(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 July 2009 (23.07.2009)

PCT

(10) International Publication Number
WO 2009/090182 A1

(51) International Patent Classification:
C07H 21/00 (2006.01) *C12N 15/11* (2006.01)

(21) International Application Number:
PCT/EP2009/050349

(22) International Filing Date: 14 January 2009 (14.01.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2008 00053 14 January 2008 (14.01.2008) DK

(71) Applicant: **SANTARIS PHARMA A/S** [DK/DK]; Kogle Alle 6, DK-2970 Hørsholm (DK).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **WENGEL, Jesper** [DK/DK]; Promenadebyen 2, 8.sal, lejlighed 4, DK-5000 Odense (DK).

(74) Agent: **TURNER, Mark**; Santaris Pharma A/S, Kogle Allé 6, DK-2970 Hørsholm (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

WO 2009/090182 A1

(54) Title: C4'-SUBSTITUTED - DNA NUCLEOTIDE GAPMER OLIGONUCLEOTIDES

(57) Abstract: The present invention relates to gapmer antisense oligonucleotides which contain non bicyclic 4'modified nucleotide analogues, such as 4'hydroxymethyl DNA, within the central gap region which are capable of recruiting RNAseH when formed in a duplex with a target RNA sequence.

C4'-SUBSTITUTED - DNA NUCLEOTIDE GAPMER OLIGONUCLEOTIDES

FIELD OF INVENTION

The present invention relates to gapmer antisense oligonucleotides which contain C4' substituted nucleotide analogues within the central gap region.

RELATED CASES

Danish patent application PA 2008 00053, filed on 14th January 2008 is hereby incorporated by reference in its entirety.

BACKGROUND

In the antisense technology, RNA is targeted by Watson-Crick hybridization of a complementary antisense oligonucleotide (AON). The goal of inhibiting gene expression in a specific way may be accomplished by preventing mRNA maturation, blocking translation or more commonly by induction of degradation. To be effective the AON has to be able to enter the cell, be stable toward nucleases, be non-toxic and show high binding affinity and specificity toward the target mRNA. Considerable progress with respect to stability and binding has been made by use of chemically modified AONs, with LNA (locked nucleic acid) being a prominent example. An LNA monomer contains an O2'-C4' linkage that locks the furanose ring in an *N*-type conformation which exhibits an unprecedented binding affinity toward complementary RNA for AONs composed of a mixture of *e.g.* LNA and DNA nucleotides. Remarkably, LNA in both the α-L and β-D conformation, induce very high RNA binding affinities of AONs with increases in thermal denaturation temperatures (T_m values) of ~2-8 °C per modification. The efficiency of AONs containing modified nucleotides is often limited by their inability to induce degradation of target mRNA by the ubiquitous RNase H enzyme. Specifically, RNase H is incompatible with substrate duplexes with *N*-type nucleotides like β-D-LNA or O2'-alkylated-RNA nucleotides dispersed throughout the AON. Such O2'-alkylated-RNA nucleotides can for example be 2'-O-methyl-RNA nucleotides or 2'-O-methoxyethyl-RNA (2'-MOE-RNA) nucleotides. The limitation described in the paragraph above has been circumvented by the use of so-called gapmers, which are chimeric AONs with a central continuous stretch of RNase H recruiting nucleotides (typically DNA or phosphorothioate DNA nucleotides but alternatively *e.g.* phosphorothioate FANA nucleotides flanked by affinity-enhancing modified nucleotides (*e.g.* LNA, α-L-LNA or O2'-alkylated RNA nucleotides). Noteworthy, it has been found that the optimal gap size is motif-dependent,

that a right balance between gap size and affinity is required and that the presence of one or two DNA-mimicking α-L-LNA monomers within the gap is compatiblewith RNase H activity [Sørensen, M. D.; Kvaerno, L.; Bryld, T.; Hakansson, A. E.; Verbeure, B.; Gaubert, G.; Herdewijn, P.; Wengel, J. *J. Am. Chem. Soc.* 2002, *124*, 2164-2176; Frieden, M.; Christensen, S. M.; Mikkelsen, N. D.; Rosenbohm, C.; Thrue, C. A.; Westergaard, M.; Hansen, H. F.; Orum, H.; Koch, T. *Nucleic Acids Res.* 2003, *31*, 6365-6372]. In order to improve the properties of antisense oligonucleotides there is a need for nucleotide modification for the gap-segment (central segment) of gapmers. These nucleotides should display properties like increased protection against nucleases, efficient hybridization to a target RNA sequence, compatibility with phosphorothioate linkages and compatibility with efficient RNase-H mediated degradation of a RNA target sequence. C4'-alkylated nucleotides is one potential class of nucleotide modification for the gap-segment of gapmers, and in one paper has 4'-*C*-methylthymidine (C4'-methyl-DNA) been studied for its compatibility with RNase-H mediated degradation of a RNA target sequence [Lima, W. F.; Nichols, J. G.; Wu, H.; Prakash, T. P.; Migawa, M. T.; Wyrzykiewicz, T. K.; Bhat, B.; Crooke, S. T. *J. Biol. Chem.* 2004, *279*, 36317-36326]. This study included strands having the C4'-methyl-DNA monomer positioned within the central segment of those strands. It was concluded that the duplexes containng the C4'-methyl-DNA nucleotide modification were poor substrates for human RNase with initial cleavage rates 2-3-fold slower than those of the unmodified heteroduplex, and that this modification leads to a loss in RNase H activity [Lima, W. F.; Nichols, J. G.; Wu, H.; Prakash, T. P.; Migawa, M. T.; Wyrzykiewicz, T. K.; Bhat, B.; Crooke, S. T. *J. Biol. Chem.* 2004, *279*, 36317-36326]. The C4'-hydroxymethyl-DNA monomers have previously been incorporated into DNA strands, and therefore procedures for preparation of their phosphoramidite building blocks for automated oligonucleotide synthesis have been reported as well as procedures for their incorporation into oligonucleotides [K. D. Nielsen et al., *Bioorg. Med. Chem.* **1995**, *3*, 1493; H. Thrane et al., *Tetrahedron* **1995**, *51*, 10389; P. Nielsen et al., *Bioorg. Med. Chem.* **1995**, *3*, 19].

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: The RNase H cleavage patterns are depicted in the figure 1 for ON0 (ON0:RNA) and ON3 (ON3:RNA) (Example 2).

Figure 2: Cleavage reactions with NAC2091 to the left (first four lanes, increasing time towards the right), then NAC2092, then NAC2093, then NAC2094 and to the right NAC2095 (Example 3).

SUMMARY OF THE INVENTION

The invention provides a gapmer oligomer which comprises one or more C4'-substituted nucleotides incorporated into the gap-segment of the gapmer oligomer, wherein said C4' substituted nucleotides, such as one or more C4' substituted-DNA nucleotides, is, optionally independently, selected from the group consisting of C4'-hydroxymentyl-DNA nucleotides, C4'-mercaptomethyl-DNA nucleotides, and C4'-aminomethyl-DNA nucleotides.

The gapmer oligomer may, in some embodiments, consist of a contiguous sequence of nucleotides, 5' X-Y-Z 3', wherein regions X and Z are, independently, 1-8 nucleotides in length, such as 2, 3, 4, 5, 6 or 7, and consist of affinity enhancing nucleotides or affinity enhancing LNA or 2'O-alkyl-RNA nucleotides, optionally mixed with DNA or C4'hydroxymethyl-DNA nucleotides; wherein region Y is 6 – 12 nucleotides in length, such as 7, 8, 9, 10, or 11; and wherein region Y consists of DNA nucleotides and one or more of said C4'-substituted nucleotides; and wherein optionally region Y comprises acyclic nucleotides, arabino-configured nucleotides, oxepane nucleic acid nucleotides or alpha-L-LNA nucleotides.

In some aspects the invention provides a gapmer oligomer of 10 – 30 nucleotides in length which comprises one or more C4'- substituted-DNA nucleotides incorporated into the gap-segment of the gapmer oligomer. Suitably, in some exemplary aspects, the C4' substituted-DNA nucleotides are selected from the group consisting of C4'-hydroxymentyl-DNA nucleotides, C4'-mercaptomethyl-DNA nucleotides, C4'-aminomethyl-DNA nucleotides.

In some embodiments, the gapmer oligomer consists or comprises of a contiguous sequence of nucleotides of formula (5' to 3'), X-Y-Z, or optionally X-Y-Z-D or D-X-Y-Z, wherein; region X (5' region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as between 1-6 nucleotide analogues, such as LNA units, and; region Y consists or comprises of at least five consecutive nucleotides which are capable of recruiting RNAse when formed in a duplex with a complementary RNA molecule, such as the mRNA target, such as DNA nucleotides, and; region Z (3'region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as between 1-6 nucleotide analogues, such as LNA units, and; region D, when present consists or comprises of 1, 2 or 3 nucleotide units, such as DNA nucleotides; wherein region Y comprises one or more of said C4'-substituted nucleotides.

The invention provides for the use of one or more C4'-substituted nucleotides, such as C4'-hydroxymethyl-DNA nucleotides, incorporated into the gap-segment of the gapmer oligomer to improve the stability of the oligomer towards enzymatic degradation in cell cultures or *in vivo*, such as in human blood serum.

The invention provides for a gapmer oligomer according to the invention, wherein the oligomer is conjugated to at least one at least one non-nucleotide or non-polynucleotide moiety covalently attached to said oligomer.

The invention provides for a method of mediating gene silencing of a target nucleic acid in a cell or an organism comprising contacting said cell or organism with a gapmer oligomer according to the invention under conditions sufficient to induce gene-silencing of said target nucleic acid. Such a method may be performed *in vivo* or *in vitro*.

The invention provides for an *in vitro* method of mediating gene silencing of a target nucleic acid in a cell or an organism comprising contacting said cell or organism with a gapmer oligomer according to the invention under conditions sufficient to induce gene-silencing of said target nucleic acid in said cell.

The invention provides for an *in vivo* method of mediating gene silencing of a target nucleic acid in a cell or an organism comprising contacting said cell or organism with a gapmer oligomer according to the invention under conditions sufficient to induce gene-silencing of said target nucleic acid in vivo in said cell or said organism.

In some aspects the invention provides antisense gapmer oligonucleotides with one or more C4'-modified nucleotide monomers, such as C4'-hydroxymethyl-DNA nucleotide monomers, incorporated into the gap-segment of the antisense oligonucleotide to be used in relation to RNA-guided gene regulation or gene analysis. The C4' substituent of the C4'-substituted nucleotide monomer may be an un-derivatised hydroxymethyl group or a derivatised R-O-CH_2 group with R being for example alkyl or R'NHC(O) groups, but the C4'-substituent group may also be converted into corresponding mercaptomethyl or aminomethyl groups, or derivatised R-S-CH_2 or R-N(R'')-CH_2 derivatives thereof. The antisense oligonucleotides of the invention contain in the flanks preferably affinity enhancing nucleotides like LNA or 02'-alkylated-RNA nucleotides. In an exemplary aspect the present invention provides antisense oligonucleotides which, when bound (hybridised) to (fully complementary) RNA target sequences, are efficient substrates of RNase H type enzymes, such as human RNaseH. In an exemplary aspect the invention provides antisense oligonucleotides which, when bound to an RNA target sequence, are more efficient substrates of RNase H type enzymes, such as human RNaseH, than the corresponding gapmer antisense oligonucleotides having in the gap-segment exclusively

DNA or phosphorothioate-DNA nucleotides. In an exemplary aspect the invention provides gapmer antisense oligonucleotides with improved properties with regard to stability towards enzymatic degradation in cell cultures or in vivo. an exemplary aspect the invention provides gapmer antisense oligonucleotides that display enhanced gene regulatory function, e.g. gene silencing effect, in cell cultures or *in vivo*, relative to the corresponding gapmer antisense oligonucleotides having in the gap-segment exclusively DNA or phosphorothioate-DNA nucleotides.

In some aspects, the invention provides gapmer antisense oligonucleotides with improved properties with regard to stability towards enzymatic degradation in cell cultures or *in vivo*, such as in human blood serum, relative to the corresponding gapmer antisense oligonucleotides having in the gap-segment exclusively DNA nucleotides. Indeed, whilst not wishing to be limited to any specific theory, it is thought the inclusion of the 4'modified nucleotide in the gap region can enhance protection against endo-nucleases.

In some aspects, it is an object of the present invention to provide antisense oligonucleotides which, when bound to RNA target sequences, are efficient substrates of RNase H type enzymes, such as human RNase H.

DETAILED DESCRIPTION OF THE INVENTION

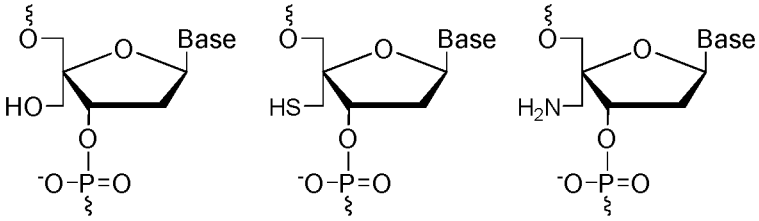
The Oligomer

The present invention provides antisense gapmer oligonucleotides with one or more C4'-substituted-DNA nucleotide monomers, such as C4'-alkyl-DNA nucleotide monomers, incorporated into the gap-segment of the antisense oligonucleotide, for example to be used in relation to gene regulation or gene analysis. Such oligomers are typically used in modulating, such as down-regulation the function of nucleic acid molecules, such as mammalian mRNA or viral RNA. The term nucleotide is used interchangeably with the term monomer as used herein. The term gapmer antisense oligonucleotide is used interchangeably with the term gapmer oligomer as used herein.

The term C4'- substituted nucleotide refers to a nucleotide which contains a substitution, such as an alkyl substitution, to one or more of the hydrogen atoms present at the 4' carbon. In this respect, the term C4'- substituted nucleotide or C4'-substituted-DNA nucleotide does not include bicyclic nucleotides which comprise a covalent bridge between the C4' and C2' groups of the sugar ring. In other words the C4' substituted nucleotide as incorporated into the gap-segment according to the present invention is a monocyclic rather than a bicyclic nucleotide. In some embodiments the C4'- substituted nucleotide, is a C4'- substituted DNA nucleotide.

The one or more C4'-substituted nucleotides may be, optionally substituted, C4'alkyl DNA nucleotides, wherein the term alkyl refers to C_{1-6} alkyl, such as methyl, ethyl, propyl, butyl, pentyl or hexyl. In some embodiments, the alkyl group of the C4'-substituted nucleotide is optionally substituted, such as with one or more, such as 2, or 3 substituent groups, selected from the group consisting of hydroxyl, mercapto or amino. In some embodiments the alkyl group is methyl which may optionally be substituted with one or more, such as 2, or 3 substituent groups, such as substituent groups selected form the group consisting of hydroxyl, mercapto (S) or amino.

In some embodiments, the C4'-substituted monomers (nucleotides) are selected among C4'-hydroxymethyl-DNA nucleotide, C4'-mercaptomethyl-DNA nucleotide, C4'-aminomethyl-DNA nucleotide as illustrated below as monomers A, B and C respectivly:



Monomer A Monomer B Monomer C

wherein the term base is uracil, thymine, cytosine, 5-methylcytosine, adenine, guanine, or another known natural or synthetic nucleobase or nucleobase analogue. In some embodiments, the base may be purine or pyrimidine, or a substituted purine or substituted pyrimidine, such as a nucleobase referred to herein, such as a nucleobase selected from the group consisting of adenine, cytosine, thymine, adenine, uracil, and/or a modified or substituted nucleobase, such as 5-thiazolo-uracil, 2-thio-uracil, 5-propynyl-uracil, 2'thio-thymine, 5-methyl cytosine, 5-thiozolo-cytosine, 5-propynyl-cytosine, and 2,6-diaminopurine.

In some embodiments the hydroxymethyl substituent of the C4'-substituted monomers, such as C4'-hydroxymethyl-DNA monomers, is functionalised by an ether linkage between a conjugated group and the methylene group of the hydroxymethyl substituent.

In some embodiments the hydroxymethyl substituent of the C4'-substituted monomers, such as C4'-hydroxymethyl-DNA monomers, is converted into a thioether functionality before incorporation into the antisense oligonucleotide of the invention using methods known to a person skilled in the art.

In some embodiments the hydroxymethyl substituent of the of the C4'-substituted monomers, such as C4'-hydroxymethyl-DNA monomers, is converted into a

mercaptomethyl functionality before incorporation into the antisense oligonucleotide of the invention using methods known to a person skilled in the art. This mercapto functionality is properly protected as e.g. its acetyl derivative during oligonucleotide synthesis using methods know to a person skilled in the art.

In some embodiments the hydroxymethyl substituent of of the C4'-substituted monomers, such as C4'-hydroxymethyl-DNA monomers, is converted into an amine functionality before incorporation into the antisense oligonucleotide of the invention using methods known to a person skilled in the art. This amine functionality is properly protected as e.g. its trifluoroacetyl or Fmoc derivative during oligonucleotide synthesis using methods know to a person skilled in the art.

In some embodiments the hydroxymethyl substituent of the C4'-substituted monomers, such as C4'-hydroxymethyl-DNA monomers, is acting as a handle for attachment of amide-linked conjugating groups. This involves conversion of the hydroxyl unit of the hydroxymethyl substituent into an amine unit, for example as described above, and further derivatisation of this amino group by e.g. a conjugating group by amide bond formation using methods known to a person skilled in the art. This may take place before oligonucleotide synthesis or after oligonucleotide synthesis using methods known to a person skilled in the art.

In some embodiments the hydroxymethyl substituent of the C4'-substituted monomers, such as C4'-hydroxymethyl-DNA monomers, is acting as a handle for attachment of amino-linked conjugating groups. This involves conversion of the hydroxyl unit of the hydroxymethyl substituent into an amine unit, for example as described above, and further derivatisation of this amino group by e.g. a conjugating group by amine bond formation using methods known to a person skilled in the art. This may take place before oligonucleotide synthesis or after oligonucleotide synthesis using methods known to a person skilled in the art.

In some embodiments, the oligomer consists or comprises of a contiguous nucleotide sequence of 10 – 30 nucleotides in length, The oligomers may, in some embodiments, comprise or consist of a contiguous nucleotide sequence of a total of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 contiguous nucleotides in length.

In some embodiments, the oligomers comprise or consist of a contiguous nucleotide sequence of a total of 10 – 22, such as 12 – 18, such as 13 – 17 or 12 – 16, such as 13, 14, 15, 16 contiguous nucleotides in length.

In some embodiments, the oligomers comprise or consist of a contiguous nucleotide sequence of a total of 10, 11, 12, 13, or 14 contiguous nucleotides in length.

In some embodiments, the oligomer according to the invention consists of no more than 22 nucleotides, such as no more than 20 nucleotides, such as no more than 18 nucleotides, such as 15, 16 or 17 nucleotides. In some embodiments the oligomer of the invention comprises less than 20 nucleotides.

In various embodiments, the compound of the invention does not comprise RNA (units). It is preferred that the compound according to the invention is a linear molecule or is synthesised as a linear molecule. The oligomer is a single stranded molecule, and preferably does not comprise short regions of, for example, at least 3, 4 or 5 contiguous nucleotides, which are complementary to equivalent regions within the same oligomer (*i.e.* duplexes) - in this regards, the oligomer is not, in some embodiments, (essentially) double stranded. In some embodiments, the oligomer is essentially not double stranded, such as is not a siRNA. In various embodiments, the oligomer of the invention may consist entirely of the contiguous nucleotide region. Thus, the oligomer is, in some embodiments, not substantially self-complementary.

In an exemplary aspect, the present invention provides antisense gapmer oligonucleotides with one or more C4'-hydroxymethyl-DNA nucleotide monomers incorporated into the gap-segment of the antisense oligonucleotide, for example to be used in relation to gene regulation or gene analysis. In some embodiments the C4'-hydroxymethyl substituent of the C4'-hydroxymethyl-DNA nucleotide monomers of the invention may be an un-derivatised hydroxymethyl group. In some embodiments the C4'-hydroxymethyl groups of the C4'-hydroxymethyl-DNA nucleotide monomers of the invention are alkyloxymethyl groups. In some embodiments the C4'-hydroxymethyl groups of the C4'-hydroxymethyl-DNA nucleotide monomers of the invention are mercaptomethyl or aminomethyl groups, or derivatised derivatives thereof.

Gapmer Design

The oligomer of the invention is a gapmer. Typically, a gapmer oligomer is an oligomer which comprises a contiguous stretch of nucleotides which is capable of recruiting an RNAse, such as RNAseH, such as a region of at least 6 or 7 DNA nucleotides, referred to herein in as region Y, wherein region Y (the gap-segment) is flanked both 5' and 3' by regions of affinity enhancing nucleotide analogues, such as between 1 – 6 nucleotide analogues 5' and 3' to the contiguous stretch of nucleotides which is capable of recruiting RNAse – these regions are referred to as regions X and Z respectively (the flanks).

The gapmer antisense oligonucleotides of the invention may in some embodiments be constructed as 5'-X-Y-Z gapmers, with X and Y as flanks around a gap-segment Y. The flanks X and Z may contain affinity enhancing monomers or a selected number of affinity enhancing LNA or O2'-alkyl-RNA (e.g. 2'-*O*-CH_3-RNA or 2'-*O*-methoxyethyl-RNA) monomers mixed with other monomers (e.g. DNA or C4'-hydroxymethyl-DNA monomers). The flanks X and Z may be have a of length 1 - 20 nucleotides, preferably 1-8 nucleotides and even more preferred 1 - 5 nucleotides. The flanks X and Z may be of similar length or of dissimilar lengths. The gap-segment Y may be a nucleotide sequence of length 5 - 20 nucleotides, preferably 6-12 nucleotides and even more preferred 7 - 10 nucleotides. In some aspects, the gap-segment of the gapmer antisense oligonucleotides of the invention may contain modified nucleotides known to be acceptable for efficient RNase H action in addition to DNA nucleotides and C4'-substituted nucleotides, such as acyclic nucleotides, arabino-configured nucleotides [Damha, M. J.; Wilds, C. J.; Noronha, A.; Brukner, I.; Borkow, G.; Arion, D.; Parniak, M. A. *J. Am. Chem. Soc.* 1998, *120*, 12976–12977; Christensen, N. K.; Petersen, M.; Nielsen, P.; Jacobsen, J. P.; Olsen, C. E.; Wengel, J. *J. Am. Chem. Soc.* 1998, *120*, 5458-5463], oxepane nucleic acid [Sabatino, D; Damha, M. J, *J. Am. Chem. Soc.* 2007, *129*, 8259-8270] or □-L-LNA nucleotides [Sørensen, M. D.; Kvaerno, L.; Bryld, T.; Hakansson, A. E.; Verbeure, B.; Gaubert, G.; Herdewijn, P.; Wengel, J. *J. Am. Chem. Soc.* 2002, *124*, 2164-2176].

The gapmers of the present invention are characterized in that they contain one or more C4'-substituted nucleotides, such as C4'-substituted DNA nucleotides within the gap-segment, referred to as region Y.

Preferably the gapmer comprises a (poly)nucleotide sequence of formula (5' to 3'), X-Y-Z, or optionally X-Y-Z-D or D-X-Y-Z, wherein; region X (5' region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as 1 - 6 nucleotide analogues, such as LNA units, and; region Y consists or comprises of at least five consecutive nucleotides which are capable of recruiting RNAse, such as RNaseH (when formed in a duplex with a complementary RNA molecule, such as the mRNA target), such as DNA nucleotides, and at least one 4' substituted nucleotide, such as 4'hycroxymethyl DNA nucleotide monomers; region Z (3'region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as 1 - 6 nucleotide analogues, such as LNA units, and; region D, when present consists or comprises of 1, 2 or 3 nucleotide units, such as DNA nucleotides.

In some embodiments, region X consists of 1, 2, 3, 4, 5 or 6 nucleotide analogues, such as LNA units, such as 2 - 5 nucleotide analogues, such as 2 - 5 LNA units, such as 3

or 4 nucleotide analogues, such as 3 or 4 LNA units; and/or region Z consists of 1, 2, 3, 4, 5 or 6 nucleotide analogues, such as LNA units, such as 2 - 5 nucleotide analogues, such as 2 - 5 LNA units, such as 3 or 4 nucleotide analogues, such as 3 or 4 LNA units.

In some embodiments region Y consists or comprises of 5, 6, 7, 8, 9, 10, 11 or 12 consecutive nucleotides which are capable of recruiting RNAse, such as RNaseH, or 6 - 10, or 7 - 9, such as 8 consecutive nucleotides which are capable of recruiting RNAse, such as RNaseH. In some embodiments region Y consists or comprises at least one DNA nucleotide unit, such as 1-12 DNA units, preferably 4 - 12 DNA units, more preferably 6 - 10 DNA units, such as 7 - 10 DNA units, such as 8, 9 or 10 DNA units, wherein one or more the the DNA units are substituted with a C4'-substituted nucleotide, such as 1, 2, 3 or 4 of the DNA units are substituted with a C4'-substituted nucleotide, such as C4'hydroxymethyl DNA nucleotide(s).

In addition to the DNA and C4'-substituted nucleotides, the gap-segment, may, in some embodiments comprises one or more, such as 1, 2, 3 or 4, other nucleotides which do not prevent the efficient cleavage of the mRNA target when formed in a duplex with a complementary RNA molecule, such as the mRNA target, such as via RNAse, such as RNaseH. Such other nucleotides may be selected from the group consisting of acyclic nucleotides, arabino-configured nucleotides, oxepane nucleic acid nucleotides or alpha-L-LNA nucleotides. Therefore in some embodiments, region Y consists of DNA nucleotides and one or more, such as 1, 2 or 3, of said C4'-substituted nucleotides; and wherein optionally region Y further comprises comprises one or more further nucleotides, such as 1, 2 or 3 further nucleotides, such as a nucleotide, optionally independently, selected from the group consisting of acyclic nucleotides, arabino-configured nucleotides, oxepane nucleic acid nucleotides or alpha-L-LNA nucleotides.

In some embodiments the the gap-region consists of DNA nucleotides and one or more C4'-substituted nucleotides, and optionally one or more alpha-L-LNA nucleotides, such as 1, 2, or 3 alpha-L-LNA nucleotide.

In some embodiments, the oligomer consists or comprises of a contiguous sequence of nucleotides, 5' X-Y-Z 3', wherein regions X and Z are, independently, 1-8 nucleotides in length, and consist of affinity enhancing nucleotides or affinity enhancing LNA or 2'O-alkyl-RNA nucleotides, optionally mixed with DNA or C4'hydroxymethyl-DNA nucleotides; wherein region Y is 6 – 12 nucleotides in length; and wherein region Y consists of DNA nucleotides and one or more of said C4'-substituted nucleotides; and wherein optionally region Y comprises acyclic nucleotides, arabino-configured nucleotides, oxepane nucleic acid nucleotides or alpha-L-LNA nucleotides.

In some embodiments, the oligomer of the invention consists or comprises of a contiguous sequence of nucleotides of formula (5' to 3'), X-Y-Z, or optionally X-Y-Z-D or D-X-Y-Z, wherein; region X (5' region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as between 1-6 nucleotide analogues, such as LNA units, and; region Y consists or comprises of at least five consecutive nucleotides which are capable of recruiting RNAse when formed in a duplex with a complementary RNA molecule, such as the mRNA target, such as DNA nucleotides, and; region Z (3'region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as between 1-6 nucleotide analogues, such as LNA units, and; region D, when present consists or comprises of 1, 2 or 3 nucleotide units, such as DNA nucleotides; wherein region Y comprises one or more of said C4'-substituted nucleotides. In some embodiments, region Y comprises of a total of 1, 2, 3 or 4 of said C4'-substituted nucleotides.

In some embodiments, the gap-segment (region Y) comprises of only DNA nucleotides and said one or more C4'-substituted nucleotides, such as 1, 2, 3 or 4 C4'-substituted nucleotides.

In some embodiments, the gap-segment (region Y) comprises of only DNA nucleotides and only one C4'-substituted nucleotide. In some embodiments, the C4'-substituted nucleotides is positioned one nucleotide away from one of the nucleotides of one of the flanks that is positioned next to the gap-segment. In some embodiments, the C4'-substituted nucleotides is juxtapositioned to one of the nucleotides of one of the flanks that is positioned next to (immediately adjacent to) the gap-segment. In some embodiments, at least one (such as 1 or 2) of the C4'-substituted nucleotides are positioned either (immediately) adjacent to or one nucleotide away from the 3' nucleotide of the 5' flank (region X), and/or the 5' nucleotide of the 3' flank (region Z) In some embodiments, at least one (such as 1, 2, or 3) of the C4'-substituted nucleotide(s) is positioned within three, two or one nucleotides of the center of the gap-segment (region Y), such as, in the case of a gap-segment which consists of an odd number of nucleotides, the central nucleotide is a C4'-substituted nucleotide.

Typically, the flanks contain affinity enhancing nucleotides like LNA or 2'-O alkylated-RNA nucleotides. In some embodiments, the flanks consist or comprise of (affinity enhancing) LNA nucleotides. In some embodiments, the flanks consist or comprise of affinity enhancing 2'O-alkyl-RNA, such as 2'O-methoxyethyl-RNA nucleotides. In some embodiments, the affinity enhancing nucleotides present in the flanks (regions X and Z) are independatly selected from the group consisting of 2'-O-alkyl-

RNA nucleotides, 2'-amino-DNA nucleotides, 2'-fluoro-DNA nucleotides, LNA nucleotides, arabino nucleic acid (ANA) nucleotides, 2'-fluoro-ANA nucleotides, HNA nucleotides, INA nucleotides, and 2'O-methoxyethyl-RNA nucleotides. In some embodiments, the flanks (regions X and Z) consist or comprise of LNA nucleotides. In some embodiments, the flanks (regions X and Z) consist or comprise of 2'O-alkyl-RNA, such as 2'O-methoxyethyl-RNA (2'MOE) nucleotides. In some embodiments, the flanks (regions X and Z) consist or comprise of 2'-fluoro-DNA nucleotides.

In some embodiments, the two flanks (X and Z) consist of 1 – 6 affinity enhancing nucleotides and the gap-segment (Y) consists of 6 – 12 DNA nucleotides wherein one or more the the DNA units are substituted with a C4'-substituted nucleotide, such as 1, 2, 3 or 4 of the DNA units are substituted with a C4'-substituted nucleotide.

In some embodiments, the two flanks (X and Z) consist of 2 – 5 affinity enhancing nucleotides and the gap-segment (Y) consists of 8 – 10 DNA nucleotides wherein one or more the the DNA units are substituted with a C4'-substituted nucleotide, such as 1, 2, 3 or 4 of the DNA units are substituted with a C4'-substituted nucleotide.

In some embodiments, the oligomer is constructed as a 5-10-5, 4-10-4, 3-10-3, 2-10-2, 1-10-1, 5-9-5, 4-9-4, 3-9-3, 2-9-2, 1-9-1, 5-8-5, 4-8-4, 3-8-3, 2-8-2, 1-8-1, 5-7-7, 4-7-4, 3-7-3, or 2-7-2 gapmer.

In some embodiments, the oligomer has improved RNase, such as RNase-H activity compared to the corresponding gapmer oligomer which has a gap (Y) segment which either consists of only DNA nucleotides, or an identical gapmer oligomer with the exception that the C4'-substituted nucleotides present in region Y are substituted with DNA units. Improved RNase-H activity may be determined using the assays referred to herein. RNase activity may be used by determining the down-regulation in a cell assay system, such as in a human cell assay system. See WO2007/031091 for examples of human cells and assays which may be used, for example.

In some embodiments region X consist of 3 or 4 nucleotide analogue units (monomers), such as LNA units, region Y consists of 7, 8, 9 or 10 units which are capable of recruiting RNAse, such as RNaseH, and region Z consists of 3 or 4 nucleotide analogue units, such as LNA units. Such designs include (X-Y-Z) 3-10-3, 3-10-4, 4-10-3, 3-9-3, 3-9-4, 4-9-3, 3-8-3, 3-8-4, 4-8-3, 3-7-3, 3-7-4, 4-7-3, and may, optionally further include region D, which may have one or 2 nucleotide units, such as DNA units.

Further gapmer designs are disclosed in WO2004/046160 and are hereby incorporated by reference, wherein the gap-segment comprises one or more C4'-substituted nucleotides as referred to herein..

US provisional application, 60/977409, hereby incorporated by reference, refers to 'shortmer' gapmer oligomers, which, in some embodiments may be the gapmer oligomer according to the present invention, wherein the gap-segment comprises one or more C4'-substituted nucleotides as referred to herein.

In some embodiments the oligomer is consisting of a contiguous nucleotide sequence of a total of 10, 11, 12, 13 or 14 nucleotide units, wherein the contiguous nucleotide sequence is of formula (5' – 3'), X-Y-Z, or optionally X-Y-Z-D or D-X-Y-Z, wherein; X consists of 1, 2 or 3 nucleotide analogue units, such as LNA units; Y, as deifned previously, consists of 7, 8 or 9 contiguous nucleotide units; and Z consists of 1, 2 or 3 nucleotide analogue units, such as LNA units. When present, D consists of a single DNA unit.

In some embodiments X consists of 1 LNA unit. In some embodiments X consists of 2 LNA units. In some embodiments X consists of 3 LNA units. In some embodiments X consists of 4 LNA units. In some embodiments Z consists of 1 LNA unit. In some embodiments Z consists of 2 LNA units. In some embodiments Z consists of 3 LNA units. In some embodiments Z consists of 4 LNA units. In some embodiments Y consists of 7 nucleotide units. In some embodiments Y consists of 8 nucleotide units. In some embodiments Y consists of 9 nucleotide units. In some embodiments Y comprises of between 1 – 9 such as 3 – 9 or DNA units, such as 3, 4, 5, 6, 7 or 8 DNA units, wherein one or more the the DNA units are substituted with a C4'-substituted nucleotide, such as 1, 2, 3 or 4 of the DNA units are substituted with a C4'-substituted nucleotide.

In some embodiments Y comprises of at least one LNA unit which is in the alpha-L configuration, such as 2, 3, or 4 LNA units in the alpha-L-configuration, such as alpha-L-oxy LNA. In some embodiments Y comprises of at least one alpha-L-oxy LNA unit and/or all the LNA units in the alpha-L- configuration are alpha-L-oxy LNA units. In some embodiments the number of nucleotides present in X-Y-Z are selected from the group consisting of (nucleotide analogue units – region Y – nucleotide analogue units): 1-8-1, 1-8-2, 2-8-1, 2-8-2, 3-8-3, 2-8-3, 3-8-2, 4-8-1, 4-8-2, 1-8-4, 2-8-4, or;1-9-1, 1-9-2, 2-9-1, 2-9-2, 2-9-3, 3-9-2, 1-9-3, 3-9-1, 4-9-1, 1-9-4, or; 1-10-1, 1-10-2, 2-10-1, 2-10-2, 1-10-3, 3-10-1. In some embodiments the number of nucleotides in X-Y-Z are selected from the group consisting of: 2-7-1, 1-7-2, 2-7-2, 3-7-3, 2-7-3, 3-7-2, 3-7-4, and 4-7-3. In some embodiments both A and C consists of two LNA units each, and B consists of 8 or 9 nucleotide units, preferably DNA units.

In some aspects it is preferable that the oligomer, or contiguous nucleotide sequence, comprises of a region of at least 6, such as at least 7 consecutive nucleotide

units, such as at least 8 or at least 9 consecutive nucleotide units (residues), including 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 consecutive nucleotides, which, when formed in a duplex with the complementary target RNA is capable of recruiting RNase, such as RNaseH. The contiguous sequence which is capable of recruiting RNAse may be region Y as referred to in the context of a gapmer as described herein. In some embodiments the size of the contiguous sequence which is capable of recruiting RNAse, such as region Y, may be higher, such as 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotide units.

In some embodiments the gap-segment of the gapmer antisense oligonucleotides of the invention contains only DNA nucleotides and the C4'-substituted nucleotide(s), such as C4' hydroxymethyl nucleotide(s).

In some embodiments the gap-segment of the gapmer antisense oligonucleotides of the invention contains DNA nucleotides and only one of the C4'-substituted nucleotides, such as C4' hydroxymethyl nucleotides.

In some embodiments the gap-segment of the gapmer antisense oligonucleotides of the invention contains DNA nucleotides and only one of the C4'-substituted nucleotides, such as C4' hydroxymethyl nucleotides,, all linked by phosphorothioate linkages.

In some embodiments the gap-segment of the gapmer antisense oligonucleotides of the invention contains DNA nucleotides and only one of the C4'-substituted nucleotides, such as C4' hydroxymethyl nucleotides, and LNA nucleotides in the flanks, all linked by phosphorothioate linkages.

In some embodiments the gap-segment of the gapmer antisense oligonucleotides of the invention contains DNA nucleotides and only one of the C4'-substituted nucleotides, such as C4' hydroxymethyl nucleotides, and O2'-alkyl-RNA nucleotides in the flanks, all linked by phosphorothioate linkages.

In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 5-10-5 gapmer. Such designs are routinely used in the production of 2'substituted gapmers, for example using 2'OME chemistry.

In some embodiments, the gapmer antisense oligonucleotides of the invention is constructed as a 4-10-4 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 3-10-3 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 2-10-2 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 1-10-1 gapmer. In some embodiments, the gapmer antisense oligonucleotides of the invention is constructed as a 5-9-5 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a

4-9-4 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 3-9-3 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 2-9-2 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 1-9-1 gapmer. In some embodiments, the gapmer antisense oligonucleotides of the invention is constructed as a 5-8-5 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 4-8-4 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 3-8-3 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 2-8-2 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 1-8-1 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 5-7-5 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 4-7-4 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 3-7-3 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 2-7-2 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 5-6-5 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 4-6-4 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 3-6-3 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 2-6-2 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 5-5-5 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 4-5-4 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 3-5-3 gapmer. In some embodiments the gapmer antisense oligonucleotides of the invention is constructed as a 2-5-2 gapmer. In some embodiments, the lengths of the two flanks differ but vary between 1 and 5 nucleotides. In some embodiments the C4'-hydroxymethyl nucleotides of the invention is juxta-positioned to one of the nucleotides of one of the flanks that is positoned next to the gap-segment. In some embodiments the C4'-substituted nucleotides, such as C4' hydroxymethyl nucleotides is/are positioned one nucleotide away of one of the nucleotides of one of the flanks that is positoned next to the gap-segment.

RNAse recruitment

It is recognised that an oligomeric compound may function via non RNase mediated degradation of target mRNA, such as by steric hindrance of translation, or other methods, however, the in a preferred aspect, the oligomers of the invention are capable of recruiting an endoribonuclease (RNase), such as RNase H.

EP 1 222 309 provides *in vitro* methods for determining RNaseH activity, which may be used to determine the ability to recruit RNaseH. A oligomer is deemed capable of recruiting RNase H if, when provided with the complementary RNA target, it has an initial rate, as measured in pmol/l/min, of at least 1 %, such as at least 5%, such as at least 10% or less than 20% of the equivalent DNA only oligonucleotide, with no 2' substitutions, with phosphorothioate linkage groups between all nucleotides in the oligonucleotide, using the methodology provided by Example 91 - 95 of EP 1 222 309.

In some embodiments, an oligomer is deemed essentially incapable of recruiting RNaseH if, (such as resulting in efficient cleavage of the target RNA) when provided with the complementary RNA target, and RNaseH, the RNaseH initial rate, as measured in pmol/l/min, is less than 1%, such as less than 5%,such as less than 10% or less than 20% of the initial rate determined using the equivalent DNA only oligonucleotide, with phosphorothioate linkage groups between all nucleotides in the oligonucleotide, using the methodology provided by Example 91 - 95 of EP 1 222 309.

In other embodiments, an oligomer is deemed capable of recruiting RNaseH if, when provided with the complementary RNA target, and RNaseH, the RNaseH initial rate, as measured in pmol/l/min, is at least 20%, such as at least 40 %, such as at least 60 %, such as at least 80 % of the initial rate determined using the equivalent DNA only oligonucleotide with phosphorothioate linkage groups between all nucleotides in the oligonucleotide, using the methodology provided by Example 91 - 95 of EP 1 222 309.

Typically the region of the oligomer which forms the consecutive nucleotide units which, when formed in a duplex with the complementary target RNA is capable of recruiting RNase consists of nucleotide units which form a DNA/RNA like duplex with the RNA target – and include both DNA units and the C4'-substituted nucleotides, and optionally other units which do not prevent or significantly hinder the activity of RNaseH, such as LNA units which are in the alpha-L configuration, particularly preferred being alpha-L-oxy LNA.

Internucleotide Linkages

The terms "linkage group" or "internucleotide linkage" are intended to mean a group capable of covalently coupling together two nucleotides, two nucleotide analogues, and a

nucleotide and a nucleotide analogue, etc. Specific and preferred examples include phosphate groups and phosphorothioate groups.

The nucleotides of the oligomer of the invention or contiguous nucleotides sequence thereof are coupled together via linkage groups. Suitably each nucleotide is linked to the 3' adjacent nucleotide via a linkage group.

Suitable internucleotide linkages include those listed within PCT/DK2006/000512, for example the internucleotide linkages listed on the first paragraph of page 34 of PCT/DK2006/000512 (hereby incorporated by reference).

It is, in some embodiments, preferred to modify the internucleotide linkage from its normal phosphodiester to one that is more resistant to nuclease attack, such as phosphorothioate or boranophosphate – these two, being cleavable by RNase H, also allow that route of antisense inhibition in reducing the expression of the target gene.

Suitable sulphur (S) containing internucleotide linkages as provided herein may be preferred. Phosphorothioate internucleotide linkages are also preferred, particularly for the gap region (Y) of gapmers. Phosphorothioate linkages may also be used for the flanking regions (X and Z, and for linking X or Z to D, and within region D, as appropriate).

In some embodiments, the linkages between the monomers of the oligomer are selected from the group consisting of phosphordiester linkages, phosophorothioate linkages, boranophsophate linkages, methylphosphonate linkages, phosphoramidate linkages, phosphortriester linkages, or phosphorodithioate linkages, or a mixture of two or more of these linkages. In some embodiments, the linkages between the monomers of the oligomer are all phosphorothioate linkages.

Regions X, Y and Z, may however comprise internucleotide linkages other than phosphorothioate, such as phosphodiester linkages, particularly, for instance when the use of nucleotide analogues protects the internucleotide linkages within regions X and Z from endo-nuclease degradation – such as when regions X and Z comprise LNA nucleotides. The use of C4' substituted nucleotides within the gap-segment as according to the present invention may enhance the endonuclease protection.

The internucleotide linkages in the oligomer may be phosphodiester, phosphorothioate or boranophosphate so as to allow RNase H cleavage of targeted RNA. Phosphorothioate is preferred, for improved nuclease resistance and other reasons, such as ease of manufacture.

In one aspect of the oligomer of the invention, the nucleotides and/or nucleotide analogues are linked to each other by means of phosphorothioate groups.

It is recognised that the inclusion of phosphodiester linkages, such as one or two linkages, into an otherwise phosphorothioate oligomer, particularly between or adjacent to nucleotide analogue units (typically in region X and or Z) can modify the bioavailability and/or bio-distribution of an oligomer – see WO2008/053314, hereby incorporated by reference.

In some embodiments, such as the embodiments referred to above, where suitable and not specifically indicated, all remaining linkage groups are either phosphodiester or phosphorothioate, or a mixture thereof.

In some embodiments all the internucleotide linkage groups are phosphorothioate. When referring to specific gapmer oligonucleotide sequences, such as those provided herein it will be understood that, in various embodiments, when the linkages are phosphorothioate linkages, alternative linkages, such as those disclosed herein may be used, for example phosphate (phosphodiester) linkages may be used, particularly for linkages between nucleotide analogues, such as LNA, units. Likewise, when referring to specific gapmer oligonucleotide sequences, such as those provided herein, when the C residues are annotated as 5'methyl modified cytosine, in various embodiments, one or more of the Cs present in the oligomer may be unmodified C residues.in some embodimentsin some embodiments

The gapmer antisense oligonucleotides of the invention may in some embodiments be constructed such that it contains natural phosphordiester linkages. However, in some aspects, it is preferred that oligomers containing phosporothioate linkages between all of the nucleotides of the oligomer. Another option is constructs containing a mixture of phosphorodiester and phosphorothioate linkages. Yet another option is constructs containg other modified linkages as known to a person skilled in the art [see also: Current Protecols in Nucleic Acid Chemistry, John Wiley & Sons, Volumes I-III; Editors S. L. Beaucage, D. E. Bergstrom, P. Herdewijn, A. Matsuda].

Nucleotide analogues

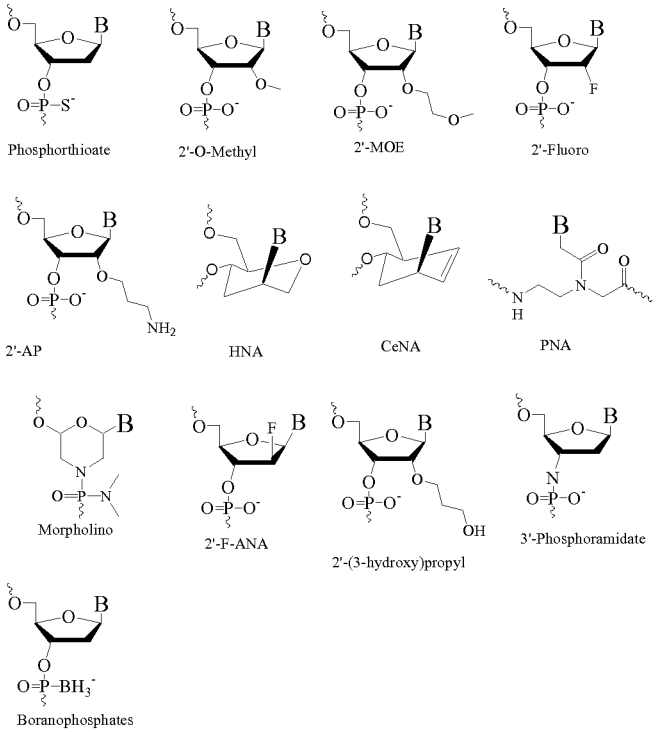
In addition to the C4'-substituted nucleotides within the gap-segment, the flanks of the gapmer antisense oligonucleotides of the invention contain nucleotide analogues, and preferably affinity enhancing modified nucleotides, particularly within the flanking regions X and Z, but may, to the extent such nucleotide analogues do not prevent cleavage of the target nucelotide acid, such as via RNaseH activity, also within the gap-segment (Y). Examples of affinity enhancing nucleotide analogues are provided herein – see also, for example, see Nawrot and Sipa, *Curr. Topics Med. Chem.* **2006**, *6*, 913-925; Current Protecols in Nucleic Acid Chemistry, John Wiley & Sons, Volumes I-III; Editors S. L.

Beaucage, D. E. Bergstrom, P. Herdewijn, A. Matsuda. Affinity enhancement can be routinely determined *in vitro* by assessing whether a nucleotide analogue enhanced the melting temperature (T_m) of an oligomer and is complementary target sequence, such as a RNA or DNA sequence.

The term "nucleotide" as used herein, refers to a glycoside comprising a sugar moiety, a base moiety and a covalently linked phosphate group and covers both naturally occurring nucleotides, such as DNA or RNA, preferably DNA, and non-naturally occurring nucleotides comprising modified sugar and/or base moieties, which are also referred to as "nucleotide analogues" herein.

Non-naturally occurring nucleotides include nucleotides which have modified sugar moieties, such as bicyclic nucleotides or 2' modified nucleotides, such as 2' substituted nucleotides.

"Nucleotide analogues" are variants of natural nucleotides, such as DNA or RNA nucleotides, by virtue of modifications in the sugar and/or base moieties. Analogues could in principle be merely "silent" or "equivalent" to the natural nucleotides in the context of the oligonucleotide, *i.e.* have no functional effect on the way the oligonucleotide works to inhibit target gene expression. Such "equivalent" analogues may nevertheless be useful if, for example, they are easier or cheaper to manufacture, or are more stable to storage or manufacturing conditions, or represent a tag or label. Preferably, however, the analogues will have a functional effect on the way in which the oligomer works to inhibit expression; for example by producing increased binding affinity to the target and/or increased resistance to intracellular nucleases and/or increased ease of transport into the cell. Specific examples of nucleoside analogues are described by *e.g.* Freier & Altmann; *Nucl. Acid Res.*, 1997, 25, 4429-4443 and Uhlmann; *Curr. Opinion in Drug Development*, 2000, 3(2), 293-213, and in Scheme 1:



Scheme 1

The oligomer may thus comprise or consist of a simple sequence of natural occurring nucleotides – preferably 2'-deoxynucleotides (referred to here generally as "DNA"), but also possibly ribonucleotides (referred to here generally as "RNA"), or a combination of such naturally occurring nucleotides and one or more non-naturally occurring nucleotides, *i.e.* nucleotide analogues. Such nucleotide analogues may suitably enhance the affinity of the oligomer for the target sequence.

Examples of suitable nucleotide analogues are provided by PCT/DK2006/000512 or are referenced therein.

Incorporation of affinity-enhancing nucleotide analogues in the oligomer, such as LNA or 2'-substituted nucleotides, can allow the size of the specifically binding oligomer to be reduced, and may also reduce the upper limit to the size of the oligomer before non-specific or aberrant binding takes place.

In some embodiments the oligomer comprises at least 2 nucleotide analogues. In some embodiments, the oligomer comprises from 3-8 nucleotide analogues, *e.g.* 6 or 7 nucleotide analogues. In the by far most preferred embodiments, at least one of said nucleotide analogues is a locked nucleic acid (LNA); for example at least 3 or at least 4, or at least 5, or at least 6, or at least 7, or 8, of the nucleotide analogues may be LNA. In some embodiments all the nucleotides analogues may be LNA.

It will be recognised that, when referring to a preferred nucleotide sequence motif or nucleotide sequence which consists of only nucleotides, the oligomers of the invention which are defined by that sequence may comprise a corresponding nucleotide analogue in place of one or more of the nucleotides present in said sequence, such as LNA units or other nucleotide analogues, which raise the duplex stability/T_m of the oligomer/target duplex (*i.e.* affinity enhancing nucleotide analogues).

In some embodiments, any mismatches between the nucleotide sequence of the oligomer and the target sequence are preferably found in regions outside the affinity enhancing nucleotide analogues, such as region Y as referred to herein, and/or region D as referred to herein, and/or at the site of non modified such as DNA nucleotides in the oligonucleotide, and/or in regions which are 5' or 3' to the contiguous nucleotide sequence.

Examples of such modification of the nucleotide include modifying the sugar moiety to provide a 2'-substituent group or to produce a bridged (locked nucleic acid) structure which enhances binding affinity and may also provide increased nuclease resistance.

A preferred nucleotide analogue is LNA, such as oxy-LNA (such as beta-D-oxy-LNA, and alpha-L-oxy-LNA), and/or amino-LNA (such as beta-D-amino-LNA and alpha-L-amino-LNA) and/or thio-LNA (such as beta-D-thio-LNA and alpha-L-thio-LNA) and/or ENA (such as beta-D-ENA and alpha-L-ENA). Most preferred is beta-D-oxy-LNA.

In some embodiments the nucleotide analogues in regions X and Z mentioned herein are independently selected from, for example: 2'-O-alkyl-RNA units, 2'-amino-DNA units, 2'-fluoro-DNA units, LNA units, arabino nucleic acid (ANA) units, 2'-fluoro-ANA units, HNA units, INA (intercalating nucleic acid -Christensen, 2002. Nucl. Acids. Res. 2002 30: 4918-4925, hereby incorporated by reference) units and 2'MOE units. In some embodiments there is only one of the above types of nucleotide analogues present in regions X and Z of the oligomer of the invention.

In some embodiments the nucleotide analogues present in regions X and Z are 2'-O-methoxyethyl-RNA (2'MOE), 2'-fluoro-DNA monomers or LNA nucleotide analogues, and as such the oligonucleotide of the invention may comprise (affinity enhancing) nucleotide analogues which are independently selected from these three types of analogue, or may comprise only one type of analogue selected from the three types. In some embodiments at least one of said nucleotide analogues is 2'-MOE-RNA, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 2'-MOE-RNA nucleotide units. In some embodiments at least one of said nucleotide analogues is 2'-fluoro DNA, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 2'-fluoro-DNA nucleotide units.

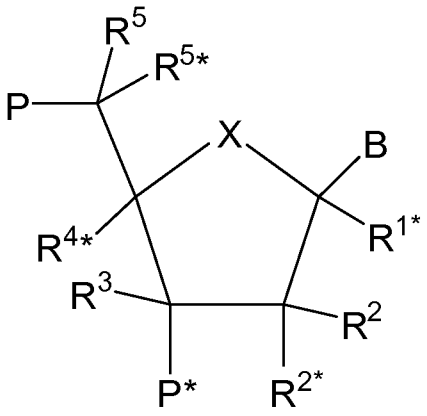
In some embodiments, the oligomer according to the invention comprises at least one Locked Nucleic Acid (LNA) unit, such as 1, 2, 3, 4, 5, 6, 7, or 8 LNA units, such as between 3 – 7 or 4 to 8 LNA units, or 3, 4, 5, 6 or 7 LNA units. The LNA units are suitably located within regions X and Z, but may also be present in region Y – for example as described herein - alpha-L LNA may be used in region Y. In some embodiments, all the nucleotide analogues of regions X and Z are LNA. In some embodiments, regions X and Z may consist or comprise both beta-D-oxy-LNA, and, optionally, one or more of the following LNA units: thio-LNA, amino-LNA, oxy-LNA, and/or ENA in either the beta-D or alpha-L configurations or combinations thereof. In some embodiments all LNA cytosine units are 5'methyl-Cytosine. In some embodiments of the invention, the oligomer may comprise both LNA and DNA units, as well as the one or more C4'-substituted nucleotides present in the gap-segment (Y). In some aspects the combined total of LNA and DNA and C4'-substituted nucleotide units is 10-25, such as 10-20 or 12-16. In some embodiments of the invention, the nucleotide sequence of the oligomer, such as the contiguous nucleotide sequence consists of at least one C4'-substituted nucleotide and the remaining nucleotide units are selected from DNA and LNA units. The term "nucleobase" refers to the base moiety of a nucleotide and covers both naturally occuring a well as non-naturally occurring variants. Thus, "nucleobase" covers not only the known purine and pyrimidine heterocycles but also heterocyclic analogues and tautomeres thereof.

Examples of nucleobases include, but are not limited to adenine, guanine, cytosine, thymidine, uracil, xanthine, hypoxanthine, 5-methylcytosine, isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, and 2-chloro-6-aminopurine.

LNA

The term "LNA" refers to a bicyclic nucleotide analogue, known as "Locked Nucleic Acid". It may refer to an LNA monomer, or, when used in the context of an "LNA oligonucleotide", LNA refers to an oligonucleotide containing one or more such bicyclic nucleotide analogues. LNA nucleotides are characterised by the presence of a biradical 'bridge' between C2' and C4' of the ribose sugar ring – for example as shown as the biradical R^{4*} - R^{2*} as described below.

The LNA used in the oligonucleotide compounds of the invention preferably has the structure of the general formula I



Formula 1

wherein for all chiral centers, asymmetric groups may be found in either R or S orientation; wherein X is selected from -O-, -S-, -N(R^{N*})-, -C(R^6R^{6*})-, such as, in some embodiments –O-; B is selected from hydrogen, optionally substituted C_{1-4}-alkoxy, optionally substituted C_{1-4}-alkyl, optionally substituted C_{1-4}-acyloxy, nucleobases including naturally occurring and nucleobase analogues, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands; P designates an internucleotide linkage to an adjacent monomer, or a 5'-terminal group, such internucleotide linkage or 5'-terminal group optionally including the substituent R^5 or equally applicable the substituent R^{5*}; P* designates an internucleotide linkage to an adjacent monomer, or a 3'-terminal group; R^{4*} and R^{2*} together designate a biradical consisting of 1 - 4 groups/atoms selected from -C(R^aR^b)-, -C(R^a)=C(R^b)-, -C(R^a)=N-, -O-, -Si(R^a)$_2$-, -S-, -SO_2-, -N(R^a)-, and >C=Z, wherein Z is selected from -O-, -S-, and -N(R^a)-, and R^a and R^b each is independently selected from hydrogen, optionally substituted C_{1-12}-alkyl, optionally substituted C_{2-12}-alkenyl, optionally substituted C_{2-12}-alkynyl, hydroxy, C_{1-12}-alkoxy, C_{2-12}-alkoxyalkyl, C_{2-12}-alkenyloxy, carboxy, C_{1-12}-alkoxycarbonyl, C_{1-12}-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted and where two geminal substituents R^a and R^b together may designate optionally substituted methylene (=CH_2), wherein for all chiral centers, asymmetric groups may be found in either *R* or *S* orientation, and; each of the substituents R^{1*}, R^2, R^3, R^5, R^{5*}, R^6 and R^{6*}, which are

present is independently selected from hydrogen, optionally substituted C_{1-12}-alkyl, optionally substituted C_{2-12}-alkenyl, optionally substituted C_{2-12}-alkynyl, hydroxy, C_{1-12}-alkoxy, C_{2-12}-alkoxyalkyl, C_{2-12}-alkenyloxy, carboxy, C_{1-12}-alkoxycarbonyl, C_{1-12}-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, hetero-aryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted, and where two geminal substituents together may designate oxo, thioxo, imino, or optionally substituted methylene; ; wherein R^N is selected from hydrogen and C_{1-4}-alkyl, and where two adjacent (non-geminal) substituents may designate an additional bond resulting in a double bond; and R^{N*}, when present and not involved in a biradical, is selected from hydrogen and C_{1-4}-alkyl; and basic salts and acid addition salts thereof. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation.

In some embodiments, R^{4*} and R^{2*} together designate a biradical consisting of a groups selected from the group consisting of $C(R^aR^b)$-$C(R^aR^b)$-, $C(R^aR^b)$-O-, $C(R^aR^b)$-NR^a-, $C(R^aR^b)$-S-, and $C(R^aR^b)$-$C(R^aR^b)$-O-, wherein each R^a and R^b may optionally be independently selected. In some embodiments, R^a and R^b may be, optionally independently selected from the group consisting of hydrogen and $_{C1-6}$alkyl, such as methyl, such as hydrogen.

In some embodiments, R^{1*}, R^2, R^3, R^5, R^{5*} are independently selected from the group consisting of hydrogen, halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl or substituted C_{2-6} alkynyl, C_{1-6} alkoxyl, substituted C_{1-6} alkoxyl, acyl, substituted acyl, C_{1-6} aminoalkyl or substituted C_{1-6} aminoalkyl. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation. In some embodiments, R^{1*}, R^2, R^3, R^5, R^{5*} are hydrogen.

In some embodiments, R^{1*}, R^2, R^3 are independently selected from the group consisting of hydrogen, halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl or substituted C_{2-6} alkynyl, C_{1-6} alkoxyl, substituted C_{1-6} alkoxyl, acyl, substituted acyl, C_{1-6} aminoalkyl or substituted C_{1-6} aminoalkyl. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation. In some embodiments, R^{1*}, R^2, R^3 are hydrogen.

In some embodiments, R^5 and R^{5*} are each independently selected from the group consisting of H, $-CH_3$, $-CH_2-CH_3$,- CH_2-O-CH_3, and $-CH=CH_2$. Suitably in some embodiments, either R^5 or R^{5*} are hydrogen, where as the other group (R^5 or R^{5*} respectively) is selected from the group consisting of C_{1-5} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, substituted C_{1-6} alkyl, substituted C_{2-6} alkenyl, substituted C_{2-6} alkynyl or substituted acyl (-C(=O)-); wherein each substituted group is mono or poly substituted with substituent groups independently selected from halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl, substituted C_{2-6} alkynyl, OJ_1, SJ_1, NJ_1J_2, N_3, $COOJ_1$, CN, $O-C(=O)NJ_1J_2$, $N(H)C(=NH)NR,R_2$ or $N(H)C(=X)N(H)J_2$ wherein X is O or S; and each J_1 and J_2 is, independently, H, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl, substituted C_{2-6} alkynyl, C_{1-6} aminoalkyl, substituted C_{1-6} aminoalkyl or a protecting group. In some embodiments either R^5 or R^{5*} is substituted C_{1-6} alkyl. In some embodiments either R^5 or R^{5*} is substituted methylene wherein preferred substituent groups include one or more groups independently selected from F, NJ_1J_2, N_3, CN, OJ_1, SJ_1, $O-C(=O)NJ_1J_2$, $N(H)C(=NH)NJ$, J_2 or $N(H)C(O)N(H)J_2$. In some embodiments each J_1 and J_2 is, independently H or C_{1-6} alkyl. In some embodiments either R^5 or R^{5*} is methyl, ethyl or methoxymethyl. In some embodiments either R^5 or R^{5*} is methyl. In a further embodiment either R^5 or R^{5*} is ethylenyl. In some embodiments either R^5 or R^{5*} is substituted acyl. In some embodiments either R^5 or R^{5*} is $C(=O)NJ_1J_2$. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation. Such 5' modified bicyclic nucleotides are disclosed in WO 2007/134181, which is hereby incorporated by reference in its entirety.

In some embodiments B is a nucleobase, including nucleobase analogues and naturally occurring nucleobases, such as a purine or pyrimidine, or a substituted purine or substituted pyrimidine, such as a nucleobase referred to herein, such as a nucleobase selected from the group consisting of adenine, cytosine, thymine, adenine, uracil, and/or a modified or substituted nucleobase, such as 5-thiazolo-uracil, 2-thio-uracil, 5-propynyl-uracil, 2'thio-thymine, 5-methyl cytosine, 5-thiozolo-cytosine, 5-propynyl-cytosine, and 2,6-diaminopurine.

In some embodiments, R^{4*} and R^{2*} together designate a biradical selected from $-C(R^aR^b)-O-$, $-C(R^aR^b)-C(R^cR^d)-O-$, $-C(R^aR^b)-C(R^cR^d)-C(R^eR^f)-O-$, $-C(R^aR^b)-O-C(R^cR^d)-$, $-C(R^aR^b)-O-C(R^cR^d)-O-$, $-C(R^aR^b)-C(R^cR^d)-$, $-C(R^aR^b)-C(R^cR^d)-C(R^eR^f)-$, $-C(R^a)=C(R^b)-C(R^cR^d)-$, $-C(R^aR^b)-N(R^c)-$, $-C(R^aR^b)-C(R^cR^d)-N(R^e)-$, $-C(R^aR^b)-N(R^c)-O-$, and $-C(R^aR^b)-S-$, $-C(R^aR^b)-C(R^cR^d)-S-$, wherein R^a, R^b, R^c, R^d, R^e, and R^f each is independently selected from hydrogen, optionally substituted C_{1-12}-alkyl, optionally

substituted C_{2-12}-alkenyl, optionally substituted C_{2-12}-alkynyl, hydroxy, C_{1-12}-alkoxy, C_{2-12}-alkoxyalkyl, C_{2-12}-alkenyloxy, carboxy, C_{1-12}-alkoxycarbonyl, C_{1-12}-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted and where two geminal substituents R^a and R^b together may designate optionally substituted methylene (=CH_2). For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation.

In a further embodiment R^{4*} and R^{2*} together designate a biradical (bivalent group) selected from -CH_2-O-, -CH_2-S-, -CH_2-NH-, -CH_2-N(CH_3)-, -CH_2-CH_2-O-, -CH_2-CH(CH_3)-, -CH_2-CH_2-S-, -CH_2-CH_2-NH-, -CH_2-CH_2-CH_2-, -CH_2-CH_2-CH_2-O-, -CH_2-CH_2-CH(CH_3)-, -CH=CH-CH_2-, -CH_2-O-CH_2-O-, -CH_2-NH-O-, -CH_2-N(CH_3)-O-, -CH_2-O-CH_2-, -CH(CH_3)-O-, and –CH(CH_2-O-CH_3)-O-, and/or, -CH_2-CH_2-, and -CH=CH- For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation.

In some embodiments, R^{4*} and R^{2*} together designate the biradical C(R^aR^b)-N(R^c)-O-, wherein R^a and R^b are independently selected from the group consisting of hydrogen, halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl or substituted C_{2-6} alkynyl, C_{1-6} alkoxyl, substituted C_{1-6} alkoxyl, acyl, substituted acyl, C_{1-6} aminoalkyl or substituted C_{1-6} aminoalkyl, such as hydrogen, and; wherein R^c is selected from the group consisting of hydrogen, halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl or substituted C_{2-6} alkynyl, C_{1-6} alkoxyl, substituted C_{1-6} alkoxyl, acyl, substituted acyl, C_{1-6} aminoalkyl or substituted C_{1-6} aminoalkyl, such as hydrogen.

In some embodiments, R^{4*} and R^{2*} together designate the biradical C(R^aR^b)-O-C(R^cR^d) -O-, wherein R^a, R^b, R^c, and R^d are independently selected from the group consisting of hydrogen, halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl or substituted C_{2-6} alkynyl, C_{1-6} alkoxyl, substituted C_{1-6} alkoxyl, acyl, substituted acyl, C_{1-6} aminoalkyl or substituted C_{1-6} aminoalkyl, such as hydrogen.

In some embodiments, R^{4*} and R^{2*} form the biradical –CH(Z)-O-, wherein Z is selected from the group consisting of C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, substituted C_{1-6} alkyl, substituted C_{2-6} alkenyl, substituted C_{2-6} alkynyl, acyl, substituted acyl, substituted

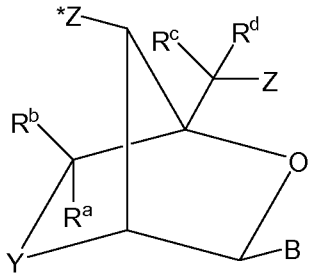
amide, thiol or substituted thio; and wherein each of the substituted groups, is, independently, mono or poly substituted with optionally protected substituent groups independently selected from halogen, oxo, hydroxyl, OJ_1, NJ_1J_2, SJ_1, N_3, $OC(=X)J_1$, $OC(=X)NJ_1J_2$, $NJ^3C(=X)NJ_1J_2$ and CN, wherein each J_1, J_2 and J_3 is, independently, H or C_{1-6} alkyl, and X is O, S or NJ_1. In some embodiments Z is C_{1-6} alkyl or substituted C_{1-6} alkyl. In some embodiments Z is methyl. In some embodiments Z is substituted C_{1-6} alkyl. In some embodiments said substituent group is C_{1-6} alkoxy. In some embodiments Z is CH_3OCH_2-. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation. Such bicyclic nucleotides are disclosed in US 7,399,845 which is hereby incorporated by reference in its entirety. In some embodiments, R^{1*}, R^2, R^3, R^5, R^{5*} are hydrogen. In some some embodiments, R^{1*}, R^2, R^{3*} are hydrogen, and one or both of R^5, R^{5*} may be other than hydrogen as referred to above and in WO 2007/134181.

In some embodiments, R^{4*} and R^{2*} together designate a biradical which comprise a substituted amino group in the bridge such as consist or comprise of the biradical $–CH_2$-N(R^c)-, wherein R^c is C_{1-12} alkyloxy. In some embodiments R^{4*} and R^{2*} together designate a biradical $–Cq_3q_4$-NOR -, wherein q_3 and q_4 are independently selected from the group consisting of hydrogen, halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl or substituted C_{2-6} alkynyl, C_{1-6} alkoxyl, substituted C_{1-6} alkoxyl, acyl, substituted acyl, C_{1-6} aminoalkyl or substituted C_{1-6} aminoalkyl; wherein each substituted group is, independently, mono or poly substituted with substituent groups independently selected from halogen, OJ_1, SJ_1, NJ_1J_2, $COOJ_1$, CN, O-C(=O)NJ_1J_2, N(H)C(=NH)N J_1J_2 or N(H)C(=X=N(H)J_2 wherein X is O or S; and each of J_1 and J_2 is, independently, H, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-6} aminoalkyl or a protecting group. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation. Such bicyclic nucleotides are disclosed in WO2008/150729 which is hereby incorporated by reference in its entirity. In some embodiments, R^{1*}, R^2, R^3, R^5, R^{5*} are independently selected from the group consisting of hydrogen, halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl or substituted C_{2-6} alkynyl, C_{1-6} alkoxyl, substituted C_{1-6} alkoxyl, acyl, substituted acyl, C_{1-6} aminoalkyl or substituted C_{1-6} aminoalkyl. In some embodiments, R^{1*}, R^2, R^3, R^5, R^{5*} are hydrogen. In some embodiments, R^{1*}, R^2, R^3 are hydrogen and one or both of R^5, R^{5*} may be other than hydrogen as referred to above and in WO 2007/134181.

In some embodiments, R^{4*} and R^{2*} form the biradical - Q -, wherein Q is $C(q_1)(q_2)C(q_3)(q_4)$, $C(q_1)=C(q_3)$, $C[=C(q_1)(q_2)]-C(q_3)(q_4)$ or $C(q_1)(q_2)-C[=C(q_3)(q_4)]$; q_1, q_2, q_3, q_4 are each independently. H, halogen, C_{1-12} alkyl, substituted C_{1-12} alkyl, C_{2-12} alkenyl,

substituted C_{1-12} alkoxy, OJ_1, SJ_1, SOJ_1, SO_2J_1, NJ_1J_2, N_3, CN, $C(=O)OJ_1$, $C(=O)$-NJ_1J_2, $C(=O)$ J_1, $-C(=O)NJ_1J_2$, $N(H)C(=NH)NJ_1J_2$, $N(H)C(=O)NJ_1J_2$ or $N(H)C(=S)NJ_1J_2$; each J_1 and J_2 is, independently, H, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-6} aminoalkyl or a protecting group; and, optionally wherein when Q is $C(q_1)(q_2)(q_3)(q_4)$ and one of q_3 or q_4 is CH_3 then at least one of the other of q_3 or q_4 or one of q_1 and q_2 is other than H. In some embodiments, R^{1*}, R^2, R^3, R^5, R^{5*} are hydrogen. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation. Such bicyclic nucleotides are disclosed in WO2008/154401 which is hereby incorporated by reference in its entirity. In some embodiments, R^{1*}, R^2, R^3, R^5, R^{5*} are independently selected from the group consisting of hydrogen, halogen, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{2-6} alkenyl, substituted C_{2-6} alkenyl, C_{2-6} alkynyl or substituted C_{2-6} alkynyl, C_{1-6} alkoxyl, substituted C_{1-6} alkoxyl, acyl, substituted acyl, C_{1-6} aminoalkyl or substituted C_{1-6} aminoalkyl. In some embodiments, R^{1*}, R^2, R^3, R^5, R^{5*} are hydrogen. In some embodiments, R^{1*}, R^2, R^3 are hydrogen and one or both of R^5, R^{5*} may be other than hydrogen as referred to above and in WO 2007/134181.

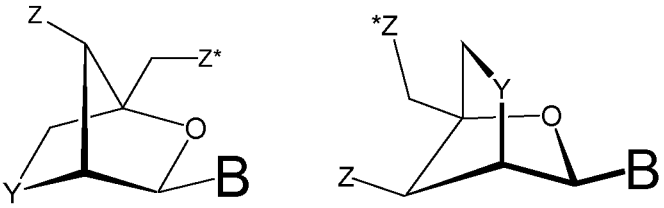
In some embodiments the LNA used in the oligonucleotide compounds of the invention preferably has the structure of the general formula II:



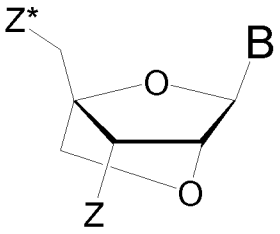
Formula II

wherein Y is selected from the group consisting of -O-, $-CH_2O$-, -S-, -NH-, $N(R^e)$ and/or $-CH_2$-; Z and Z* are independently selected among an internucleotide linkage, R^H, a terminal group or a protecting group; B constitutes a natural or non-natural nucleotide base moiety (nucleobase), and R^H is selected from hydrogen and C_{1-4}-alkyl; R^a, R^b R^c, R^d and R^e are, optionally independently, selected from the group consisting of hydrogen, optionally substituted C_{1-12}-alkyl, optionally substituted C_{2-12}-alkenyl, optionally substituted C_{2-12}-alkynyl, hydroxy, C_{1-12}-alkoxy, C_{2-12}-alkoxyalkyl, C_{2-12}-alkenyloxy, carboxy, C_{1-12}-alkoxycarbonyl, C_{1-12}-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro,

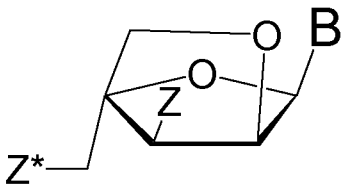
azido, sulphanyl, C_{1-6}-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted and where two geminal substituents R^a and R^b together may designate optionally substituted methylene (=CH_2); and R^H is selected from hydrogen and C_{1-4}-alkyl. In some embodiments R^a, R^b R^c, R^d and R^e are, optionally independently, selected from the group consisting of hydrogen and C_{1-6} alkyl, such as methyl. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation, for example, two exemplary stereochemical isomers include the beta-D and alpha-L isoforms, which may be illustrated as follows:



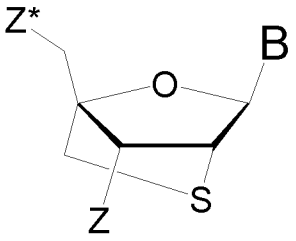
Specific exemplary LNA units are shown below:



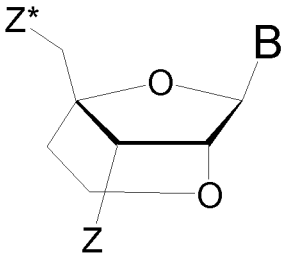
β-D-oxy-LNA



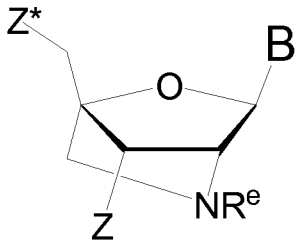
α-L-Oxy-LNA



β-D-thio-LNA



β-D-ENA



β-D-amino-LNA

The term "thio-LNA" comprises a locked nucleotide in which Y in the general formula above is selected from S or -CH_2-S-. Thio-LNA can be in both beta-D and alpha-L-configuration.

The term "amino-LNA" comprises a locked nucleotide in which Y in the general formula above is selected from -N(H)-, N(R)-, CH_2-N(H)-, and -CH_2-N(R)- where R is selected from hydrogen and C_{1-4}-alkyl. Amino-LNA can be in both beta-D and alpha-L-configuration.

The term "oxy-LNA" comprises a locked nucleotide in which Y in the general formula above represents –O-. Oxy-LNA can be in both beta-D and alpha-L-configuration.

The term "ENA" comprises a locked nucleotide in which Y in the general formula above is -CH_2-O- (where the oxygen atom of –CH_2-O- is attached to the 2'-position relative to the base B). R^e is hydrogen or methyl.

In some exemplary embodiments LNA is selected from beta-D-oxy-LNA, alpha-L-oxy-LNA, beta-D-amino-LNA and beta-D-thio-LNA, in particular beta-D-oxy-LNA.

Conjugates

In some embodiments the gapmer antisense oligonucleotides of the invention is conjugated to groups that are known to mediate improved biodistribution, cell-membrane permeability, tissue distribution etc. Examples of such groups that are known to a person skilled in the art are peptides or cholesterol.

Generally in the art of antisense oligomers, the term "conjugate" is intended to indicate a heterogenous molecule formed by the covalent attachment ("conjugation") of an oligomer to one or more non-nucleotide, or non-polynucleotide moieties. Examples of non-nucleotide or non- polynucleotide moieties include macromolecular agents such as proteins, fatty acid chains, sugar residues, glycoproteins, polymers, or combinations thereof. Typically proteins may be antibodies for a target protein. Typical polymers may be polyethylene glycol.

Therefore, in various embodiments, the oligomer of the invention may comprise both a polynucleotide region which typically consists of a contiguous sequence of nucleotides, and a further non-nucleotide region. When referring to the oligomer of the invention consisting of a contiguous nucleotide sequence, the compound may comprise non-nucleotide components, such as a conjugate component.

In various embodiments of the invention the oligomeric compound is linked to ligands/conjugates, which may be used, e.g. to increase the cellular uptake of oligomeric

compounds. WO2007/031091 provides suitable ligands and conjugates, which are hereby incorporated by reference.

The invention also provides for a conjugate comprising the compound according to the invention as herein described, and at least one non-nucleotide or non-polynucleotide moiety covalently attached to said compound. Therefore, in various embodiments where the compound of the invention consists of a specified nucleic acid or nucleotide sequence, as herein disclosed, the compound may also comprise at least one non-nucleotide or non-polynucleotide moiety (e.g. not comprising one or more nucleotides or nucleotide analogues) covalently attached to said compound.

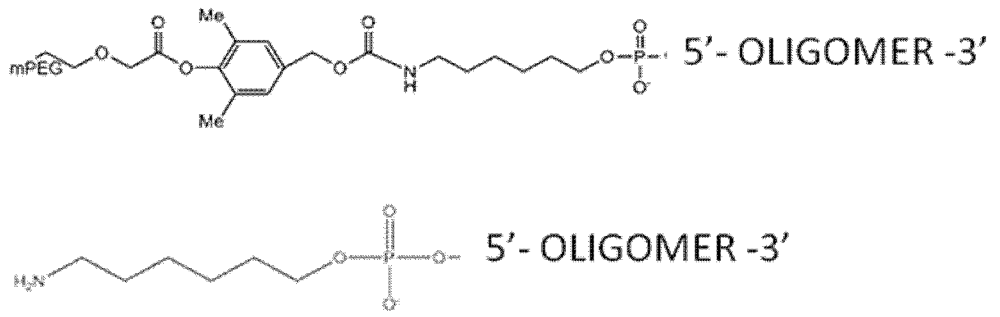
Conjugattion (to a conjugate moiety) may enhance the activity, cellular distribution or cellular uptake of the oligomer of the invention. Such moieties include, but are not limited to, antibodies, polypeptides, lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g. Hexyl-s-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipids, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-o-hexadecyl-rac-glycero-3-h-phosphonate, a polyamine or a polyethylene glycol chain, an adamantane acetic acid, a palmityl moiety, an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

The oligomers of the invention may also be conjugated to active drug substances, for example, aspirin, ibuprofen, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

In certain embodiments the conjugated moiety is a sterol, such as cholesterol.

In various embodiments, the conjugated moiety comprises or consists of a positively charged polymer, such as a positively charged peptides of, for example between 1 -50, such as 2 – 20 such as 3 – 10 amino acid residues in length, and/or polyalkylene oxide such as polyethylglycol(PEG) or polypropylene glycol – see WO 2008/034123, hereby incorporated by reference. Suitably the positively charged polymer, such as a polyalkylene oxide may be attached to the oligomer of the invention via a linker such as the releasable inker described in WO 2008/034123.

By way of example, the following conjugate moieties may be used in the conjugates of the invention:



Activated oligomers

The term "activated oligomer," as used herein, refers to an oligomer of the invention that is covalently linked (i.e., functionalized) to at least one functional moiety that permits covalent linkage of the oligomer to one or more conjugated moieties, i.e., moieties that are not themselves nucleic acids or monomers, to form the conjugates herein described. Typically, a functional moiety will comprise a chemical group that is capable of covalently bonding to the oligomer via, e.g., a 3'-hydroxyl group or the exocyclic NH_2 group of the adenine base, a spacer that is preferably hydrophilic and a terminal group that is capable of binding to a conjugated moiety (e.g., an amino, sulfhydryl or hydroxyl group). In some embodiments, this terminal group is not protected, e.g., is an NH_2 group. In other embodiments, the terminal group is protected, for example, by any suitable protecting group such as those described in "Protective Groups in Organic Synthesis" by Theodora W Greene and Peter G M Wuts, 3rd edition (John Wiley & Sons, 1999). Examples of suitable hydroxyl protecting groups include esters such as acetate ester, aralkyl groups such as benzyl, diphenylmethyl, or triphenylmethyl, and tetrahydropyranyl. Examples of suitable amino protecting groups include benzyl, alpha-methylbenzyl, diphenylmethyl, triphenylmethyl, benzyloxycarbonyl, tert-butoxycarbonyl, and acyl groups such as trichloroacetyl or trifluoroacetyl. In some embodiments, the functional moiety is self-cleaving. In other embodiments, the functional moiety is biodegradable. See e.g., U.S. Patent No. 7,087,229, which is incorporated by reference herein in its entirety.

In some embodiments, oligomers of the invention are functionalized at the 5' end in order to allow covalent attachment of the conjugated moiety to the 5' end of the oligomer. In other embodiments, oligomers of the invention can be functionalized at the 3' end. In still other embodiments, oligomers of the invention can be functionalized along the backbone or on the heterocyclic base moiety. In yet other embodiments, oligomers of the invention can be functionalized at more than one position independently selected from the 5' end, the 3' end, the backbone and the base.

In some embodiments, activated oligomers of the invention are synthesized by incorporating during the synthesis one or more monomers that is covalently attached to a functional moiety. In other embodiments, activated oligomers of the invention are synthesized with monomers that have not been functionalized, and the oligomer is functionalized upon completion of synthesis. In some embodiments, the oligomers are functionalized with a hindered ester containing an aminoalkyl linker, wherein the alkyl portion has the formula $(CH_2)_w$, wherein w is an integer ranging from 1 to 10, preferably about 6, wherein the alkyl portion of the alkylamino group can be straight chain or branched chain, and wherein the functional group is attached to the oligomer via an ester group (-O-C(O)-$(CH_2)_w$NH).

In other embodiments, the oligomers are functionalized with a hindered ester containing a $(CH_2)_w$-sulfhydryl (SH) linker, wherein w is an integer ranging from 1 to 10, preferably about 6, wherein the alkyl portion of the alkylamino group can be straight chain or branched chain, and wherein the functional group attached to the oligomer via an ester group (-O-C(O)-$(CH_2)_w$SH)

In some embodiments, sulfhydryl-activated oligonucleotides are conjugated with polymer moieties such as polyethylene glycol or peptides (via formation of a disulfide bond).

Activated oligomers containing hindered esters as described above can be synthesized by any method known in the art, and in particular by methods disclosed in PCT Publication No. WO 2008/034122 and the examples therein, which is incorporated herein by reference in its entirety.

In still other embodiments, the oligomers of the invention are functionalized by introducing sulfhydryl, amino or hydroxyl groups into the oligomer by means of a functionalizing reagent substantially as described in U.S. Patent Nos. 4,962,029 and 4,914,210, i.e., a substantially linear reagent having a phosphoramidite at one end linked through a hydrophilic spacer chain to the opposing end which comprises a protected or unprotected sulfhydryl, amino or hydroxyl group. Such reagents primarily react with hydroxyl groups of the oligomer. In some embodiments, such activated oligomers have a functionalizing reagent coupled to a 5'-hydroxyl group of the oligomer. In other embodiments, the activated oligomers have a functionalizing reagent coupled to a 3'-hydroxyl group. In still other embodiments, the activated oligomers of the invention have a functionalizing reagent coupled to a hydroxyl group on the backbone of the oligomer. In yet further embodiments, the oligomer of the invention is functionalized with more than one of the functionalizing reagents as described in U.S. Patent Nos. 4,962,029 and

4,914,210, incorporated herein by reference in their entirety. Methods of synthesizing such functionalizing reagents and incorporating them into monomers or oligomers are disclosed in U.S. Patent Nos. 4,962,029 and 4,914,210.

In some embodiments, the 5'-terminus of a solid-phase bound oligomer is functionalized with a dienyl phosphoramidite derivative, followed by conjugation of the deprotected oligomer with, e.g., an amino acid or peptide via a Diels-Alder cycloaddition reaction.

In various embodiments, the incorporation of monomers containing 2'-sugar modifications, such as a 2'-carbamate substituted sugar or a 2'-(O-pentyl-N-phthalimido)-deoxyribose sugar into the oligomer facilitates covalent attachment of conjugated moieties to the sugars of the oligomer. In other embodiments, an oligomer with an amino-containing linker at the 2'-position of one or more monomers is prepared using a reagent such as, for example, 5'-dimethoxytrityl-2'-O-(e-phthalimidylaminopentyl)-2'-deoxyadenosine-3'-- N,N-diisopropyl-cyanoethoxy phosphoramidite. See, e.g., Manoharan, et al., Tetrahedron Letters, 1991, 34, 7171.

In still further embodiments, the oligomers of the invention may have amine-containing functional moieties on the nucleobase, including on the N6 purine amino groups, on the exocyclic N2 of guanine, or on the N4 or 5 positions of cytosine. In various embodiments, such functionalization may be achieved by using a commercial reagent that is already functionalized in the oligomer synthesis.

Some functional moieties are commercially available, for example, heterobifunctional and homobifunctional linking moieties are available from the Pierce Co. (Rockford, Ill.). Other commercially available linking groups are 5'-Amino-Modifier C6 and 3'-Amino-Modifier reagents, both available from Glen Research Corporation (Sterling, Va.). 5'-Amino-Modifier C6 is also available from ABI (Applied Biosystems Inc., Foster City, Calif.) as Aminolink-2, and 3'-Amino-Modifier is also available from Clontech Laboratories Inc. (Palo Alto, Calif.).

Other Benefits

In some embodiments the antisense oligonucleotide of the invention, when bound to an RNA target sequence, is a more efficient substrates of RNase H type enzymes than the corresponding gapmer antisense oligonucleotides having in the gap-segment exclusively DNA or phosphorothioate-DNA nucleotides.

In some embodiments, the invention provides gapmer antisense oligonucleotides that display enhanced gene regulatory function, e.g. gene silencing effect, in cell cultures

or *in vivo*, relative to the corresponding gapmer antisense oligonucleotides having in the gap-segment exclusively DNA or phosphorothioate-DNA nucleotides.

In some embodiments of the invention, the gapmer antisense oligonucleotides produce a reduced immune response relative to the corresponding gapmer antisense oligonucleotides having in the gap-segment exclusively DNA or phosphorothioate-DNA nucleotides.

In some embodiments of the invention, the gapmer antisense oligonucleotides have a prolonged effect relative to the corresponding gapmer antisense oligonucleotides having in the gap-segment exclusively DNA or phosphorothioate-DNA nucleotides.

In some embodiments, the gapmer antisense oligonucleotides of the invention are delivered efficiently to specific organs or tissues of a human or an animal.

In some embodiments, the gapmer antisense oligonucleotides of the invention are able to penetrate the cell membrane efficiently.

In some embodiments, the gapmer antisense oligonucleotides of the invention are able to bind to plasma proteins which increases the retention of the RNA complexes in the human body.

Other Aspects

The invention further provides for a method of mediating nucleic acid modification of a target nucleic acid in a cell or an organism comprising the steps:

a. Contacting a cell or organism with a gapmer antisense oligonucleotide of the invention under conditions wherein modification of a target nucleic acid can occur
b. Thereby mediating modification of a target nucleic acid

In some embodiments, the method of mediating nucleic acid modification of a target nucleic acid is performed in vitro. In some embodiments, the method of mediating nucleic acid modification of a target nucleic acid is performed in vivo, i.e. in animals or in humans. In some embodiments, the method of mediating nucleic acid modification of a target nucleic acid is performed in cell cultures. In some embodiments, the method is performed on an isolated cell. In some embodiments, the nucleic acid modification of the method is gene silencing (= down regulation of gene expression), preferably degradation of target mRNA or translational inhibition of target mRNA or inhibition of other types of RNA, e.g. non-coding RNA.

The invention further provides a method of examining the function of a gene in a cell or organism comprising:

a. Introducing a gapmer antisense oligonucleotide of the invention corresponding to said gene into the cell or organism, thereby producing a test cell or test organism
b. Maintaining the test cell or test organism under conditions under which modification of a target nucleic acid can occur
c. Observing the phenotype of the test cell or organism produced in step b and optionally comparing the observed phenotype with the phenotype of an appropriate control cell or control organism, thereby providing information about the function of the gene.

The the gapmer antisense oligonucleotides of the invention can be introduced into cells e.g. using transfection or natural update (gymnosis*), a*s known to a person skilled in the art.

The information obtained about the function of a gene may be used to determine whether a gene product is a suitable target for therapeutic intervention in relation to a particular disease. Thus, if it is demonstrated that a certain gene product act in a certain biochemical pathway known to be affected in e.g. a specific subtype of cancer, the gene product might be a suitable target for therapeutic intervention for treatment of the aforementioned subtype of cancer.

In some embodiments of the method of examining the function of a gene in a cell or organism, the nucleic acid modifications of the method is gene silencing (= down regulation of gene expression), preferably degradation of target mRNA or translational inhibition of target RNA.

In some embodiments of the method of the invention, such as the method for examining the function of a gene in a cell or organism, the method is performed in cell cultures, in vitro or in vivo. In some embodiments, the method is performed on an isolated cell.

The invention further provides for a method of assessing whether an agent acts on a gene product comprising the steps:

a. Introducing a gapmer antisense oligonucleotide of the invention corresponding to said gene into a cell or organism, thereby producing a test cell or test organism
b. Maintaining the test cell or test organism under conditions under which modification of a target nucleic acid occurs
c. Introducing the agent into the test cell or test organism
d. Observing the phenotype of the test cell or organism produced in step c and optionally comparing the observed phenotype with the phenotype of an

appropriate control cell or control organism, thereby providing information about whether the agent acts on the gene product

In some embodiments of the method of assessing whether an agent acts on a gene or gene product, the nucleic acid modification of the method is gene silencing (= down regulation of gene expression), preferably degradation of target RNA or translational inhibition of target RNA.

In some embodiments of the method of assessing whether an agent acts on a gene product, the method is performed in cell cultures, in vitro or in vivo. In some embodiments, the method is performed on an isolated cell.

Compositions

The oligomer of the invention may be used in pharmaceutical formulations and compositions. Suitably, such compositions comprise a pharmaceutically acceptable diluent, carrier, salt or adjuvant. PCT/DK2006/000512 provides suitable and preferred pharmaceutically acceptable diluent, carrier and adjuvants - which are hereby incorporated by reference. Suitable dosages, formulations, administration routes, compositions, dosage forms, combinations with other therapeutic agents, pro-drug formulations are also provided in PCT/DK2006/000512 - which are also hereby incorporated by reference.

In some aspects of the invention provides a pharmaceutical composition comprising the gapmer antisense oligonucleotide and a pharmaceutically acceptable diluent, carrier or adjuvant. It will be apparent to the skilled man that the gapmer antisense oligonucleotides of the invention can be designed to target specific genes and gene products. It is to be understood that the gapmer antisense oligonucleotides will target an RNA sequence, and not a protein. However, the level of a gene product such as a protein may be affected indirectly, if its mRNA or a non-coding RNA is modified e.g. by RNA degradation or translational inhibition. Also the expression of the gene encoding the protein may be affected, e.g. because of DNA methylation.

Thus, the invention further provides for the gapmer antisense oligonucleotide of the invention for use as a medicament. Once a therapeutic target has been validated, the skilled man can design the gapmer antisense oligonucleotides that affect the level and the activity of the target, because the specificity of the gapmer antisense oligonucleotides lies exclusively within the sequence of the antisense oligonucleotide.

Most often, the gapmer antisense oligonucleotides of the invention will be prepared by automated oligonucleotide synthesis as known to a person skilled in the art. The incorporation of the C4'-substituted nucleotides, such as C4' hydroxymethyl nucleotides,

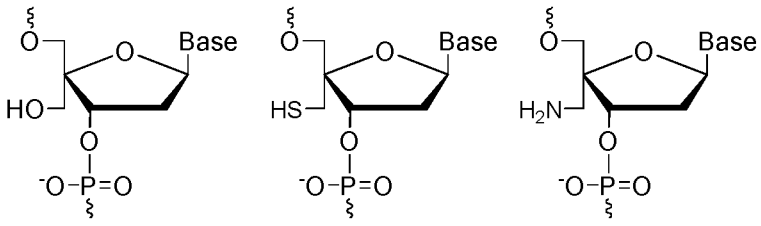
into of the gapmer antisense oligonucleotides of the invention follows standard methods for oligonucleotide synthesis, work-up, purification and isolation [F. Eckstein, Oligonucleotides and Analogues, IRL Press, Oxford University Press, 1991].

C4'-substituted nucleotides, such as C4' hydroxymethyl nucleotides, have previously been incorporated into DNA strands, and therefore procedures for preparation of their phosphoramidite building blocks for automated oligonucleotide synthesis have been reported as well as procedures that can be used for synthesis fo the gapmer antisense oligonucleotides of the invention [K. D. Nielsen et al., *Bioorg. Med. Chem.* **1995**, *3*, 1493; H. Thrane et al., *Tetrahedron* **1995**, *51*, 10389; P. Nielsen et al., *Bioorg. Med. Chem.* **1995**, *3*, 19].

Embodiments

The following embodiments of the invention as disclosed in Danish patent application PA 2008 00053, filed on 14th January 2008 describe further embodiments of aspects of the invention and may therefore be combined with the aspects of the invention described and claimed herein.

1. A gapmer antisense oligonucleotide with one or more C4'-hydroxymethyl-DNA nucleotide monomers incorporated into the gap-segment of the gapmer antisense oligonucleotide in which the one or more C4'-hydroxymethyl-DNA nucleotide monomers are selected among Monomers A-C shown in the figure below:



Monomer A Monomer B Monomer C

in which Monomer A is C4'-hydroxymentyl-DNA nucleotide monomers,
in which Monomer B is C4'-mercaptomethyl-DNA nucleotide monomers,
in which Monomer C is C4'-aminomethyl-DNA nucleotide monomers,
in which "Base" = uracil, thymine, cytosine, 5-methylcytosine, adenine, guanine or another known natural or synthetic nucleobase or nucleobase analogue,

in which the monomers of the gapmer antisense oligonucleotide can be linked by phosphordiester linkages, phosophorothioate linkages, boranophsophate linkages, methylphosphonate linkages, phosphoramidate linkages, phosphortriester linkages, or phosphorodithioate linkages, or a mixture of two or more of these linkages,
in which the hydroxymethyl substituent can be functionalised, i.e. transformed into alkyloxymethyl, like methyloxymethyl, ethyloxymethyl, propyloxymetyl, hydroxyethyloxymethyl, aminoethyloxymethyl or mecaptoethyloxymethyl, or transformed into alkylthiomethyl, alkylaminomethyl, dialkylaminomethyl, acyloxymethyl, etc, or into substituted derivatives thereof,
and in which the hydroxymethyl-substituent can be converted into aminomethyl, mercaptomethyl, etc., or into substituted derivatives thereof carrying conjugating groups like cholesteryl or long chain fatty acid residues.

2. A gapmer antisense oligonucleotide according to embodiment 1 able to mediate gene silencing (= reduction in gene expression) by RNase-H mediated antisense RNA targeting or steric block antisense RNA targeting.
3. A gapmer antisense oligonucleotide according to embodiment 1 able to mediate gene regulation by RNase-H mediated antisense RNA targeting.
4. A gapmer antisense oligonucleotide according to embodiment 1 wherein said gapmer is composed of affinity-enhancing nucleotides in the two flanks and DNA nucleotides in the gap-segment.
5. A gapmer antisense oligonucleotide according to embodiment 4 wherein said gapmer is composed of affinity-enhancing nucleotides in the two flanks and DNA nucleotides in the gap-segment with at least one linkage being a phosphorothioate linkage.
6. A gapmer antisense oligonucleotide according to embodiment 4 wherein said gapmer is composed of affinity-enhancing nucleotides in the two flanks and DNA nucleotides in the gap-segment with all linkages being phosphorothioate linkages.
7. A gapmer antisense oligonucleotide according to embodiment 6 wherein said gapmer contains affinity-enhancing LNA nucleotides in the two flanks and DNA nucleotides in the gap-segment with all linkages being phosphorothioate linkages.
8. A gapmer antisense oligonucleotide according to embodiment 6 wherein said gapmer contains affinity-enhancing O2'-alkyl-RNA nucleotides in the two flanks and DNA nucleotides in the gap-segment with all linkages being phosphorothioate linkages.

9. A gapmer antisense oligonucleotide according to embodiment 6 wherein said gapmer contains affinity-enhancing O2'-methoxyethyl-RNA nucleotides in the two flanks and DNA nucleotides in the gap-segment with all linkages being phosphorothioate linkages.

10. A gapmer antisense oligonucleotide according to any of the embodiments 4-9 wherein said gapmer contains 1-6 affinity-enhancing nucleotides in the two flanks and 6-12 DNA nucleotides in the gap-segment.

11. A gapmer antisense oligonucleotide according to any of the embodiments 4-9 wherein said gapmer contains 2-5 affinity-enhancing nucleotides in the two flanks and 8-10 DNA nucleotides in the gap-segment.

12. A gapmer antisense oligonucleotide according to any of the preceding embodiments wherein the gap-segment of the gapmer antisense oligonucleotide comprises nucleotide analogues.

13. A gapmer antisense oligonucleotide according to embodiment 12, wherein nucleotide analogues are selected from the group of arabino-configured monomers, acyclic monomers, oxepane nucleic acid monomers or □-L-LNA monomers.

14. A gapmer antisense oligonucleotide according to any of embodiments 1-13, which has improved RNase-H activity compared to the corresponding gapmer antisense oligonucleotide not containing one or more C4'-hydroxymethyl-DNA nucleotide monomers.

15. A gapmer antisense oligonucleotide according to any of embodiments 1-13, which has prolonged gene silencing effect compared to the corresponding gapmer antisense oligonucleotide not containing one or more C4'-hydroxymethyl-DNA nucleotide monomers.

16. A gapmer antisense oligonucleotide according to any of embodiments 1-13, which has an increased gene silencing effect compared to the corresponding gapmer antisense oligonucleotide not containing one or more C4'-hydroxymethyl-DNA nucleotide monomers.

17. A method of mediating gene silencing in a cell or an organism comprising contacting said cell or organism with a gapmer antisense oligonucleotide of any of embodiments 1-16 under conditions wherein gene silencing can occur.

18. A method according to embodiment 17, said method being performed in vitro.

19. A method according to embodiment 17, said method being performed on an isolated cell.

20. A method according to embodiment 17, said method being performed in vivo in a whole animal or in a human.

21. A method of examining the function of a gene in a cell or organism comprising:

a. Introducing a gapmer antisense oligonucleotide of any of embodiments 1-16 that targets RNA for gene silencing into the cell or organism, thereby producing a test cell or test organism
b. Maintaining the test cell or test organism under conditions under which gene silending occurs, thereby producing a test cell or test organism in which RNA levels of the gene is reduced
c. Observing the phenotype of the test cell or organism produced and optionally comparing the observed phenotype with the phenotype of an appropriate control cell or control organism, thereby providing information about the function of the gene.

22. A method according to embodiment 21, used for determination of whether a gene product is a suitable target for therapeutic intervention.

23. A method according to embodiments 21 and 22, said method being performed in vitro.

24. A method according to embodiment 23, said method being performed on an isolated cell.

25. A method according to embodiment 21 and 22, said method being performed in vivo in a whole animal or in a human.

26. A method of assessing whether an agent acts on a gene product comprising the steps:

a. Introducing a gapmer antisense oligonucleotide of any of embodiments 1-16 that targets RNA for mediating gene silencing into the cell or organism, thereby producing a test cell or test organism
b. Maintaining the test cell or test organism under conditions under which gene silencing occurs, thereby producing a test cell or test organism in which RNA levels of the gene is reduced
c. Introducing the agent into the test cell or test organism
d. Observing the phenotype of the test cell or organism and optionally comparing the observed phenotype with the phenotype of an appropriate control cell or control organism, thereby providing information about whether the agent acts on the gene product.

27. A method according to embodiment 26, said method being performed in vitro.

28. A method according to embodiment 27, said method being performed on an isolated cell.

29. A method according to embodiment 26, said method being performed in vivo in a whole animal or in a human.

30. A pharmaceutical composition comprising the gapmer antisense oligonucleotide of any of embodiments 1-16 and a pharmaceutically acceptable diluent, carrier or adjuvant.

31. The gapmer antisense oligonucleotide of any of embodiments 1-16 for use as a medicament.

32. The gapmer antisense oligonucleotide of any of embodiments 1-16 for use within molecular diagnostics.

33. The gapmer antisense oligonucleotide of any of embodiments 1-16 for use for disease prognostics.

34. The gapmer antisense oligonucleotide of any of embodiments 1-16 for research use.

35. The gapmer antisense oligonucleotide of any of the preceding embodiments in which the one or more C4'-hydroxymethyl-DNA nucleotide monomer is Monomer A.

36. The gapmer antisense oligonucleotide of any of the preceding embodiments in which the oligonucleotide contains a conjugating group attached either internally or at the 3'- or 5'-end of the oligonucleotide.

37. The gapmer antisense oligonucleotide of any of the preceding embodiments in which the oligonucleotide contains at least two conjugating groups attached either internally and/or at the 3'- and/or 5'-end of the oligonucleotide.

Experimental procedures and Examples

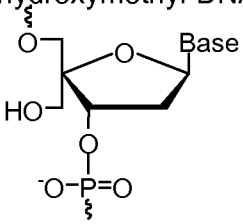
Example 1. Synthesis of the C4'-hydroxymethyl nucleotide monomers and oligonucleotides of the invention.

The incorporation of the C4'-hydroxymethyl-DNA monomers into of the gapmer antisense oligonucleotides of the invention follows standard methods for oligonucleotide synthesis, work-up, purification and isolation [F. Eckstein, Oligonucleotides and Analogues, IRL Press, Oxford University Press, 1991].

The C4'-hydroxymethyl-DNA monomers have previously been incorporated into DNA strands, and therefore procedures for preparation of their phosphoramidite building blocks for automated oligonucleotide synthesis have been reported as well as procedures that can be used for synthesis fo the gapmer antisense oligonucleotides of the invention [K. D. Nielsen et al., *Bioorg. Med. Chem.* **1995**, *3*, 1493; H. Thrane et al., *Tetrahedron* **1995**, *51*, 10389; P. Nielsen et al., *Bioorg. Med. Chem.* **1995**, *3*, 19]. It should be noted that these

published procedures can be used for synthesis of not only the thymine derivative but also the corresponding derivatives of other natural and non-natural nucleobases like cytosine, uracil, 5-methylcytosine, guanine and adenine derivatives, with minor standard changes in procedures that are known to a person skilled in the art.

LNA is an oligonucleotide containing one or more 2'-*O*,4'-*C*-methylene-linked ribonucleotides (LNA nucleotides) [M. Petersen and J. Wengel, *Trends Biotechnol.* **2003**, *21*, 74-81]. Known methods have been used to incorporate LNA nucleotides into the gapmer antisense oligonucleotides of the invention by use of commercially available LNA phosphoramidites [Pfundheller, Sørensen, Lomholt, Johansen, Koch and Wengel, J. "Locked Nucleic Acid Synthesis", *Methods Mol. Biol.* **2004**, vol. 288 (Oligonucleotide Synthesis), 127-145., P. Herdewijn, Ed., Humana Press Inc.] The structure of the C4'-hydroxymethyl-DNA monomers is exemplified below:



Example 2. Study of RNase H cleavage – preliminary data

Oligonucleotides - Antisense strands:

ON0: 5'-GTCTCTATGGACCT

ON3: 5'-GTCTC**X**ATGGACCT

- All linkages are natural phosphodiester linkages.
- A, C, G and T are DNA nucleotides
- **X** is a C4'-hydroxymethyl-DNA monomer of the invention, in this example a 4'-C-(hydroxymethyl)thymidine monomer (see structure displayed in Example 1 above; Base = thymin-1-yl).

The compatibility of **ON0** (control) and **ON3** with respect to RNase H cleavage of the corresponding duplexes formed with complementary RNA was investigated using an RNA sequence 5'-AGGUCCAUAGAGAC-3' that was [^{32}P]-labelled at its 5'-end. The radioactive RNA was mixed with unlabelled RNA (1 pmol/final sample) and a four-fold excess of the ON strand to be studied in a solution containing 20 mM Tris–HCl, pH 7.5 and 100 mM KCl. The reactions were incubated at 65 °C for 2 min followed by slow cooling to 37 °C. An equal volume of a solution containing 20 mM Tris–HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$, 2 mM DTT with 0.2 U of *E. coli* RNase H (Amersham) per final sample was added and incubation was continued at 37 °C. In control samples, RNase H was not added. Aliquots were withdrawn at the time points 2, 10 and 30 min after RNase H addition. A

basic hydrolysis of labelled RNA were performed by heating to 90 °C for 15 min in 100 mM Na_2CO_3, pH 9.0, 2 mM EDTA followed by cooling on ice and addition of formamide dye. All reactions were analyzed by PAGE (20% polyacrylamide containing 8.3 M urea) followed by autoradiography.

The RNase H cleavage patterns are depicted in the figure 1 for **ON0** (**ON0**:RNA) and **ON3** (**ON3**:RNA). Remarkably and surprisingly in light of earlier reports on the incompatibility of C4'-methyl-DNA monomers for efficient RNase H activity [Lima, W. F.; Nichols, J. G.; Wu, H.; Prakash, T. P.; Migawa, M. T.; Wyrzykiewicz, T. K.; Bhat, B.; Crooke, S. T. *J. Biol. Chem.* 2004, *279*, 36317-36326], the experiment show that the duplex containing **ON3** was more efficiently cleaved than the control duplex containing **ON0**. This testifies to the strong compatibility of the C4'-hydroxymenthyl-DNA nucleotide monomers of the invention as constituents of antisense oligonucleotides. This experiment further demonstrates that the C4'-hydroxymenthyl-DNA nucleotide monomers of the invention are compatible with highly efficient RNase H activity when incorporated into an antisense oligonucleotide having phosphordiester linkages throughout the sequence.

Example 3. Study of RNase H cleavage – data with gapmer antisense oligonucleotides.

Gapmer antisense oligonucleotides:

NAC2091: 5'-**T^{L}C^{MeL}C^{MeL}**GTCATCGCT**C^{MeL}C^{MeL}T^{L}**C

NAC2092: 5'-**T^{L}C^{MeL}C^{MeL}**G**X**CATCGCT**C^{MeL}C^{MeL}T^{L}**C

NAC2093: 5'-**T^{L}C^{MeL}C^{MeL}**GTCA**X**CGCT**C^{MeL}C^{MeL}T^{L}**C

NAC2094: 5'-**T^{L}C^{MeL}C^{MeL}**GTCATCGC**X**C^{MeL}C^{MeL}T^{L}**C

NAC2095: 5'-**T^{L}C^{MeL}C^{MeL}**G**X**CA**X**CGC**X**C^{MeL}C^{MeL}T^{L}**C

- All linkages are natural phosphorothioate linkages.
- A, C, G and T are DNA nucleotides
- **X** is a C4'-hydroxymethyl-DNA monomer of the invention, in this example a 4'-C-(hydroxymethyl)thymidine monomer (see structure displayed in Example 1 above; Base = thymin-1-yl).
- "L" in superscript indicates that the residue is an LNA nucleotide (shown in bold face letters).
- "MeL" in superscript indicates that the residue is an LNA nucleotide with a 5-methylcytosine base (shown in bold face letters).
- NAC2091 is a control gapmer antisense oligonucleotide (3-9-4 gapmer) having 3 LNA nucleotides in each of the two flanks (with on DNA nucleotide at the 3'-end of one of the flanks).

The compatibility of NAC2092-NAC-2095 with respect to RNase H cleavage of the corresponding duplexes formed with complementary RNA was investigated using a fully complementary 16-mer RNA target sequence that was [^{32}P]-labelled at its 5'-end. Experimental procedures as those described in Example 2 were used (RNA/antisense oligonucleotide ratio: ¼; 0.035 RNase H pr. oligo) with reaction samples studied at time points 30 sec, 2 min, 10 min and 30 min.

The results are depiced In Figure 2 (20% acryl amide 7M urea gels) for the cleavage reactions with NAC2091 to the left (first four lanes, increasing time towards the right), then NAC2092, then NAC2093, then NAC2094 and to the right NAC2095:

With respect to RNase H cleavage this experiment shows that:

- The control gapmer antisense oligonucleotide NAC2091 induces efficient cleavage of the RNA target;
- A single incorporation in the gap-segment of a C4'-hydroxymethyl-DNA monomer in the 5'-end of the gap-segment (NAC2092) or in the 3'-end of the gap-segment (NAC2094) is compatible with very efficient RNase H cleavage of the RNA target;
- NAC2094 seems to induce more efficient cleavage than the control NAC2091;
- One incorporation of a C4'-hydroxymethyl-DNA monomer in the centre of the gap-segment (NAC2093) is compatible with RNase H cleavage of the RNA target, albeit with slightly reduced efficiency relative to the NAC2091 control oligonucleotide;
- Three incorporations of the C4'-hydroxymethyl-DNA monomer dispersed in the gap-segment (NAC2095) is compatible with RNase H cleavage of the RNA target, albeit with reduced efficiency relative to the NAC2091 control oligonucleotide;

The excellent compatibility of the C4'-hydroxymethyl-DNA nucleotide monomer is despite of the the fact that the C4'-substituent is oriented towards the minor groove of a nucleic acid duplex where the RNase H enzyme is known to bind.

Example 4. RNA binding affinity of the gapmer antisense oligonucleotides.

Below are listed the RNA thermal denaturation temperatures recorded for the gapmer antisense oligonucleotides:

NAC2091:	5'-**T^{L}C^{MeL}C^{MeL}**GTCATCGCT**C^{MeL}C^{MeL}T^{L}**C	80.0 °C
NAC2092:	5'-**T^{L}C^{MeL}C^{MeL}**G**X**CATCGCT**C^{MeL}C^{MeL}T^{L}**C	81.0 °C
NAC2093:	5'-**T^{L}C^{MeL}C^{MeL}**GTCA**X**CGCT**C^{MeL}C^{MeL}T^{L}**C	79.0 °C
NAC2094:	5'-**T^{L}C^{MeL}C^{MeL}**GTCATCGC**X**C^{MeL}C^{MeL}T^{L}**C	79.0 °C
NAC2095:	5'-**T^{L}C^{MeL}C^{MeL}**G**X**CA**X**CGC**X**C^{MeL}C^{MeL}T^{L}**C	80.0 °C

As can be seen the C4'-hydroxymethyl-DNA nucleotide monomer is fully compatible with excellent targeting of RNA by the gapmer antisense oligonucleotides of the invention.

Example 5. Antisense effect of the gapmer antisense oligonucleotides.

The experiments above have revealed how the gapmer antisense oligonucleotides of the invention display excellent RNA targeting capabilities and surprisingly excellent RNase H substrate properties when bound to RNA. These properties in addition to increased biostability [reported for DNA oligogonucleotides containing C4'-hydroxymethyl-DNA; Fensholdt, J; Thrane, H; Wengel, J, *Tetrahedron Lett.* 1995, *36*, 2535-2538] make the gapmer antisense oligonucleotides of the invention improved antisense molecules for gene silencing in standard in vitro and in vivo experiments and improved antisense molecules as therapeutic agents.

CLAIMS

1. A gapmer oligomer which comprises one or more C4'-substituted-DNA nucleotides incorporated into the gap-segment of the gapmer oligomer, wherein said C4'-substituted-DNA nucleotides are selected from the group consisting of C4'-hydroxymentyl-DNA nucleotides, C4'-mercaptomethyl-DNA nucleotides, and C4'-aminomethyl-DNA nucleotides.
2. The gapmer oligomer according to claim 1 which consisting of a contiguous sequence of nucleotides, 5' X-Y-Z 3', wherein regions X and Z are, independently, 1-8 nucleotides in length, and consist of affinity enhancing nucleotides or affinity enhancing LNA or 2'O-alkyl-RNA nucleotides, optionally mixed with DNA or C4'hydroxymethyl-DNA nucleotides; wherein region Y is 6 – 12 nucleotides in length; and wherein region Y consists of DNA nucleotides and one or more of said C4'-substituted nucleotides; and wherein optionally region Y comprises acyclic nucleotides, arabino-configured nucleotides, oxepane nucleic acid nucleotides or alpha-L-LNA nucleotides.
3. The gapmer oligomer according to claim 1 or 2, wherein the gap-segment (region Y) comprises of only DNA nucleotides and said one or more C4'-substituted nucleotide(s).
4. The gapmer oligomer according to claim 3, wherein the gap-segment (region Y) comprises of only DNA nucleotides and only one C4'-substituted nucleotide.
5. The gapmer oligomer according to any one of claims 1 – 4, wherein the C4'-substituted nucleotide(s) is positioned one nucleotide away from one of the nucleotides of one of the flanks that is positioned next to the gap-segment.
6. The gapmer oligomer according to any one of claims 1 – 4, wherein the C4'-substituted nucleotides is juxtapositioned to one of the nucleotides of one of the flanks that is positioned next to the gap-segment.
7. The gapmer oligomer according to any one of claims 1 – 6, wherein the flanks consist or comprise of affinity enhancing LNA nucleotides.
8. The gapmer oligomer according to any one of claims 1 – 6, wherein the flanks consist or comprise of affinity enhancing 2'O-alkyl-RNA, such as 2'O-methoxyethyl-RNA nucleotides.
9. The gapmer oligomer according to any one of claims 1 – 8, wherein the linkages between the monomers of the oligomer are selected from the group consisting of phosphordiester linkages, phosophorothioate linkages, boranophsophate linkages, methylphosphonate linkages, phosphoramidate linkages, phosphortriester

linkages, or phosphorodithioate linkages, or a mixture of two or more of these linkages.

10. The gapmer oligomer according to claim 9, wherein the linkages between the monomers of the oligomer are all phosphorothioate linkages.

11. The gapmer oligomer according to any one of claims 1 – 10, wherein the two flanks (X and Z) consist of 1 – 6 affinity enhancing nucleotides and the gap-segment (Y) consists of 6 – 12 DNA nucleotides.

12. The gapmer oligomer according to any one of claims 1 – 10, wherein the two flanks (X and Z) consist of 2 – 5 affinity enhancing nucleotides and the gap-segment (Y) consists of 8 – 10 DNA nucleotides.

13. The gapmer oligomer according to any one of claims 1 – 12, wherein the oligomer is constructed as a 5-10-5, 4-10-4, 3-10-3, 2-10-2, 1-10-1, 5-9-5, 4-9-4, 3-9-3, 2-9-2, 1-9-1, 5-8-5, 4-8-4, 3-8-3, 2-8-2, 1-8-1, 5-7-7, 4-7-4, 3-7-3, or 2-7-2 gapmer.

14. The gapmer oligomer according to any one of claims 1 – 13, wherein said oligomer has improved RNase-H activity compared to the corresponding gapmer oligomer which has a gap (Y) segment which consist of DNA nucleotides.

15. The gapmer oligomer according to any one of claim 1 – 14, wherein the oligomer is conjugated, such as to a peptide or cholesterol.

16. Use of one or more C4'-hydroxymethyl-DNA nucleotides incorporated into the gap-segment of the gapmer oligomer to improve the stability of the oligomer towards enzymatic degradation in cell cultures or *in vivo*.

17. An *in vitro* method of mediating gene silencing of a target nucleic acid in a cell or an organism comprising contacting said cell or organism with a gapmer oligomer according to any one of claims 1 – 15 under conditions sufficient to induce gene-silencing of said target nucleic acid.

18. An *in vivo* method of mediating gene silencing of a target nucleic acid in a cell or an organism comprising contacting said cell or organism with a gapmer oligomer according to any one of claims 1 – 15 under conditions sufficient to induce gene-silencing of said target nucleic acid.

19. A gapmer oligomer of 10 – 30 nucleotides in length which comprises one or more C4'-substituted-DNA nucleotides incorporated into the gap-segment of the gapmer oligomer, wherein said C4'-substituted-DNA nucleotide(s) is/are, optionally independently selected from the group consisting of C4'-hydroxymentyl-DNA nucleotides, C4'-mercaptomethyl-DNA nucleotides, C4'-aminomethyl-DNA nucleotides.

20. The gapmer oligomer according to claim 19 which consisting or comprising of a contiguous sequence of nucleotides of formula (5' to 3'), X-Y-Z, or optionally X-Y-Z-D or D-X-Y-Z, wherein; region X (5' region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as between 1-6 nucleotide analogues, such as LNA units, and; region Y consists or comprises of at least five consecutive nucleotides which are capable of recruiting RNAse when formed in a duplex with a complementary RNA molecule, such as the mRNA target, such as DNA nucleotides, and; region Z (3'region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as between 1-6 nucleotide analogues, such as LNA units, and; region D, when present consists or comprises of 1, 2 or 3 nucleotide units, such as DNA nucleotides; wherein region Y comprises one or more of said C4'-substituted nucleotides .
21. The gapmer oligomer according to claim 20, wherein region Y comprises of a total of 1, 2, 3 or 4 of said C4'-substituted nucleotides
22. The gapmer oligomer according to claim 20, wherein the gap-segment (region Y) comprises of only DNA nucleotides and only one of said C4'-substituted nucleotides.
23. The gapmer oligomer according to any one of claims 19 – 22, wherein C4'-substituted nucleotides are positioned either adjacent to or one nucleotide away from the 3' nucleotide of the 5' flank (region X), and/or the 5' nucleotide of the 3' flank (region Z)
24. The gapmer oligomer according to any one of claims 19 – 23, wherein a C4'-substituted nucleotide(s) is positioned within three, two or one nucleotides of the center of the gap-segment (region Y), such as, in the case of a gap-segment which consists of an odd number of nucleotides, the central nucleotide is a C4'-substituted nucleotide.
25. The gapmer oligomer according to any one of claims 19 - 24, wherein the gap-segment (region Y) comprises of only DNA nucleotides and the one or more of said C4'-substituted nucleotides.
26. The gapmer oligomer according to any one of claims 19 – 25, wherein the gap-segment comprises DNA nucleotides, the one or more of said C4'-substituted nucleotides, and at least one, such as one, two or three nucleotide analogue units selected from the group consisting of acyclic nucleotide, arabino-configured nucleotide, oxepane nucleic acid nucleotide and alpha-L-LNA nucleotide.

27. The gapmer oligomer according to any one of claims 19 – 26, wherein the affinity enhancing nucleotides present in the flanks (regions X and Z) are independatly selected from the group consisting of 2'-O-alkyl-RNA nucleotides, 2'-amino-DNA nucleotides, 2'-fluoro-DNA nucleotides, LNA nucleotides, arabino nucleic acid (ANA) nucleotides, 2'-fluoro-ANA nucleotides, HNA nucleotides, INA nucleotides, and 2'O-methoxyethyl-RNA nucleotides.
28. The gapmer oligomer according to claim 27, wherein the flanks (regions X and Z) consist or comprise of LNA nucleotides.
29. The gapmer oligomer according to claim 27, wherein the flanks (regions X and Z) consist or comprise of 2'O-alkyl-RNA, such as 2'O-methoxyethyl-RNA (2'MOE) nucleotides.
30. The gapmer oligomer according to claim 27, wherein the flanks (regions X and Z) consist or comprise of 2'-fluoro-DNA nucleotides.
31. The gapmer oligomer according to any one of claims 19 – 30, wherein the linkages between the monomers of the oligomer are selected from the group consisting of phosphordiester linkages, phosophorothioate linkages, boranophsophate linkages, methylphosphonate linkages, phosphoramidate linkages, phosphortriester linkages, or phosphorodithioate linkages, or a mixture of two or more of these linkages.
32. The gapmer oligomer according to claim 31, wherein the linkages between the monomers of the oligomer are all phosphorothioate linkages.
33. The gapmer oligomer according to any one of claims 19 – 32, wherein the two flanks (X and Z) independently consist of 1, 2, 3, 4, 5 or 6 affinity enhancing nucleotides and the gap-segment (Y) consists of 6, 7, 8, 9, 10, 11 or 12 DNA nucleotides.
34. The gapmer oligomer according to claim 33, wherein the two flanks (X and Z) independently consist of 1 – 6, such as 2 – 5 affinity enhancing nucleotides and the gap-segment (Y) consists of 8 – 14 such as 8 – 10 DNA nucleotides.
35. The gapmer oligomer according to any one of claims 19 – 34, wherein the oligomer is constructed as a 5-10-5, 4-10-4, 3-10-3, 2-10-2, 1-10-1, 5-9-5, 4-9-4, 3-9-3, 2-9-2, 1-9-1, 5-8-5, 4-8-4, 3-8-3, 2-8-2, 1-8-1, 5-7-7, 4-7-4, 3-7-3, or 2-7-2 gapmer, optionally further comprising region D at the 5' or 3' position.
36. The gapmer oligomer according to any one of claims 19 – 35, wherein said oligomer has improved RNase-H activity compared to the corresponding gapmer oligomer which has a gap (Y) segment which consists of DNA nucleotides.

37. The gapmer oligomer according to any one of claim 19 – 36, wherein the oligomer is conjugated to at least one at least one non-nucleotide or non-polynucleotide moiety covalently attached to said oligomer..

38. Use of one or more C4'-hydroxymethyl-DNA nucleotides incorporated into the gap-segment of the gapmer oligomer to improve the stability of the oligomer towards enzymatic degradation in cell cultures or *in vivo*, such as in human blood serum.

39. An *in vitro* method of mediating gene silencing of a target nucleic acid in a cell or an organism comprising contacting said cell or organism with a gapmer oligomer according to any one of claims 19 – 37 under conditions sufficient to induce gene-silencing of said target nucleic acid.

40. An *in vivo* method of mediating gene silencing of a target nucleic acid in a cell or an organism comprising contacting said cell or organism with a gapmer oligomer according to any one of claims 19 – 37 under conditions sufficient to induce gene-silencing of said target nucleic acid in said cell to said organism.

FIGURES

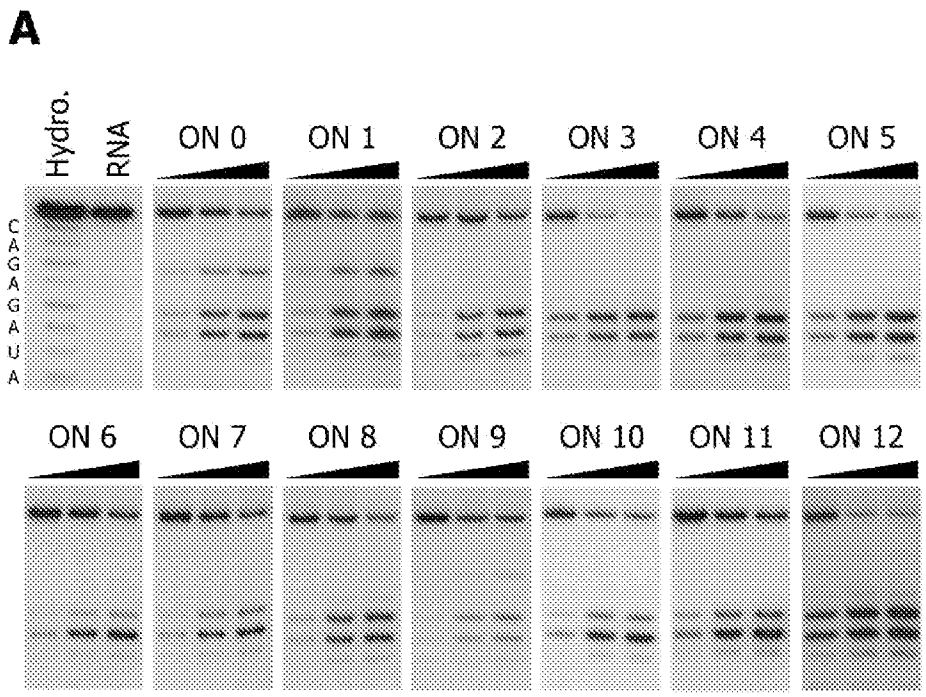


Figure 1

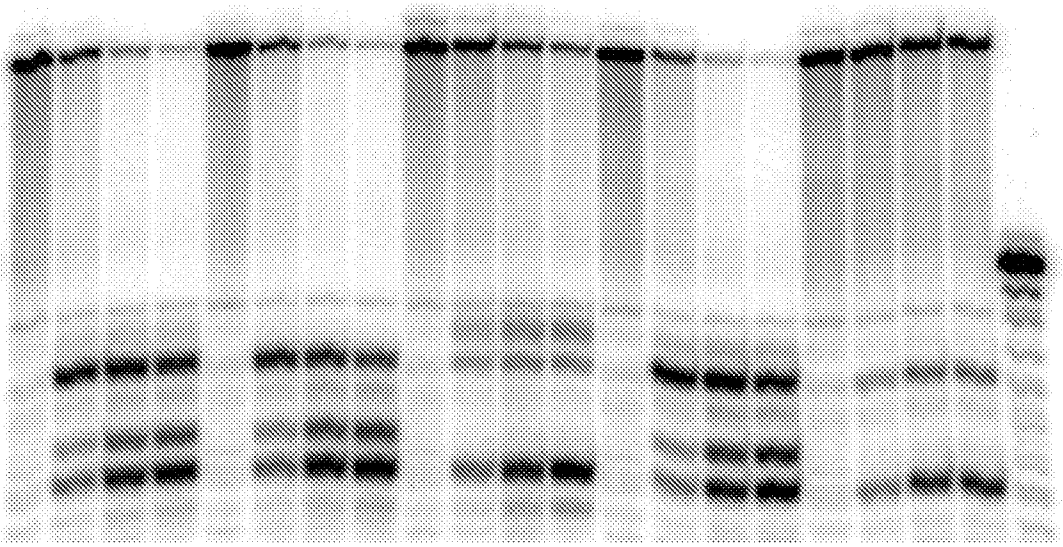


Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/050349

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H21/00 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07H C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, COMPENDEX, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIMA W F ET AL: "Structural requirements at the catalytic site of the heteroduplex substrate for human RNase H1 catalysis" JOURNAL OF BIOLOGICAL CHEMISTRY 20040827 AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY INC. US, vol. 279, no. 35, 27 August 2004 (2004-08-27), pages 36317-36326, XP002519925 cited in the application figure 2; table 1A ----- -/--	1-40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 March 2009	01/04/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Griesinger, Irina

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/050349

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FENSHOLDT J ET AL: "Synthesis of Oligodeoxynucleotides Containing 4'-C-(Hydroxymethyl)thymidine: Novel Promising Antisense Molecules" TETRAHEDRON LETTERS, ELSEVIER, AMSTERDAM, vol. 36, no. 14, 3 April 1995 (1995-04-03), pages 2535-2538, XP004028332 ISSN: 0040-4039 cited in the application abstract	1-40
A	MA D D F ET AL: "Synthetic oligonucleotides as therapeutics: the coming of age" BIOTECHNOLOGY ANNUAL REVIEW, XX, XX, vol. 5, 1 July 2000 (2000-07-01), pages 155-196, XP002965527 abstract; figure 2	1-40
A	MIHO TAKAGI-SATO ET AL: "Design of ENA gapmers as fine-tuning antisense oligonucleotides with sequence-specific inhibitory activity on mouse PADI4 mRNA expression" NUCLEIC ACIDS SYMPOSIUM SERIES, IRL PRESS, OXFORD, GB, no. 50, 1 January 2006 (2006-01-01), pages 319-320, XP003021650 ISSN: 0261-3166 abstract; figure 1	1-40
P,X	VESTER ET AL: "Chemically modified oligonucleotides with efficient RNase H response" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, ELSEVIER SCIENCE, GB, vol. 18, no. 7, 6 March 2008 (2008-03-06), pages 2296-2300, XP022574949 ISSN: 0960-894X Figure 1: Structure A page 2299, left-hand column, paragraph 2	1-40