Perspectives for Medicinal Applications of Synthetic Oligonucleotides: Antisense Technology

Wojciech J. Stec
Piotr Guga
Centre of Molecular and Macromolecular Studies
Department of Bioorganic Chemistry
Polish Academy of Sciences
Łódź, Poland

Most people don't appreciate what an enormous development it was to be able to synthesize oligonucleotides. It's playing out now in every possible disease. Everybody's research in molecular biology depends upon solid-state synthesis of nucleic acids. Every single lab you go to uses that technology.

Harold E. Varmus (C&EN, 1994, January 3, p. 18-21)

1. Introduction

The other half of this century brought a number of significant discoveries in the field of modern biology such as the elucidation of the structure and function of nucleic acids (1), the discovery of transfer RNA and the recognition of its sequence-specific hybridization to transient messenger RNA *via* an anticodon segment (2), deciphering of genetic code (3) and the introduction of nucleic acids sequencing methods (4). Discoveries of fundamental importance also included the discovery of small circular DNA molecules (plasmids) (5), the understanding of the function of polymerases (6), the discovery of restriction enzymes (7), cloning of synthetic DNA and expression of foreign "genes" in bacteria and eukaryotic cells (DNA-recombinant technology) (8), the discovery of enzymatic properties of RNA (ribozymes) (9). Efforts of geneticists, immunologists and enzymologists were supported by molecular approach of chemists and physicists, and, undoubtedly, the design of efficient chemical methods of synthesis of oligonucleotides (10) had a tremendous impact on the whole molecular biology. One particular aspect of broad availability of synthetic oligonucleotides will be discussed in this

review. Their primary application concerns the enzymatic construction of "synthetic" genes and their expression leading to the bulk production of natural proteins (some of them, such as erythropoietin (11), granulocyte-colony-stimulating factor (12), insulin (13) and human growth hormone (14) have been shown to be effective in several therapeutic applications). Synthetic oligonucleotides have also proven to be indispensable tools for biologists as hybridization probes and PCR-primers (15), useful not only for studies of cell biology, but also for practical detection of genetic disorders (16). These pioneering studies paved a way to *gene therapy* (17), one of the most challenging problems of medicine of the end of this century. *Gene therapy* is discussed in accompanying papers presented during this Symposium (18-21). Another broad field of application of synthetic oligonucleotides, generally accepted today as *antisense technology* (22), will be discussed in this and accompanying presentations (23-28).

The concept of antisense technology evolved after seminal discovery by Zamecnik and Stephenson (29), that the treatment of cells infected with Rous Sarcoma virus with short oligonucleotides complementary to selected fragments of viral mRNA inhibits viral replication and cell transformation (1978). In his pioneering experiments, Zamecnik used oligonucleotides (13 mers) at concentration 20 μM (30). Such high initial concentration of oligonucleotides, unacceptable from the perspective of medicinal application, resulted from the fact that oligonucleotides in biological media undergo nucleolytic degradation, and their activity as specific gene suppressor requires massive excess of oligonucleotides. The solution to this problem, resulting from the work of many researchers, involved the applications of chemically modified oligonucleotides.

2. Antisense Strategies

To understand the principle of antisense technology, some basic knowledge of protein biosynthesis is necessary. The structures of all proteins, functional and constitutional for every living cell, are encoded in natural biopolymers known as DNA. Deoxyribonucleic acids (the size of these biopolymers ranges from a few million nucleotides for bacteria to ca. five billion for humans) are polyphosphodiesters with 3',5'-(2'-deoxyribose-phosphate) backbone with four nucleobases: thymine (Thy), cytosine (Cyt), guanine (Gua) and adenine (Ade) attached to 2'-deoxyribose at 1'-position. The sequence of nucleobases constitutes the so called genetic code which can be translated onto the sequence of amino acids in proteins via a multistep intracellular process (31). First, the sequence of the DNA strand is transcribed into the sequence of RNA (which differs from DNA by replacement of 2'-deoxyribose by ribose and thymine by uracil). The RNA, which is much less stable than DNA, constitutes a matrix on which the ribosomal machinery, with the participation of small transfer ribonucleic acids, amino acids, nucleoside triphosphates, and several proteins known as elongations

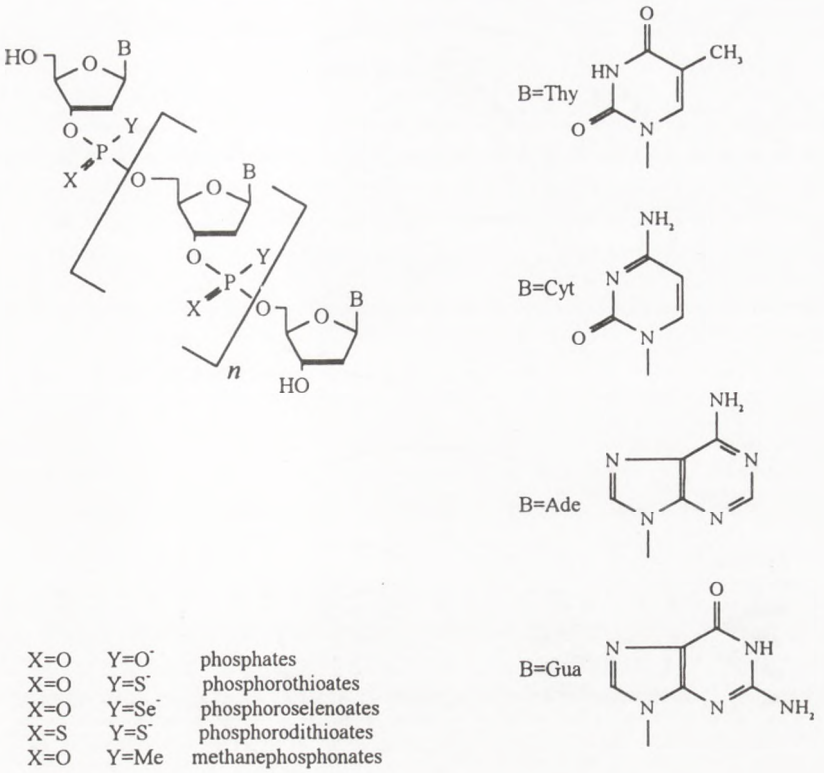

Fig. 1. Schematic representation of natural and modified oligonucleotides.

factors, assembles corresponding proteins. The molecules of RNA are known as the *sense strands*. The nucleobases in DNA and RNA molecules are able to interact with one another *via* hydrogen bonds to form duplex-, triplex-, or tetraplex-structures (32). Molecules of DNA mostly exist as antiparallel dimers, while molecules of RNA do possess secondary structures formed by single stranded RNA, resulting in double stranded-, loops- and hair-pin-motifs. The hydrogen-bond type interactions between nucleobases, one of the most powerful recognition systems created by nature, are crucial for the **antisense technology**. If the synthetic oligonucleotide possessing the base sequence complementary to a segment of mRNA (a mature form of RNA, messenger RNA) enters the cell and binds to that mRNA target sequence, it may bring about selective inhibition of gene expression. If a product of such gene expression (protein) is vital for the organism or cell, the inhibition may result in the organism's cell's death. If the biological system is a cancer cell, and the target mRNA derives from a cancer-specific oncogene, then the cancer cell may selectively be killed. Such approach provides that selectivity is maintained and exogenous oligonucleotide does not inhibit the expression

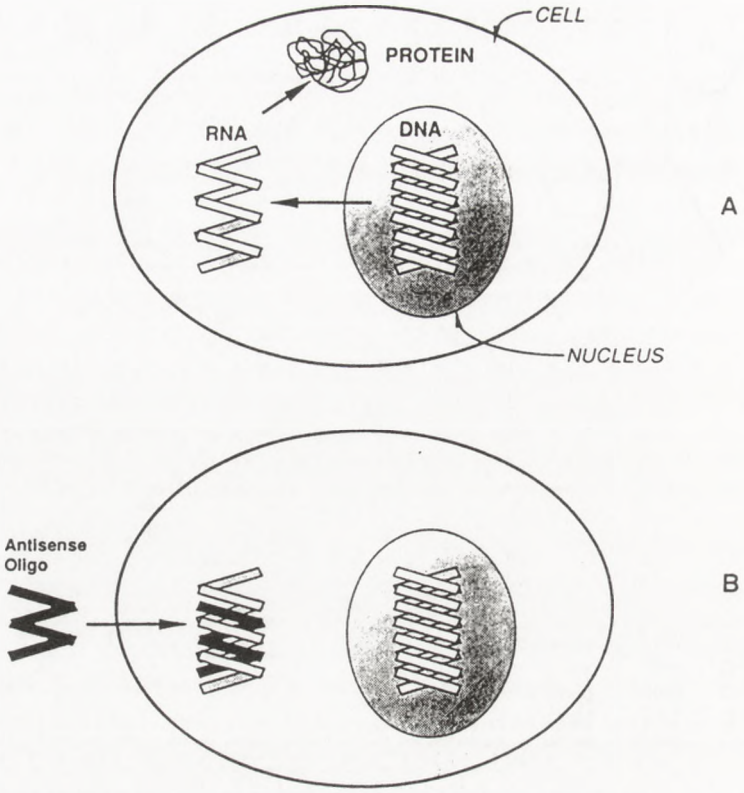

Fig. 2. **A.** Schematic representation of the process of protein synthesis, proceeding from DNA to mRNA (*transcription*) to protein (*translation*). **B.** The intervention of an antisense oligodeoxyribonucleotide, that crosses the cell membrane, binds to a complementary sequence on the target mRNA and prevents translation (*translation arrest*).

of any normal genes; therefore normal cells are spared. If the target is a viral gene, the complementary oligonucleotide should act as a selective antiviral drug (33).

Basic assumptions built into this approach are as follows:

1. Oligonucleotide can cross the cell membrane and is able to reach its target sequence in the cell (**cellular uptake**).
2. Oligonucleotide is stable under *in vivo* conditions and reaches the target sequence in sufficient quantity (**stability**).
3. Oligonucleotide recognizes and hybridizes with the target RNA sequence so the DNA-RNA hybrid is produced (**hybridization**).
4. Formation of this hybrid prevents the expression of gene(s) coded by the hybridized mRNA (**inhibition of expression**).
5. Oligonucleotides should not bind non-selectively to any other sites, particularly proteins, (**selectivity of binding**).

3. Oligonucleotide Analogues

The aforementioned observation by Zamecnik and Stephenson that sequence-specific oligonucleotides inhibit viral replication and cell transformation preceded the effective phosphoroamidite approach to oligonucleotide synthesis. Synthetic oligonucleotides have become available since 1982 (34). Soon it became clear that high concentration of administered oligonucleotides is necessary for effective inhibition of viral replication, because oligonucleotides are hydrolyzed by nucleases present in cells and body fluids. It was also shown that the uptake of ionic oligonucleotides occurs *via* both fluid-phase pinocytosis and adsorptive endocytosis, but the cellular plasma membrane proteins involved in the process of uptake and action of antisense oligonucleotides are still not well characterized (35). Only recently two oligodeoxyribonucleotide binding proteins on plasma membranes of human cells have been identified (36). It has also become obvious that certain alterations within oligonucleotide structures are necessary to prevent the process of enzymatic hydrolysis. These modifications have had to meet the requirements of cellular uptake, hybridization, inhibition of expression, selectivity of binding and non-toxicity to target cells. The principle of *biocompatibility* has excluded modification of nucleobases and their sequence to keep them able to hybridize to the target mRNA. Similarly, the sugar-phosphate backbone, responsible for conformational properties and hybridization ability of oligonucleotide, should not be changed. Therefore, the most obvious site of modification was the phosphate moiety. There are two classes of phosphate modified oligonucleotide, most significant to the development of antisense technology:

1) phosphorothioate analogues of oligonucleotides, oligo(nucleoside phosphorothioate)s, (**PS-Oligo**, Fig. 1. X=O, $Y=S^-$) (37),

2) methanephosphonate analogues of oligonucleotides, oligo(nucleoside methanephosphonate)s, (**Me-Oligo**, Fig. 1. X=O, Y=Me) (38).

PS-Oligos, usually 15-30 nucleotides in length, have each a phosphate moiety modified by replacement of one-of-two nonbridging oxygen atoms by sulphur. They retain the negative charge, and therefore, like natural oligonucleotides, are polyelectrolytes. Eckstein (39), Benkovic (40) and Frey (41) showed that phosphorothioate analogues of DNA are much more resistant to nucleolytic action of cellular enzymes. In recent studies it has been found that they are relatively non-toxic towards primates (42). Their cellular uptake is similar to that of unmodified oligonucleotides, while their hybridization to target mRNA is a bit less effective (lower T_M) compared to natural oligonucleotides (43). This failure can be compensated by size extension (more hydrogen-bonding type interaction). Their chemical synthesis, for the first time performed in 1984 (44), is relatively simple and has been scaled-up to multigram quantities per day (45). PS-Oligos can be synthesized (Fig. 3) by a relatively minor adaptation of the standard procedure using exactly the same phosphoramidite monomers (34). Instead of the oxidation step, a sulfurization procedure is performed, typically with 3H-1,2-benzodithiole-3-one

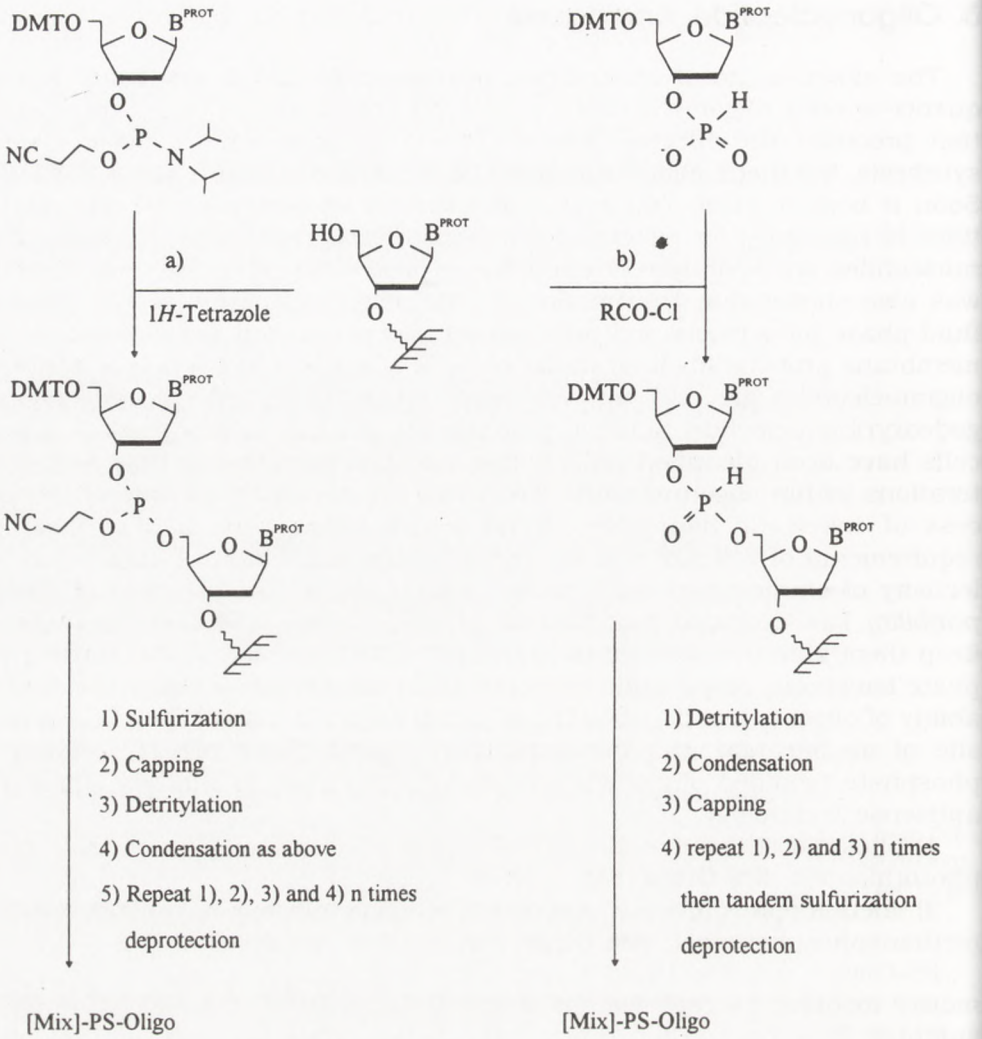

Fig. 3. Phosphoramidite a) and H-phosphonate b) methodologies routinely used in the non-stereocontrolled synthesis of PS-Oligos.

1,1-dioxide (*Beaucage reagent*) (46), tetraethylthiuram disulfide (TETD) (47), or bis(diisopropoxyphosphinothioyl)disulphide (*S-Tetra*) (48), although plain solution of elemental sulphur in pyridine or lutidine can also be used (44). PS-Oligos can also be obtained *via* H-phosphonate approach and in this case the sulphurization step is performed only once after the elongation of oligomer is complete (45).

Recent studies on the mechanism of protein biosynthesis inhibition have shown that hybrids formed between PS-Oligos and target mRNAs are the substrates accepted by Ribonuclease-H (RNase H) (49), the enzyme which

recognizes RNA-DNA heteroduplexes and selectively cleaves mRNA, destroying the matrix for the biosynthesis of corresponding proteins.

There are numerous examples of efficacy of PS-Oligos in *in vitro* systems (50), and recently several reports appeared on their *in vivo* activity. First clinical trials are underway for combating AIDS and several types of cancers (51). According to the 1995 survey by *The Pharmaceutical Research and Manufactures of America* (Washington) (52), these are as follows (see Tab. 1):

TABLE 1
BIOTECHNOLOGY MEDICINES IN DEVELOPMENT

Antisense			
Product Name	Company	Indication	U.S. Development Status
afivirsen (ISIS 2105)	ISIS Pharmaceutical (Carlsbad, CA)	genital warts	Phase II
GEM 91	Hybridon (Worcester, MA)	primary HIV infection and AIDS	Phase II
ISIS 2302	ISIS Pharmaceutical (Carlsbad, CA)	inflammatory diseases	Phase I
ISIS 2922	ISIS Pharmaceutical (Carlsbad, CA)	cytomegalovirus (CMV) retinitis in AIDS patients	Phase III
LR-3001	Lynx Therapeutics (Hayward, CA)	chronic myelogenous leukemia (CML) in accelerated phase or blast crisis	Phase I
LR-3280	Lynx Therapeutics (Hayward, CA)	anti-vestenosis post-PTCA	Phase I

Me-Oligos, like PS-Oligos, are up to 15 nucleobases in length and they possess methyl groups directly bound to the phosphorus atoms. Unlike natural oligonucleotides and PS-Oligos, Me-Oligos are non-ionic species, and are completely resistant to enzymatic degradation. They are also more lipophilic which should facilitete their cellular uptake. However, the lipophilicity gain has to be balanced with limited solubility of Me-Oligos in physiological media. There are numerous reports on their *anti-herpes* and *anti-ras* oncogene activity (53). Interestingly, hybrids formed between Me-Oligos and mRNA are not substrates for RNase H (38), so the mode of their biological activity is unclear, albeit simplified picture implies that by "bulk effect" Me-Oligos stop ribosomal activity. Very recently, however, Kole et al. (54) reported that RNA hybridized to the modified oligonucleotides of a different type, namely 2'-O-methyloligoribonucleotides, and incubated in nuclear ex-

tracts from HeLa cells, is truncated, and the activity responsible for this effect is not RNase H but another nuclease degrading RNA in the 3' to 5' direction. Thus, a spectrum of intracellular mechanisms potentially applicable for the cleavage of selected mRNA in cells cannot be considered as fully recognized.

Like PS-Oligos, Me-Oligos are produced by means of "gene-machines" (automated solid phase synthesis) (55), and they are now available in bulk quantities, enabling preclinical and clinical studies.

4. Stereocontrolled Synthesis of PS-Oligonucleotides and Me-Oligonucleotides

The common feature of PS-Oligos and Me-Oligos is their **diastereoisomerism** (56). The elemental replacement of a nonbridging oxygen at internucleotide phosphate moiety creates the centre of asymmetry. Standard synthetic methods are slightly stereoselective and they typically lead to a nonequimolar mixture of two diastereoisomeric forms (see Tab. 2). The oligonucleotide containing **n** modified internucleotide bonds consists of 2^n diastereoisomers.

TABLE 2
PERCENT OF THE [R_P] ISOMER IN THE DINUCLEOSIDE(3',5')PHOSPHOROTHIOATES (NpsN')

N'/N	dA	dG	dC	dT
dA	51.7	42.6	53.4	53.8
dG	55.3	51.0	55.2	56.0
dC	57.0	48.6	59.7	62.7
dT	58.6	50.8	60.0	59.6

Dinucleoside (5',3')phosphorothioates (NpsN' where N is at the 5'-end) were prepared using the standard coupling method (DNA synthesizer - Applied Biosystems — model 380B), followed by sulphurization with a 0.08M solution of bis(diisopropoxyphosphinothioyl)disulphide (48) in acetonitrile:pyridine (2:1 v/v) for 45s (approx. 400 µl). 5'-Dimethoxytrityl group was removed in a final step of the synthesis. The crude mixtures were analyzed by means of HPLC using a Supelcosil RP-18 column (5, 2.3mm x 250mm) and a gradient of acetonitrile in 0.1M TEAB from 0%, 0.5% CH_3CN/min, flow rate 1ml/min. Coupling efficiency calculated from the chromatogram was not lower than 98%.

The calculated ratio of particular diastereoisomers in the mixture can be derived from the relevant polynomial equation (57). Furthermore, it has been found that diastereoisomers of PS-Oligos, at least up to a pentamer, synthesized under conditions of the routine synthesis of PS-Oligos, are formed in nonequal quantities. For example, in the case of [mix]-d[$(C_{PS})_4$C], containing total 2^4 = 16 diastereomers, the ratio of [All-R_P] to [All-S_P] diastereoisomer is 5:1. Since each single component of this mixture constitutes a

unique molecule characterized by its chirality, in terms of molecular recognition different diastereoisomers may interact with target RNA, or proteins, in different ways. It has to be emphasized that in that respect the problem of *diastereoisomerism* of PS-Oligos and Me-Oligos was generally unappreciated, and most so-far reported results on the biological efficacy of PS-Oligos and Me-Oligos were obtained using the so called "random mixtures of diastereoisomers". The possibility that only a fraction of diastereoisomers is responsible for the sequence-specific activity of PS-Oligo, while the rest of them constitute a ballast responsible for **non-specific activity** (58) or some side effects (toxicity) was not addressed. It was assumed (59) that such mixture contains close to proportional ratio of diastereoisomers of PS-Oligos or Me-Oligos (what is not true — *vide supra*), and there is no selectivity during their transport to the cells. There are several questions at this point: 1) whether the transport is or is not stereoselective; 2) if interactions of diastereoisomers with cellular proteins are stereoselective; 3) if the efficacy of interaction with target mRNA depends upon chirality of modified oligonucleotide and, 4) whether the stability of particular diastereoisomers towards cell nucleases is comparable. Recently Arnold et al. presented results indicating that hybrids containing [R_P]-dinucleoside 3', 5' methanephosphonates incorporated in longer oligonucleotide constructs possess much more promising properties as antisense probes than analogous molecules with random stereochemistry of methanephosphonate fragments (60). This observation indicates that stereochemistry of the antisense probe plays a certain role in the molecular recognition process. Nonetheless, all questions mentioned above can be answered only if methods enabling stereocontrolled synthesis of PS-Oligos and/or Me-Oligos are established.

4.1. Stereocontrolled synthesis of Me-Oligos

In the Authors' Laboratory some efforts were undertaken to develop stereocontrolled methods of synthesis of both PS-Oligos and Me-Oligos (61-64). Yet, the efficient stereocontrolled synthesis of Me-Oligos is still in its infancy (65). An earliest effective stereocontrolled method reported in the literature is the one designed in this Laboratory (63); its concept is based on stereospecific nucleophilic substitution at phosphorus atom involved in diastereomerically pure 5'-*O*-MMT-nucleoside 3'-*O*-[*O*-4-nitrophenyl methanephosphonates] as the substrates. Their reaction with 5'-hydroxy-3'-protected nucleosides(tides) occurs in the presence of t-butyl magnesium chloride with high stereospecificity (>95%) and moderate chemical yield (>70%). Using this approach the dimers, trimers and tetramers of predetermined stereochemistry (sense of P-chirality) were obtained. Although with poor efficiency, stereoregular tetrathymidylic components were condensed *via* non-stereospecific process and octa(thymidine methanephosphonate)s and [All-R_P-except-one]- and [All-S_P-except-one] configuration were obtained (66). These constructs were used for demonstration, that their avidity to the complementary dA_{15} is stereodependent, as measured by melting temperature (T_M) of the corre-

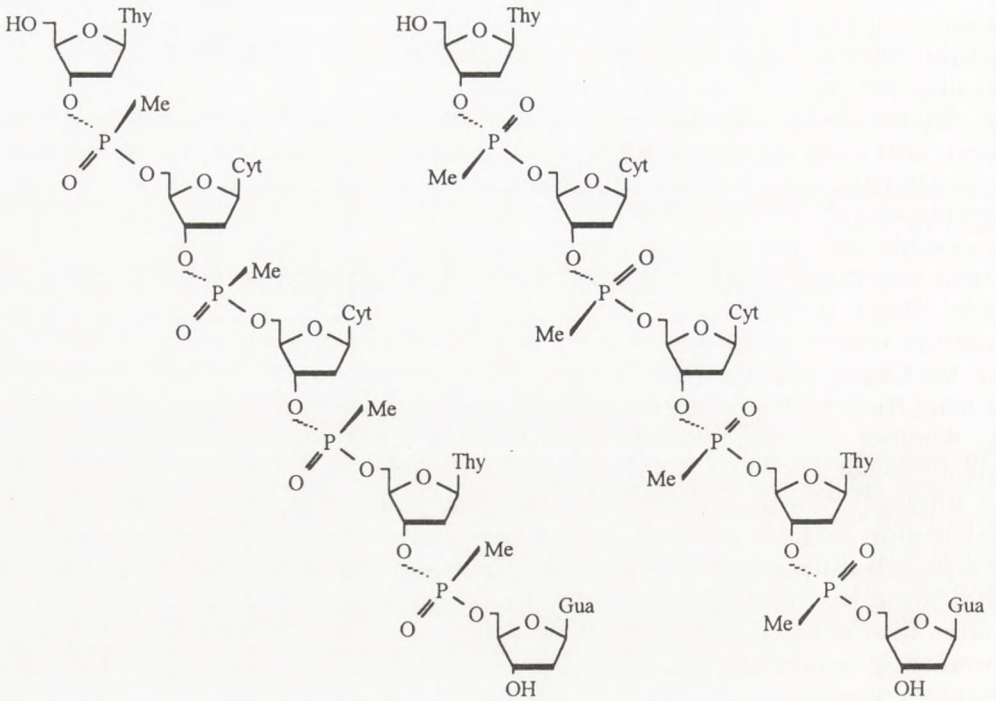

Fig. 4. Diastereoisomerically pure [All-R_P]- and [All-S_P]- penta(nucleoside methanephosphonate)s of sequence TCCTG obtained by Woźniak et al.

sponding heterodimers. A significant increase of T_M in the case of [All-R_P-except-one]-octamer [T_M 38°C], as compared with that for [All-S_P-except-one]-octamer [T_M<5°C], and for [Mix]-d[$(T_{PMe})_7$T] (a mixture of 128 diastereoisomers, [T_M 13°C]) strongly supports a hypothesis that appropriate chirality of Me-Oligos may enhance the hybridization potential and in this way it may increase the chance for the specific binding of antisense constructs to the target mRNA.

Modification of the methodology presented above was published by Cormier (67). He was looking for a replacement for the p-nitrophenoxy group that would be stable to chromatographic purification of monomers, but at the same time be a sufficiently good leaving group to allow the coupling reaction to proceed. Cormier recommended the use of 1,1,1,3,3,3-hexafluoro-2-propanoxy substituent which increased the yield and efficiency of resolution of desired monomers. Unfortunately, in this particular case the methodology employing a Grignard reagent could not be applied to the solid phase synthesis of Me-Oligos. The major limitation was the fact that the mixture of the Grignard reagent with alcohol, which was to be activated, was a typical viscous suspension or slurry, while a successful coupling reaction requires

rapid diffusion of the reactants into the pores of the support. However, recent work by Wickstrom and Le Bec indicates that the replacement of routinely used solid support such as LCA-CPG with non-porous polystyrene beads coated with a surface layer of polyethylene glycol and loaded with deoxynucleoside may enable stereocontrolled solid phase synthesis of Me-Oligos (65). Unfortunately, the experimental evidence for feasibility of this modification is limited to a description of stereocontrolled synthesis of dinucleotide. Another method elaborated in the authors' laboratory (64) is based upon the process of nucleophilic substitution at methanephosphonate phosphorus atom with methylselenyl as the leaving: group it allowed to obtain a mixed sequence penta(nucleoside methanephosphonate) in [All-R_P]- and [All-S_P]-diastereoisomerically pure forms (Fig. 4). However, this method so far has not been adapted to the requirements of the solid phase synthesis (68).

4.2. Stereocontrolled synthesis of PS-Oligos

Current approaches to stereocontrolled synthesis of PS-Oligos are summarized in recent reviews (56,69-71). Space limitation does not allow us to present them here in detail, but the only effective method, so far presented in the chemical literature, is based on a novel methodology developed in authors' laboratory (62,72). This method employs (Fig. 5) the reaction of 2-alkoxy-2-thiono-1.3.2-oxathiaphospholanes with alcohols in the presence of strong organic bases such as DBU to give O,O-dialkyl phosphorothioates (73).

The corresponding nucleotide monomers, 5'-*O*- and base-protected nucleoside 3'-*O*-[2-thiono-(1.3.2-oxathiaphospholanes)], were prepared and separated into diastereomerically pure forms. Their reaction with nucleosides bound *via* 3'-oxygen to solid support appeared to be stereospecific (>98%) and gave dinucleoside (3',5')-phosphorothioates with preparative yield >94%. Using a DNA synthesizer, several stereoregular ([All-R_P]- or [All-S_P]-configuration) oligonucleotides such as d[$(C_{PS})_9$C], d[$(T_{PS})_{11}$T], d[$(A_{PS})_{11}$A] and d[$G_{PS}G_{PS}G_{PS}A_{PS}A_{PS}T_{PS}T_{PS}C_{PS}C_{PS}$C] (Eco RI sequence) were obtained. With these oligomers in hands, it became possible to investigate the influence of chirality of PS-Oligos on the stability of their corresponding duplexes. Theoretical considerations (74) suggested that double stranded PS-Oligo/DNA helices (presumably existing in B conformation) involving [All-S_P]-PS-Oligos should be more stable than those with [All-R_P] chirality. This idea has been rationalized in terms of destabilizing interactions caused by higher steric demands of the sulfur atom (compared to the oxygen atom) directed *inward* in the double helix with the [R_P]-PS-Oligo. Furthermore, if the negative charge in the phosphorothioate anion is localized on the sulfur atom, its *inward* orientation may further destabilize the duplex by causing stronger repulsion of negative charges on both complementary strands. Those intuitive predictions, based on few experimental results from mono phosphorothioate-modified oligonucleotide models, were supported by the results of molecular modeling and theoretical calculations performed by Jaroszewski et al. (75). Indeed, we found that the duplex formed with [All-S_P]-d[$(A_{PS})_{11}$A]

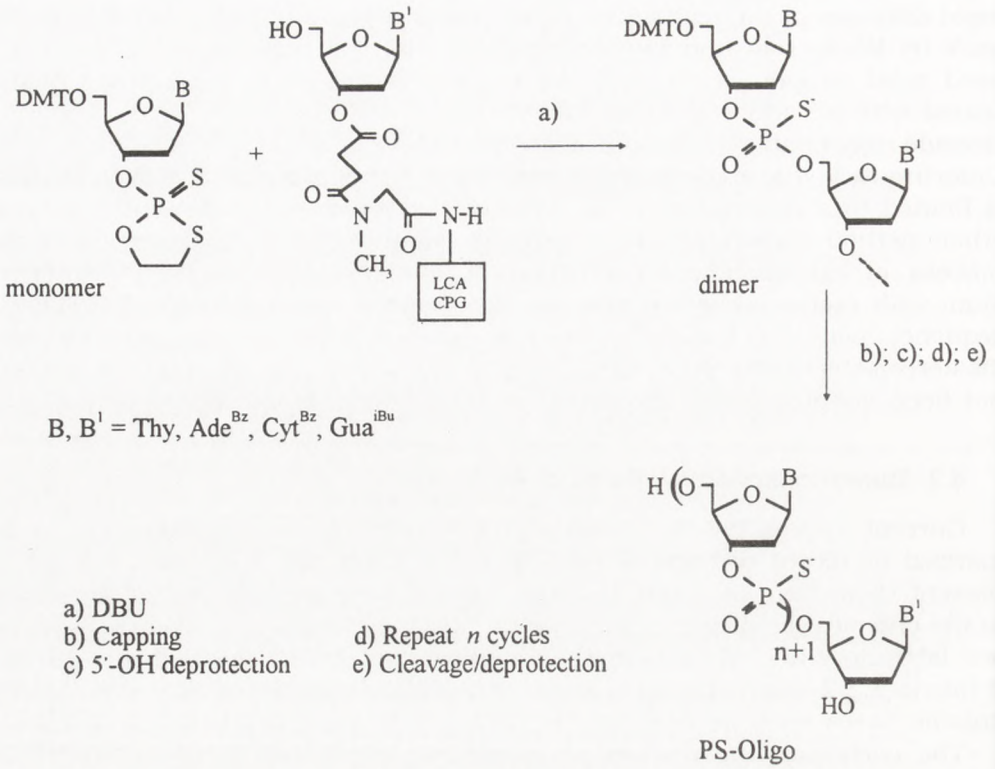

Fig. 5. Schematic representation of stereocontrolled "oxathiaphospholane method" of synthesis of PS-Oligo.

and dT_{12} is more stable (Tm=34°C) than either duplex MIX-d[$(A_{PS})_{11}$A]/dT_{12} (Tm=31°C) or [All-R_P]-d[$(A_{PS})_{11}$A]/dT_{12} (Tm=29°C). However, the duplex consisting of [All-S_P]-d[$(T_{PS})_{11}$T] and dA_{12} is, surprisingly, somewhat less stable than duplexes [All-R_P]-d[$(T_{PS})_{11}$T]/dA_{12} and MIX-d[$(T_{PS})_{11}$T]/dA_{12}. It is worth emphasizing that within the pairs of corresponding duplexes (e.g. [All-S_P]-d[$(A_{PS})_{11}$A]/dT_{12} and [All-S_P]-d[$(T_{PS})_{11}$T]/dA12, which differ only in regard to the location of phosphorothioates within particular strand of heteroduplex) the number and nature of hydrogen bonds involved in the duplex structure, as well as the orientation of the sulfur atoms within the grooves of the helix, are the same, while the duplex stabilities significantly differ (72).

Another important feature of PS-Oligos as the antisense probes is their better stability against cellular nucleases as compared to natural oligonucleotides. The difference in stability between stereoregular PS-Oligos of opposite configuration at P-atoms is particulary significant. It has been found that PS-Oligos of [R_P]-configuration are good substrates for cellular 3'-exonucleases, while those of [S_P]-configuration are resistant towards these enzymes (76).

As it was mentioned earlier, the PS-Oligo/RNA duplexes are recognized and hydrolyzed by RNase H. P-Stereoregular phosphorothioate analogues of

pentadecamer $^{5'}$d(AGATGTTTGAGCTCT)$^{3'}$ complementary to the synthetic pentadecaribonucleotide $^{5'}$r(AGAGCUCAAACAUCU)$^{3'}$, and to the fragment of the polyribonucleotide (475 nt) obtained by *in vitro* transcription using plasmid pT7-7* containing the Interleukin-2 gene inserted between Eco RI and Hind III recognition sites were prepared *via* oxathiaphospholane method (72). The DNA/RNA duplex containing the PS-Oligo of [All-R_P]-configuration was found to be more susceptible towards RNase H dependent degradation of pentadecaribonucleotide compared to the hybrids containing either the [All-S_P]-counterpart or the so called "random mixture of diastereomers" of the pentadeca(nucleoside phosphorothioate). This stereodependence of RNase H action was also observed for the polyribonucleotide hybridized with these phosphorothioate oligonucleotides. The results of melting studies of PS-Oligo/RNA hybrids allowed for the rationalization of the observed stereodifferentiation in terms of the higher stability of heterodimers formed between oligoribonucleotides and [All-R_P]-oligo(nucleoside phosphorothioate)s, compared with the less stable heterodimers formed with [All-S_P]-oligo(nucleoside phosphorothioate)s or the random mixture of diastereomers (77).

Currently, PS-Oligos of specific base-sequence are tested for their anti-leukemic and anti-viral activity.

5. Conclusions

Although antisense PS-Oligos have been reported in hundreds of articles, there are still many inconsistencies with respect to the mechanism and specificity of their action (78). For several years, the Authors' approach has been focused on structural aspects of P-chiral analogues of oligonucleotides and many efforts have been made to explain the stereocontrolled synthesis of those PS-Oligos and Me-Oligos which have shown significant activity as the "random mixture" of diastereomers. A "random mixture of diastereomers" containing **n** internucleotide phosphorothioate functions consists of 2^n diastereomers [e.g. dodacemer of d[$(A_{PS})_{11}$A] consists of 2^{11} = 2048 diastereomers]. Each particular diastereomer constitutes an individual compound possessing its own chirality and individual ability to interact with other biomolecules. Therefore, especially in the search for potential drugs, it seems indispensable to generate diastereomerically pure, or "nearly-pure", specimens and use them in comparative studies together with mixtures containing combination of all possible diastereoisomers.

The stereocontrolled synthesis of any chiral molecule has posed the challenging problem to organic chemists since Pasteur's discovery of the consequences of the asymmetry of carbon atom surrounded by four different ligands. The problem of stereocontrolled synthesis of proteins has been investigated for nearly a century and still chemists are searching for more efficient methods enabling the synthesis of oligopeptides without racemization (79). One has to notice that the C-chiral oligopeptides, synthesized using a non-stereospecific method, albeit do contain the fraction of appro-

priate chirality (ca. 2^{-n}, where **n** is a number of C-chiral centers), are very often biologically inactive. Therefore, the design of stereocontrolled methods of synthesis of P-chiral analogues of oligonucleotides is challenging and demands more efforts. It is appropriate to mention that nowadays pharmaceutical industry is entering an era of "chirotechnology revolution" (80): in many countries including the USA, Japan and the European Community, any potential drug containing asymmetry centers has to be tested in its stereochemically pure form before registration (81). Therefore, organic synthesis more and more often makes use of biocatalysts for stereocontrolled transformations of organic molecules. Unfortunately, the enzyme assisted synthesis of Me-Oligos is inefficient (82), while PS-Oligos can be prepared enzymatically with the limitation that produced poly- (83) and oligo(nucleoside phosphorothioate)s (84) are exclusively of [R_P]-configuration.

Other strategies leading to more potent antisense oligonucleotides try to avoid P-chirality by further modifications of phosphates (e.g. phosphorodithioates, Fig. 1, X=S, $Y=S^-$) (85) or to replace phosphates by achiral moieties like formacetal, sulphones, diethylsiloxanes (86) etc. To overcome the hurdle is the objective of hundreds of research establishments. Reported results give promise that the new and exciting analogue will be forthcoming for both research and therapeutic applications.

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Perspectives for Medicinal Applications of Synthetic Oligonucleotides: Antisense Technology

Summary

Pharmaceutical industry is entering the era of "chirotechnology revolution": in many countries any potential drug containing asymmetry centres has to be tested in its stereochemically pure forms. In the authors' laboratory many efforts have been made, with a certain extent of success, to stereocontrolled chemical synthesis of oligonucleoside phosphorothioates and methanephosphonates. Stability of stereoregular oligonucleotides towards selected nucleases, as well as the thermal stability of duplexes formed with DNA and RNA were investigated. Other strategies leading to antisense oligonucleotides try to avoid P-chirality by replacement of phosphates with achiral phosphorodithioates or moieties like formacetal, sulphones, diethylsiloxanes etc.

Key words:
oligo(nucleoside phosphorothioate)s, oligo(nucleoside methanephosphonate)s, stereocontrolled synthesis, antisense technology.

Address for correspondence:
Wojciech J. Stec, Centre of Molecular and Macromolecular Studies, Department of Bioorganic Chemistry, Polish Academy of Sciences, Sienkiewicza St. 112, 90-363 Łódź, Poland, fax: (042) 84-71-26, 81-54-83.