value of conjugation chemistry has been clearly demonstrated both *in vitro* and *in vivo.*

1.3 Open questions on the conjugation of biocompatible units to oligonucleotides

Current research in oligonucleotides conjugation is especially aimed at conjugating AS-ODNs to biocompatible lipophilic molecular moieties derived from natural compounds; this would provide, at the same time the maximum benefit expected from the antisense activity and the lowest toxicity effects. Indeed, considering that the transport of these molecules into cells has been shown to be facilitate by linking them to lipophilic carriers, antisense oligonucleotides bearing a terminal biodegradable lipophilic group attached through a phosphoester bond could be useful pro-drugs with improved cellular uptake.

At this regard, the phosphatidyl group appears to be one of the most suitable lipophilic groups, on account of its widespread occurrence in the molecular structure of many lipid constituents of cell membranes. But, this group has never been covalently linked to oligonucleotides .

In fact, the lability of the carboester bond in the phosphatidyl group, under the strongly basic conditions routinely used in standard phosphoramidite chemistry procedures [67] (see Fig. 3) to remove all protecting groups and to detach oligos from the solid support, has been the major obstacle to overcome until now.

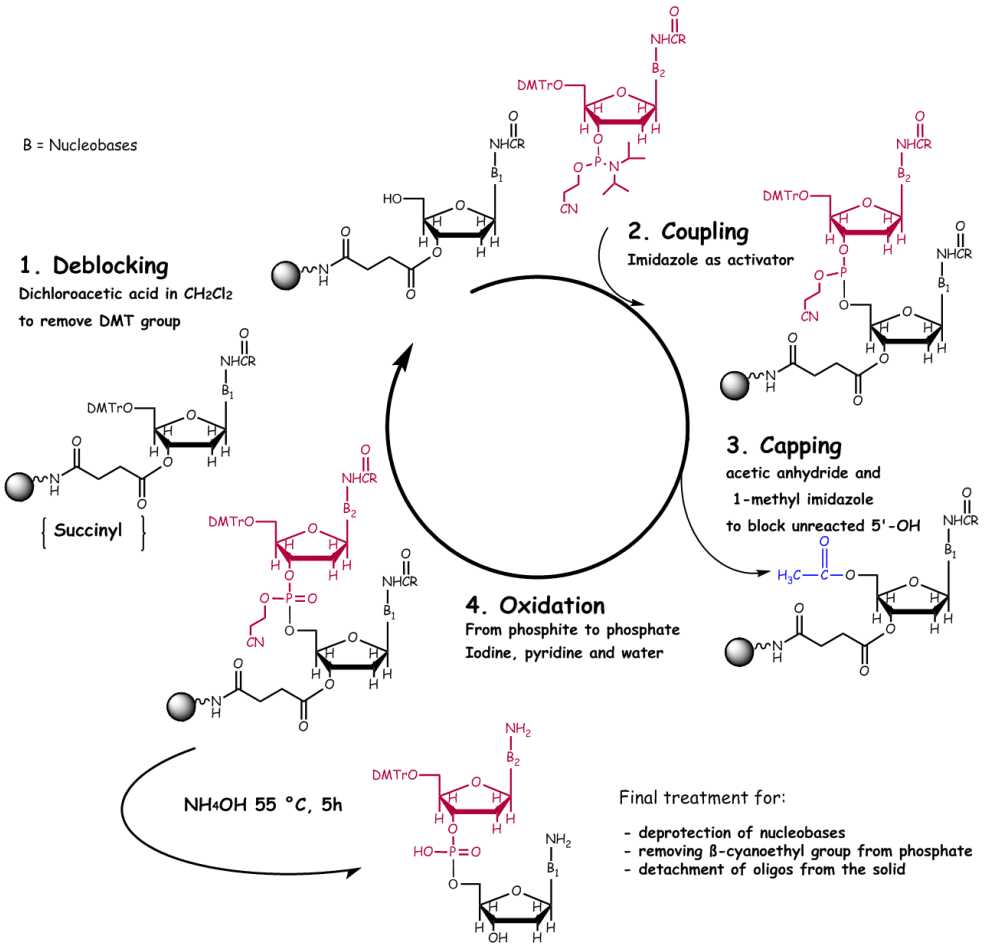


Fig.3 Current protocol for solid phase synthesis of oligonucleotides using phosphoramidite chemistry.

2. AIM OF THE WORK

New chemical procedures have been recently reported regarding the synthesis of oligonucleotides. In particular, some of these methodologies do not require the final treatment with ammonium hydroxide (Fig. 3) used in current solid-phase synthesis of oligonucleotides. So, the application of these new procedures would be in principle useful for the preparation of oligonucleotides conjugated to base-labile moieties such as the phosphatidyl one.

Indeed, by exploiting these new synthetic opportunities, it was possible, during my graduate thesis, to obtain, as a first approach, a 5'-*O*-phosphatidyl-tetranucleotide.

Of course, the chemical and bio-pharmacological properties of this new class of compounds have not been studied yet. So, if we want to prepare phosphatidyloligonucleotides to be used as antisense, their actual antisense activity should be assessed.

To evaluate the influence of the lipophilic moiety in improving membrane permeability and, consequently, its effect on the antisense activity, it is necessary that a fair number of phosphatidyl-ODNs are available, differing from each other either in their phosphatidyl moiety (consisting of different fatty acids) or in their sequence.

To carry out these kind of studies the coding sequence against which we want exert the antisense activity should be selected. So, to this purpose, we have chosen to prepare some phosphatidyl-tetradecanucleotides all having the *antisense* sequence against a region of the Vascular Endothelial Growth

Factor (VEGF) gene, but differing from each other in their phosphatidyl moiety.

These, whose structures are shown in Fig. 4, are the following:

- 5'-*O*-[1,2-*O*-dimyristoyl-*sn*-glycero-3-phosphoryl]-d(TGGCTTGA-AGATGT-3')
- 5'-*O*-[1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphoryl]-d(TGGCTTGA-AGATGT-3')
- 5'-*O*-[1,2-*O*-distearoyl-*sn*-glycero-3-phosphoryl]-d(TGGCTTGAA-GATGT-3')

The acyl chains in the phosphatidyl moiety have been selected within those which mostly occur in membrane lipids, choosing them so that we could also study possible relationships between the total number of carbon atom in the acyl chain and chemical and bio-pharmacological properties of these compounds.

As regards the sequence of the ODN moiety, this was chosen targeted to a coding sequence of the VEGF gene. This gene is widely studied because the protein it codifies plays a key role in angiogenesis; then, the relevant mRNA sequence is well known and extensively used in many studies. Several of these studies [68-69] have shown that the better sequence toward which to target antisense activity is contained in the 261-281 region. For this reason the oligonucleotide sequence of the antisense moiety of our phosphatidyl-ODNs was chosen to be complementary to a specific part of this region.

Aim of this work was to synthesize for the first time these new phosphatidyl *antisense* molecules, to analyze their spectroscopic properties, to evaluate their ability to give duplexes with the target sequence, and to test the desired biological activity**.**

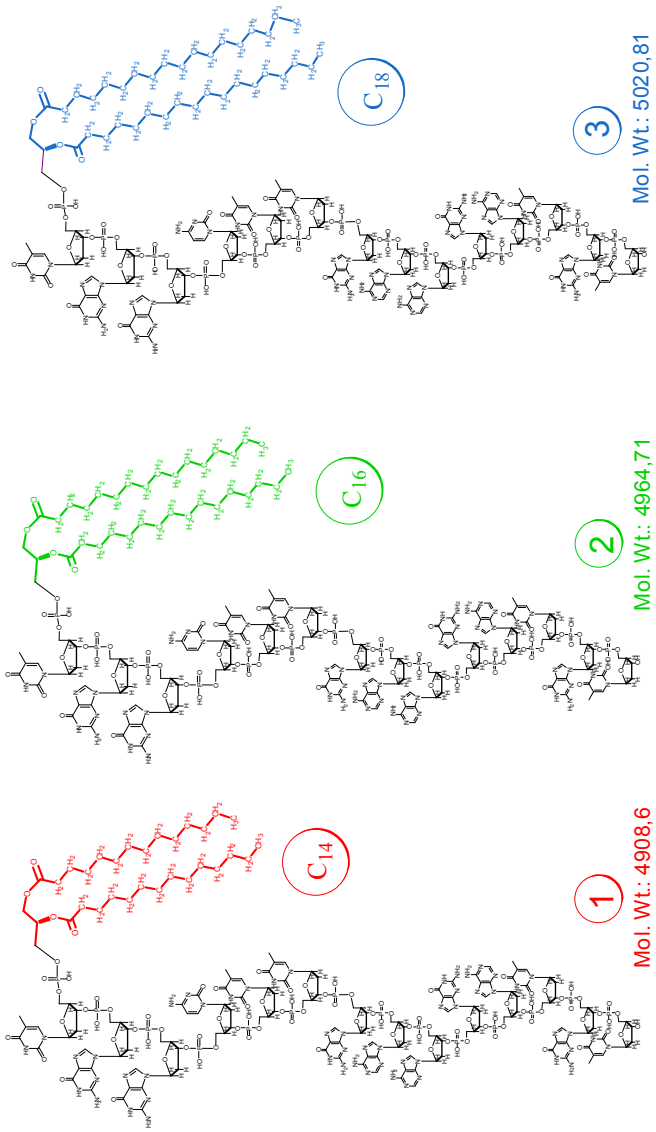


Fig. 4

❶*5'-O-[1,2-O-**dimyristoyl**-sn-glycero-3-phosphoryl]-d(TGGCTTGAAGATGT)-3'*

❷*5'-O-[1,2-O-**dipalmitoyl**-sn-glycero-3-phosphoryl]-d(TGGCTTGAAGATGT)-3'*

❸*5'-O-[1,2-O-**distearoyl**-sn-glycero-3-phosphoryl]-d(TGGCTTGAAGATGT)-3'*

3. METHODS AND RESULTS

As a previously mentioned, to achieve the synthesis of the three phosphatidyloligonucleotides, it was necessary to avoid the use of ammonium hydroxide in the final step of the current solid phase synthesis of oligonucleotides. (Fig 3)

However, this step allows us to carry out in a single operation:

a) deprotection of nucleobases,

b) removing β-cyanoethyl group from phosphate bridges

c) detachment of oligos from the solid support.

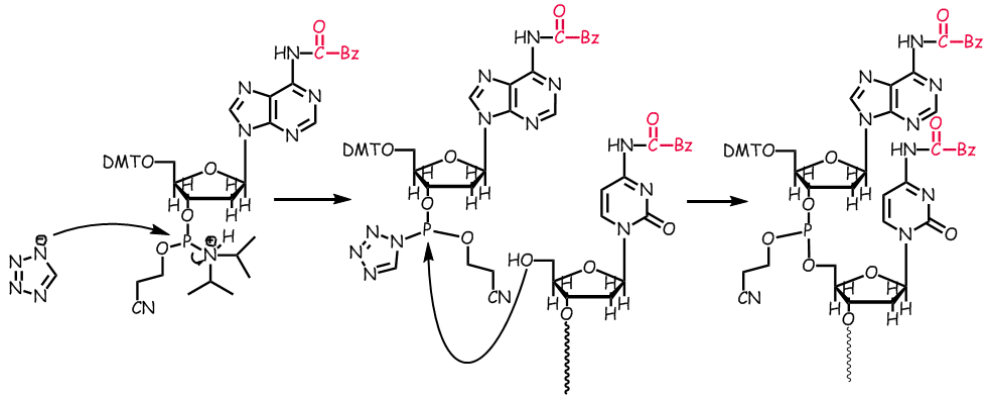
To meet this limitation, it was necessary to modify the classical oligonucleotide synthetic pathway taking into account any recent developments in the oligonucleotide synthesis

New opportunities in oligonucleotide synthesis

The use of unprotected nucleobases

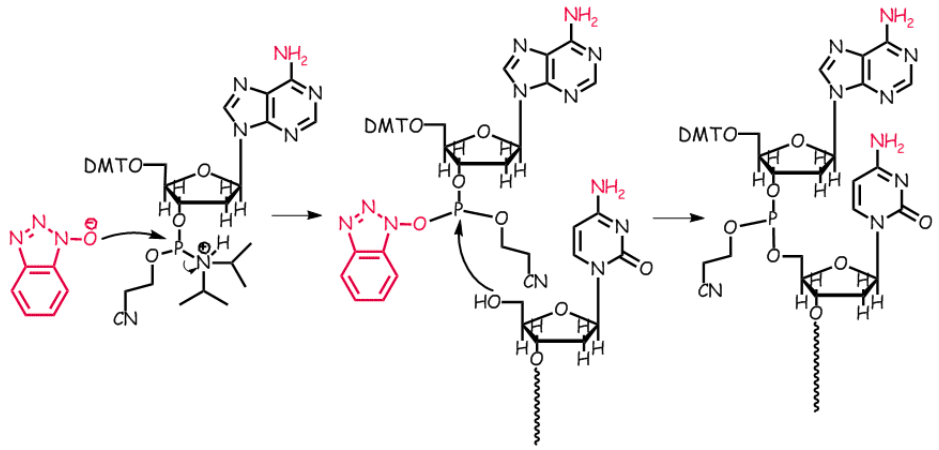
For more than two decades, various methods and reactions for the chemical synthesis of oligodeoxynucleotides have been developed [70, 71].

The key reaction in the classical solid phase synthesis of oligonucleotides is the coupling of 3'-*O*- phosphoramidite derivatives of nucleosides (appropriately protected at the nucleophilic groups of nucleobases) to the 5'-hydroxyl group of another anchored nucleoside residue. For this reaction tetrazole is used as an activator (Scheme 1).



Scheme 1.

It has been recently reported [72] that it is possible to carry out an efficient DNA synthesis without any protection of nucleobases, even in the phosphoramidite chemistry, by using 1-hydroxy-benzotriazole (HOBt) as a promoter for the activation of deoxynucleoside 3'-*O*-phosphoramidite building blocks. This procedure, called "activated phosphite method", is based on the formation of bulky phosphite-type intermediates which can be generated from phosphoramidite derivatives of deoxynucleotides by an alcohol-type activator and would not react with free amino groups of nucleobases (Scheme 2) .



Scheme 2

Such protocols have been used in practice for solid phase synthesis of alkali-labile modified DNAs such as oligonucleotides containing DNA lesions [73]. In our case, the possibility to carry out the oligonucleotide elongation using 3'-*O*- phosphoramidite derivatives of nucleosides unprotected at nucleobases would allow us to avoid the ammonia treatment that the use of protected nucleobases would have required.

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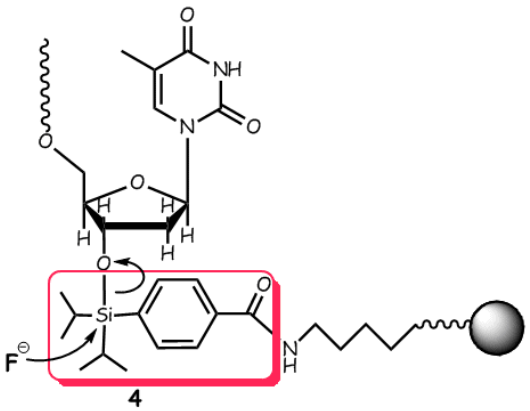
Anchoring by "silyl linkers"

Although the use of the "*activated phosphite method*" would allow us to avoid the ammonia treatment for removal of the protecting groups of nucleobases, this treatment should have been still necessary for the detachment of the oligonucleotide from the solid support.

In fact, in the current automated solid-phase synthesis of DNA, oligonucleotides are bound to solid support through an ester bond with a succinyl linker; the removing from the support being accomplished by hydrolysis with aqueous ammonia. Therefore, as previously stated, DNA oligomers having base-labile functional derivatives cannot be synthesized using this standard linker. Therefore, we decided to use one of the silyl-linkers recently reported in the literature and, in particular, we looked at the linker [74] reported in scheme 3.



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Scheme 3

The use of this linker, would allow us to detach oligonucleotides from the solid support following mild treatment with tetrabutylammonium fluoride (TBAF).

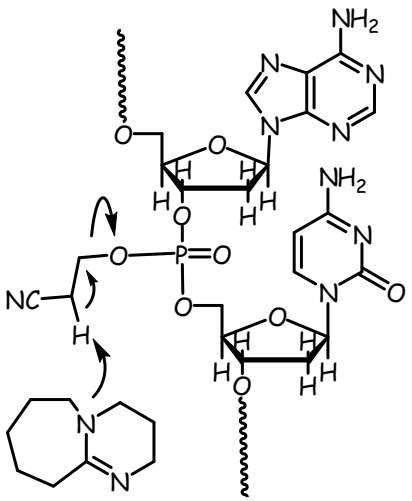
So, at least with respect the two operations for the deprotection of nucleobases and the removal of oligonucleotide from the support, it seemed possible , in principle,to avoid the final ammonia treatment by combined use of the "activated phosphite method" and the just said silyl-type linker

Removing of cyanoethyl groups by the use of strong bases in organic solvents.

At this point the last unresolved problem was the one on how to remove cyanoethyl groups from phosphate bridges without using ammonia.

In this regard, we considered to apply a useful innovation in oligonucleotide synthesis [75] whereby the final deprotection of phosphate group is performed in anhydrous solvent with 1,8-diazabicycloundec-7-ene (DBU) (Scheme 4).

In summary , the opportunities so far mentioned, if properly developed within a synthetic plan, would allow us to carry out the solid phase synthesis of modified oligonucleotides bearing alkali-labile moieties (like as phosphatidyl-ODNs), overcoming the drawbacks in the current phosphoramidite procedure.



Scheme 4

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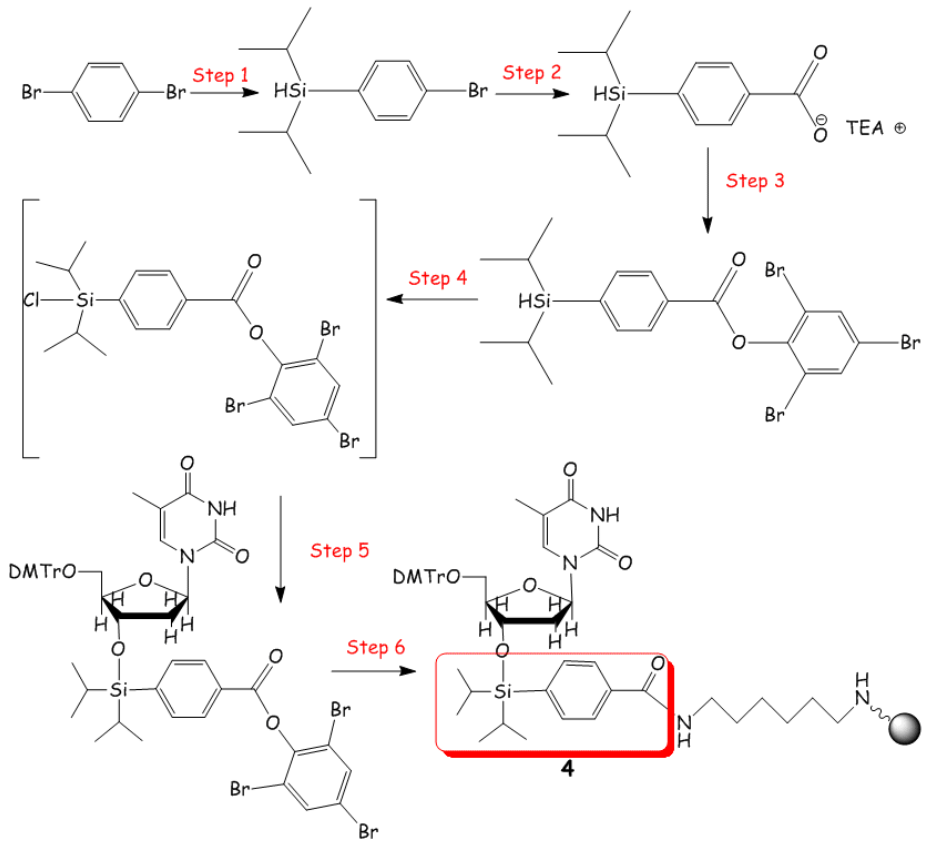
3.1 Synthesis of the linker and solid support functionalization

The various silyl-type linkers proposed in recent literature [76-80], are usually anchored to either siliceous (controlled pore glass, CPG) or organopolymeric (high crosslinked polystyrene, HCP) solid supports. Simply for reason of expediency, we chose to use the silyl linker 4 (Scheme 5), whose synthesis and loading to aminopropyl-CPG (AP-CPG) had been previously described by a Japanese research group [74].



Step 1: But-Li 1 eq, - 78°C, THF, 5 min; then Cl-DIS 1 eq, 15 min. Step 2: But-Li 1 eq,- 78°C, THF, 5 min; then CO_2 1 eq, 15 min. Step 3: TBP 2 eq, BOPCl 4 eq, Pyridine, rt, 30 min. Step 4: DCDMI 2 eq, CH_2Cl_2, rt, 1 h. Step5: DMTrT 2 eq, Imidazole 10 eq, CH_2Cl_2, rt, 1 h. Step 6 : LCAA-CPG, CH_2Cl_2, 36 h, rt.

Scheme 5- Synthesis of silyl linker **4** and functionalization of the solid support.

Scheme 5 shows the synthetic route reported in the literature for obtaining the silyl-type linker 4 and anchoring the first nucleoside to solid support. According to this route, triethylammonium 4-(diisopropylsilyl)-benzoate is synthesized from 1,4-dibromobenzene by using stepwise halogen-metal exchange reaction (steps 1 and 2). The benzoate is then converted into the relevant tribromophenyl ester (step 3) by condensation with tribromophenol in the presence of bis(2-oxo-3-oxazolidinyl) phosphinyl chloride (BOPCl). The tribromophenyl ester is converted by treatment with 1,3-dichloro-5,5-dimethylhydantoine into the corresponding chlorosilanedyl derivative (step 4) which is allowed to react with 5'-*O*-DMTr-thymidine in the presence of imidazole to give the anchor nucleoside (step 5).

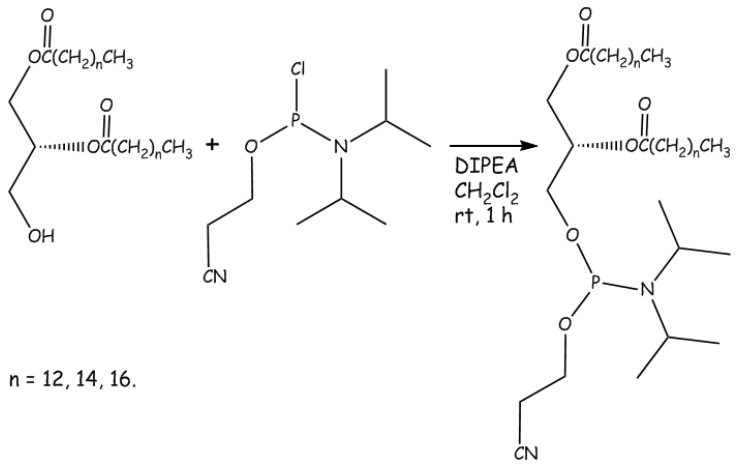
The final amidation of the activated benzoate group by amino groups of long chain aminopropyl controlled pore glass (AP-CPG) furnish the desired support for solid phase elongation of ODNs (step 6). This step allow the Authors to obtain the 5'-DMT-T loaded AP-CPG with a loading capacity of 20.8μmol/g.

Aiming to improve the yield of this crucial step of the synthesis, we made some changes to the reported protocol. In particular, to favour the nucleophilic acyl substitution reaction we used as a component of the suspending solution the highly polar aprotic solvent dimethylformamide (DMF), and used also, in the presence of a tertiary amine, long chain amino alkyl-CPG (LCAA-CPG) instead AP-CPG. Indeed, suspending LCAA-CPG in a mixture of 50:50 (v/v) CH_2Cl_2/DMF containing 1 equivalent of triethylamine per equivalent of active ester, it was possible to obtain, after 30 hours, 5'-DMT-T-(LCCA-CPG) whose loading capacity proved to be 30.6 μmol/g.

With this support, the elongated ODNs were detached from the solid phase following a treatment with tetrabutylammonium fluoride (TBAF) in neutral conditions.

3.2 Synthesis of 1,2 diacyl-sn-glycero-3-(2-cyanoethyl)-N,N--diisopropylphosphoramidites

To introduce the desired phosphatidyl group at the 5'-hydroxyl of the elongated olgonucleotide, it was necessary that the relevant diacylglycerophosporoamidite would be available to be used in a final coupling step of the synthesis. To this purpose we prepared each of the required 1,2-*O*-diacyl-*sn*-glycero-3-*O*-phosphoramidites, which are not commercially available, by reaction of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite with the pertinent diacylglycerol (scheme 6). 1,2-Ditetra, 1,2-dihexa- and 1,2-dioctadecanoylglycerol were used for this purpose having all the *S* configuration at the stereogenic carbon, so that the resulting phosphoramidites would have had the same stereochemistry at the chiral center as in naturally occurring phospholipids (*R*). After chromatography purification, each diacylglycerophosphoramidite was characterized on the basis of its spectroscopic (^{1}H-^{13}C-NMR) properties.

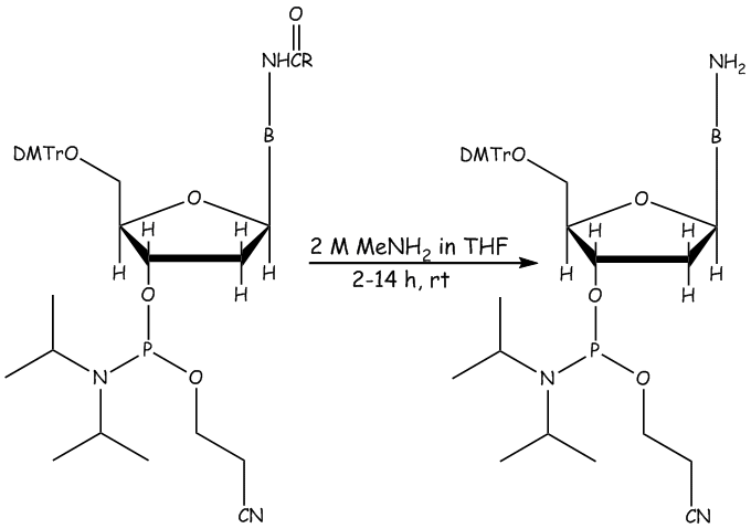


Scheme 6

3.3 Preparation of unprotected Nucleoside phosphoramidites

Wanting to carry out the synthesis of phosphatidyl-oligonucleotides by applying the *phosphite activated method*, it was necessary to have N-unprotected deoxynucleoside-3'-*O*-phosphoramidites. A convenient method for the synthesis of these building blocks has been reported [81] that makes the commercially available *N*-protected deoxynucleoside-3'-*O*-phosphoramidites reacting with methylamine (Scheme 7).

Following this procedure, we prepared N-unprotected guanosine, cytidine and adenosine phosphoramidites, which were purified and spectroscopically characterized before use (see experimental section).



B = Guanine, Cytosine and Adenine

Scheme 7.

3.4 Synthesis of 5'-O-(1,2-O-diacyl-sn-glycero-3-phosphoryl)-d(TGGCTTGAAGATGT) **(1-3)**

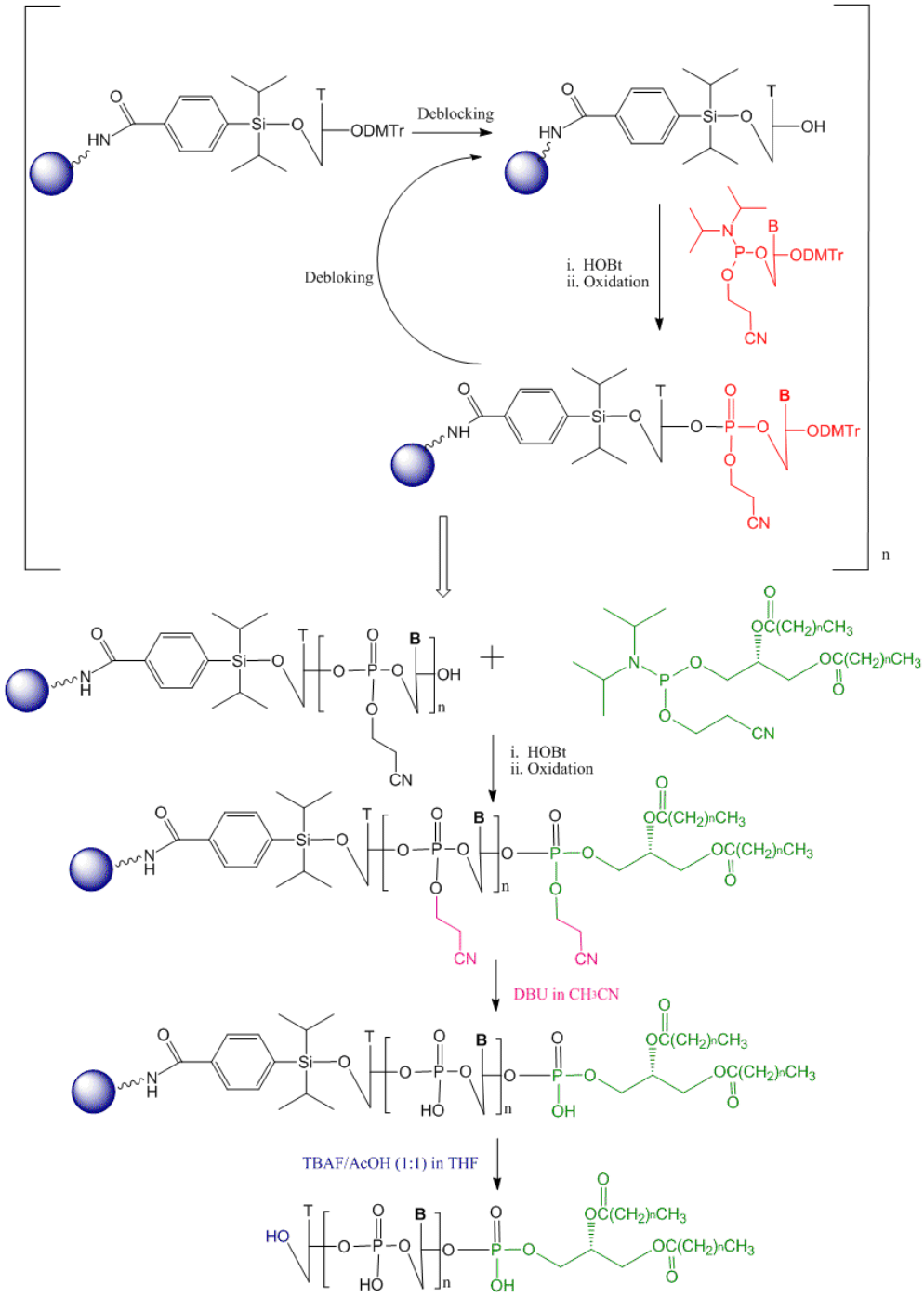
The title synthesis was carried out on a common DNA synthesizer equipped with a column filled with the previously prepared 5'-DMT-T-(LCAA-CPG). The utilized protocol was the same as commonly used in solid phase synthesis by standard phosphoramidite chemistry except that HOBt was used as activator and *N*-unprotected nucleoside 3'-*O*-phosphoramidites were used as building blocks.

Of course, no capping step was performed throughout the synthesis.

After the oligonucleotide elongation, a final coupling was performed with the appropriate diacylglycerophoshoroamidite. As in this step we encountered a problem in using acetonitrile to dissolve the relevant diacylglycerophosphoramidite, anhydrous dichloromethane was used as the solvent for this purpose. Furthermore, to ensure the maximum efficiency of this last coupling step, this was repeated twice.

As above mentioned, to preserve the ester bond in the conjugated phosphatidyl moiety, removal of phosphate protecting group (β-cyanoethyl) was accomplished by passing through the reaction column a solution of 1,8-diazabicycloundec-7ene (DBU) in anhydrous acetonitrile. Detachment of phosphatidyloligonucleotides from the solid support was then carried out by flushing the column with a solution of a mixture of tetrabultyl ammonium fluoride / acetic acid (TBAF-AcOH) in anhydrous tetrahydrofuran (THF).

The overall synthetic sequence followed to achieve the solid phase synthesis of each of phosphatidyloligodeoxynucleotides **1-3 (**Fig. 4) is depicted in Scheme 8.



Scheme 8: Synthetic route for preparing 1,2-diacyl-*sn*-glycero-phosphatidyl-oligonucleotides.

The high excess of TBAF necessary to achieve complete detachment of the oligos from the reaction column gave rise to serious problems in the subsequent purification steps. After several trials, removing of TBAF excess was accomplished by membrane ultrafiltration.

In order to carry out HPLC further purification of the three newly synthesized phosphatidyl-oligonucleotides, some preliminary chromatographic runs were performed on RP-18 analitical column which did not give satisfactory results. After many attempts, optimal chromatographic conditions were found using a C-8 reverse phase column eluted with a gradient of 2-propanol in triethylammonium acetate buffer. Fig. 5 reports the overlapped chromatograms obtained for each of the three phosphatidyltetradecanucleotides (**1-3**).

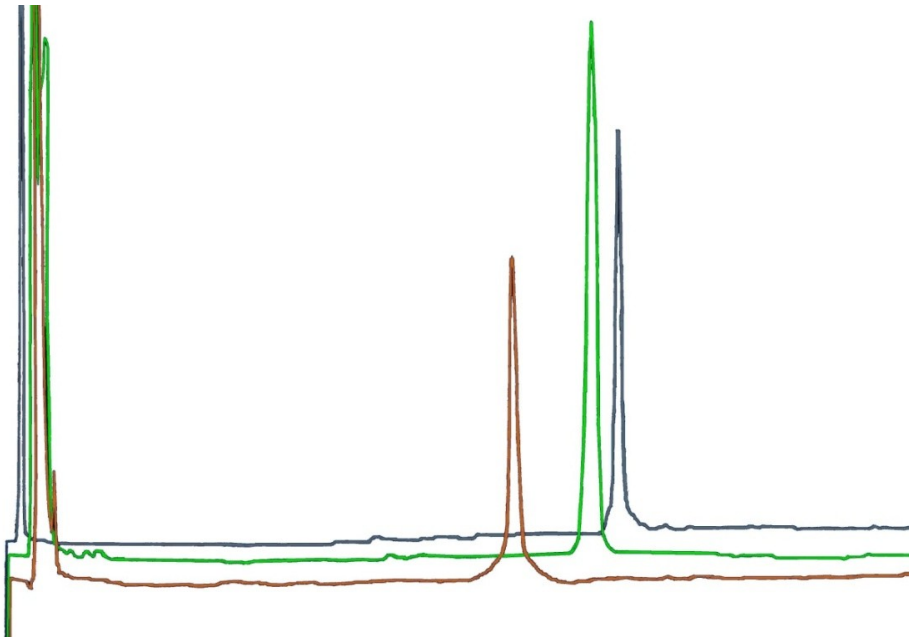


Fig. 5: Overlapped chromatograms of ■ compound **1**, ■ compound **2** and ■ compound **3**

Retention times of the three analytes, which are strictly related to their lipophilicity, increased with the number of carbon atoms in the acyl chains of

the phosphatidyl moiety. More, it was found a linear correlation between the percentage of 2-propanol needed for elution and the total number of carbon atoms in the hydrophobic tails of the phosphatidyl group.

Final purification of the three phosphatidyloligonucleotides, was then carried out by transferring to a semi-preparative scale the experimental conditions optimized for the analytical chromatographic separation. Unfortunately, it was not possible to apply the standard procedures for the scaling-up of the analytical parameters. In fact, the increased amounts of the phosphatidyl-ODNs which had to be injected into the semi-preparative chromatographic system, due to their strong tendency to self-aggregate, gave rise to a serious broadening of the chromatographic peaks. On the other hand it is known [82] that lipo-ODNs tend not only to aggregate into micelles but also to exhibit a strong interaction with stationary reversed phase, therefore, HPLC purification of these compounds is difficult to achieve. Among other things, the conversion of the optimized analytical parameters into new parameters for the semi-preparative chromatography was also complicated because in the latter we replaced triethylammonium acetate in the eluent solution with ammonium acetate, in order to get the three phosphatidyl-ODNs coupled with counter-ions compatible with the subsequent spectroscopic analyses.

Anyway, after a number of trials, the best semi-preparative chromatographic conditions were found by setting the solvent flow up to the highest value allowed by the maximum pressure limit in the chromatographic apparatus and adequately lowering the concentration of the injected solution of lipo-ODN. In practice, it was necessary to use intermediate conditions between analytical and semi-preparative scale.

After the chromatographic purification, the compounds **1**-**3** were undertaken to lyophilization in order to remove all residual ammonium acetate and stored for further analyses.

3.5 Spectroscopic characterization of 5'-O-(1,2-O-diacyl-sn-glycero-3-phosphoryl)-d(TGGCTTGAAGATGT-3')(1-3)

The compounds **1-3**, after purification, were each analyzed for their MS, UV and NMR spectroscopic properties.

Mass Spectra

MALDI-TOF mass spectrometry performed with the use of 3-hydroxypicolinic acid as a matrix, is one of the most commonly used methods [83] to obtain mass spectra of oligonucleotides. So, the three lipo-ODNs were first undertaken to this kind of analysis, but, it was not in ay way possible to detect the expected signals in the relevant spectra. This is probably due to the presence of the conjugated lipophilic moiety in these modified oligonucleotides.

Taking into account that recent literature in this field [84,85] reported mass spectra of modified oligonucleotides obtained by using 2,4,6-trihydroxyacetophenone or 3,4-diaminobenzophenone as matrices, these have been even used but all attempts did not lead to any results.

Recent reports on the application of HPLC-ESI mass spectrometry to oligonucleotides [86] prompted us to try this opportunity too. By applying this method and suitably adapting it to our case, mass spectra of the compounds were finally obtained. The HPLC chromatographic separation before the ESI/MS analysis was performed on a RP-C18 column using a gradient of 2-propanol in ammonium acetate buffer.

The first compound to be analyzed was 5'-*O*-[1,2-*O*-distearoyl-*sn*-glycero-3-phosphoryl]-d(TGGCTTGAAGATGT-3') which gave the expected multi charge signals but all of them were accompanied by the relevant Na^+/K^+ adducts. Using methanol instead of 2-propanol as the

chromatographic eluent, the adducts no longer appeared in the spectrum but only signals from multi-charged ions were present (Fig. 6). HPLC-ESI/MS analysis of the remaining two lipo-ODNs, performed under these last experimental conditions, gave mass spectra showing signals from the expected multi-charged ions (see Experimental).

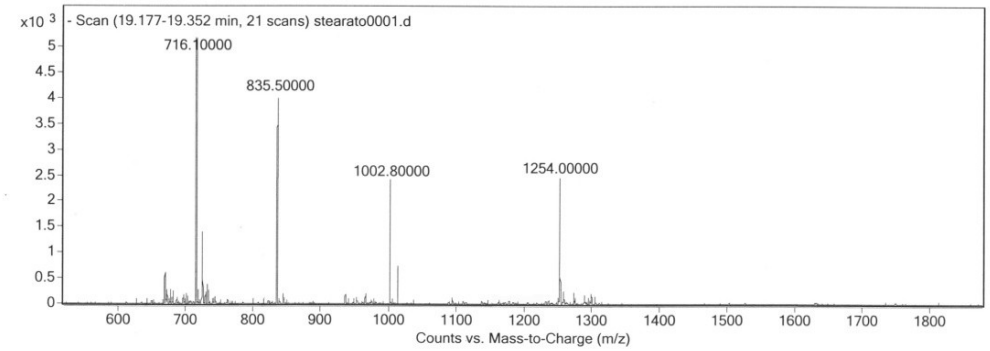


Fig. 6 – ESI-MS(-) of compound **3**.

UV Spectra

UV spectra of aqueous solutions of each compound (**1**-**3**) showed the classical absorption curve of ODNs (max at 260 nm) allowing in principle their quantification. These spectra showed in some cases in the region between 300 and 450 nm absorbance values higher than the expected ones, indicating possible scattering phenomena. These could have been associated to the formation of aggregates and/or supramolecular complexes, about which it would be interesting to carry out further investigation.

However, taking into consideration this fact, when UV spectra were run for quantification purposes, properly diluted solutions had to be prepared for the spectral analyses.

NMR Spectra

^{1}H-NMR spectra of **1**, **2** or **3**, when performed in D_2O at room temperature, looked flattened and poorly resolved; to some extent, this result was to be expected given the strong tendency of these compounds to self-aggregate. In Fig. 7 the ^{1}H-NMR spectrum of **1** is reported to give an example.

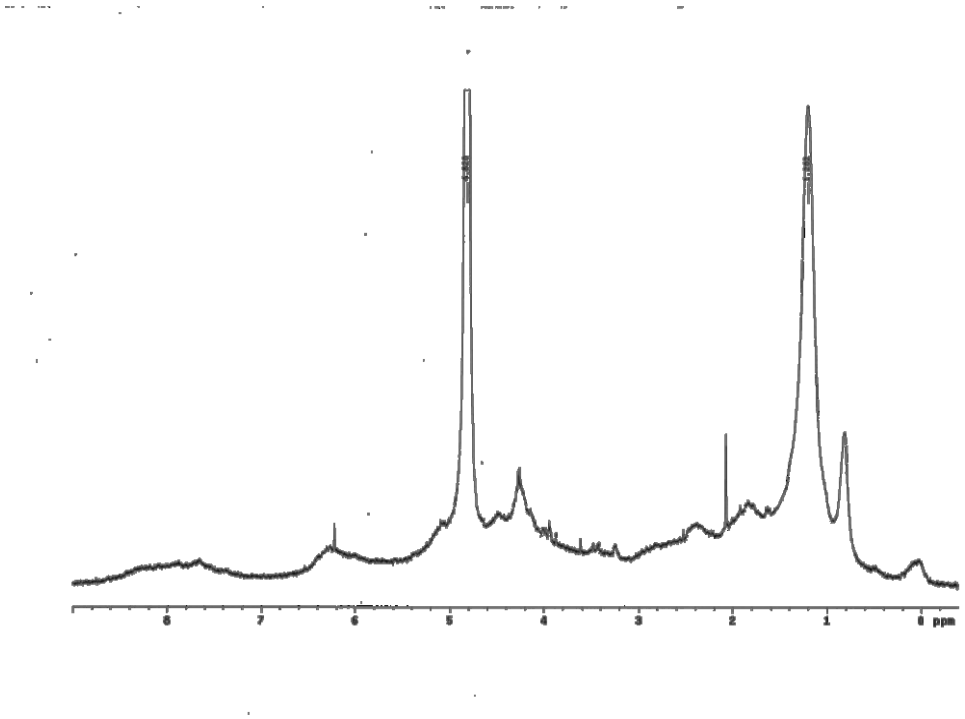


Fig. 7. ^{1}H-NMR of compound **1** run at 25°C.

When ^{1}H-NMR experiments were carried out at 60 °C all peaks became sharper and spectra compatible with the expected molecular structures were obtained, which were also quite similar to each other. Fig. 8 shows again the ^{1}H-NMR spectrum of **1** obtained in the latter experimental conditions.

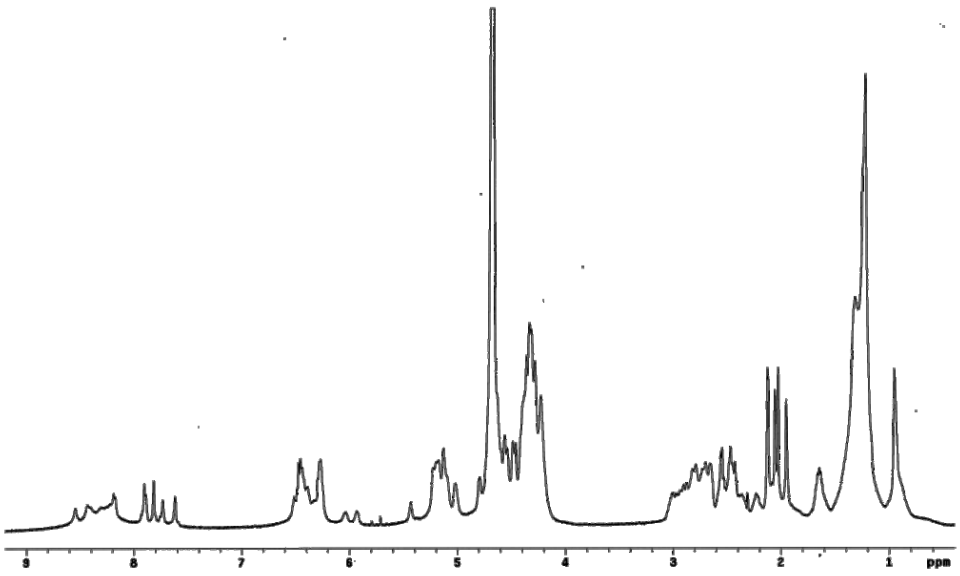


Fig.8. ^{1}H-NMR of compound **1** run at 60°C.

^{1}H-NMR spectra of compounds **1**-**3**, although sharpened by the increase of temperature, were however quite complex ones; so, unequivocal assignments of ^{1}H resonances had to be supported by ^{1}H-^{1}H COSY experiments which were performed, these too, at 60 °C (Fig. 9). Since ^{1}H-NMR spectra from **1**, **2** and **3** were quite similar to each other, we'll discuss in detail only the spectrum of compound **1**, taken just for example. The overall ^{1}H-NMR data for compounds **1**-**3** are reported under experimental.

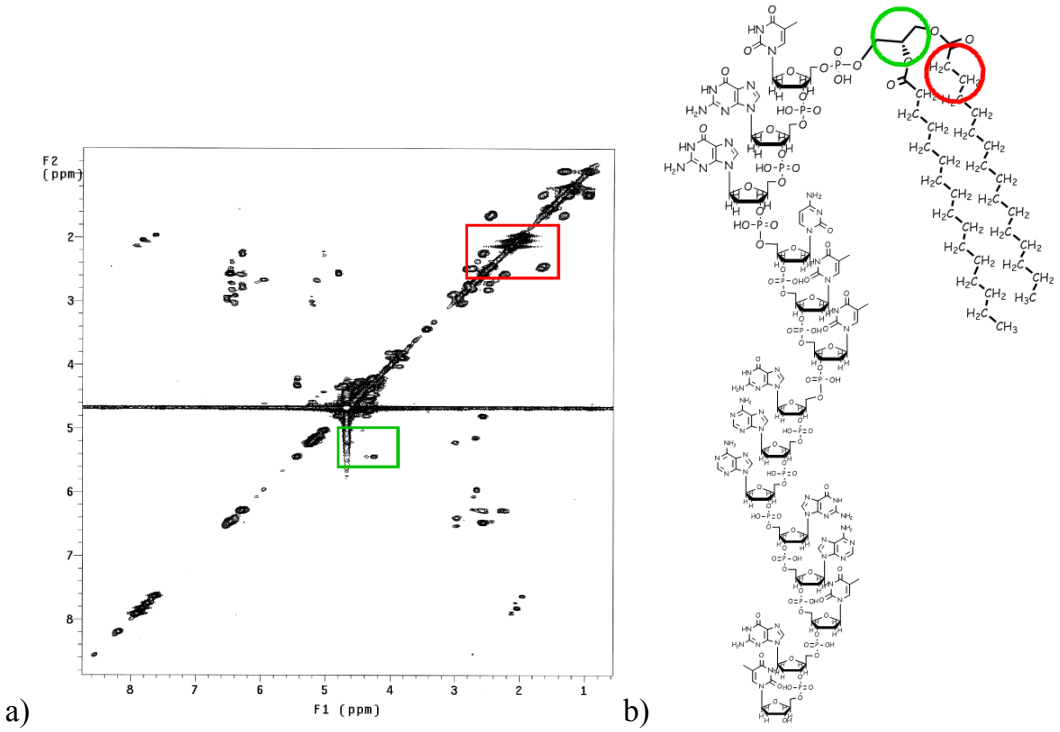


Fig. 9: a) ^{1}H-^{1}H COSY spectrum of compound **1**; b) molecular structure of compound **1**. Areas with color edge in the graph are used to highlight the regions of the spectrum where diagnostically important cross peaks fail.

In the spectrum of **1** (δ_{HOD} 4.67 ppm) methyls of myristoyl chains appeared as a broad triplet at δ 0.95, accompanied by two broad signals centered at δ 1.22 and 1.31 for all γ- to μ-CH_2 of the same alkyl chains, and a multiplet for the two β-CH_2 at δ 1.65. The two α-CH_2 resonances fell within partially overlapped multiplets (δ 2.17÷3.06) attributable to all H2' and H2" of the sugar rings; on the basis of the α-CH_2/β-CH_2 correlation peak showed in the COSY spectrum it was possible to assign the resonance of α–CH_2 at δ 2.47 ppm (Fig. 10 a).

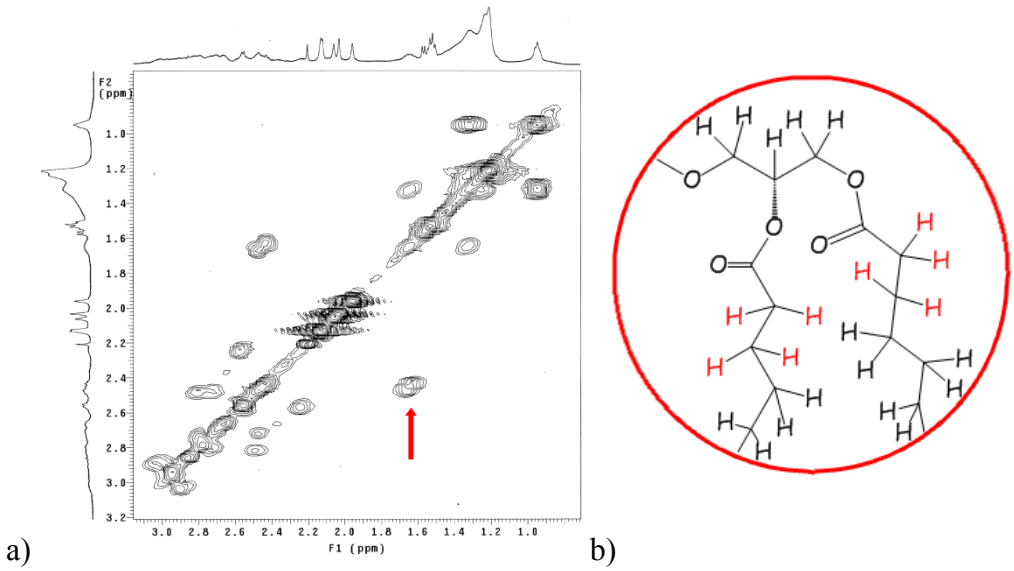
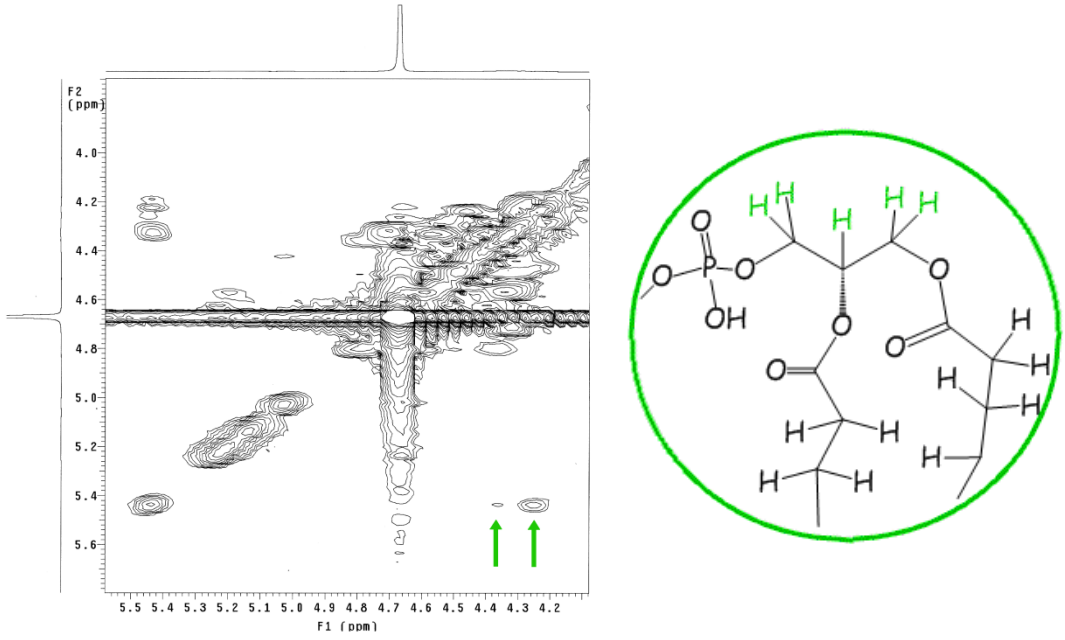


Fig. 10. A enlarged section of ^{1}H-^{1}H NMR COSY spectrum of **1**, showing a cross-correlation peak (a) of the α-CH_2/β-CH_2 in the acyl chain (b).

Methyls of the thymine nucleobases T12, T14 and T1 gave singlets at δ 1.95, 2.03 and 2.06 respectively, while methyls of T5 and T6 gave two partially overlapped singlets at δ 2.12 and 2.13. H6 of tymines appeared as singlets at δ 7.62, 7.73, 7.81, and two partially overlapped ones at 7.89 and 7.90, for T12, T1, T14, T6 and T5 respectively. These assignments were unequivocally done on the basis of the thymine H6/CH3 cross-peaks clearly observable in the COSY spectrum .

H2 and H8 of adenines, together with H8 of guanines and H6 of cytosine, gave multiplets at δ 8.10÷8.60, while the signal from the H5 of cytosine was superimposed on multiplets at δ 6.19÷6.59 from most of H1'of the deoxyribose rings. Signals relating to the latter protons appeared, in fact, as multiplets at δ 5,93 (1 H), 6.04 (1 H) and 6.19÷6.59 (12 H). Other signals from the protons allocated on 3'-carbons of the sugar backbone appeared as multiplets at δ 4.79 (1 H) and 4.97÷5.28 (12 H), while the H3'resonance of the T14 unit was obscured by the residual HOD signal. The remaining H5',

H5" and H4' of the deoxyribose rings gave multiplets at δ 4.15÷4.60 altogether with H3 (2 H) and H1 (2 H) of glycerol, whose H2 appeared as a multiplet at δ 5.44. On the basis of the H1a/H2, H1b/H2 and $H_2$3/H2 cross-peaks in the COSY spectrum it was possible to assign the resonance of H1a of glycerol at δ 4.35 and H1b and $H_2$3 of glycerol at δ 4.26 ppm. (Fig. 11 a).



a) b)

Fig. 11: A enlarged section of ^{1}H-^{1}H NMR COSY spectrum of **1**, showing cross-correlation peaks (a) of the protons of glycerol backbone (b).

The above spectroscopic data, in addition to the results previously obtained from MS analysis, allowed us to unequivocally confirm the expected structure for the new synthesized compounds **1-3**.

3.6 Melting temperatures of 'antisense' phosphatidyl-ODN / 'sense' ODN duplexes.

Once the main structural properties of the new synthesized phosphatidyl-ODNs were determined, we went to evaluate some of their bio-molecular and biological features as well.

Among these properties, the ability of these *antisense* phosphatidyl - ODNs to form duplexes with the complementary *sense* sequence it seemed interesting to be assessed. In general, the higher this ability, the higher should be the antisense activity. As it is known, duplex stability is strictly related to its thermal stability; the latter being currently expressed in terms of melting temperature (*Tm*) that is in turn deduced from absorbance *vs* temperature (melting and annealing curves) profiles of oligonucleotides, recorded on a temperature-programmable UV-spectrophotometer.

To this end, first we proceeded to synthesize the complementary *native* ODN *sense* sequence (5'-ACATCTTCAAGCCA-3') necessary to obtain the desired duplex melting curves. The synthesis was carried out on solid phase by applying, in this case, the currently used phosphoramidite chemistry (see above Fig. 3).

On the other hand, the *not-modified* ODN *antisense* sequence should have been also available because it would be used as a control in parallel annealing experiments. So, after having synthesized the above cited *native* ODN *sense* sequence, we used once again the classical phosphoramidite chemistry to synthesize, on solid phase, the *not-modified* ODN *antisense* sequence (5'-TGGCTTGAAGATGT-3').

At this point, because of the impossibility to have access to an adequate variable temperature UV spectrophotometer, we decided to determine the desired melting temperatures by another suitable technique. To this end, the

ability of our phosphatidyl-ODNs to give duplexes with the complementary *sense* oligonucleotide was tested by Differential Scanning Calorimetry (DSC) analysis [87].

It is known in fact that DNA/DNA binding thermodynamic quantities may be determined from DSC measurements on the thermal dissociation or 'melting' of DNA/DNA duplexes at high temperatures. DSC measurements on the heat absorbed in the transition from a duplex to the single-strand states yield direct determinations of the binding enthalpy that are model-independent and direct determinations of the heat capacity change for the transition. So, we went to determine, from DSC measurements the thermodynamic quantities describing the melting or dissociation of our three *antisense* phosphatidyl-ODN/*sense* ODN duplexes and their corresponding *antisense* ODN/*sense* ODN duplexes. In particular the determined thermodynamic quantities were: a) the temperature at half the transition peak area (*Tm*) and b) the heat capacity change accompanying the transition (ΔCp) from the difference in the pre-transitional baselines at *Tm*.

The following pairs of oligonucleotides, appropriately dissolved 100 mM NaCl buffered solution (pH 7.4), were used for DSC experiments:

- (5'-TGGCTTGAAGATGT-3') / (5'-ACATCTTCAAGCCA-3')
- 5'-*O*-[1,2-*O*-dimyristoyl-*sn*-glycero-3-phosphoryl]-(5'-TGGCTTG-AAGATGT-3') / (5'-ACATCTTCAAGCCA-3')
- 5'-*O*-[1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphoryl]-(5'-TGGCTTG-AAGATGT-3') / (5'-ACATCTTCAAGCCA-3')
- 5'-*O*-[1,2-*O*-distearoyl-*sn*-glycero-3-phosphoryl]-(5'-TGGCTTGA-AGATGT-3') / (5'-ACATCTTCAAGCCA-3')

Typical DSC scans obtained from ODN/ODN and phosphatidyl-ODN/ODN duplex solutions are shown in Figure 12, along with their

repeated scans. In particular, the phosphatidyl-ODN referred to in the figure is the dimyristoyl-glycerophosphoryl-tetradecamer **1**.

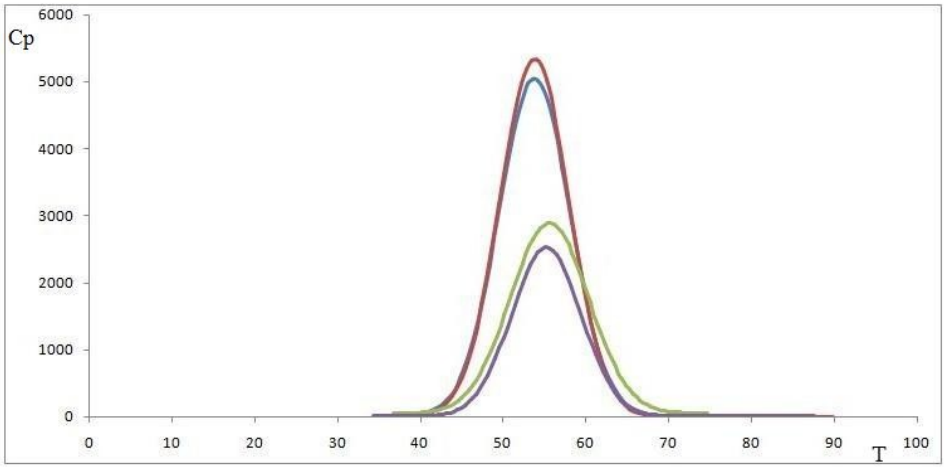


Fig.12: Overlapped DSC scans of ■ unmodified *antisense/sense* duplex and ■re-scan of the same; ■compound **1**/*sense* duplex and ■ it re-scan.

The temperature of 55,9 °C corresponding to the peak maximum of the *antisense* phosphatidyl-ODN **1**/*sense* ODN duplex is a few degrees higher than that of the peak maximum of the *antisense* ODN/*sense* ODN duplex (53,5°C), indicating that the former duplex is thermally stable at least as much as the corresponding *antisense* ODN/*sense* ODN duplex is. Also, the reappearance of the transition peaks upon a re-scan of the samples shows that the transitions are reversible and that there is not a significant degradation of the sample after heating to 90°C. Dipalmitoyl-glycerophosphoryl-tetradecamer **2** showed a similar behavior (*Tm*= 53.2 °C), while the *Tm* value of the duplex of distearoyl-glycerophosphoryl-tetradecamer **3** was 9.1 °C lower than that of the reference duplex.

Considering that, in the case of phosphatidyl-ODNs **1** and **2**, the *Tm* values obtained for *antisense* phosphatidyl-ODN/*sense* ODN duplexes were not very different from the reference value of unmodified *antisense* ODN/*sense* ODN duplex, it can be inferred that the duplexes formed by *antisense*

phosphatidyl-ODNs **1** and **2** with the *sense* counterpart have stabilities similar to that of the relevant unmodified (*native*) duplex.

The result obtained for phosphatidyl-ODN **3** is probably due to the larger size of the stearoyl chain in the phosphatidyl moiety, somewhat disturbing the base pairing near the phosphatidyl.

In all the thermograms obtained for *antisense* phosphatidyl-ODNs (**1-3**)/*sense* ODN duplexes the observed heat capacity changes (ΔCp) were lower than those measured in thermograms from the reference unmodified duplex. In the light of the observed tendency of these molecules to self-assemble, these results may not be surprising; in fact, if certain number of molecules of phosphatidyl-ODNs are involved in the formation of aggregates, they will be subtracted from taking part in duplex formation and therefore, the heat exchanged for the transition duplex/single strand will be lower. Anyway, further investigations are needed to clarify these points.

3.7 Biological assays

Considering that the sequence of the oligonucleotide moiety of phosphatidyl tetradecamers **1-3** had been chosen as to be that of an *antisense* oligonucleotide from the region 261 to 274 of human $VEGF_{165}$ coding region, some preliminary tests were carried out to ascertain the *antisense* effectiveness of these compounds.

VEGF expresses in a lot of solid tumor cells. Shi et al. [88] has studied the influence of the VEGF AS-ODN on the blood vessel generation behavior and proliferation of renal carcinoma cell line-Caki-1cell, which manifested that AS-ODN can down-regulate the expression of VEGF, and restrain the proliferation and immigration of the endothelial cells. Since has been reported [89] that VEGF and its receptors are expressed also in human

neuroblastoma, We decided to evaluate the effect of VEGF *antisense* phosphatidyl-ODNs on the expression of VEGF at mRNA level in Human Neuroblastoma cell line SH-SY5Ycells, which could provide experimental foundation for the potential clinical application of the newly synthesized VEGF phosphatidyl-ODNs.

In this regard, wanting to perform the *antisense* tests in the experimental conditions of maximum activity of VEGF expression by cells, we evaluated the possibility of carrying out the experiments on cell cultures undertaken to the so called "hypoxic conditioning". It's know [90-91] in fact that administration to cells of cobalt chloride ($CoCl_2$) produces hypoxia which induces in turn VEGF production. For this purpose, the cellular response (in terms of VEGF production) to different concentrations of $CoCl_2$ was investigated. The best results were obtained following administration of 25μM $CoCl_2$.

At this point, cells of neuroblastoma SH-SY5Y line were incubated, in the presence of 25μM $CoCl_2$, with the two phosphatidyl antisense 5'-*O*-[1,2-*O*-dimyristoyl-*sn*-glycero-3-phosphoryl]-(TGGCTTGAAGATGT-3') and 5'-*O*-[1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphoryl]-(TGGCTTGAAGATGT-3'), both at various concentrations. In parallel experiments, the relevant unmodified *antisense* ODN was administered as a control. After 60 h incubation, cells were treated with TRIZol™ reagent and mRNA was extracted (see experimental section). Transcription from mRNA to cDNA and PCR were then performed and the amplified DNA was electrophoretically analyzed on agarose gel.

As can be deduced from Fig. 13 reported below, unmodified (native) *antisense* ODN, when administered at concentration of 20 μM or higher is able to lower the cellular VEGF m-RNA concentration, but is not able to do it at 10 μM concentration. Interestingly, both phosphatidyl tetradecamers **1-2**

were able to significantly decrease the cellular VEGF m-RNA already at 10 μM concentration.

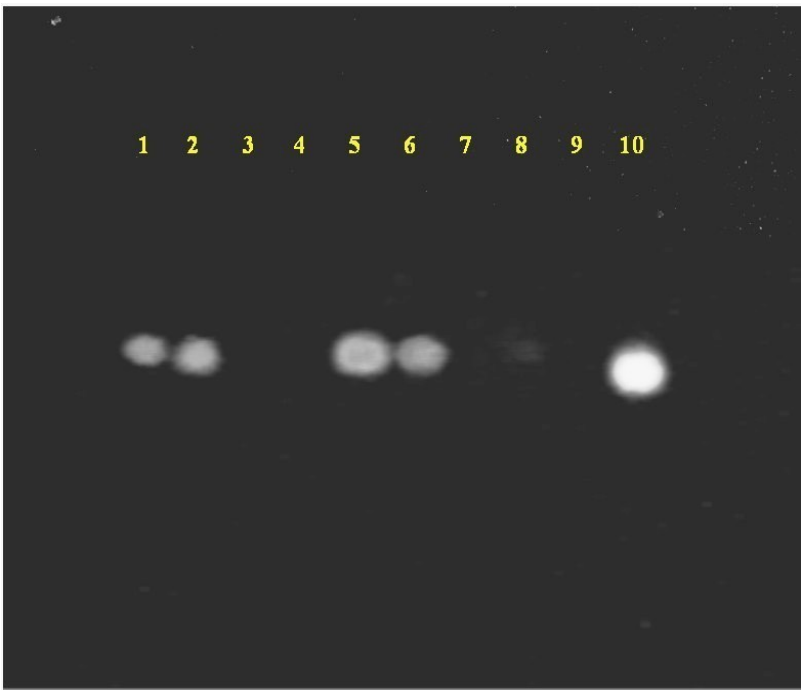


Fig. 13. Agarose gel of cDNA from Neuroblastoma cells. - 1: Untreated; 2: 10 μM unmodified ODNs+25 μM $CoCl_2$; 3: 20 μM unmodified ODNs+25 μM $CoCl_2$; 4: 30 μM unmodified ODNs+25 μM $CoCl_2$; 5: 5 μM of **2**+25 μM $CoCl_2$; 6: 5 μM of **1**+25 μM $CoCl_2$; 7: 10 μM of **2**+25 μM $CoCl_2$; 8: 10 μM of **1**+25 μM $CoCl_2$; 9: 15 μM of **1**+25 μM $CoCl_2$; 10: 25 μM $CoCl_2$.

To ascertain the cell viability in the course of our experiments, and in particular to verify possible cell toxicity of our phosphatidyl-ODN, all cell cultures undertaken to the antisense tests were controlled by MTT assay [92]. Further, MTT assay was performed also on cell cultures treated with 10, 20 and 30 μM unmodified *antisense* ODN.

From the results of MTT assays (Fig. 14) it was possible to state that phosphatidyl-ODNs used in our antisense tests did not exert any cellular toxicity at the used concentrations.

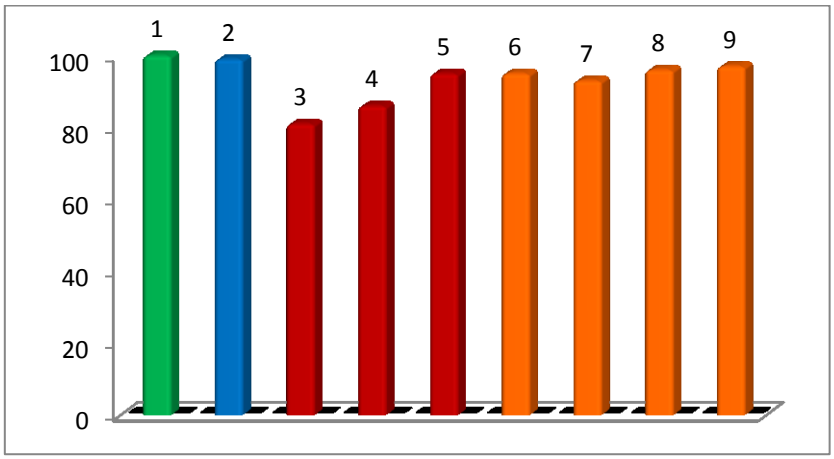


Fig. 14. Results of MTT assay on Neuroblastoma cells. - 1: Untreated; 2: 25 µM $CoCl_2$; 3: 30 µM unmodified ODNs+25 µM $CoCl_2$; 4: 20 µM unmodified ODNs+25 µM $CoCl_2$; 5: 10 µM unmodified ODNs+25 µM $CoCl_2$; 6: 10 µM of **2**+25 µM $CoCl_2$; 7: 15 µM of **1**+25 µM $CoCl_2$; 8: 10 µM of **1**+25 µM $CoCl_2$; 9: 5 µM of **2**+25 µM $CoCl_2$.

Although the preliminary results above described were very encouraging ones, further work is currently underway to better define the molecular aspects underlying the observed data.

4 CONCLUSIVE REMARKS

As has been said in the introduction, oligonucleotides bearing a terminal lipophilic and biocompatible group attached possibly through a biodegradable bond could be useful as antisense pro-drugs with improved cellular uptake. Nevertheless, despite various synthetic strategies have been addressed to the lipophilic modification of oligonucleotides, the phosphatidyl group, which is widely distributed among the cell membrane lipids, has never been used for this purpose. This is mainly due to the lability of the ester bond in the phosphatidyl during the treatment for the deprotection of synthetic oligonucleotides.

In the course of this research work, exploiting various synthetic opportunities, and making also some appropriate changes, we succeeded to synthesize for the first time 5'-*O*-phosphatidyloligodeoxynucleotides. The versatility of the synthetic route we developed allows the preparation of phosphatidyl-oligonucleotides differing in their phosphatidyl tail and/or in the sequence and the length of the oligonucleotide moiety. We would like to emphasize that the synthesis was designed so that it can be easily carried out by using a common automated DNA synthesizer.

In particular we turned our attention toward the preparation of some phosphatidyl-tetradecadeoxynucleotides all having the antisense sequence against a coding region of the VEGF gene but differing from each other in the fatty acid composition of their phosphatidyl moiety. To this purpose, phosphatidyl-ODNs **1**, **2** and **3** were prepared having 1,2-*O*-dimyristoyl-, 1,2-*O*-dipalmitoyl- and 1,2-*O*-distearoyl-*sn*-glycero-3-phosphoryl group as their phosphatidyl moiety respectively.

The structure of the newly synthesized compounds was then confirmed on the basis of their spectroscopic properties (UV, MS, NMR).

Both UV and NMR measurements have highlighted a strong tendency of these compounds to give supramolecular aggregates whose features (liposomes, micelles, etc.) it has not been possible to establish because of lack of time. Further studies aimed at characterization of these aggregates are now under way.

Thermal melting profiles of different duplexes formed by each of the *antisense* phosphatidyl-ODNs (**1**-**3**) and the unmodified complementary *sense* ODN were obtained by DSC in order to evaluate the annealing properties of these new compounds.

Melting temperatures *(Tm)* have been extracted and compared to that found in parallel experiment with the relevant *native* duplex. Phosphatidyl-ODNs **1** and **2** showed *Tm* very close to that of *native* duplex, while the *Tm* value of phosphatidyl-ODN **3** dropped by 9.1 °C above that of the unmodified sequences. This is probably due to the larger size of the stearoyl chain in the phosphatidyl moiety, somewhat disturbing the base pairing near the phosphatidyl. How the length of the acyl chain and/or that of the nucleotide sequence may influence the duplex stability of these phosphatidyl-ODNs are interesting topics that deserve to be investigated in the near future.

Further, the actual effectiveness of our *antisense* phosphatidyl-ODNs in inhibiting the expression of VEGF mRNA was assessed following administration of phosphatidyl-ODNs **1** and **2** to Neuroblastoma cell lines. As a result, both **1** and **2** were effective in lowering drastically the cellular VEGF m-RNA, and they have done so at concentrations significantly lower than those required to produce the same effect by the unmodified *antisense* ODN.

As a whole, the results of this work confirm the idea that *antisense* oligonucleotides with native molecular structure, but covalently linked to a natural lipid moiety such as phosphatidyl, may be subject to an enhanced cellular uptake compared to the non-conjugated ones, such requiring lower concentrations than the latter to exert the same desired biological effect. In other words this means that, if these compounds are used *in vivo* to modulate gene expression for pharmacological purposes, the possibility that systemic toxicity occurs would be greatly reduced.

Considering that the phosphatidyl-ODNs in the first instance we synthesized all have saturated acyl chains in their phosphatidyl group, it would be interesting to study the influence of the presence of unsaturated acyl chains in the same group on the ability of these compounds to interact with bio-membranes and, ultimately, on their cellular uptake. So, the synthesis of this kind of new molecules and the evaluation of their interaction with membrane models (mono- and bilayers, LUV, etc.) will be interesting research topics.

Finally, one more consideration.

Albert Einstein is said to have said this sentence: “imagination is more important than knowledge”. So, let me imagine an *antisense* oligonucleotide that bears two phosphatidyl moieties covalently linked to its 3’- and 5’-end; in addition to a more pronounced cellular uptake and an increased nuclease resistance (both to 3’- and 5’-exonucleases), a compound of this type may have, especially, a marked tendency to self sick its lipid ends, thus giving rise to a plasmid-like ODN. It is not difficult to imagine the pharmacological potential of a compound of this kind.

We think that, making the appropriate changes to the synthetic route designed by us for the preparation of 5’-*O*-phosphatidyl-ODNs, it will be possible to realize this dream.

5 EXPERIMENTAL

5.1 Synthesis of silyl linker.

Materials

1,4-Dibromobenzene was purchased from Sigma Aldrich and, before using, it was dried under vacuum for 24 h in the presence of P_2O_5. The butyl-lithium solution and chlorodiisopropylsilane were obtained from Fluka and Sigma Aldrich respectively.

Solid CO_2 was freshly prepared following gas expansion.

2,4,6-Tribromophenol and bis(2-oxo-3-oxazolidinyl) phosphinyl chloride (BOPCl) were purchased from Sigma Aldrich; before using, they were kept under vacuum for 24 h in the presence of P_2O_5.

1,4-Dichloro-5,5-dimethyl hydantoine was obtained from Fluka and dried as above.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxythymidine and imidazole were purchased from Sigma Aldrich and Fluka respectively, and they were dried too.

LCAA-CPG (Long Chain Amino Alkyl Controlled Pore Glass) was obtained from Sigma Aldrich.

Synthesis of silyl-linker and anchoring of the first nucleoside to CPG.

To 10 ml of anhydrous THF (-78 ºC) 1,4-dibromobenzene (0.5 g; 2.12 mmol) was added under Ar and then 0.785 ml (1 equivalent) of butyl-lithium (2.7 M in heptane solution) were added to the solution. The mixture was

allowed to react for 5 minutes and then was added with chloro-diisopropyl silane (0.360 ml; 2.12 mmol). After 15 minutes at -78°C, 0.785 ml (1 equivalent) of butyl-lithium (2.7 M solution in heptane) were added to the reaction mixture which, 5 minutes after, was treated with a large excess of solid CO_2. One hour later, the mixture was evaporated from the solvent and was purified by liquid chromatography. The elution conditions were taken from TLC [silica gel, CH_2Cl_2/MeOH/TEA (95:4:1)], where the product showed $R_f = 0.12$. Column chromatography on silica gel was carried out with a methanol gradient from 4 to 20% in CH_2Cl_2 in the presence of 1% TEA. Triethylammonium 4-(diisopropylsilyl)benzoate (250 mg), was then analyzed for its spectroscopic (^{1}H-NMR) properties which confirmed the structure.

^{1}H NMR ($CDCl_3$): δ 7.89 (d, J_{ortho} = 8 Hz, 2 H, H-2 e H-6), 7.43 (d, J_{ortho} = 8 Hz, 2 H, H-3 e H-5), 3.83 (s, $J_{^{29}Si,H}$ = 6 Hz, 1 H altogether, Si-H), 3.02 (q, J = 7 Hz, 6 H, NC***H***$_2$CH$_3$), 1.25 (t, J = 7 Hz, 9 H, NCH$_2$C***H***$_3$), 1.10 (m, 2 H, CH isopropyl), 0.94 (d, J = 7.5 Hz , 6 H, CH_3 of -CH(CH$_3$)$_2$), 0.86 (d, J = 7.5 Hz , 6 H, CH_3 of -CH(CH$_3$)$_2$).

In the next step triethylammonium 4-(diisopropylsilyl)benzoate (0.250 g; 1.06 mmol) was dissolved in anhydrous pyridine (20 ml) and to the solution 4 equivalent of BOPCl (4.24 mmol; 1.079 g) and 2 equivalent of 2,4,6-tribromophenol (2.12 mmol, 0.701 g) were added under Ar. After 1 h the solvent was evaporated and the product was purified by liquid choromatography, using hexane/ethyl acetate/TEA (96:3:1) as the eluent mixture. 419 mg of 2,4,6-tribromophenyl 4-(diisopropylsilyl)benzoate were obtained and analyzed by NMR spectroscopy which confirmed its structure .

^{1}H NMR ($CDCl_3$): δ 8.22 (d, J = 8.0 Hz , 2 H, H-2 e H-6), 7.78 (s, 2 H, H-3' e H-5'), 7.74 (d, J = 8.0 Hz, 2 H, H-3 e H-5), 4.06 (s, $J_{^{29}Si,H}$ = 6.5 Hz, 1 H altogether, Si-H), 1.32 (m, 2 H, CH of -CH(CH$_3$)$_2$), 1.13 (d, J = 7.5 Hz , 6 H, CH_3 of-CH(CH$_3$)$_2$), 1.06 (d, J = 7.5 Hz, 6 H, $CH_3$of -CH(CH$_3$)$_2$).

The third synthetic step was carried out dissolving 250 mg (0.531 mmol) of 2,4,6-tribromophenyl 4-(diisopropylsilyl)benzoate in 10 ml of anhydrous CH_2Cl_2 and adding to 2 equivalents of 1,3-dichloro-5,5-dimethyl hydanthoine (1.062 mmol, 210 mg) to the solution. After 1 h at room temperature, 10 equivalents of imidazole (5.31 mmol, 361 mg) and 2 equivalents of 5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine (1,062 mmol, 579 mg) were added to the mixture and allowed to react. After 2 h the organic solvent was evaporated and the product was purified by liquid chromatography on silica gel column using hexane/*tert*-butanol/TEA (92:7:1) as the eluent mixture. After the isolation the structure of 3'-*O*-{diisopropyl[4-(2,4,6-tribromophenoxycarbonyl)phenyl]silyl}-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine (362 mg) was confirmed by ^{1}H NMR.

^{1}H NMR ($CDCl_3$): δ 8.18 (d, J = 8 Hz, 2 H, H-2 e H-6), 7.77 (s, 2 H, H-3' e H-5'), 7.65 (d, J = 8 Hz, 2 H, H-3 e H-5), 7.65 (partially overlapped s, 1 H, H-6 of thymine), 7.26 (m, 9 H, DMT), 6.83 e 6.81 (d, overlapped, J = 9 Hz, 4 H altogether, H in *ortho* to CH_3O of DMT), 6.48 (dd, $J_{1',2'}$= 8 Hz $J_{1',2''}$= 6 Hz, 1 H, H-1'), 4.69 (m, 1 H, H-3'), 4.17 (m, 1 H, H-4'), 3.78 (s, 6 H, CH_3O- of DMT), 3.51 (dd, $J_{5',5''}$ = -11 Hz, $J_{5',4'}$= 3 Hz, 1 H, H-5'), 3.31 (dd, $J_{5''-5'}$ = -11 Hz, $J_{5'',4'}$= 2.5 Hz, 1 H, H-5''), 2.47 (m, 1 H, H-2''), 2.29 (m, 1 H, H-2'), 1.51 (br s, 3 H, CH_3 thymine), 1.26 (m, 2 H, CH of -$CH(CH_3)_2$), 1.05-0.98 (d, overlapped 12 H, CH_3 of -$CH(CH_3)_2$).

Finally, the anchoring of 3'-*O*-{diisopropyl[4-(2,4,6-tribromophenoxycarbonyl)phenyl]silyl}-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine to CPG was performed as follows. 1.22 g of CPG (166 μmol, loading capacity(NH_2)=136 μmol/g), 2 equivalents (362 mg, 332 μmoli) of compound to be anchored and 2 equivalents of dry TEA (332 μmoli, 48 μL) were placed in a CH_2Cl_2/DMF (1:1 v/v, dry) solution and allowed to stir for 36 h. After this period, the reaction mixture was

introduced in an empty reactor column currently used in automated synthesizers and it was stepwise washed with CH_2Cl_2, THF and ACN (all dry). After drying the siliceous support, an aliquot of it (21.2 mg) was treated with 2,5% CCl_3COOH solution in CH_2Cl_2 to release the dimethoxytrityl group which was spectrophotometrically quantified; the loading capacity resulted to be 30.6 μmol/g.

5.2 Synthesis of 1,2-Diacyl-sn-glycero-3-(2-cianoethyl)-N,N-diisopropyl phosphoramidite.

Materials

1,2-*O*-Dimyristoyl-*sn*-glycerol, 1,2-*O*-Dipalmitoyl-*sn*-glycerol and 1,2-*O*-Distearoyl-*sn*-glycerol were purchased from Bachem; before using, they were dried under vacuum for 24 h in the presence of P_2O_5.
β-Cyanoethyl-N,N-diisopropyl chloro-phosphoramidite was obtained from Sigma Aldrich.
N,N-Diisopropylethylamine, was obtained from Fluka, distilled before use and stored on molecular sieves 3 A type (Fluka).

Synthesis of 1,2-diacyl-sn-glicero-3-(2-cyanoethyl)-N,N-diisopropyl phospho -ramidites, general procedure.

All the three 1,2-diacyl-sn-glicero-3-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidites necessary for the synthesis of phosphatidyl-ODNs **1-3** were obtained through the same procedure.

To a solution containing 500 mg of the appropriate 1,2-Diacyl-*sn*-glycerol in 15 ml of anhydrous CH_2Cl_2, 2 equivalents of *N,N*-diisopropylethylammine (0.6 ml, 3.52 mmol) and β-cyanoethyl-*N,N*-

diisopropylchlorophosphoramidite (0.39 ml 1.76 mmol) were added under Ar.

The mixture was left to stir for 1 h and, after addition of 20 ml of CH_2Cl_2, it was extracted with 10 % aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The residue was fractionated by column chromatography on SiO_2, eluting with petroleum ether/ethyl ether/*tert*-buthanol/TEA (88:6:5:1); fractions containing the expected 1,2-diacyl-*sn*-glycero-3-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite were detected by Rhodamine B reagent and pooled. 1,2-*O*-Dimyristoyl-*sn*-glycero-3-(2-cyanoethyl)- *N,N*- diisopropylphosphoramidite, 1,2-*O*-dipalmitoyl-*sn*-glycero-3-(2-cyanoethyl)- *N,N*-diisopropylphosphoramidite and 1,2-*O*-distearoyl-*sn*-glycerol-3-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite so obtained were analyzed for their ^{1}H NMR spectroscopic properties.

5.2.1 1,2-dimyristoyl-sn-glycero-3-(2-cyanoethyl)-N,N-diisopropylphosphoramidite

^{1}H NMR (C_6D_6): δ 5.41 (m, 1H, H-2 of glycerol), 4.52 and 4.44 (dd, $J_{1a,1b}$ = -11.9 Hz, $J_{1a,2}$ = 3.7 Hz; 1 H altogether, H-1a of glycerol), 4.26 and 4.24 (dd, $J_{1b,1a}$ = -11.9, $J_{1b,2}$ = 6.5 Hz, 1H altogether, H-1b of glycerol), 3.85-3.79 (overlapped m, 1H, H-3a of glycerol), 3.75 and 3.70 (ddd, $J_{3b,3a}$ = -10.5 Hz, $J_{3b,2}$ = 3.8 Hz, $J_{3b,P}$ = 8 Hz; ddd, $J_{3b,3a}$ = -10.5 Hz, $J_{3b,2}$ = 3.7 Hz, $J_{3b,P}$ = 7.5 Hz; altogether 1H, H-3b of glycerol), 3.51 (m, 2 H, OC***H***$_2$$CH_2CN$), 3.43-3.26 (overlapped m, 2 H, CH of -$CH(CH_3)_2$), 2.233 and 2.230 (t, $J_{\alpha,\beta}$ = 7.5 Hz, altogether 2 H, α-CH_2 of myristoyl), 2.19 (t, $J_{\alpha,\beta}$ = 7.4 Hz, 2 H, α-CH_2 of myristoyl), 1.88-1,77 (overlapped m, 2 H, OCH_2C***H***$_2$CN), 1.61 (overlapped m, 4 H, β-CH_2 of myristoyl), 1.31, 1.30, 1.24 and 1.22 (s broad , 40 H altogether, from γ- to ξ- CH_2 of myristoyl), 1.125, 1.121, 1.114 and 1.111 (d,

J = 6.6, 6.7, 6.2, 6.8 Hz 12 H altogether, CH_3 of-$CH(CH_3)_2$), 0.92 (t, J = 6.9 Hz, 6 H, CH_3 of myristoyl).

^{13}C NMR (C_6D_6): δ 173.20 e 173.18 (COO), 118.09 and 118.07 (CN), 71.91 and 71.87 (d, J_{CCOP} = 7.2 e 7.0 Hz respectively, C-2 of glycerol), 63.38 (C-1 of glycerol), 62.87 and 62,77 (d, J_{COP}= 15.8 and 15.7 Hz respectively, C-3 of glycerol), 59.43 and 57.54 (d, J_{COP}= 18.7 and 18.3 Hz respectively, O***C***H_2CH_2CN), 44.11 and 44.09 (d, J_{CNP}= 12.5 and 12.3 Hz respectively, CH of -$CH(CH_3)_2$), 35.16 and 34,90 (α-CH_2 of myristoyl), 33.01 (ν-CH_2 of myristoyl), 30.86, 30.82, 30.80, 30.77, 30.61, 30.49, 30.40 and 30. 16 (from γ- to μ-CH_2 of myristoyl), 25.98 and 25.94 (β-CH_2 of myristoyl), 25.37 and 25.31 (CH_3 of -$CH(CH_3)_2$), 23.78 (ξ-CH_2 of myristoyl), 20.72 (d, J_{CCOP} = 6.6 Hz, OCH_2***C***H_2CN), 15.03 (CH_3 of myristoyl).

5.2.2 1,2-dipalmitoyl-sn-glycero-3-(2-cyanoethyl)-N,N-diisopropylphosphoramidite

^{1}H NMR (C_6D_6): δ 5.41 (m, 1H, H-2 of glycerol), 4.52 and 4.44 (dd, $J_{1a,1b}$ = -11.9 Hz, $J_{1a,2}$ = 3.7 Hz; 1 H altogether, H-1a of glycerol), 4.26 and 4.24 (dd, $J_{1b,1a}$ = -11.9, $J_{1b,2}$ = 6.5 Hz, 1H altogether, H-1b of glycerol), 3.85-3.79 (overlapped m, 1H, H-3a of glycerol), 3.75 and 3.70 (ddd, $J_{3b,3a}$ = -10.5 Hz, $J_{3b,2}$ = 3.8 Hz, $J_{3b,P}$ = 8 Hz; ddd, $J_{3b,3a}$ = -10.5 Hz, $J_{3b,2}$ = 3.7 Hz, $J_{3b,P}$ = 7.5 Hz; 1H altogether, H-3b of glycerol), 3.51 (m, 2 H, OC***H***$_2CH_2CN$), 3.43-3.26 (overlapped m, 2 H, CH of -$CH(CH_3)_2$), 2.233 and 2.230 (t, $J_{\alpha,\beta}$ = 7.5 Hz, 2 H altogether, α-CH_2 of palmitoyl), 2.19 (t, $J_{\alpha,\beta}$ = 7.4 Hz, 2 H, α-CH_2 of palmitoyl), 1.88 - 1.77 (overlapped m, 2 H, OCH_2C***H***$_2CN$), 1.61 (overlapped m, 4 H, β-CH_2 of palmitoyl), 1.31, 1.30, 1.24 and 1.22 (s broad , 48 H altogether, from γ- to ξ- CH_2 of palmitoyl), 1.125, 1.121, 1.114 and 1.111 (d, J = 6.6, 6.7, 6.2, 6.8 Hz 12 H altogether, CH_3 of -$CH(CH_3)_2$), 0.92 (t, J = 6.9 Hz, 6 H, CH_3 of palmitoyl).

^{13}C NMR (C_6D_6): δ 173.20 and 173,18 (COO), 118.09 e 118.07 (CN), 71.91 and 71.87 (d, J_{CCOP} = 7.2 and 7.0 Hz respectively, C-2 of glycerol), 63.38 (C-1 of glycerol), 62.87 and 62.77 (d, J_{COP}= 15.8 and 15.7 Hz respectively, C-3 of glycerol), 59.43 and 57.54 (d, J_{COP}= 18.7 and 18.3 Hz respectively, O***C***H_2CH_2CN), 44.11 and 44.09 (d, J_{CNP}= 12.5 and 12.3 Hz respectively, CH of -$CH(CH_3)_2$), 35.16 e 34.90 (α-CH_2 of palmitoyl), 33.01 (ν-CH_2 of palmitoyl), 30.86, 30.82, 30.80, 30.77, 30.61, 30.49, 30.40 and 30. 16 (da γ- a μ-CH_2 of palmitoyl), 25.98 and 25.94 (β-CH_2 of palmitoyl), 25.37 and 25.31 (CH3 of -$CH(CH_3)_2$), 23.78 (ξ-CH_2 of palmitoyl), 20.72 (d, J_{CCOP} = 6.6 Hz, OCH_2***C***H_2CN), 15.03 (CH_3 of palmitoyl).

5.2.3 1,2-distearoyl-sn-glycero-3-(2-cyanoethyl)-N,N-diisopropylphosphoramidite

^{1}H NMR (C_6D_6): δ 5.41 (m, 1H, H-2 of glycerol), 4.52 and 4.44 (dd, $J_{1a,1b}$ = -11.9 Hz, $J_{1a,2}$ = 3.7 Hz; 1 H altogether, H-1a of glycerol), 4.26 and 4.24 (dd, $J_{1b,1a}$ = -11.9, $J_{1b,2}$ = 6.5 Hz, 1H altogether, H-1b of glycerol), 3.85-3.79 (overlapped m, 1H, H-3a of glycerol), 3.75 and 3.70 (ddd, $J_{3b,3a}$ = -10.5 Hz, $J_{3b,2}$ = 3.8 Hz, $J_{3b,P}$ = 8 Hz; ddd, $J_{3b,3a}$ = -10.5 Hz, $J_{3b,2}$ = 3.7 Hz, $J_{3b,P}$ = 7.5 Hz; 1H altogether, H-3b of glycerol), 3.51 (m, 2 H, OC***H***$_2CH_2CN$), 3.43-3.26 (overlapped m, 2 H, CH of -$CH(CH_3)_2$), 2.233 and 2.230 (t, $J_{\alpha,\beta}$ = 7.5 Hz, 2 H altogether, α-CH_2 of stearoyl), 2.19 (t, $J_{\alpha,\beta}$ = 7.4 Hz, 2 H, α-CH_2 of stearoyl), 1.88 - 1.77 (overlapped m, 2 H, OCH_2C***H***$_2CN$), 1.61 (overlapped m, 4 H, β-CH_2 of stearoyl), 1.31, 1.30, 1.24 and 1.22 (s broad , 56 H altogether, from γ- to ξ- CH_2 of stearoyl), 1.125, 1.121, 1.114 and 1.111 (d, J = 6.6, 6.7, 6.2, 6.8 Hz 12 H altogether, CH_3 of -$CH(CH_3)_2$), 0.92 (t, J = 6.9 Hz, 6 H, CH_3 of stearoyl).

^{13}C NMR (C_6D_6): δ 173.20 e 173.18 (COO), 118.09 and 118.07 (CN), 71.91 and 71.87 (d, J_{CCOP} = 7.2 and 7.0 Hz respectively, C-2 of glycerol),

63.38 (C-1 of glycerol), 62.87 and 62.77 (d, J_{COP}= 15.8 and 15.7 Hz respectively, C-3 of glycerol), 59.43 e 57.54 (d, J_{COP}= 18.7 and 18.3 Hz respectively, O***C***H_2CH_2CN), 44.11 and 44.09 (d, J_{CNP}= 12.5 and 12.3 Hz respectively, CH of -$CH(CH_3)_2$), 35.16 and 34.90 (α-CH_2 of stearoyl), 33.01 (ν-CH_2 of stearoyl), 30.86, 30.82, 30.80, 30.77, 30.61, 30.49, 30.40 and 30. 16 (from γ- to μ-CH_2 of stearoyl), 25.98 and 25.94 (β-CH_2 of stearoyl), 25.37 and 25.31 (CH_3 of -$CH(CH_3)_2$), 23.78 (ξ-CH_2 of stearoyl), 20.72 (d, J_{CCOP} = 6.6 Hz, OCH_2***C***H_2CN), 15.03 (CH_3 of stearoyl).

5.3 Deacylation of N-protected 3'-deoxynucleoside phosphoramidite

Materials

5'-O-(4,4'-Dimethoxytrityl)-N^6-benzyl-2'-deoxyadenosine-3'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite, 5'-O-(4,4'-dimethoxytrityl)-N^6-benzyl-2'-deoxycytidine-3'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite and the 5'-O-(4,4-Dimethoxytrityl)-N-isobutyryl-2'-deoxyguanosine-3'-(2-cyanoethyl-N,N-diisopropyl) phosphoramidite were all purchased from Primm.
2 M Solution of $MeNH_2$ in THF was obtained from Sigma Aldrich.

5.3.1 Debenzoylation of 5'-O-(4,4'-dimethoxytrityl)-N^6-benzoyl-2'-deoxyadenosine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite

5'-*O*-(4,4'-dimethoxytrityl)-N^6-benzoyl-2'-deoxyadenosine-3'-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (1 g, 1.168 mmol) was added to 11,6 ml of a solution of 2 M CH_3NH_2 in THF (23,2 mmol) the under Ar and the mixture was left under stirring for 2 h. The solution was then taken to

dryness and the residue was chromatographed on silica gel column eluting with CH_2Cl_2/MeOH/TEA (96:3:1). Fractions containing the deprotected nucleoside phosphoramidite were pooled, taken to dryness and analyzed by NMR spectroscopy.

^{1}H NMR ($CDCl_3$): δ 8.29 (s, 1 H, H-8 adenine), 8.03 and 8.0 (s, 1 H altogether, H-2 adenine), 7.41-7.17 (m, 9 H altogether, DMT), 6.79 (m, 4 H, H *ortho* to CH_3O- of DMT), 6.46 (m, 1 H, H-1'), 6.20 (br s, 2 H, NH_2), 4.78 (m, 1 H, H-3'), 4.29 (m, 1 H, H-4'), 3.88 - 3.52 (partially overlapped multiplets and broad s, 10 H altogether, CH_3O of DMT, OC***H***$_2CH_2CN$, CH of -$CH(CH_3)_2$), 3.46 - 3.32 (m, 2 H, H-5' and H-5"), 2.88 (m, 1 H, H-2"), 2.70 - 2.44 (m, 3 H altogether, H-2" and OCH_2C***H***$_2CN$), 1.20 - 1.117 (d, 8 H, CH_3 of -$CH(CH_3)_2$), 1.11 (d, J = 6.5 Hz, 4 H, CH_3 of -$CH(CH_3)_2$.

5.3.2 Debenzoylation of 5'-O-(4,4'-dimethoxytrityl)-N^6-benzoyl-2'-deoxycytidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite

N-Unprotected 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite was obtained from the relevant *N*^{6}-Benzoyl - 5'-*O*-(4,4'-dimethoxytrityl)-*N*^{6}-benzoyl-2'-deoxycytidine-3'-*O*-(2-cyanoethyl)-N,N-diisopropylphosphoramidite following the same procedure already described under section *5.3.1*. The NMR spectrum of the purified compound was as follows.

^{1}H NMR ($CDCl_3$): δ 7.85 and 7.77 (d, J = 7 Hz, 1 H altogether, H-6 of cytosine), 7.42 - 7.19 (m, 9 H altogether, DMT), 6.83 (m, 4 H, H *ortho* to CH_3O- of DMT), 6.30 (m, 1 H, H-1'), 5.573 and 5.567 (d, partially overlapped, J = 7 Hz, 1 H, H-5 cytosine), 4.57 (m, 1 H, H-3'), 4.13 (m, 1 H, H-4'), 3.80 - 3.52 (partially overlapped multiplets and broad singlet, 10 H altogether, CH_3O- of DMT, CH of -$CH(CH_3)_2$ and OC***H***$_2CH_2CN$), 3.51 -

3.31 (m, partially overlapped, 2 H altogether, H-5' and H-5"), 2.64 - 2.39 (partially overlapped multiplets, 3 H altogether, -$OCH_2C\boldsymbol{H}_2CN$ and H-2"), 2.20 (m, 1 H, H-2'), 1.17-1.13 (d, 9 H, CH_3 of -$CH(CH_3)_2$), 1.04 (d, J = 6.5 Hz, 3 H, CH_3 of -$CH(CH_3)_2$).

5.3.3 Deacylation of 5'-O-(4,4'-dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite

N-Unprotected 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyguanosine-3'-*O*-(2-cyano-ethyl)-*N*,*N*-diisopropylphosphoramidite was obtained from the title compound following a similar procedure to that described under section *5.3.1*, but allowing to react for 15 h. and performing the final chromatographic purification on silica gel column using CH_2Cl_2/EtOH/TEA (85:15:1) as solvent system. The purified compound was then analyzed by NMR spectroscopy.

^{1}H NMR ($CDCl_3$): δ 7.66 and 7.65 (s, 1 H altogether, H-8 guanine), 7.37 -7.12 (m, 9 H altogether, DMT), 6.76 (m, 4 H, H *ortho* to CH_3O- of DMT), 6.2 (m, 3 H, H-1'), 6.14 (br s, 2 H, NH_2), 4.70 (m, 1 H, H-3'), 4.23 and 4.19 (m, 1 H altogether, H-4'), 3.84 - 3.48 (partially overlapped multiplets and broad s, 10 H altogether, CH_3O- of DMT, CH of -$CH(CH_3)_2$ and $OC\boldsymbol{H}_2CH_2CN$), 3.41 - 3.22 (m, 2 H, H-5' and H-5"), 2.76 (m, 1 H, H-2"), 2.60 - 2.38 (m, overlapped, 3 H altogether, H-2" and -$OCH_2C\boldsymbol{H}_2CN$), 1.16 - 1.13 (overlapped d, 9 H, CH_3 of -$CH(CH_3)_2$), 1.07 (d, J = 7 Hz, 3 H, CH_3 of -$CH(CH_3)_2$).

5.4 Synthesis and purification of 5'-O-(1,2-O-diacyl-sn-glycero-3-phosphoryl)-d(TGGCTTGAAGATGT) (**1 – 3**)

Materials for the synthesis

Solid phase synthesis was carried out on automated synthesizer [Cyclone Plus 8400, Biosearch (Millipore)].

The following products were purchased from Primm: 5'-*O*-(4,4'-dimethoxytrityl)-2'deoxythymidine-3'-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite, *Amidite Diluent* (superdry acetonitrile), *Deblock solution*, *Oxidizer solution*.

1-Hydroxybenzotriazole (HOBT · X H_2O), was purchased from Fluka and dried on P_2O_5 under vacuum at 50º for 48 h.

1-Methyl-2-pyrrolidone (NMP), stored on molecular sieve, was obtained from Fluka.

1,8-Diazabiciclo[5.4.0]undec-7-ene (DBU) and the tetrabutylammonium fluoride (TBAF) 1 M in THF were purchased from Sigma Aldrich; they were kept dry on molecular sieves 3 A type (Fluka) for 96 h.

The solid phase synthesis of phosphatidyloligonucleotides **1-3** was accomplished on an automated synthesizer (Cyclone Plus 8400) using as "reactor" a column filled with GPC to which a thymidine residue was anchored by a silyl linker (450 mg, 13.5 μmol loaded).

Solutions and reactants were the following:

- *Deblock solution* (2.5% of $CHCl_2COOH$ in CH_2Cl_2);
- *Activator solution* [0.2 M of HOBt in ACN-NMP (15:1)];
- *Oxidizer solution* (0.02 M I_2 in THF/H_2O/Py);

- Solution of 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine-3'-*O*-(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite (50 mg/ml; 0.067 M) in ACN (*Diluent*);
- Solution of 5'-*O*-(4,4'-dimethoxytrityl)-N^6-benzoyl-2'-deoxyadenosine-3'-*O*-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite (50 mg/ml; 0.067 M) in ACN (*Diluent*)
- Solution of 5'-*O*-(4,4'-dimethoxytrityl)-N^6-benzoyl-2'-deoxycytidine-3'-*O*-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite (50 mg/ml; 0.067 M) in ACN (*Diluent*)
- Solution of 5'-*O*-(4,4-Dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine-3'-O-(2-cyanoethyl)-*N,N*-diisopropyl) phosphoramidite (50 mg/ml; 0.067 M) in ACN (*Diluent*)
- Solution of 1,2-*O*-diacyl-*sn*-glicero-3-*O*-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite (70 mg/ml; 0.1 M) in dry CH_2Cl_2 .

Solid phase synthesis of 1-3 diacyl-sn-glycero-3-phosphoryl)-d(TGGCTTGAAGATGT) (**1** – **3**)

To synthesize coumpounds **1-3** the sequence 5'-X-TGGCTTGAAGATGT-3' was set on the start program of the automated synthesizer where X was in each case the appropriate 1,2-*O*-diacyl-*sn*-glycero-3-*O*-phosphoramidite.

After the last coupling step, the column was removed from the synthesizer, filled with 10% DBU in ACN and left to stand for 1 min. This treatment allowed removing of β-cyanoethyl protecting groups from phosphate bridges. After the column was washed with ACN and the solvent removed, a solution of TBAF/AcOH (1:1) 1 M in THF was passed through the column and this was left to stand for 1 h. After this period, the detached modified ODN was recovered by eluting the column with THF. The eluate

was taken to dryness in vacuo and the residue, dissolved in aqueous ethanol was passed through ultrafiltration spin concentrators (VIVASPIN-6, Sartorius) to eliminate the excess of TBAF.

Material for purification of phosphatidyl-ODNs **1-3**

High performance liquid chromatography (HPLC) was carried out on Hewlett & Packard 1200 chromatograph, equipped with UV-visible detector fixed at λ=260 nm. LiChrospher-100, RP-18 (5 μm, 4x250 mm) and LiChrospher-100, RP-18 (10 μm, 10x250 mm) columns were used for analytical or semipreparative purpose respectively.
Ammonium acetate and 2-isopropanol were purchased from Carlo Erba and Merck, respectively.

HPLC purification of phosphatidyl ODNs **1-3**

Each of the crude compounds **1-3** coming from ultrafiltration has been undertaken to HPLC analysis. This was performed on Chromospher C-8 column (5μ, 3 x 100 mm) using an elution gradient of isopropanol in 0.1 M triethylammonium acetate buffer (pH: 7,4) from 10 to 100% in 60 min, at a flow rate of 0.6 mL min^{-1}. Compounds **1**, **2** and **3** had retention times of 31.4, 35.5 and 37.9 min respectively. In the same chromatographic conditions the relevant unmodified oligonucleotide had a retention time of 1.21 min.

Each crude **1-3** was then suspended in 5 mL of 50% aqueous ethanol and purified by semipreparative HPLC. This was performed on Chromospher C-8 column (5μ, 10 x 250 mm) using elution gradient of isopropanol in 0.1 M ammonium acetate buffer (pH: 7,4) from 10 to 100% in 60 min, at a flow rate of 3.5 mL min^{-1}. For each chromatographic run only 100 μL of solution were injected. Retention times were 36.2, 40.2 and 44.2 min for myristoyl,

palmitoyl and stearoyl derivative respectively. Fractions containing the desired lipid-oligonucleotide were pooled and taken to dryness.

5.5 Characterization of 5'-O-(1,2-O-diacyl-sn-glycero-3--phosphoryl)-d(TGGCTTGAAGATGT)

Materials and equipments used for HPLC-ESI/MS

High performance liquid chromatography (HPLC) was carried out on Agilent 6410 LC QQQ, equipped with Agilent Multimode (ESI/APCI) source. Ammonium acetate, methanol and 2-isopropanol (for mass spettroscopy) was purchased from Sigma Aldrich.

Materials and equipments used for UV spectroscopy

UV spectra were carried out on UV/VIS JASCO V-560 spectrometer. Quartz cuvettes had 1 cm path length.

Materials and equipments used for NMR spectroscopy

^{1}H NMR spectra were recorded on Varian Unity Inova spectometer at 500 MHz. The chemical shifts were reported as δ (ppm) referenced to TMS. D_2O was purchased from Sigma Aldrich.

HPLC-ESI/MS analysis

Purified 5'-*O*-[1,2-*O*-distearoyl-*sn*-glycero-3-phosphoryl]-d(TGGCTTGAAGATGT), was solubilised in 10 mL of water and 5 µL of the solution was injected in HPLC-ESI/MS apparatus. The chromatographic step was carried out on Zorbax Eclipse XDB C-18 column (1.8 µ, 4.6 x 50 mm) using eluition gradient of 2-propanol in 1 mM ammonium acetate buffer (pH: 7.4) from 0 to 100% in 20 min, at flow rate of 0.2 mL min^{-1}. Instrument

parameters were set as follow: T_{source}= 100 °C, T_{gas}= 100 °C, V= -3000 and Frag = 100

Diagnostic signals and their relating multicharge ions were:

- 1254.2 $[M - 4H^+]^{4-}$, 1270.0 $[M - 7H^+ + 3Na^+]^{4-}$, 1285.2 $[M -8H^+ + 2Na^+ + 2K^+]^{4-}$, 1301.0 $[M - 9H^+ + 5K^+]^{4-}$
- 1002.8 $[M - 5H]^{5-}$, 1016.7 $[M - 8H^+ + 3Na^+]^{5-}$, 1027.4 $[M -9H^+ + 2Na^+ + 2K^+]^{5-}$, 1041.0 $[M - 10H^+ + 5K^+]^{5-}$
- 835.5 $[M - 6H]^{6-}$, 846.0 $[M - 9H^+ + 3Na^+]^{6-}$, 856.0 $[M -10H^+ + 2Na^+ + 2K^+]^{6-}$, 867.0 $[M - 11H^+ + 5K^+]^{6-}$
- 716.1 $[M - 7H]^{7-}$,725.0 $[M - 10H^+ + 3Na^+]^{7-}$, 733.8 $[M -11H^+ + 2Na^+ + 2K^+]^{7-}$, 742.9 $[M - 12H^+ + 5K^+]^{7-}$

In the same experimental conditions but using methanol instead 2-propanol the following signals were obtained:

	$[M-3H]^{3-}$	$[M-4H]^{4-}$	$[M-5H]^{5-}$	$[M-6H]^{6-}$	$[M-7H]^{7-}$
Myristoyl -oligo	1635.0	1225.9	980.3	816.5	700.1
Palmitoyl -oligo	1653.7	1240.0	990.8	826.2	708.2
Stearoyl- oligo	--	1254.0	1022.8	835.5	716.1

UV analysis

Each of three compounds **1-3** were dissolved in 10 mL of water and 1 mL of this solution was diluted to 250mL. The UV spectra of these solutions were recorded and the absorbance value at λ_{260} were determined.

Quantifications have been achieved assigning to each phosphatidyl-ODN a molar extinction coefficient of 136.600, obtained by applying the method of neighbour-neither [93] to the common ODN sequence in all the three compounds.

The following amounts were determined for each compound:

- 5'-*O*-[1,2-*O*-dimyristoyl-*sn*-glycero-3-phosphoryl]-d(TGGCTTGAAGATGT) = 7.8 μmol (57% yield with respect to CPG loading)
- 5'-*O*-[1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphoryl]-d(TGGCTTGAAGATGT) = 3.43 μmol (25,4% yield with respect to CPG loading).
- 5'-*O*-[1,2-*O*-distearoyl-*sn*-glycero-3-phosphoryl]-d(TGGCTTGAAGATGT) = 7.22 μmol (53,4% yield with respect to CPG loading).

NMR spectroscopy analysis

Compounds **1-3** were lyophilized first and then each of them dissolved in 750μL of D_2O. Their spectra were recorded at 60°C.

Compound **1** :

^{1}H NMR (D_2O, δ_{HOD} 4.67 ppm): δ 0.95 (br t, 6 H, CH_3 of myristoyls), 1.22 and 1.31 (40 H altogether, from γ- to μ-CH_2 of myristoyls), 1.65 (m, 4 H, β–CH_2 of myristoyls), 1.95 (s, 3 H, CH_3 T12), 2.03 (s, 3 H, CH_3 T14), 2.06

(s, 3 H, CH_3 T1), 2.12 and 2.13 (partially overlapped singlets, 6 H altogether, CH_3 T5 and T6), 2.17-3.06 (multiplets, 32 H, 2'-H, 2"-H and α-CH_2 of myristoyls; on the basis of the α-CH_2/β-CH_2 cross-peak seen in the COSY spectrum it was possible to assign the resonance of α-CH_2 at δ 2.47 ppm), 4.15-4.60 (multiplets, 46 H, 5'-H, 5"-H, 4'-H, 3-H_2 and 1-H_2 of glycerol; on the basis of the 1a-H/2-H, 1b-H/2-H and 3-H_2/2-H cross-peaks showed in the COSY spectrum it was possible to assign the resonance of 1a-H of glycerol at δ 4.35 and 1b-H and 3-H_2 of glycerol at δ 4.26 ppm), 4.79 (m, 1 H, 3'-H), 4.97-5.28 (multiplets, 12 H, 3'-H), 5.44 (m, 1 H, 2-H of glycerol), 5.93 (m, 1 H, 1'-H), 6.05 (m, 1 H, 1'-H), 6.22-6.58 (multiplets, 13 H, 1'-H and 5-H C4), 7.62 (s, 1 H, 6-H T12), 7.73 (s, 1 H, 6-H T1), 7.82 (s, 1 H, 6-H T14), 7.89 and 7.90 (partially overlapped singlets, 2 H altogether, 6-H T6 and T5), 8.10-8.60 (12 H; 2-H and 8-H of adenines, 8-H of guanines, 6-H of cytosine) ppm. The 3'-H T14 resonance is obscured by the residual HOD signal.

Compound **2**:

^{1}H NMR (D_2O, δ_{HOD} 4.67 ppm): δ 0.9 (br t, 6 H, CH_3 of palmitoyls), 1.16 and 1.28 (48 H altogether, from γ- to μ-CH_2 of palmitoyls), 1.8 (m, 4 H, β–CH_2 of palmitoyls), 1.92 (s, 3 H, CH_3 T12), 2.09 (s, 3 H, CH_3 T14), 2.11 (s, 3 H, CH_3 T1), 2.14 and 2.15 (partially overlapped singlets, 6 H altogether, CH_3 T5 and T6), 2.37-3.18 (multiplets, 32 H, 2'-H, 2"-H and α-CH_2 of palmitoyls), 4.25-4.70 (multiplets, 46 H, 5'-H, 5"-H, 4'-H, 3-H_2 and 1-H_2 of glycerol), 5.10-5.48 (multiplets, 13 H, 3'-H), 5.64 (m, 1 H, 2-H of glycerol), 6.12-6.68 (multiplets, 15 H, 1'-H and 5-H C4), 7.64 (s, 1 H, 6-H T12), 7.75 (s, 1 H, 6-H T1), 7.85 (s, 1 H, 6-H T14), 7.98 and 7.99 (partially overlapped singlets, 2 H altogether, 6-H T6 and T5), 8.08-8.65 (12 H; 2-H and 8-H of adenines, 8-H of guanines, 6-H of cytosine) ppm. The 3'-H T14 resonance is obscured by the residual HOD signal.

- *Compound* **3**:

  ^{1}H NMR (D_2O, δ_{HOD} 4.67 ppm): δ 0.98 (br t, 6 H, CH_3 of stearoyls), 1.24 and 1.51 (56 H altogether, from γ– to μ-CH_2 of stearoyls), 1.87 (m, 4 H, β–CH_2 of stearoyls), 2.02 (s, 3 H, CH_3 T12), 2.11 (s, 3 H, CH_3 T14), 2.15 (s, 3 H, CH_3 T1), 2.18 and 2.19 (partially overlapped singlets, 6 H altogether, CH_3 T5 and T6), 2.33-3.14 (multiplets, 32 H, 2'-H, 2"-H and α-CH_2 of stearoyls), 4.28-4.67 (multiplets, 46 H, 5'-H, 5"-H, 4'-H, 3-H_2 and 1-H_2 of glycerol), 5.06-5.39 (multiplets, 13 H, 3'-H), 5.59 (m, 1 H, 2-H of glycerol), 6.07-6.61 (multiplets, 15 H, 1'-H and 5-H C4), 7.65 (s, 1 H, 6-H T12), 7.78 (s, 1 H, 6-H T1), 7.81 (s, 1 H, 6-H T14), 7.96 and 7.97 (partially overlapped singlets, 2 H altogether, 6-H T6 and T5), 8.12-8.75 (12 H; 2-H and 8-H of adenines, 8-H of guanines, 6-H of cytosine) ppm. The 3'-H T14 resonance is obscured by the residual HOD signal.

5.6 Synthesis of unmodified sense and antisense ODNs.

Materials

Solid phase synthesis was carried out on the automated synthesizer Cyclone Plus 8400, Biosearch (Millipore).

The following products were purchased from Primm: 5'-*O*-(4,4'-dimethoxytrityl)-2'deoxythymidine-3'-(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite, 5'-*O*-(4,4'-Dimethoxytrityl)-N^6-benzyl-2'-deoxyadenosine-3'-*O*-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite, 5'-*O*-(4,4'-dimethoxytrityl)-N^6-benzyl-2'-deoxycytidine-3'-*O*-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite, 5'-*O*-(4,4-Dimethoxytrityl)-*N*-isobutyryl-2'-deoxyguanosine-3'-(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite, *Amidite Diluent (superdry acetonitrile), Deblock solution, Oxidizer solution, Activator solution, CAP A and CAP B solution.*

Glen-Pak™ DNA Purification Cartridge 3g was purchased from Glen-Research.
Acetonitrile, ammonia, triethylamine acetate, sodium chloride, trifluoroacetic acid and ammonium hydroxide were purchased from MERCK.

Solid phase synthesis of d(TGGCTTGAAGATGT) and d(ACATCTTCAAGCCA)

The currently used phosphoramidite protocol for solid phase synthesis of oligonucleotides was applied. After complete elongation, DMT-ON oligos were detached from the support and β-cyanoethyl, togheter with the acyl groups, were removed from phosphate and nucleobases by using ammonium hydroxide (r.t. for 1 h, then 55 °C for 4 h) Each oligo was recovered in 5 mL of the ammonia solution. To this solution another 5mL of ammonia solution and further 10mL of 100 mg/mL Sodium Chloride solution were added to a total volume of 20mL. Each oligo/salt mixture, in 10mL aliquots, was taken to a Glen-Pak™ DNA Purification Cartridge previously conditioned flushing with 10mL of Acetonitrile followed by 20mL 2M Triethylammonium acetate. During the loading process, the DMT-ON oligos tend to stick to the cartridge packing material while most of the failure sequences are not retained.

The cartridge was washed with 2 x 10mL of 5% Acetonitrile in 100 mg/mL Sodium Chloride solution. After, the cartridge was rinsed with 2 x 10mL of 2% Trifluoroacetic acid (TFA) aqueous solution to remove the DMT from the oligonucleotide.

Finally the cartridge was washed with 2 x 10mL of deionized water. to rinse away the TFA and excess salts. The purified oligo was then eluted using 1 x 10mL of 50% acetonitrile in water containing 0.5% ammonium hydroxide (The dilute ammonium hydroxide was utilized to neutralize any remaining TFA).

5.7 Melting Temperature measurements by DSC

Materials

DSC scans were carried out on a Setaram micro differential scanning calorimeter (microDSC III) equipped with stainless steel 1 mL sample cells, interfaced with a BULL 200 Micral computer.
Sodium dihydrogen phosphate, Disodium hydrogen phosphate, Sodium chloride and Ethylenediaminetetracetic Acid (EDTA) were purchased from Merck.

Thermal analyses

Both the sample and reference cells were heated with a precision of 0.08°C at the scanning rate of 1°C min–1. The oligonucleotide concentration was 5 μM in all the experiments. In order to obtain the excess heat capacity curves, buffer–buffer base lines were recorded at the same scanning rate and then subtracted from sample curves. Calibration in energy was previously obtained by dissipating a well defined amount of power, electrically generated by an EJ2 Setaram Joule calibrator within the sample cell. All DSC measurements were performed in nitrogen atmosphere.
Each of the oligomers which had to be annealed (*sense* ODN and *antisense* phosphatidil-ODN) were dissolved in the appropriate volume of 10 mM sodium phosphate buffer, pH 7.0, containing 100 mM sodium chloride and 0.1 mM EDTA, at a final concentration of 10 μM. Immediately before the analysis, equal volumes of the two solutions were mixed together and the resulting solution was placed in the sample cell of the instrument.
In a series of DSC scans, both cells were first loaded with buffer solution, equilibrated at 20°C for 15 min, and scanned from 20 to 90°C at a scan rate of 60 °C/h. The buffer versus buffer scan was repeated once and upon the

second cooling, the sample cell was emptied, rinsed, and loaded with the duplex solution prior to the 15 min equilibration period. This procedure was followed for the all three duplex solutions after each previous solution sample had been scanned twice to check for reversibility. In loading of the sample cell with the duplex solutions care was taken to minimize the presence of air bubbles. The data were recorded every 2 s. After completion of a series of DSC scans, the second buffer versus buffer scan was used as the baseline scan and subtracted from the duplex versus buffer scans prior to analysis.

The *Tm* values measured for each compound were: 54,3, 55,3, 53,2 and 45,2 °C for duplexes from unmodified ODN and compounds **1**, **2** and **3** respectively.

5.8 Biological assays

5.8.1 *Evaluation of antisense activity on Neuroblastoma cells*

Materials

Neuroblastoma SHSY-5Y cells line, the buffered solution for cell culture, Trizol reagent, reverse transcriptase (M- MLV), primer and polymerase were purchased from Invitrogen.

Agarose was purchase from Merck.

Procedure

Cells of human neuroblastoma cell line SH-SY5Y were cultured in 1 : 1 Ham's F12 : Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 50 µg/ml penicillin, and 100 µg/ml streptomycin and kept at 37°C in humidified 5% CO_2/95% atmosphere. For each of the administered phosphatidyl-ODNs three well plates were used. Phosphatidyl-ODN **1** was administered at 15, 10 and 5 µM

concentration while only 10 or 5 μM of phosphatidyl-ODN **2** were used. The concentrations of native ODNs used as a controls were of 30, 20 and 10 μM.

After 60 h incubation the growth media was removed from culture dish, and 1 mL of TRIzol® reagent (guanidine isothiocyanate - phenol) per 10 cm^2 of culture dish surface area was added directly to the cells adhering to the culture dish. The cells were lysed by pipetting them up and down several times directly in the culture dish.

The lysed sample was incubated for 5 minutes at room temperature to allow complete dissociation of the nucleoprotein complex.

Next, 0.2 mL of chloroform were added per 1 mL of TRIzol® reagent previously used for cell lysis. The sample was shaken, incubated for 2–3 minutes at room temperature and then was centrifuged at 12,000 × *g* for 15 minutes at 4°C.

The mixture was separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA remained exclusively in the aqueous phase.

To this aqueous phase 0.5 mL of 100% isopropanol were added per 1 mL of TRIzol® reagent used for the previous cell lysis. The samples was incubated at room temperature for 10 minutes and then centrifuged at 12,000 × *g* for 10 minutes at 4°C. The supernatant was removed from the tube, leaving only the RNA pellet.

The pellet was washed with 3 mL of 75% aqueous ethanol solution and the RNA sample was briefly vortexed prior to be centrifuged at 7500 × *g* for 5 minutes at 4°C. The RNA pellet was dried in air dry. To re-suspend the RNA pellet 50 μL of RNase-free water were added and the solution was passed up and down several times through a pipette tip. Spectrophotometric determination of the absorbance value at 260 nm and the A_{260}/A_{280} ratio allowed us to determine the amount and purity of the isolated RNA. The concentration of RNA ranged between 0.45 and 0.5 μg/μL.

RNA reverse transcription

One μL of total RNA, 1 μL of random primer, 10 μL of DEPC-treated water were placed in a sterile tube and, after mixing well, incubated at 70 ℃ for 5 min. Into the tube were added 4 μL of 5× reaction buffer, 1 μL of inhibitor, 2 μL of 10 mM dNTP orderly. After being mixed well and incubated at 25 ℃ for 5 min, to the reaction mixture was added 1 μL (100 U) of M-MLV reverse transcriptase, incubated at 25 ℃ for 10 min and at 42 ℃ for 60 min. The reaction was stopped by heating at 95 ℃ for 10 min, and subsequently for 5 min at 4°C. The resulting solution contained the desired total cDNA.

PCR reaction

PCR was performed in a total volume of 100 μL, containing 5 μL of the previously obtained cDNA solution, 5 pmol of each upstream and downstream primer for VEGF gene , and 1.8 units of Taq polymerase. The cycle program was as follows; 30 runs of denaturation at 95°C for 45 s, annealing at 60°C for 1 min, and elongation at 72°C for 1 min. The cycle program was preceded by an initial denaturation at 95°C for 3 min and followed by a final extension at 72°C for 5 min.

PCR products were analysed by 1.0% agarose gel electrophoresis with 1x TBE buffer and visualized with SYBR (cyanine dye).

5.8.2 Viability tests (MTT ASSAY)

Materials

Neuroblastoma SHSY-5Y cells line and the buffered solution for cell culture were purchased from Invitrogen.

MTT was purchased from Sigma-Aldrich.

Procedure

To evaluate cell viability the MTT assay was used. Briefly, Neuroblastoma cells (1 x 10^5) were palced in flat-bottomed 200 µL microplates, and incubated at 37°C in a humidified 5% CO2/95% air mixture for 24 h. Then, they were treated for 60 h with either individual phoshatidyl-ODNs (**1**-**2**) (as ammonium salts) or with unmodified ODNs. Solutions of phosphatidyl-ODNs were prepared by dissolving in the culture medium each previously lyophilized compound.

Cell viability tests were performed on cells treated with a) unmodified ODNs at 30, 20 and 10 µM concentrations; b) compound **1** at 15, 10 and 5 µM concentrations; c) compound **2** at 10 and 5 µM concentrations. After incubation the culture medium was removed. Cell nuclei were stained using Hoechst 33258 (20 µg/ml diluted in assay buffer: NaCl 137 mmol/L, KCl 5 mmol/L, Na_2HPO_4 0.4 mmol/L, $NaHCO_3$ 4 mmol/L, glucose 5.5 mmol/L, $MgCl_2$ 2 mmol/L). The culture plates were then visualized under fluorescence microscopy (excitation 352 and emission 449 nm), and cell quantification was performed. Tissue area was quantified using Leica Qwin software calibrated as the manufacturer's instructions. The cellular density was expressed as the total cell number per vessel area.

After cell quantification the medium was removed and 20 µL of 0.5% MTT in phosphate buffer saline was added to each microwell containing 180 µL of fresh medium. After 4 h incubation, the supernatant was removed and replaced with 200 µL DMSO to completely dissolve the formazan precipitates. The optical density of each sample was measured by a spectrophotometer set at 570 nm. Each experiment was performed in quadruplicate and repeated twice.

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