

synthesis typically employs P(V) chemistry while DNA or RNA synthesis employs P(III) phosphoramidite chemistry. For these and other reasons, PMO and DNA syntheses have been considered incompatible.

[0009] Recent attempts at the synthesis of PMO-ASO chimeras include thiophosphoroamidate (also referred to as phosphorothioamidate) morpholinos (TMOs) – oligonucleotides that combine PS charged linkages and morpholino rings in the backbone. Synthesis of these oligonucleotides is accomplished in a 3' to 5' direction by sequential addition of morpholino 3'-phosphorodiamidite P(III) nucleotides (Langner, et al., J. Am. Chem. Soc. 2020, 142(38), 16240-16253). Caruthers reports an antisense oligonucleotide that can specifically hybridize to a target region in an exon of the human dystrophin gene to induce exon skipping that is 8 to 50 nucleotides in length, comprising a thiomorpholino nucleotide (TMO) comprising at least 8 to 10 consecutive nucleotides complementary to a target region in the human dystrophin gene exon, wherein the TMO comprises a morpholino subunit, wherein the morpholino nitrogen of the morpholino subunit is linked by a thiophosphate-containing internucleotidic linkage to a 5' exocyclic carbon of an adjacent nucleotide, or the 6'-exocyclic carbon of an adjacent morpholino subunit, or a TMO/DNA chimera, wherein and at least one nucleotide base comprises a base other than uracil (**Figures 2 and 3**).

[0010] There remains a need for a robust and flexible synthetic route for generating chimeras that contain morpholino nucleotides and other therapeutically relevant modifications, for example, for use in new therapeutic drugs with improved biological attributes.

## SUMMARY

[0011] The disclosure provides methods of making a hybrid oligonucleotide comprising assembling P(III) and P(V) nucleotide nucleotides in a 6' to 3' or 5' to 3' direction on a support, and employing PMO 6'-phosphoramidite P(III) or phosphoramidate P(V) as the first nucleotide from the support.

[0011a] In one aspect, the disclosure provides a method of making a hybrid oligonucleotide comprising assembling P(III) and P(V) nucleotides in a 6' to 3' or 5' to 3' direction on a support and employing PMO 6'-phosphoramidite P(III) or phosphoramidate P(V) as the first nucleotide from the support, wherein at least one of the assembled P(III) and P(V) nucleotides is a first morpholino nucleotide analog and at least one of the P(III) and P(V) assembled nucleotides is a first reverse RNA or a first reverse DNA nucleotide or nucleotide analog, and the method comprises connecting the first morpholino nucleotide analog to the first reverse RNA or the first reverse DNA nucleotide or nucleotide analog via a phosphorothioamidate linkage.

[0012] In one aspect, the disclosure provides a hybrid oligonucleotide synthesized by the method of the disclosure.

[0013] The disclosure also provides a hybrid oligonucleotide comprising:

(i) a first nucleotide sequence comprising a first 5' or 6' end and a first 3' end, wherein the first 3' end comprises a terminal 3' nucleic acid residue that is a morpholino nucleotide analog;

(ii) a second nucleotide sequence comprising a second 5' end and a second 3' end, wherein the second 5' end comprises a terminal 5' nucleic acid residue that is a deoxyribonucleotide or analog thereof, or a ribonucleotide or analog thereof; and

(iii) at least one phosphorothioamidate linkage that links the terminal 3' nucleic acid residue of the first nucleotide sequence and the terminal 5' nucleic acid residue of the second nucleotide sequence.

**[0013a]** In one aspect, the disclosure provides a hybrid oligonucleotide comprising:

(i) a first nucleotide sequence consisting of a series of two or more morpholino nucleotide analogs linked by phosphorodiamidate linkages and comprising a 6' end and a 3' end;

(ii) a second nucleotide sequence consisting of a series of two or more deoxyribonucleotides or analogs thereof and/or ribonucleotides or analogs thereof and comprising a 5' end and a 3' end; and

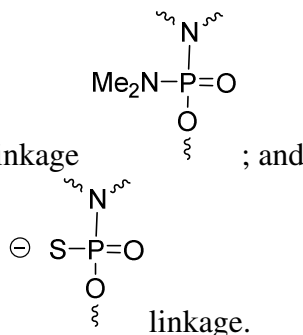
(iii) a phosphorothioamidate linkage that links the terminal 3' nucleic acid residue of the first nucleotide sequence and the terminal 5' nucleic acid residue of the second nucleotide sequence.

**[0013b]** In one aspect, the disclosure provides a hybrid oligonucleotide comprising a plurality of morpholino nucleotide analogs and a plurality of RNA or DNA nucleotides or nucleotide analogs, wherein each morpholino nucleotide analog that is linked to an adjacent RNA or DNA nucleotide or nucleotide analog is linked to the adjacent RNA or DNA nucleotide or nucleotide analog via a phosphorothioamidate linkage.

**[0014]** The disclosure also provides a hybrid oligonucleotide comprising:

(i) at least one P(III) morpholino nucleotide analog;  
(ii) at least one P(V) morpholino nucleotide analog;  
(iii) at least one P(III) ribonucleotide, at least one P(III) deoxyribonucleotide analog, or a combination thereof;

(iv) at least one phosphorodiamidate linkage



; and

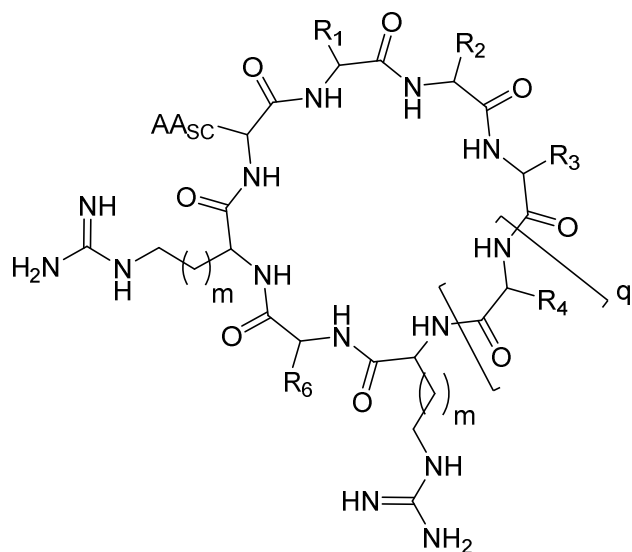
(v) at least one phosphorothioamidite linkage.

**[0015]** In examples, each 5' nucleotide adjacent to each P(V) morpholino nucleotide analog is a P(III) or P(V) morpholino nucleotide analog.

**[0016]** In examples, the hybrid oligonucleotide comprises two or more consecutive P(V) morpholino nucleotide analogs linked through phosphorodiamidate linkages.

**[0017]** In examples, any P(V) morpholino nucleotide analog is linked to any downstream nucleotide that is not a P(V) morpholino nucleotide analog through a phosphorothioamidate linkage.

**[0018]** The disclosure also relates to compound comprising: the hybrid oligonucleotide of the disclosure, and a cyclic peptide of formula (I):



(I), or a protonated form thereof,

wherein:

R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> are each independently H or an aromatic or heteroaromatic side chain of an amino acid;

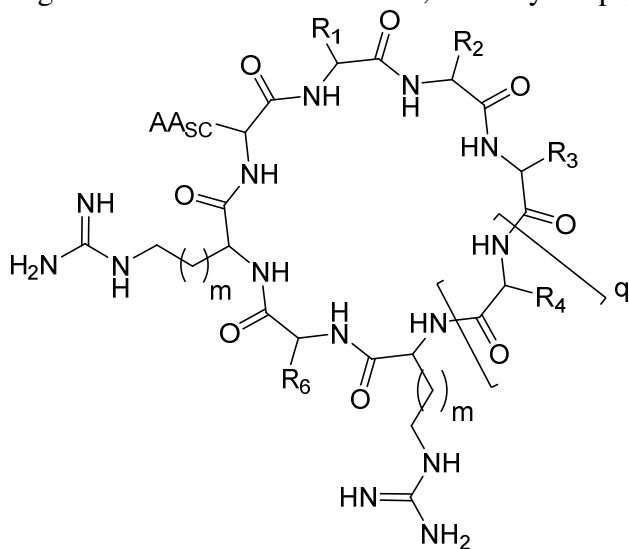
5 R<sub>4</sub> and R<sub>6</sub> are independently H or an amino acid side chain;

AA<sub>sc</sub> is an amino acid side chain;

q is 1, 2, 3 or 4; and

each m is independently an integer from 0-3.

10 **[0018a]** In one aspect, the disclosure provides a compound comprising: the hybrid oligonucleotide of the disclosure, and a cyclic peptide of formula (I)



(I), or a protonated form thereof,

wherein:

R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> are each independently H or a residue of tyrosine, phenylalanine or tryptophan;

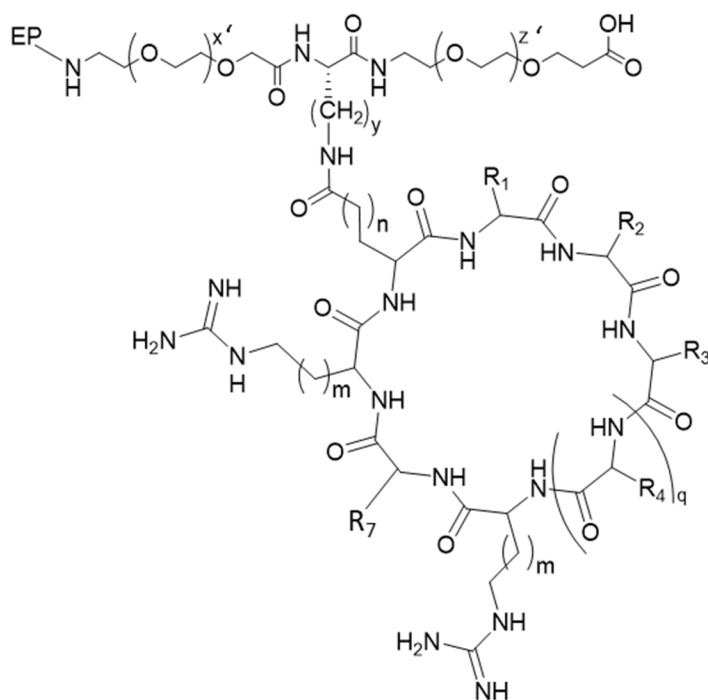
15 R<sub>4</sub> and R<sub>6</sub> are independently H or an amino acid side chain;

AA<sub>sc</sub> is an amino acid side chain;

q is 1, 2, 3 or 4; and

each m is independently an integer from 0-3.

20 **[0019]** In one aspect, the disclosure also relates to a compound comprising: the hybrid oligonucleotide of the disclosure, and an EEV of Formula (B):



(B), or a protonated form thereof,

wherein:

$R_1$ ,  $R_2$ , and  $R_3$  are each independently H or an aromatic or heteroaromatic side chain of an amino acid;

5  $R_4$  and  $R_7$  are independently H or an amino acid side chain;

EP is an exocyclic peptide as defined herein;

each  $m$  is independently an integer from 0-3;

$n$  is an integer from 0-2;

$x'$  is an integer from 1-20;

10  $y$  is an integer from 1-5;

$q$  is 1-4; and

$z'$  is an integer from 1-23.

[0020] The disclosure further relates to compositions comprising the compounds of the disclosure and method of treating a disease with such compounds and compositions. In one aspect, the disclosure provides a pharmaceutical composition comprising a hybrid oligonucleotide of the disclosure or a compound of the disclosure.

## BRIEF DESCRIPTION OF THE FIGURES

[0021] **Fig. 1A-1B.** A schematic comparing: (A) a typical gapmer structure comprising 5' and 3' ribonucleic acid (RNA) flanking regions in which the RNA monomer units are linked through phosphorothioate (PS) linkages (RNA-PS) and a deoxyribonucleotide (DNA) containing center

## CLAIMS

1. A hybrid oligonucleotide comprising:
  - (i) a first nucleotide sequence consisting of a series of two or more morpholino nucleotide  
5 analogs linked by phosphorodiamidate linkages and comprising a 6' end and a 3' end;
  - (ii) a second nucleotide sequence consisting of a series of two or more  
deoxyribonucleotides or analogs thereof and/or ribonucleotides or analogs thereof and  
comprising a 5' end and a 3' end; and
  - (iii) a phosphorothioamidate linkage that links the terminal 3' nucleic acid residue of the  
10 first nucleotide sequence and the terminal 5' nucleic acid residue of the second nucleotide  
sequence.
2. The hybrid oligonucleotide of claim 1, wherein the series of deoxyribonucleotides or  
analogs thereof and/or ribonucleotides or analogs thereof comprises a series of 2'-  
modified ribonucleotide analogs.
- 15 3. The hybrid oligonucleotide of claim 2, wherein the series of 2'-modified ribonucleotide  
analogs is a series of 2'-OMe ribonucleotide analogs.
4. The hybrid oligonucleotide of any one of claims 1 to 3, wherein the hybrid  
oligonucleotide comprises at least about 10% and up to 50% morpholino nucleotide  
analogs.
- 20 5. The hybrid oligonucleotide of any one of claims 1 to 4, wherein the hybrid  
oligonucleotide comprises from up to 25% morpholino nucleotide analogs to up to 40%  
morpholino nucleotide analogs.
6. The hybrid oligonucleotide of any one of claims 1 to 5, wherein the hybrid  
oligonucleotide is a steric block oligonucleotide.
- 25 7. The hybrid oligonucleotide of claim 6, wherein the steric block oligonucleotide is  
configured to induce exon skipping.

8. The hybrid oligonucleotide of any one of claims 1 to 7, wherein each morpholino nucleotide analog that is linked to an adjacent morpholino nucleotide analog is linked to the adjacent morpholino nucleotide analog via a phosphorodiamidate linkage.
- 5 9. The hybrid oligonucleotide of any one of claims 1 to 8, wherein each RNA or DNA nucleotide or nucleotide analog that is linked to an adjacent RNA or DNA nucleotide or nucleotide analog is linked to the adjacent RNA or DNA nucleotide or nucleotide analog via a phosphorothioate linkage.
10. The hybrid oligonucleotide of claim 9, comprising from 10 to 50 nucleotides.
- 10 11. The hybrid oligonucleotide of any one of claims 1 to 10, wherein less than 75% of the internucleotidic linkages have a negative charge.
12. The hybrid oligonucleotide of any one of claims 1 to 10, wherein less than 50% of the internucleotidic linkages have a negative charge.
13. The hybrid oligonucleotide of any one of claims 1 to 10, wherein less than 25% of the internucleotidic linkages have a negative charge.
- 15 14. The hybrid oligonucleotide of any one of claims 1 to 13, comprising a gapmer in which:
- (i) the first nucleotide sequence comprises a 5' flank of the gapmer, wherein the 5' flank comprises from 2 to 20 morpholino nucleotide analogs;
- (ii) the second oligonucleotide sequence comprises a gap oligonucleotide, wherein the gap oligonucleotide sequence comprises from 8 to 20 deoxyribonucleotides or
- 20 ribonucleotides or analogs thereof; and
- wherein hybrid oligonucleotide further comprising a 3' flank comprising from 2 to 20 morpholino nucleotide analogs.
15. The hybrid oligonucleotide of claim 14, wherein the 5' flank comprises from 2 to 10 morpholino nucleotide analogs.
- 25 16. The hybrid oligonucleotide of claim 14, wherein the 5' flank comprises from 2 to 5 morpholino nucleotide analogs.

17. The hybrid oligonucleotide of any one of claims 14 to 16, wherein the first 5' or 6' end of the 5' flank comprises a lynchpin residue comprising a PMO 6'-phosphoramidite P(III).
18. The hybrid oligonucleotide of any one of claims 14 to 17, wherein the 3' flank comprises from 2 to 10 morpholino nucleotide analogs.
- 5 19. The hybrid oligonucleotide of any one of claims 14 to 17, wherein the 3' flank comprises from 2 to 5 morpholino nucleotide analogs.
20. The hybrid oligonucleotide of any one of claims 14 to 19, wherein morpholino nucleotide analogs of the 3' flank are linked through phosphorodiamidate linkages.
21. The hybrid oligonucleotide of any one of claims 14 to 20, wherein the second 5' or 6' end  
10 of the 3' flank comprises a lynchpin residue comprising a PMO 6'-phosphoramidite P(III).
22. The hybrid oligonucleotide of any one of claims 14 to 21, comprising 10 or fewer morpholino nucleotide analogs.
23. The hybrid oligonucleotide of any one of claims 21 to 21, comprising 7 or fewer  
15 morpholino nucleotide analogs.
24. The hybrid oligonucleotide of any one of claims 14 to 23, wherein at least 50% of the deoxyribonucleotide analogs of the gap oligonucleotide sequence are linked through phosphothioate linkages.
25. A hybrid oligonucleotide comprising a plurality of morpholino nucleotide analogs and a  
20 plurality of RNA or DNA nucleotides or nucleotide analogs, wherein each morpholino nucleotide analog that is linked to an adjacent RNA or DNA nucleotide or nucleotide analog is linked to the adjacent RNA or DNA nucleotide or nucleotide analog via a phosphorothioamidate linkage.
26. A method of making a hybrid oligonucleotide comprising assembling P(III) and P(V)  
25 nucleotides in a 6' to 3' or 5' to 3' direction on a support and employing PMO 6'-phosphoramidite P(III) or phosphoramidate P(V) as the first nucleotide from the support, wherein at least one of the assembled P(III) and P(V) nucleotides is a first morpholino nucleotide analog and at least one of the P(III) and P(V) assembled nucleotides is a first reverse RNA or a

first reverse DNA nucleotide or nucleotide analog, and the method comprises connecting the first morpholino nucleotide analog to the first reverse RNA or the first reverse DNA nucleotide or nucleotide analog via a phosphorothioamidate linkage.

27. The method of claim 26, wherein PMO 6'-phosphoramidite P(III) is the first nucleotide from the support.

28. The method of claim 26, wherein PMO 6'-phosphoramidate P(V) is the first nucleotide from the support.

29. The method of any of claims 26 to 28, wherein the hybrid oligonucleotide comprises:

(a) at least one phosphorothioamidate linkage; and

(b) at least one phosphorothioester linkage, phosphorodiester linkage, phosphorodiamidate linkage or a combination thereof.

30. The method of any of claims 26 to 29, wherein the method comprises coupling a first reverse amidite to a secondary amino group of a morpholino ring to form a phosphorothioamidate linkage.

31. The method of any one of claims 26 to 30, further comprising adding at least one P(III) nucleotide.

32. The method of any one of claims 26 to 31, comprising adding at least two P(III) nucleotide.

33. The method of claim 32, wherein the P(III) nucleotides are consecutive.

34. The method of any one of claims 26 to 33, further comprising adding at least one P(V) nucleotide.

35. The method of any one of claims 26 to 34, comprising adding at least two P(V) nucleotides.

36. The method of claim 35, wherein the P(V) nucleotides are consecutive.

37. The method of any of claims 26 to 36, wherein at least two P(III) nucleotides are followed by at least two P(V) nucleotides.

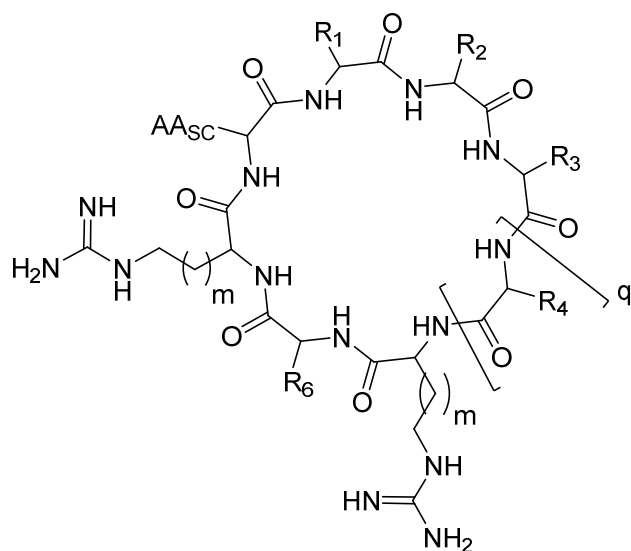
38. The method of any of claims 26 to 37, wherein at least two P(V) nucleotides are followed by at least two P(III) nucleotides.
39. The method of any of claims 26 to 38, wherein at least one P(III) nucleotide and one P(V) nucleotide alternate to form a P(III)-P(V)-P(III) or P(V)-P(III)-P(V) motif.
- 5 40. The method of any one of claims 26 to 39, wherein the method comprises deprotecting and neutralizing the 3'-terminal secondary amino group of a first DNA nucleotide monomer.
41. The method of claim 40, wherein the method comprises further adding one or more additional DNA nucleotides to the first DNA nucleotide, wherein two or more DNA nucleotides are linked via a phosphorothioate (PS) linkage.
- 10 42. The method of claim 40 or 41, wherein the method further comprises adding a second PMO 6'-phosphoramidite P(III) to a 3' terminal DNA nucleotide of the hybrid oligonucleotide.
43. The method of any one of claims 26 to 42, wherein the hybrid oligonucleotide comprises a gapmer comprising:
- 15 (i) a 6' flank comprising two or more morpholino nucleotide analogs, or a 5' flank comprising two or more ribonucleotides or ribonucleotide analogs;
- (ii) a gap region comprising 5 or more deoxyribonucleotides or deoxyribonucleotide analogs (DNA monomers); and
- (iii) a 3' flank comprising two or more morpholino nucleotide analogs, or a 3' flank comprising two or more ribonucleotides or ribonucleotide analogs,
- 20 wherein the method comprises extending the oligonucleotide by adding one or more additional DNA monomers to the first DNA monomer that are linked through a phosphorothioate bond (DNA-PS).
44. The method of claim 43, wherein the method further comprises adding PMO 6'-phosphoramidite P(III) to the 3' end of the oligonucleotide.
- 25 45. The method of claim 44, comprising adding the PMO 6'-phosphoramidite P(III) after all cytidine-based nucleotides in the 5' flank and the gap are N-benzoyl protected and Cap A is based on iso-butyric, pivalic or benzoic anhydride instead of acetic anhydride.

46. The method of claim 43, wherein the oligonucleotide comprises: 6'-PMO-DNA-PMO-3', 6'-PMO-DNA-RNA-3', 5'-RNA-DNA-PMO-3'.

47. A hybrid oligonucleotide synthesized by the method of any one of claims 26 to 46.

48. A compound comprising: the hybrid oligonucleotide of any of claims 1 to 25 or 47, and a

5 cyclic peptide of formula (I)



(I), or a protonated form thereof,

wherein:

R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> are each independently H or a residue of tyrosine, phenylalanine or tryptophan;

10 R<sub>4</sub> and R<sub>6</sub> are independently H or an amino acid side chain;

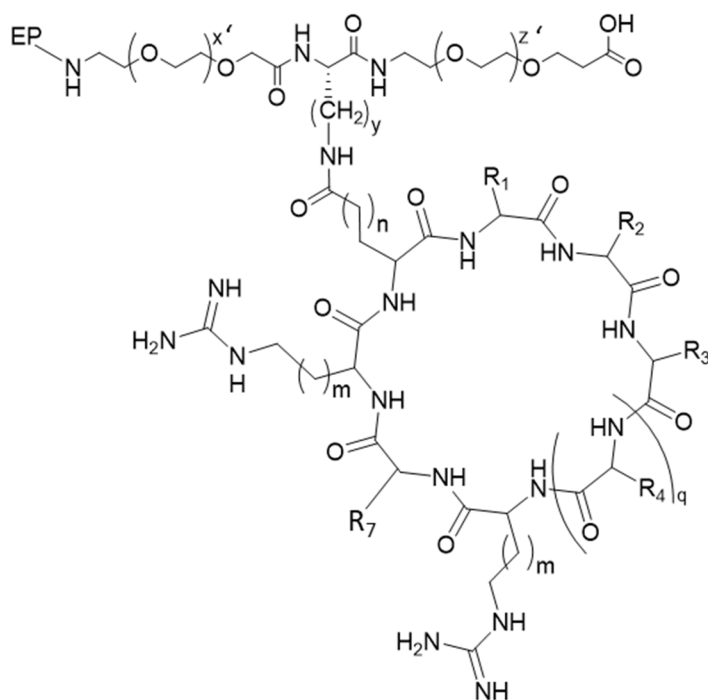
AA<sub>SC</sub> is an amino acid side chain;

q is 1, 2, 3 or 4; and

each m is independently an integer from 0-3.

49. A compound comprising: the hybrid oligonucleotide of any of claims 1 to 25 or 47, and

15 an EEV of Formula (B):



(B), or a protonated form thereof,

wherein:

R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> are each independently H or an aromatic or heteroaromatic side chain of an amino acid;

5 R<sub>4</sub> and R<sub>7</sub> are independently H or an amino acid side chain;

EP is an exocyclic peptide as defined herein;

each m is independently an integer from 0-3;

n is an integer from 0-2;

x' is an integer from 1-20;

10 y is an integer from 1-5;

q is 1-4; and

z' is an integer from 1-23.

50. A pharmaceutical composition comprising a hybrid oligonucleotide of any one of claims 1 to 25 or 47 or a compound of claim 48 or 49.