Novel oligonucleotide analogues based on morpholino nucleoside subunits – Antisense technologies: New chemical possibilities

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Even several decades after pioneer publications there is continued interest in the construction and synthesis of a variety of novel oligonucleotide analogues. The first oligonucleotide analogues which had a regular, predetermined structure containing nucleoside units joined with carbonate, carbamate, hydroxyacetate and hydroxyacetamide tethers have been developed in the 1970s. Further progress in oligonucleotide synthetic methods during the 1980s stimulated the development of a variety of oligonucleotide analogues containing modified carbohydrate and phosphate backbones. Particular attention has been given to the PNA (peptide nucleic acids), morpholino, and negatively charged PNA oligonucleotide analogues, which showed the most promise in a number of biological applications, such as diagnostics, nucleic acid analyses, and gene expression. The cost of parent compounds and oligonucleotide analogue synthesis is one of the most limiting factors to broad application. Studies that succeed in resolving this cost problem in the most effective way would be beneficial. Herein is presented a short review on oligonucleotide analogues that can be synthesized from inexpensive parent compounds — ribonucleosides — with or without the protection of heterocyclic bases, and with minimal protection of other reactive functions.

Antisense Technologies

Principles and beginnings

The antisense approach to molecular biology is based on the complementarity of the heterocyclic bases of nucleic acids – purines and pyrimidines. The "target' is a particular DNA or RNA sequence, gene, mRNA, or some heterogeneous nucleic acid (*e.g.*, viral nucleic acid). The oligonucleotide or its analogue must be complementary to the region of the target that is functionally important. Antisense oligonucleotides are designed to inhibit a biological event, such as transcription, translation or splicing in order to regulate gene expression. The idea of the so-called complementary addressed modification of nucleic acids was launched in 1967 in Novosibirsk[1]. The use of complementary oligonucleotides as antisense agents for the regulation of some biological events was also demonstrated[2,3].

Currently, the synthesis of oligonucleotides analogues is very difficult. In obtaining oligonucleotides with modified carbohydrate backbones, chemists must also preserve the biological function of the oligonucleotides — the ability to bond with the complementary nucleic acid sequence. Modifications often alter some of the properties of oligonucleotides such as their resistance to nucleases, and their ability to penetrate into cells and nuclei. Sites of possible modifications of nucleotide subunits determined during the decades following the early 1960's are schematically depicted in **Figure 1**. The modifications can also be introduced at the 3′ or 5′ end of oligonucleotide, or into the middle of the chain.

Modes of action

Two major mechanisms contribute to antisense activity: the destruction of the target nucleic acid, and the steric or covalent blockage of target sequences[4]. It seems that the main route of action is RNase H mediated cleavage of RNA. The combination of *complementary address* — oligonucleotide — and some low molecular weight RNase mimetics, so-called artificial nucleases, may be potentially interesting[5]. Ribozyme and DNA-zyme action represents another possible specific action[4]. A large number of publications are currently devoted to the action of small interfering RNAs[6]. The blockage of translation, transcription, splicing or other demonstration of antisense activity can be achieved

through unusually strong specific binding of oligo-nucleotides or analogues. Some profoundly modified oligonucleotide analogues, such as peptide nucleic acids, demonstrate increased thermal stability of complementary duplexes *in vitro*. The melting point of complementary duplexes could also be considerably increased by using an intercalator or another staple covalently joined to the addressing oligonucleotide. Chemically active functional groups such as alkylating or photoactivated structures tethered to the oligonucleotide irreversibly damage or covalently bind the target, preventing crucial biological processes[7].

Problems, chemical tasks and achievements

The antisense technology launched several decades ago encountered certain difficulties that needed to be resolved during its progress from model experiments to clinical trials. For example, the choice of the target could be made using a computer simulation of biomolecular tertiary structures, combined with knowledge of the biology of the pathogenic process or agent. Stability of the oligonucleotides and analogues in biological media and in organisms are achieved through chemical modifications of the oligo-nucleotides and their design including chimera synthesis. Researchers also apply various carriers and special physiological treatment to facilitate the penetration of the antisense agent into the cells and to the site of action. Toxicity of antisense drugs becomes
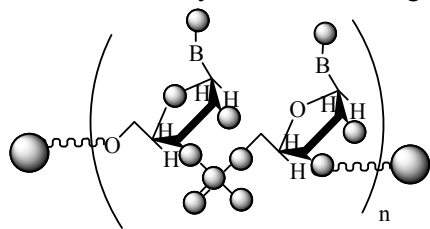
particularly important at the physiological level. Non-antisense effects such as immune response may mask or alter the true antisense mechanisms of action. All of these problems, along with a number of others, pose a serious challenge to chemists[8].

The first analogues — polyvinyl and polyacrylate-based polymers — were studied in the early 1970s. The more sophisticated constructions had a regular predetermined structure containing nucleoside units joined with carbonate, carbamate, hydroxyacetate and hydroxyacetamide tethers, and are reviewed later[9]. The progress in oligonucleotide synthetic methods during the 1980s stimulated the development of a variety of oligonucleotide analogues containing modified carbohydrate and phosphate backbones. Examples of these first generation oligonucleotide analogues — phosphotriester and phosphorothioate oligonucleotides — are depicted in **Figure 2**. To date, the phosphorothioate oligonucleotides are the most widely used, and have been studied in clinical trials and introduced in practice[10]. Common problems such as toxicity and low affinity associated with the phosphorothioate oligonucleotides have been solved to some extent by the second generation of oligonucleotide analogues shown in **Figure 2**. 2'-O-Alkylated RNA phosphorothioate analogues are the most important members of this class.

The emergence of the third generation of oligonucleotide analogues reflects the explosive progress in the search for new possibilities and new structures. Many of the challenges of antisense technologies have been solved in the last few years (**Figure 3**). Peptide nucleic acids (PNA) have a highly modified oligomer backbone. PNAs have favourable hybridization properties and high biological stability, but comparatively low water solubility and cellular uptake. "Locked" nucleic acids (LNA) also have extremely high target affinity. These types of analogues are designed primarily for the blockade of
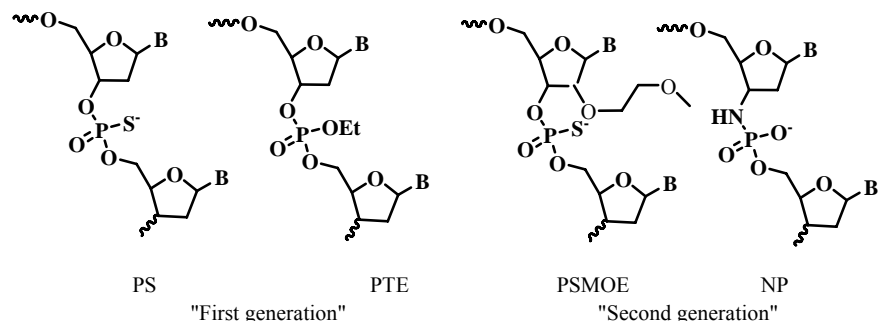

Figure 1— Possible modifications of nucleotide subunits

PS   PTE   PSMOE   NP
"First generation"   "Second generation"

Figure 2—Phosphothioate (PS), phosphotriester (PTE), phosphorothioate 2'-alkylated (PSMOE) and phosphoroamidate (NP) oligonucleotide analogues
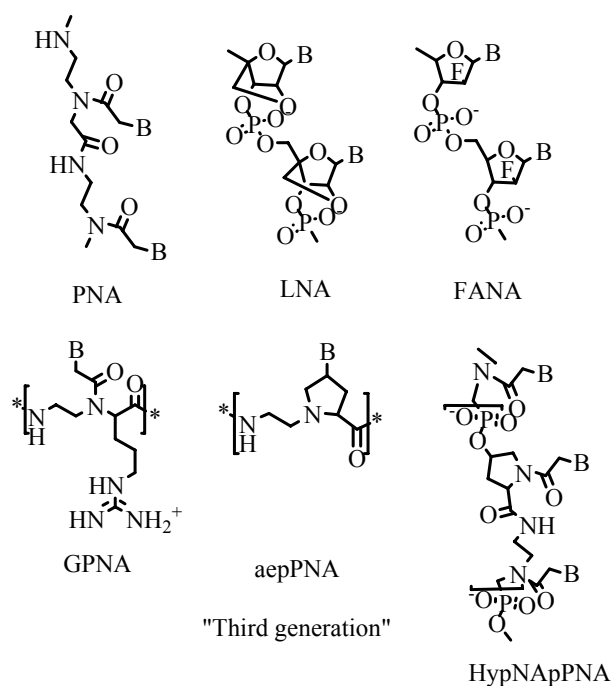
Figure 3—Peptide nucleic acid (PNA), "Locked" nucleic acid (LNA), 2′-Fluoroarabino nucleic acid (FANA), Guanidine based nucleic acid (GPNA), Aminoethylproline nucleic acid (aepPNA), and chimeric hydroxypyrrolidine-phosphate PNA (HypNA-pPNA)
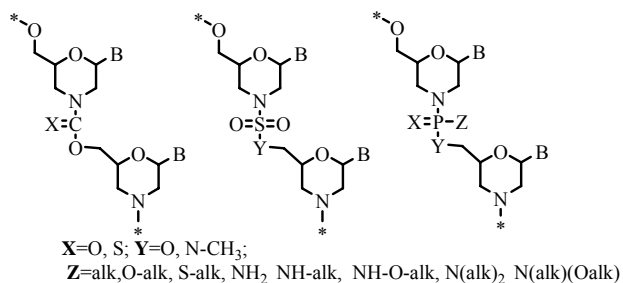

X=O, S; **Y**=O, N-CH$_3$;
Z=alk,O-alk, S-alk, NH$_2$, NH-alk, NH-O-alk, N(alk)$_2$, N(alk)(Oalk)

Figure 4—Morpholino oligonucleotide analogues

enzyme dependent processes since they do not induce RNase H activity. In contrast, 2′-fluoroarabinooligo-nucleotides (FANAs) promote RNase cleavage of their complementary RNA targets. Some examples of newly synthesized variants of PNAs are shown in **Figure 3**, such as positively charged oligomers (GPNA and aepPNA) and negatively charged chimeras (pHypNA)[11,12]. These structures are very sophisticated and usually require laborious synthetic procedures. The properties of these new PNAs depend on the stereochemistry of the subunits (not shown in the picture). Nevertheless, such oligonucleotide mimetics demonstrate excellent water solubility, high cellular uptake, increased affinity for the comple-

mentary target, and an apparent discrimination between DNA and RNA targets[13].

Another class of oligonucleotide analogues is depicted in **Figure 4**. Morpholino oligonucleotides (MO) are non-ionic DNA analogues with various linkers joining the subunits; carbamate, sulfo- or phosphor- tethers. Morpholinos have found wide applicability, patented, and are now commercially available as phosphorodiamidate derivatives[14-17]. MOs are more soluble in water than PNAs are, but have an additional chiral centre in the case of the most widely used phosphorodiamidate derivatives, and are usually available as racemates.

The antisense technology has proven its worth both in fundamental research and in medical applica-tions[18,19], although additional challenges demand highly skilled and imaginative chemists. Obviously, the integrated efficacy of the future drug or tool depends on its efficacy at different levels: *in vitro*, in cell culture, and in living organisms[20]. The cost of parent compounds and oligonucleotide analogue synthesis is the primary question addressed by scientists designing an antisense drug. Studies that succeed in resolving this cost problem in the best way are the most beneficial. Herein are presented some preliminary results concerning the chemical research respondent, as well as the outlined chemical tasks. The current investigation was based on a synthetic strategy that allows preparation of new oligonucleo-tide mimics at minimal cost, reaction stages, and protection strategies.

New chemical possibilities

Morpholino oxalyldiamide oligomers

Morpholino oligonucleotides initially attracted attention as they could be successfully synthesized from inexpensive parent substances – ribonucleosides - following some simple one pot reactions. A wide variety of ribonucleosides and amino compounds are employed successfully in this reaction sequence[21,22]. It should be noted that the stereochemistry of the ribose ring is preserved for the most part in all cases. Morpholino subunits may be further modified by introducing other functional groups into the molecule. In the search for new morpholino-based oligo-nucleotide analogues, it was at first attempted to add other functions to the morpholino subunit by changing the hydroxyl group to an amine, analogous to the reactions described earlier for nucleosides[23] (**Figure 5**). After obtaining the crucial 2′-amino-
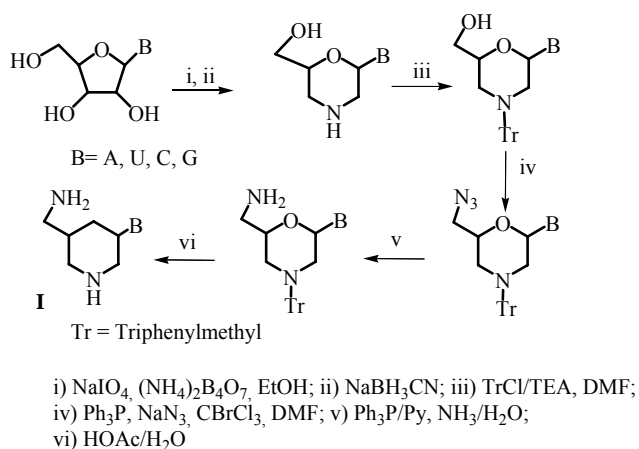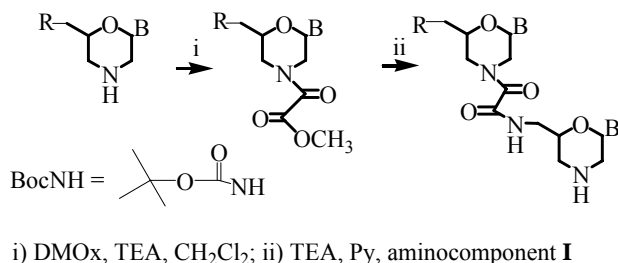
B= A, U, C, G

Tr = Triphenylmethyl

i) NaIO$_4$, (NH$_4$)$_2$B$_4$O$_7$, EtOH; ii) NaBH$_3$CN; iii) TrCl/TEA, DMF;
iv) Ph$_3$P, NaN$_3$, CBrCl$_3$, DMF; v) Ph$_3$P/Py, NH$_3$/H$_2$O;
vi) HOAc/H$_2$O

Figure 5—Synthesis of the 2′-aminomethylmorpholino nucleoside subunits **I**

i) DMOx, TEA, CH$_2$Cl$_2$; ii) TEA, Py, aminocomponent **I**

R = OH, DMTrO, BocNH, Polymer support; **B** = Ura or Ade

Figure 6—Synthesis of morpholino oxalyldiamide dimers

methylmorpholino nucleosides **I**, the obvious difference in the reactivity between the primary 2′-aminomethyl group and the morpholine nitrogen of morpholino nucleoside was determined. This difference motivated the development of a synthetic strategy based on the different reactivity of these functional groups. The best choice was to find a bifunctional reagent capable of differentiating between the two reaction centres. In this case, the use of a temporary protective group would not be necessary, and the synthesis could be considerably simplified.

The search for the linkers joining the monomer nucleoside subunits led to the use an oxalyl residue. It is important to note that the oxalic acid tether does not introduce additional chiral centres into the oligomer. Various esters of oxalic acid were investigated: bispentafluorophenyl, biscyanoethyl, bis-*p*-nitro-phenyl esters and also a bistriazolide derivative in an attempt to achieve selectivity in this reaction. In some cases, the reagent modified with the amino groups of heterocyclic bases or the oxalyl derivatives decomposed in the reaction-mixture. The selectivity

achieved between the primary aliphatic amino group and morpholine nitrogen was not sufficient.

As shown previously, the dimethyl ester of oxalic acid (DMOx) reacts readily with aliphatic amino groups and is widely used as a bifunctional reagent in precursor-based approaches[24]. The DMOx does not react with the amino groups of heterocyclic bases, rendering the protection of the nucleobases unnecessary. We succeeded in obtaining dimers following the simple procedures depicted in the (**Figure 6,** ref. 25). The anchor protective group could be removed after the completion of the synthesis if used. After studying the properties of these dimers, it was determined that these substances are stable in the presence of acids and are readily hydrolyzed in basic conditions. Fortunately, the use of heterocyclic base protective groups is not necessary in the present scheme. After the dimers were obtained, base stacking was verified in the derivatives and the circular dichroism spectra of the dimers and the mixture of corresponding monomers were recorded, depending on the temperature. The data obtained indicate that the bases of the morpholine dimers exist in a stacked conformation[25]. The shapes of the CD spectra of dimers have more similarities with the spectra of corresponding ribodinucleoside phosphates rather than deoxyribodinucleoside phosphates. Repeating the activation and condensation steps (i) and (ii) in **Figure 6**, a uracil containing hexamer was also synthesised and the thermal stability of its duplex with d(C$_2$A$_6$C$_2$), poly(dA) and poly(rA) as complementary strands was studied. dT$_6$ was used for comparison and formed complementary duplexes in all cases. The complementary duplex formation between the morpholino oxalyldiamide hexamer and any of the complementary strands used was not detected, although some hyperchromicity was observed. The length of the morpholino oligomer was probably not sufficient to form stable complexes under such conditions. Generally, the addition of positively charged moieties into the oligonucleotide mimics benefits the affinity to the complementary target, although it should be kept in mind that an excess on such residues often reduces the selectivity of binding. It was decided to add a positive charge to the oligomer and to employ the block synthesis scheme for the construction of the morpholino oxalyldiamide oligomer (**Figure 7**).

Morpholinoglycine oligomers

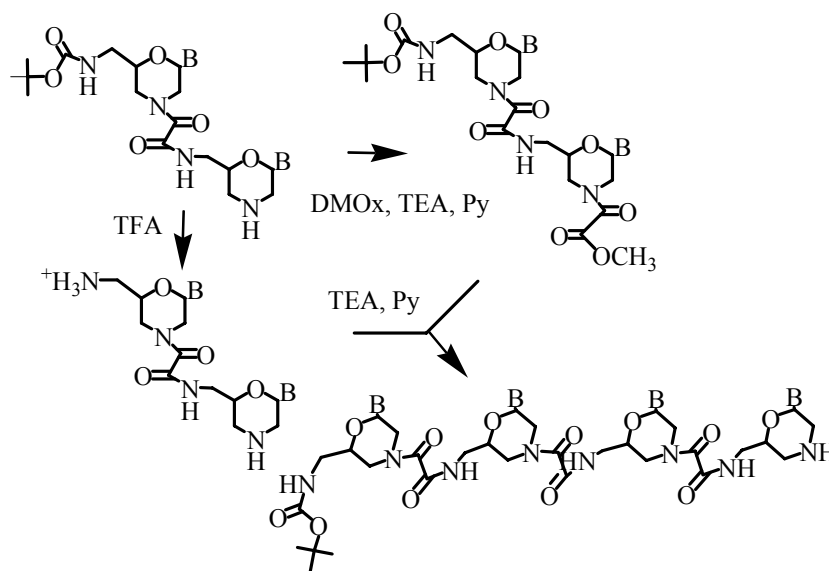In search of other constructions of morpholino-based oligomers, morpholino oligonucleotide

Figure 7—Block synthesis of morpholino oxalyldiamide oligonucleotides

i) NaN₃, Ph₃P, CBrCl₃;

ii) NaIO₄, NaCNBH₃, Gly/TEA;

iii) Ph₃P/Py, tnen NH₃/H₂O;

   or H₂/ Pd/C

iv) (Boc)₂O, 1M NaOH/iPrOH
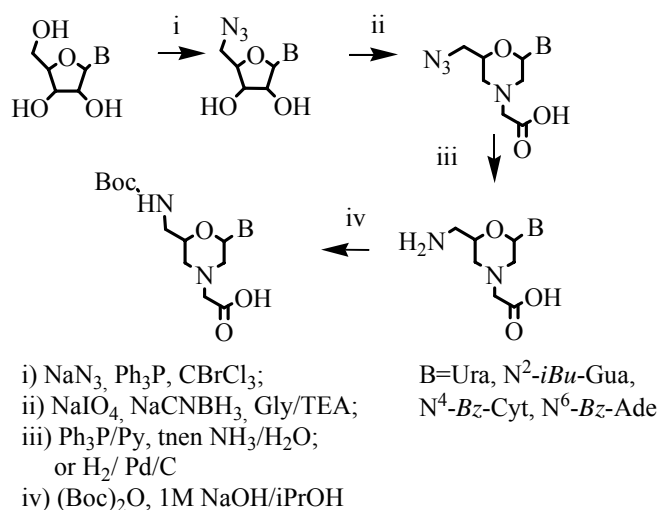
B=Ura, N²-*iBu*-Gua,

N⁴-*Bz*-Cyt, N⁶-*Bz*-Ade

Figure 8—Synthesis of morpholinoglycine nucleoside subunits

analogues where glycine residues link the subunits were also considered. This type of monomer units and corresponding oligomers were proposed earlier[26] but no experimental details have been published.

To produce the desired monomers the reaction scheme applied earlier for the synthesis of morpholino nucleosides was altered. The glycine linker was introduced concurrently with closure of the morpholine ring (**Figure 8**). In order to use simple methods of peptide chemistry to join the monomers, it was first necessary to protect amine

groups of heterocyclic bases. The scheme of the morpholino glycine oligomers synthesis is depicted in the (**Figure 9**). Oligomers of this type would have tertiary morpholine-like amino function in each subunit, and it is proposed that they will have better binding affinity to the complementary target than morpholino oxalyldiamide oligomers. We succeeded in obtaining the morpholinoglycine tetramers even without the protection of the carboxyl function. Further work in this area is under progress.
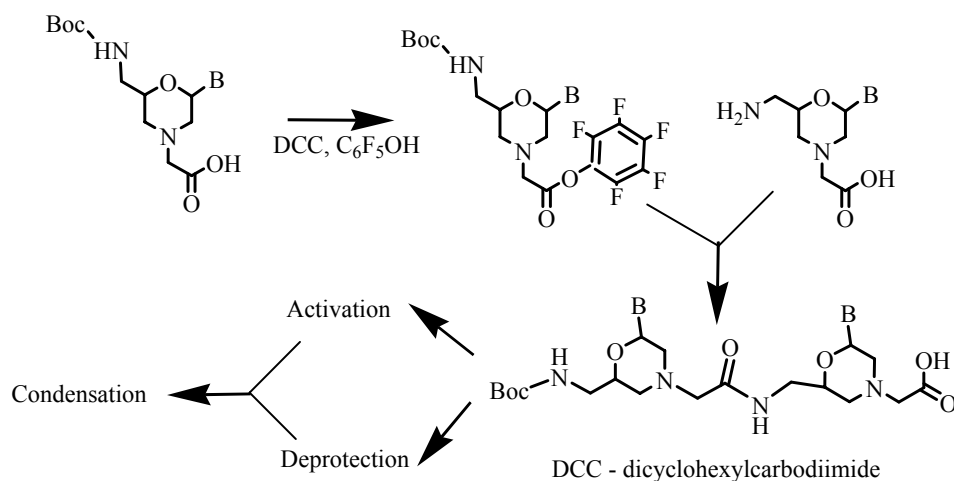
Figure 9—Block synthesis of morpholinoglycine oligonucleotides

Conclusion

Despite several decades of intensive efforts by chemists to design and synthesize an antisense drug or tool that is broadly applicable, the current level of progress cannot be defined as complete. Certain successes have propelled scientists to continue the search for a better method of synthesis. The most promising method in this connection may be to return to the beginning and start anew the search for simple constructions that may be readily obtained and tested.

Acknowledgements

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