

Trinucleotide Cap Analogs and Methods of Use Thereof

CROSS-REFERENCE TO PRIORITY APPLICATIONS

This application claims priority to U.S. Provisional Application No. 63/267,223, filed January 27, 2022; U.S. Provisional Application No. 63/382,956, filed November 9, 2022; and U.S. Provisional Application No. 63/476,787, filed December 22, 2022, the contents of which are incorporated herein by reference in their entireties.

BACKGROUND

In vitro transcribed messenger RNAs (mRNAs) have numerous *in vivo* applications, such as vaccination, where mRNA encoding specific antigen(s) is administered to elicit protective immunity in a patient; cell therapy, where mRNA is transfected into cells *ex vivo* to alter cell phenotype or function prior to delivery of these altered cells to a patient; or replacement therapy, where mRNA encoding a therapeutic protein is administered to the patient.

A primary structural element of an mRNA molecule that is utilized in *in vivo* applications includes a Cap structure on the 5'-end of the mRNA. Naturally occurring Cap structures include a 7-methylguanosine (^{7m}G or ^{m7}G) linked through a 5'- to 5'-triphosphate chain at the 5'-end of the mRNA molecule. The Cap must be present for the mRNA to retain template activity for protein synthesis. The chemical structure of the Cap can modulate translation efficiency in a cell. Therefore, effective Cap structures are necessary.

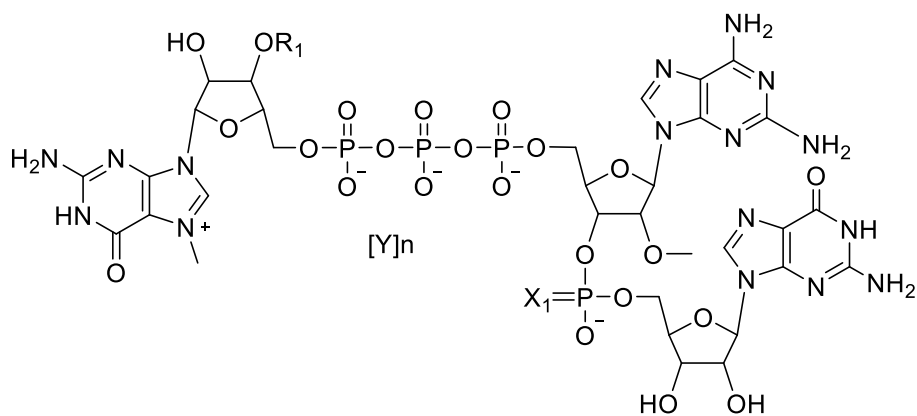
SUMMARY

This summary is a high-level overview of various examples of the disclosure and introduces some of the concepts that are further described in the Detailed Description section below. This summary is not intended to identify key or essential features of the claimed subject matter, nor is it intended to be used in isolation to determine the scope of the claimed subject matter. The subject matter should be understood by reference to appropriate portions of the entire specification, any or all drawings, and each claim.

Described herein are trinucleotide cap analogs and methods of making and using the same. In one aspect, there is provided herein is a compound of the following formula:

or any stereoisomer thereof.

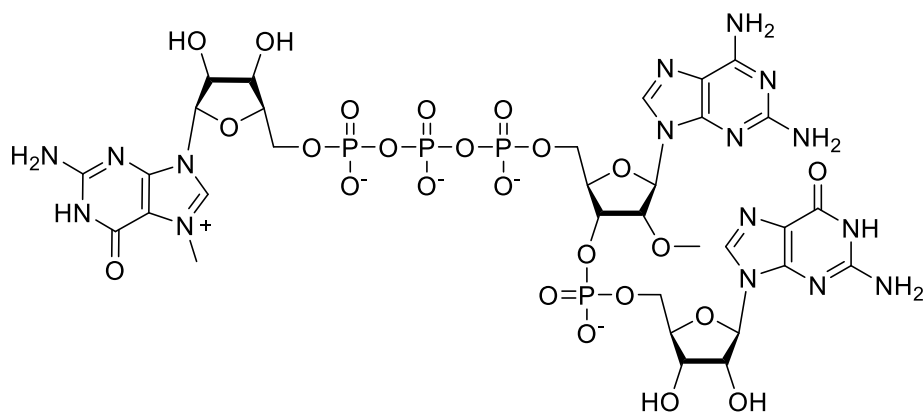
In another aspect, there is also provided herein is a compound of the following formula:



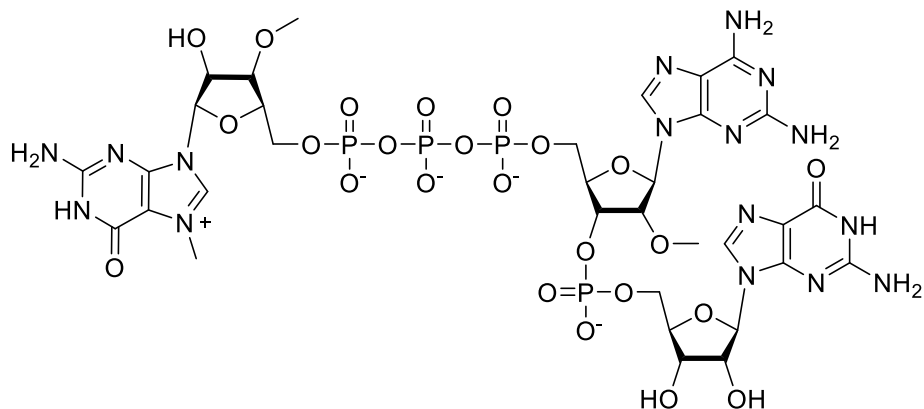
5

Formula II

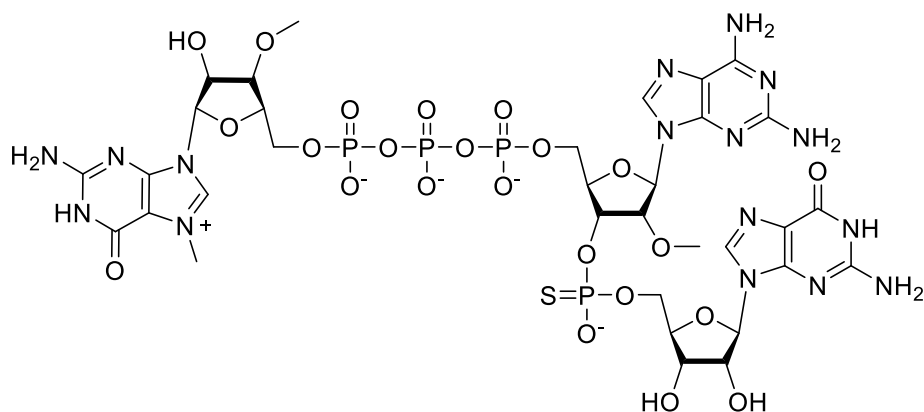
or any stereoisomer thereof, wherein: R₁ is H or CH₃, X₁ is O or S, each independent Y is H⁺ or a cation, and n is 0, 1, 2, 3, or 4, wherein when X₁ is S, R₁ is CH₃. Optionally, the compound has the following structure:



Compound 7



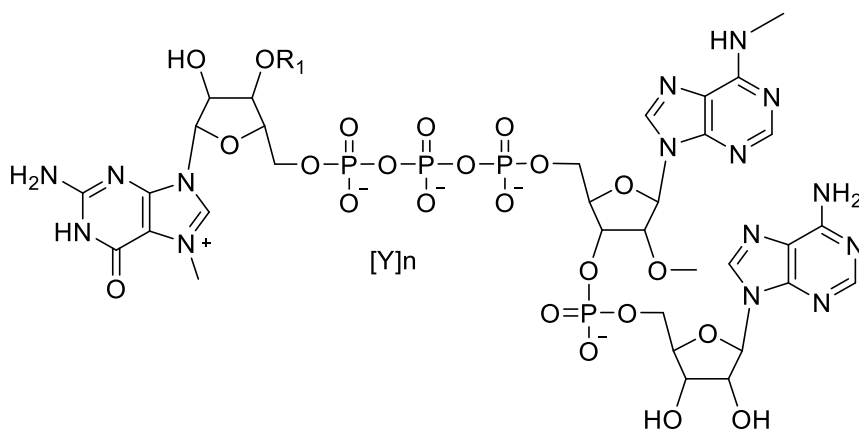
Compound 8



Compound 9

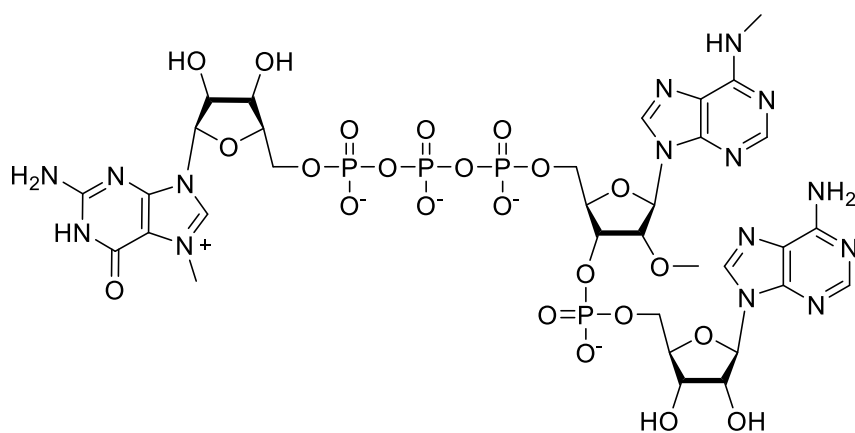
or any stereoisomer thereof.

In another aspect, there is also provided herein is a compound of the following formula:

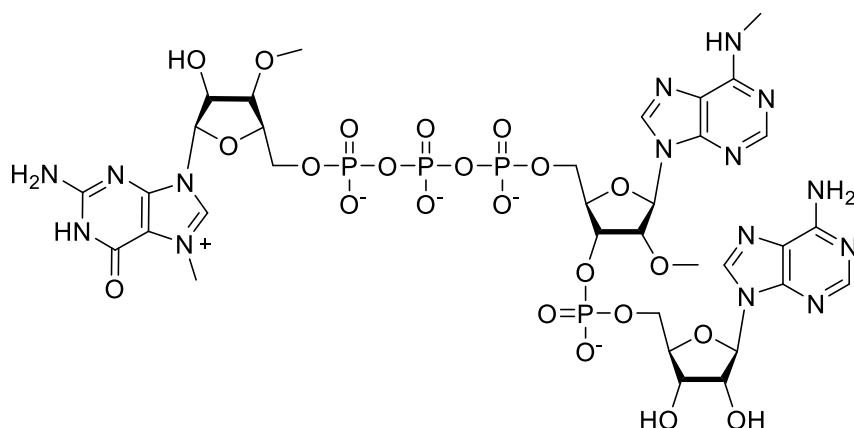


Formula III

or any stereoisomer thereof, wherein: R_1 is H or CH_3 ; each independent Y is H^+ or a cation; and n is 0, 1, 2, 3, or 4. Optionally, the compound has the following structure:



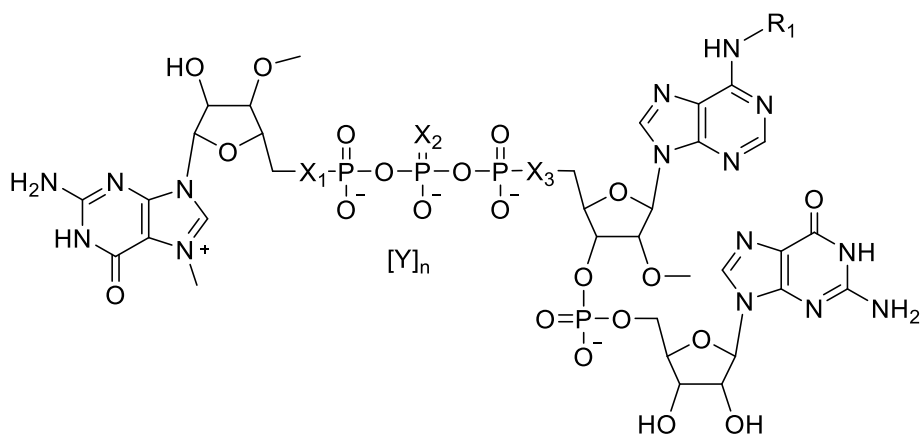
Compound 10



Compound 11

or any stereoisomer thereof.

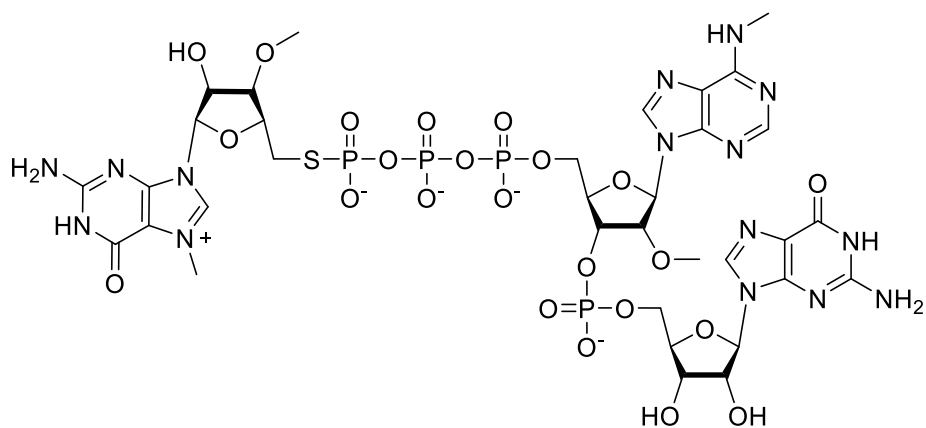
In another aspect, there is additionally provided herein is a compound of the following formula:



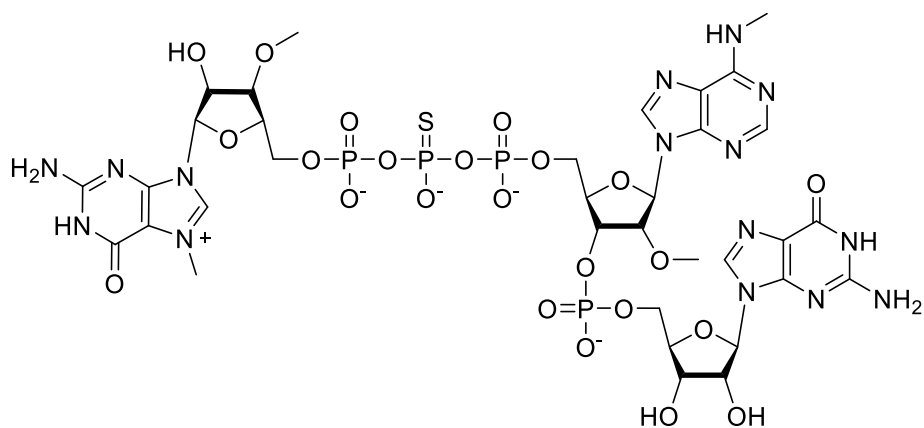
Formula IV

or any stereoisomer thereof, wherein: R_1 is H or CH_3 ; X_1 , X_2 , and X_3 are each independently selected from O and S, wherein when one of X_1 , X_2 , or X_3 is S, the remaining of X_1 , X_2 , and X_3 are O; each independent Y is H^+ or a cation; and n is 0, 1, 2, 3, or 4.

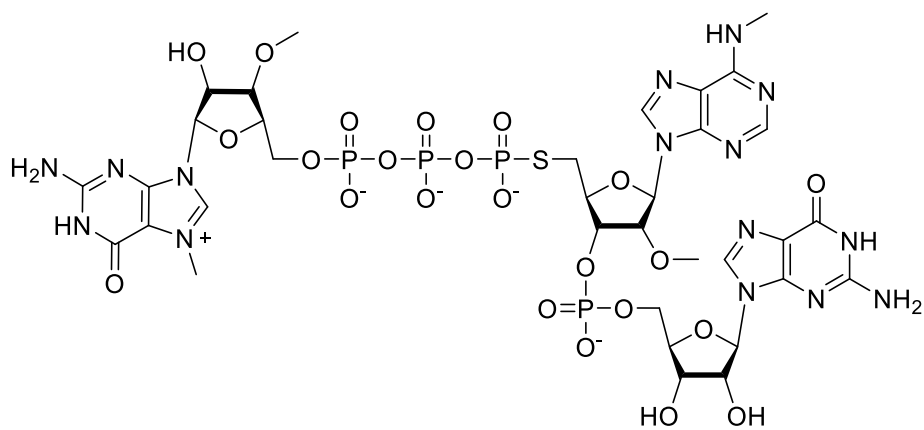
Optionally, the compound has the following structure:



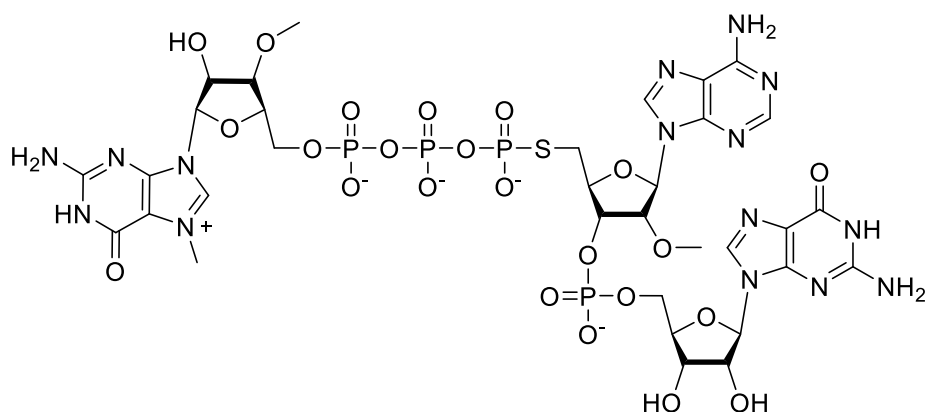
Compound 12



Compound 13



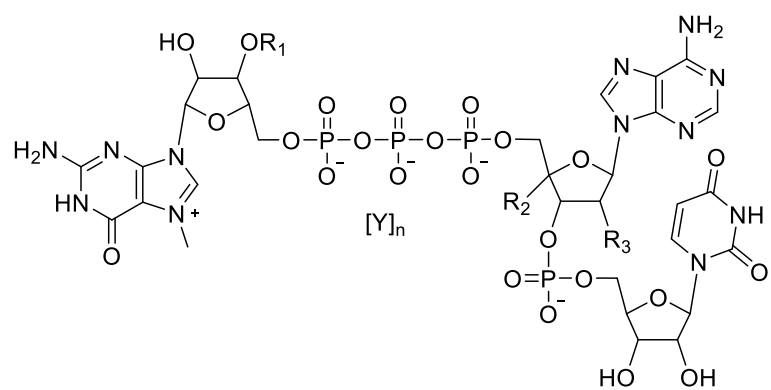
Compound 14



Compound 73

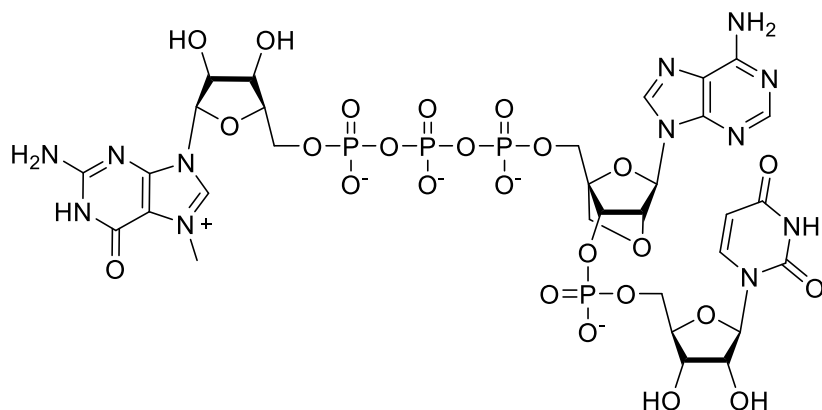
or any stereoisomer thereof.

In another aspect, there is further provided herein is a compound of the following formula:

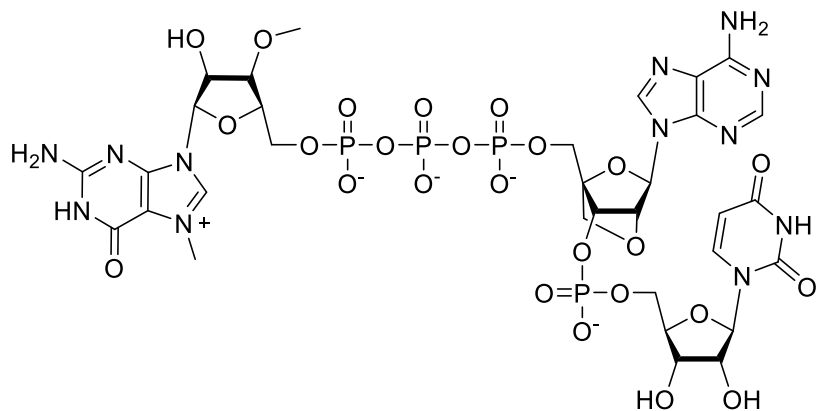


Formula V

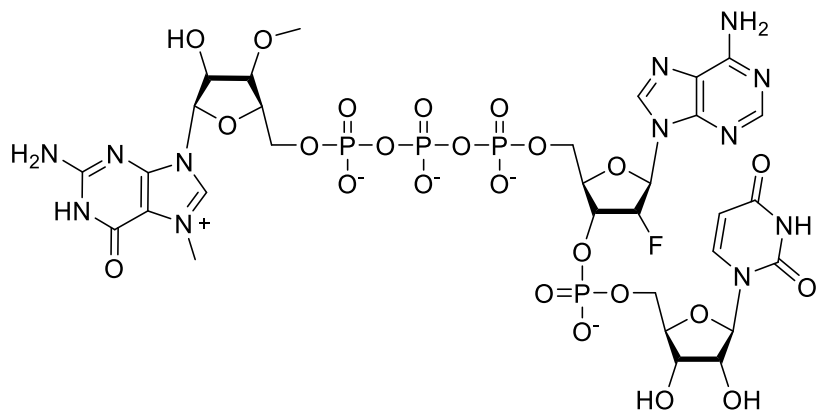
or any stereoisomer thereof, wherein: R_1 is H or CH_3 ; R_2 is H and R_3 is F, or R_2 and R_3 are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; each independent Y is H^+ or a cation; and n is 0, 1, 2, 3, or 4, wherein when R_2 is H and R_3 is F, R_1 is not H. Optionally, the compound has the following structure:



Compound 15



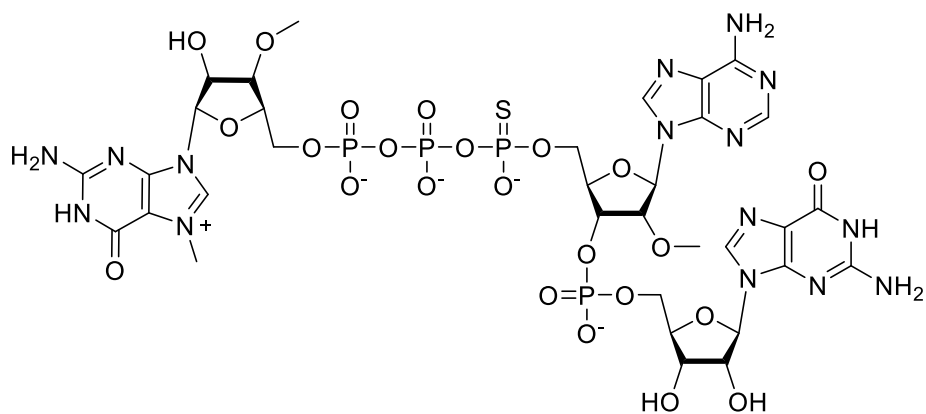
Compound 16



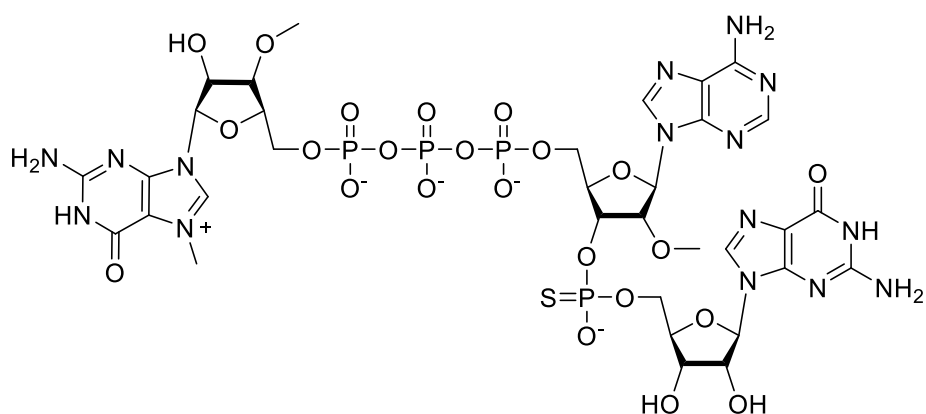
Compound 17

5 or any stereoisomer thereof.

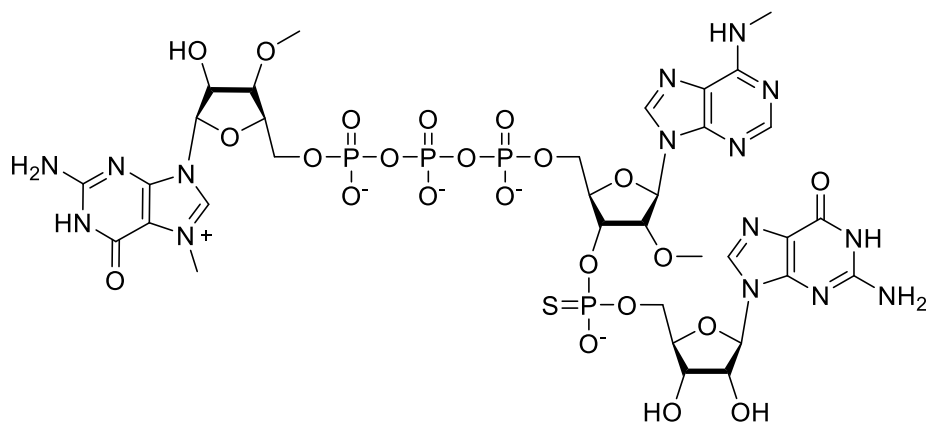
In another aspect, there is also provided herein is a compound of the following formula:



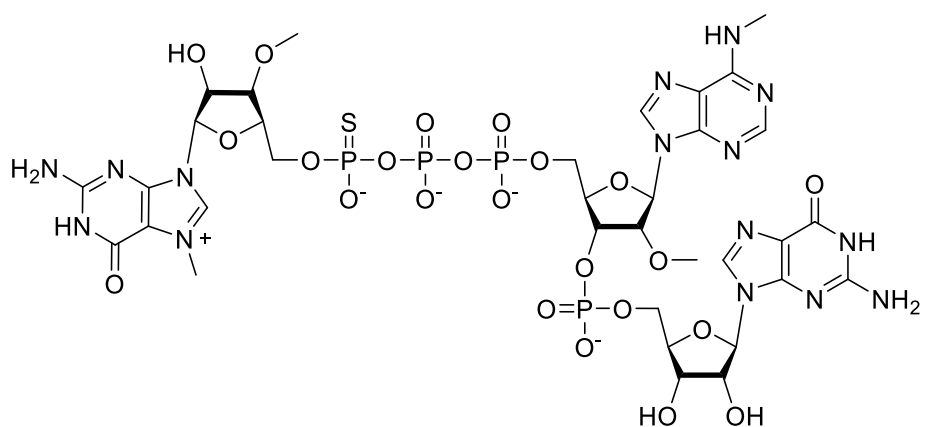
Compound 20



Compound 21



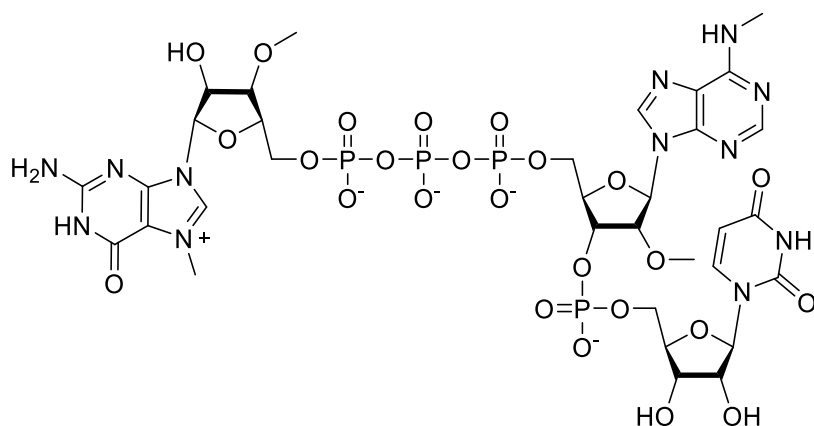
Compound 74



Compound 81

or any stereoisomer thereof.

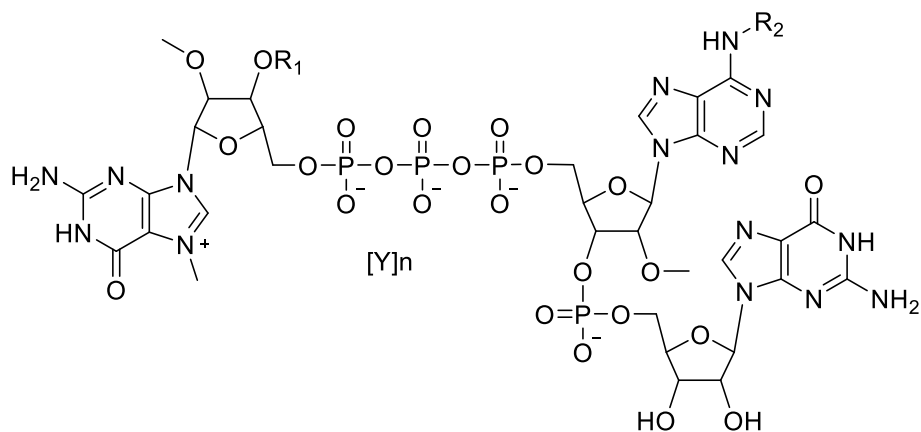
In another aspect, there is further provided herein is a compound of the following formula:



Compound 24

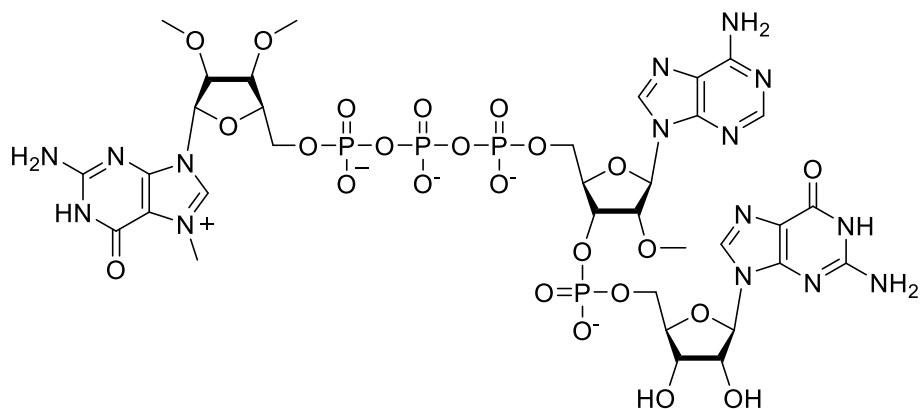
or any stereoisomer thereof.

In another aspect, there is also provided herein is a compound of the following formula:

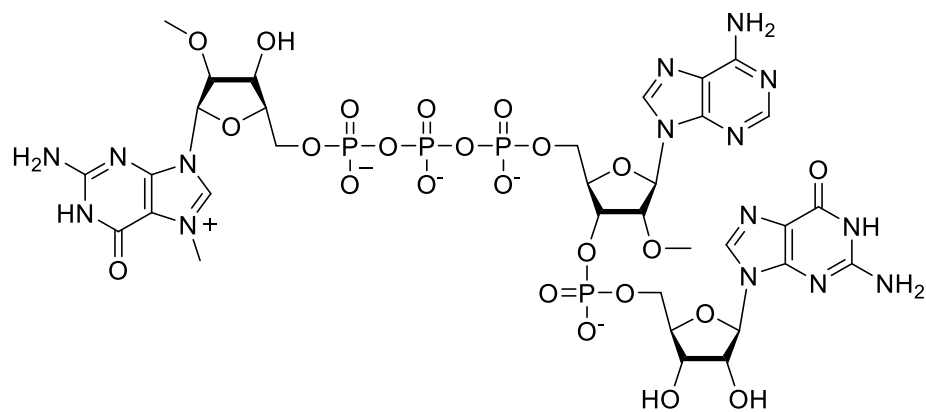


Formula VIII

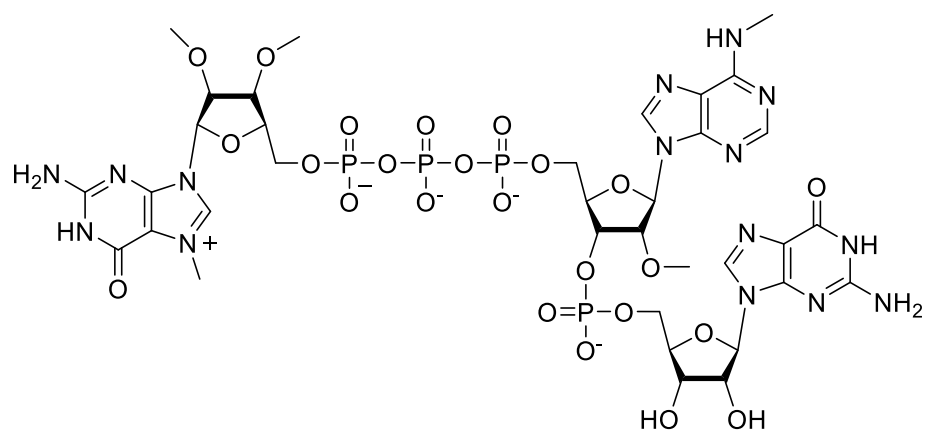
or any stereoisomer thereof, wherein: R₁ and R₂ are each independently selected from H and CH₃; each independent Y is H⁺ or a cation; and n is 0, 1, 2, 3, or 4. Optionally, the compound has the following structure:



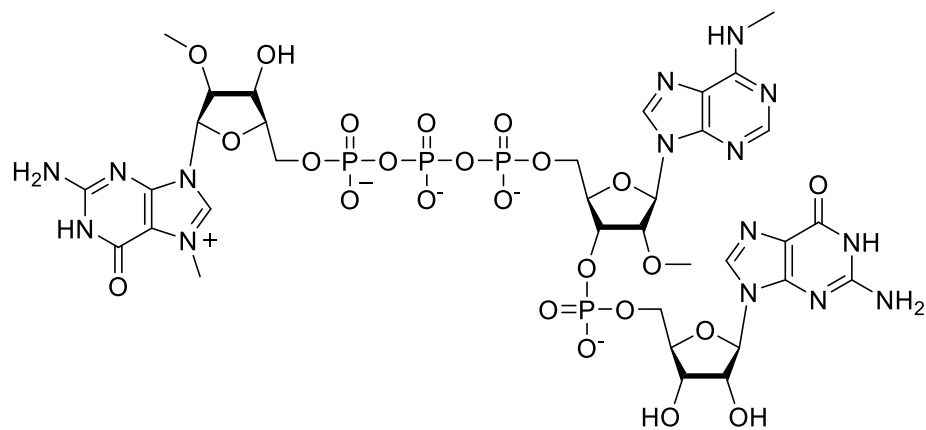
Compound 25



Compound 26



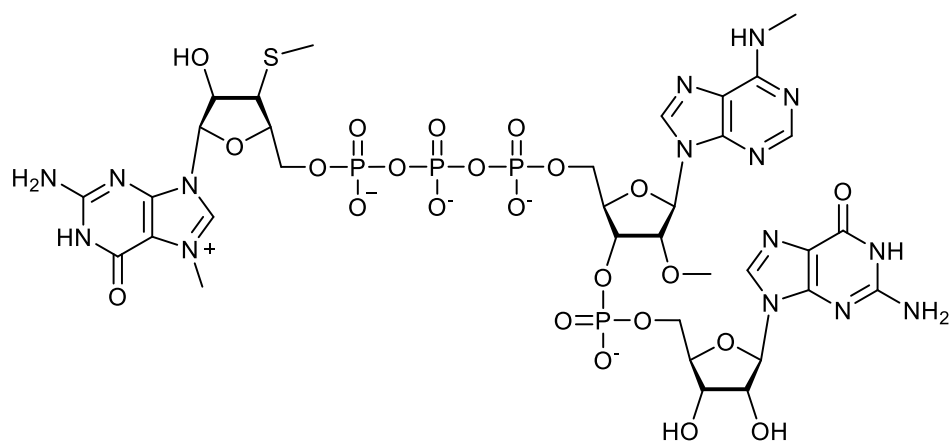
Compound 27



Compound 28

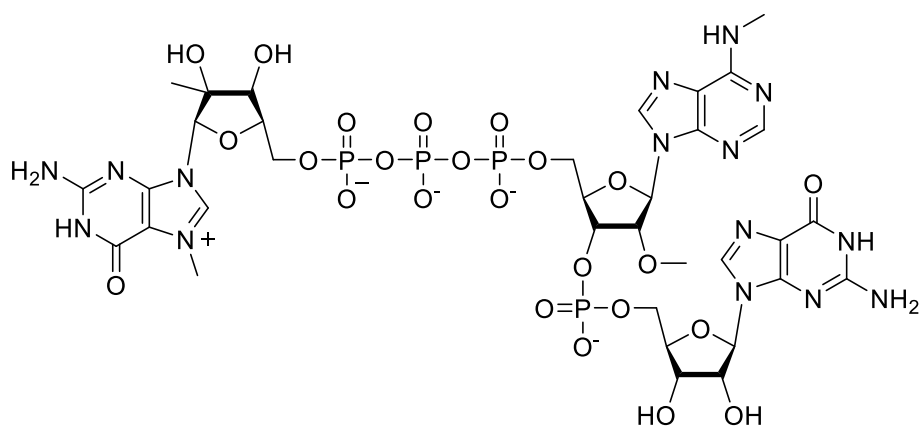
or any stereoisomer thereof.

In another aspect, there is additionally provided herein is a compound of the following formula:



Compound

52

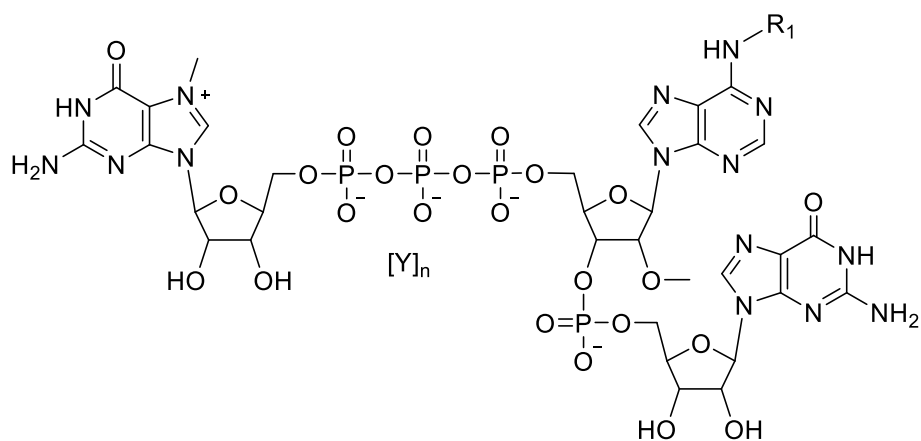


Compound

76

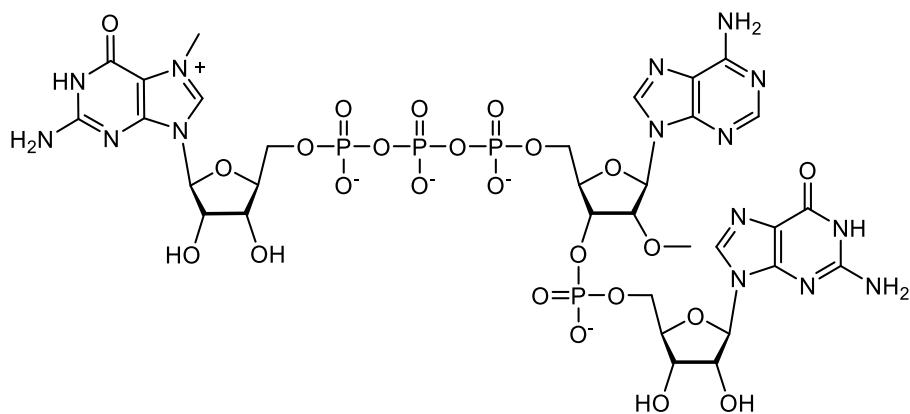
or any stereoisomer thereof.

In another aspect, there is also provided herein is a compound of the following formula:

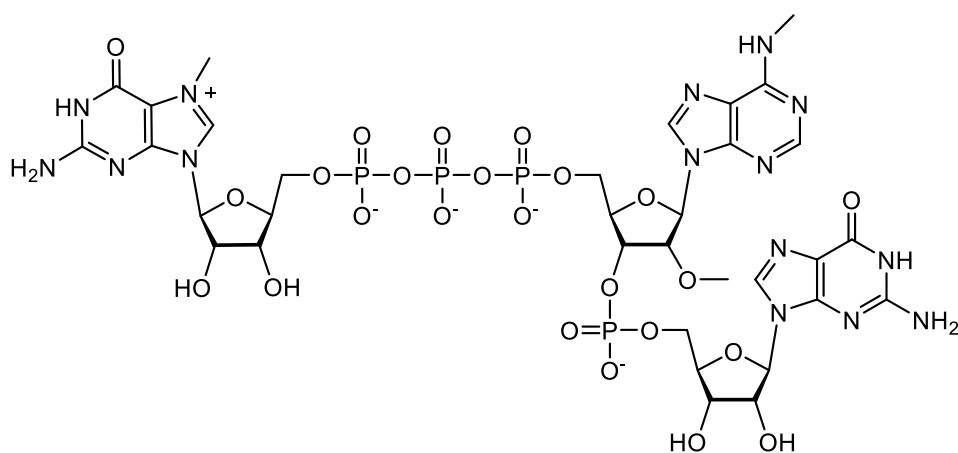


Formula X

or any stereoisomer thereof, wherein R_1 is H and CH_3 ; each independent Y is H^+ or a cation; and n is 0, 1, 2, 3, or 4. Optionally, the compound has the following structure:



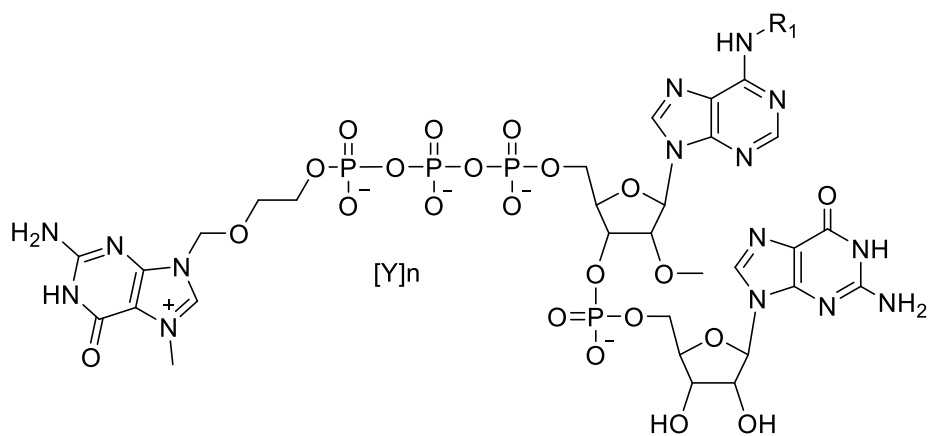
Compound 75



**Compound
77**

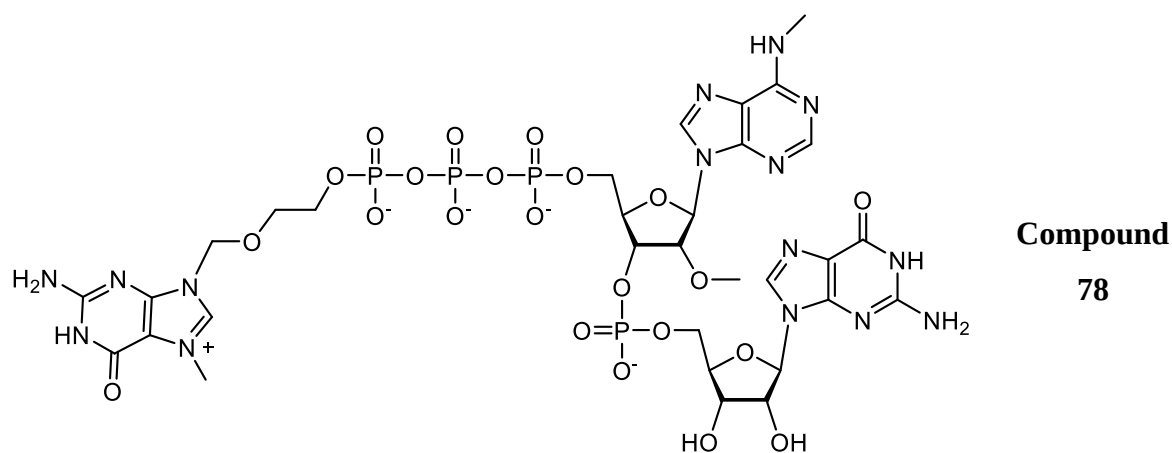
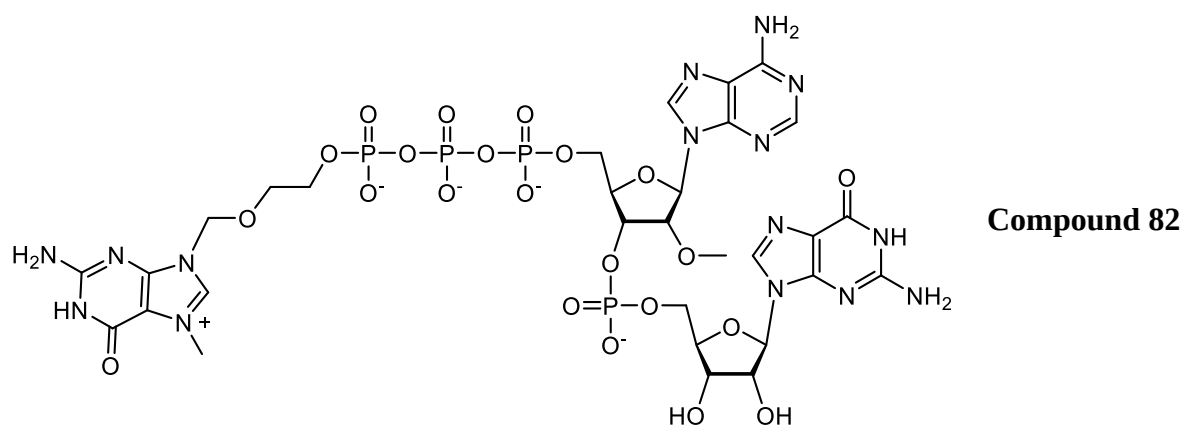
or any stereoisomer thereof.

In another aspect, there is additionally provided herein is a compound of the following formula:



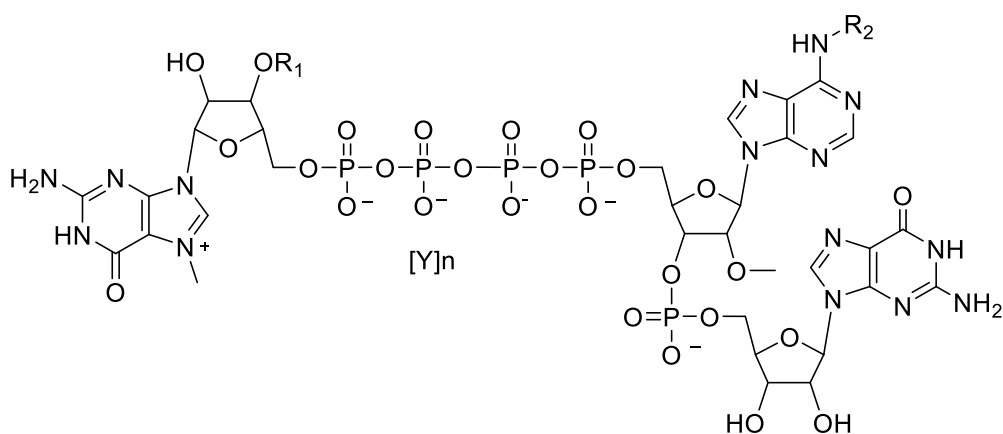
Formula XI

or any stereoisomer thereof, wherein R_1 is H and CH_3 ; each independent Y is H^+ or a cation; and n is 0, 1, 2, 3, or 4. Optionally, the compound has the following structure:

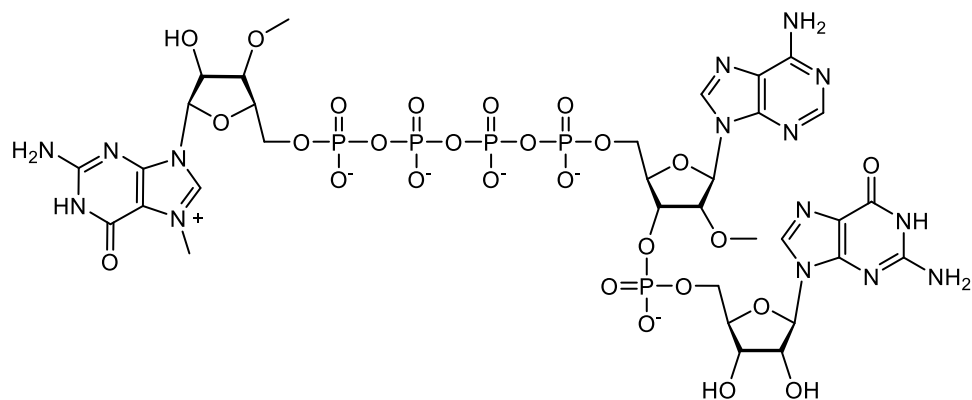


or any stereoisomer thereof.

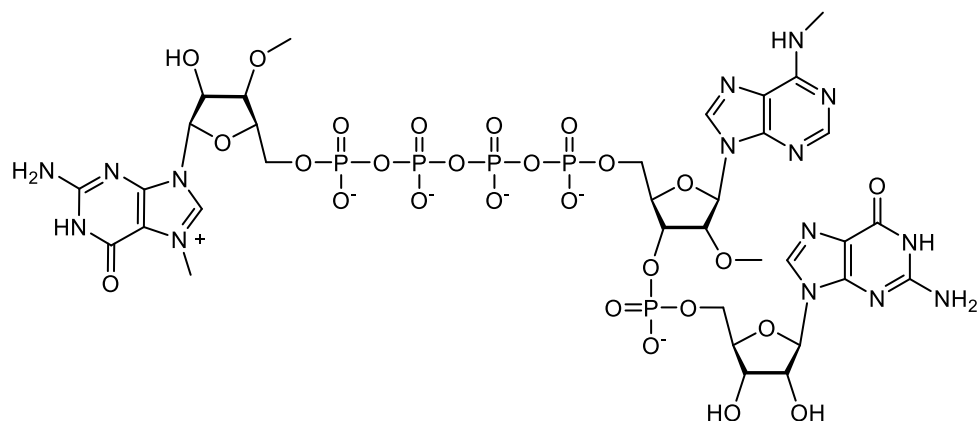
In another aspect, there is also provided herein is a compound of the following formula:



or any stereoisomer thereof, wherein R_1 and R_2 are each independently selected from H and CH_3 ; each independent Y is H^+ or a cation; and n is 0, 1, 2, 3, 4, or 5. Optionally, the compound has the following structure:



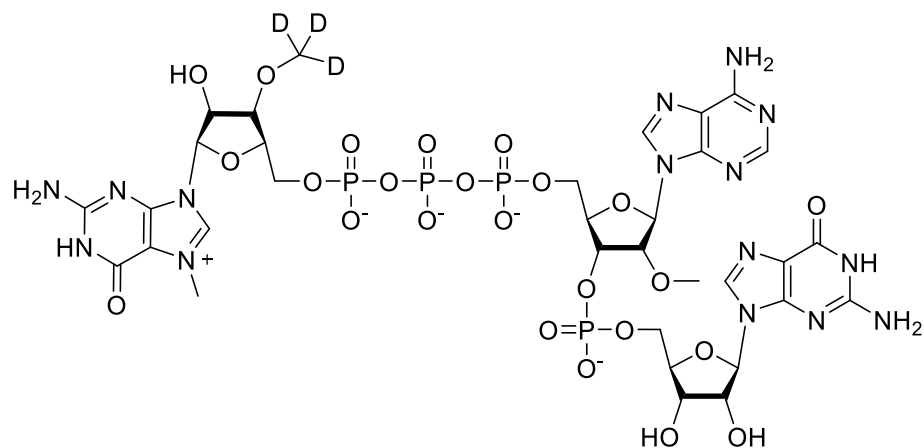
**Compound
79**



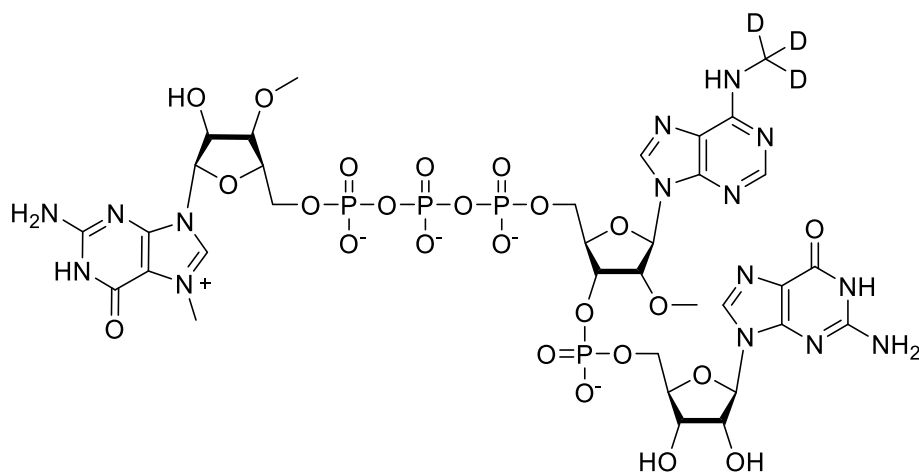
**Compound
80**

or any stereoisomer thereof.

Also described herein are deuterated forms of the disclosed compounds. In another aspect, there is provided herein is compound selected from the group consisting of:



Compound 83

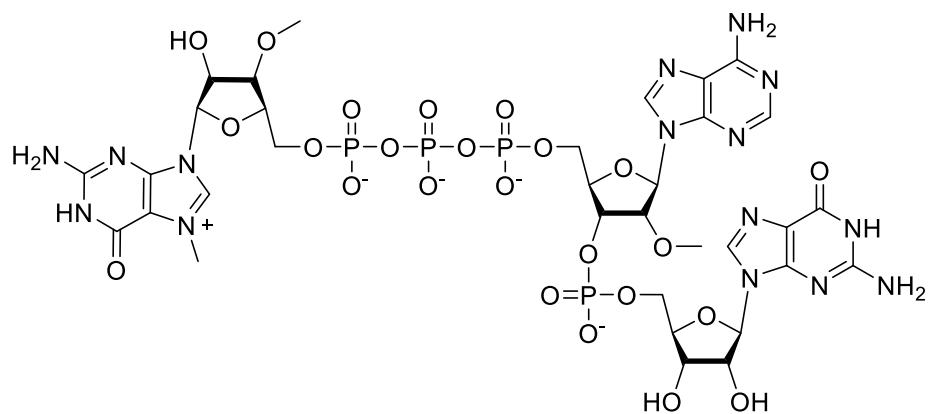


Compound 84

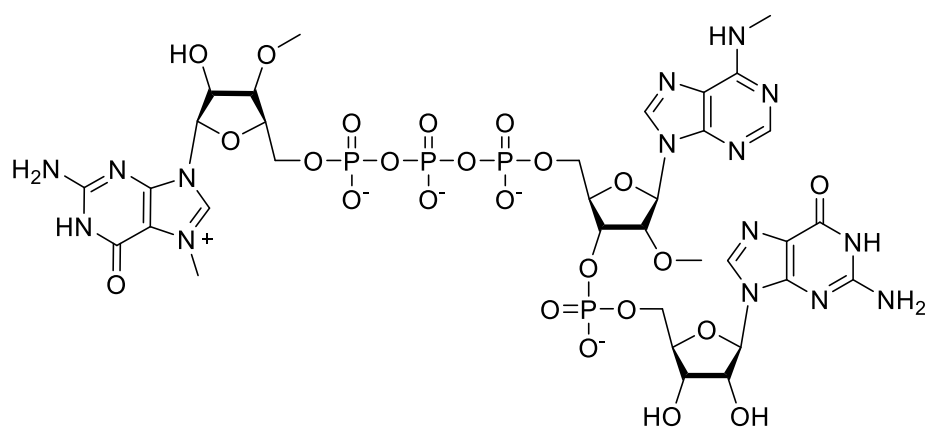
or any stereoisomer thereof.

In another aspect, there is also described herein is a pharmaceutical composition, comprising a compound as described herein and a pharmaceutically acceptable carrier. In another aspect, there is also provided an RNA molecule comprising a 5'-cap, wherein the 5'-cap comprises a compound as described herein. Optionally, the RNA molecule is a messenger RNA (mRNA) molecule or a self-amplifying RNA (saRNA) molecule. In another aspect, there is provided the use of an RNA molecule as defined above, in the manufacture of a medicament for inducing a therapeutic effect. Methods of inducing a therapeutic effect in a subject are also provided. The methods comprise administering to the subject an RNA molecule (e.g., an mRNA or a saRNA molecule) as described herein. The administered RNA may contain some amount of immunogenic double stranded RNA (dsRNA). The amount of dsRNA is calculated per each ug of administered RNA.

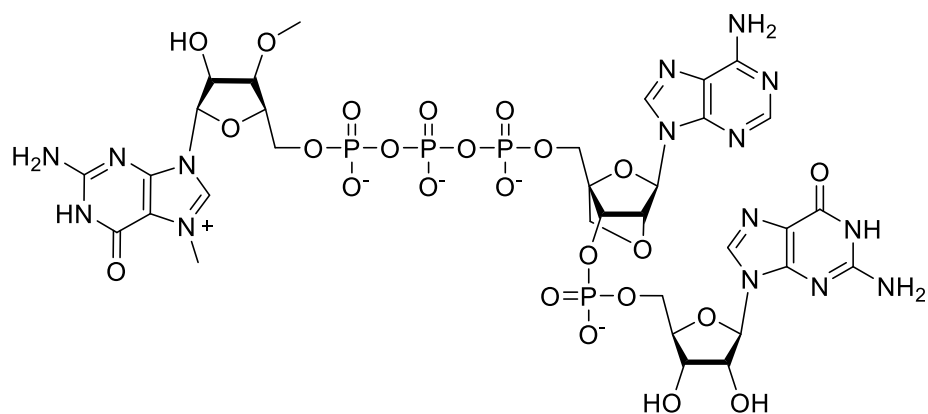
In another aspect, there is provided the use of a therapeutic dose unit of an mRNA molecule comprising a 5'-cap, in the manufacture of a medicament to be administered to a subject, wherein the 5'-cap comprises a compound as defined above. In another aspect, there is provided the use of a therapeutic dose unit of an mRNA molecule comprising a 5'-cap, in the manufacture of a medicament to be administered to a subject, wherein the 5'-cap comprises a compound selected from the group consisting of:



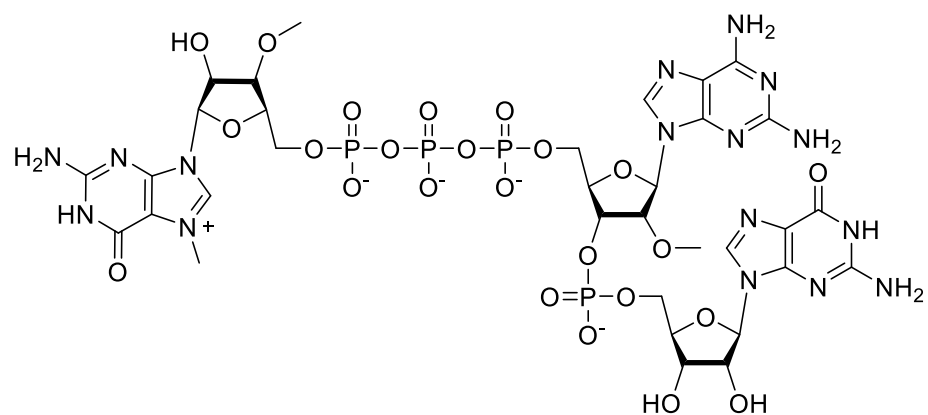
Compound 29



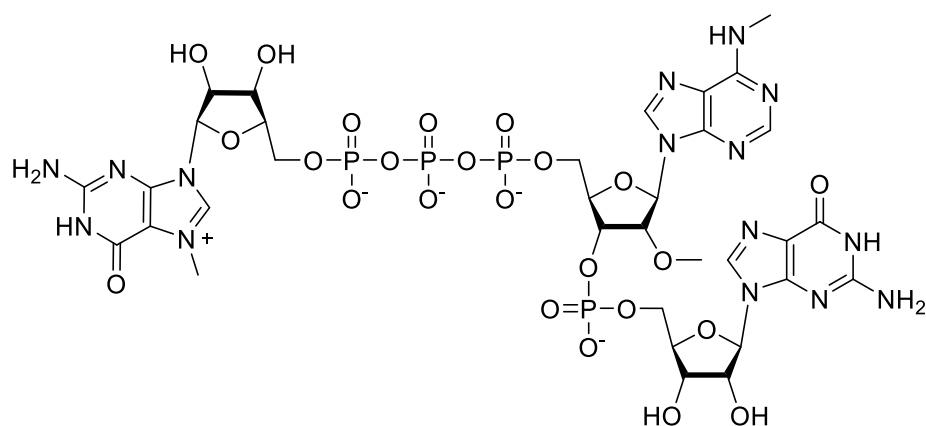
Compound 30



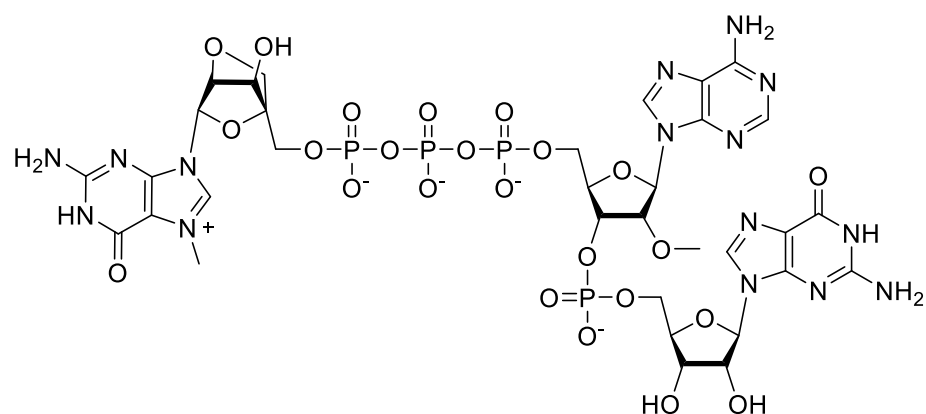
Compound 2



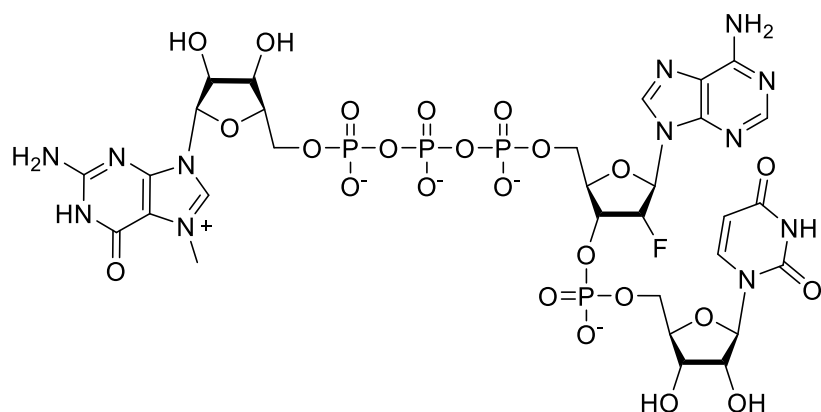
Compound 8



Compound 31



Compound 32



Compound 33

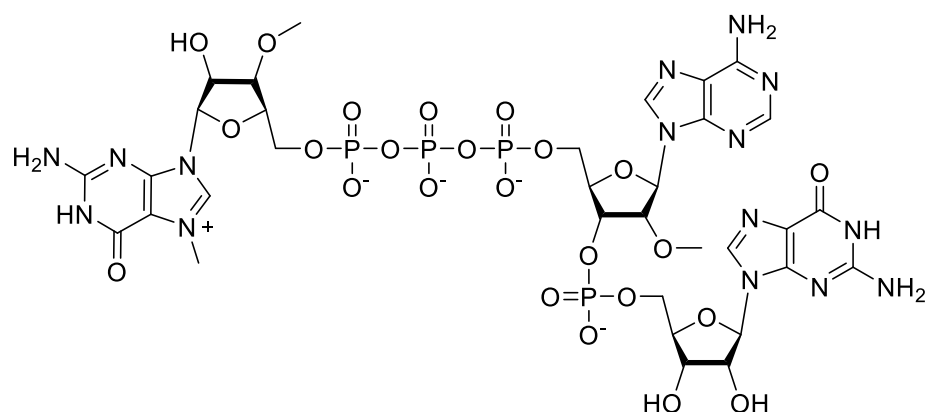
or a stereoisomer thereof,

wherein the therapeutic dose unit of the mRNA molecule comprises less than 7 ng/ μ g of double stranded RNA (dsRNA),

wherein the subject exhibits increased tolerability to the therapeutic dose unit of the mRNA molecule to be administered as compared to an equivalent therapeutic dose unit of the mRNA molecule comprising 7 ng/ μ g or greater dsRNA.

Further provided herein are methods of administering to an animal a therapeutic dose unit of an mRNA molecule comprising a 5'-cap. In some examples, the 5'-cap comprises a

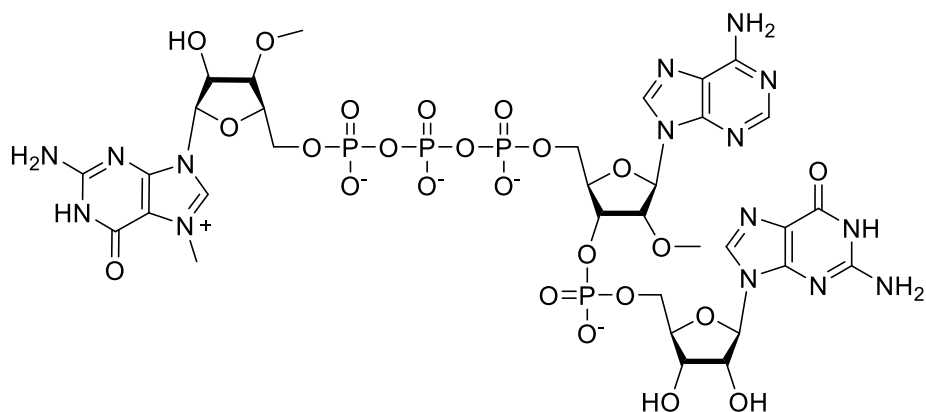
compound as described herein. In some examples, the 5'-cap comprises a compound selected from the group consisting of, or any stereoisomer thereof:



Compound 29

double stranded RNA (dsRNA), less than 3.0 ng/μg of double stranded RNA (dsRNA), less than 2.9 ng/μg of double stranded RNA (dsRNA), less than 2.8 ng/μg of double stranded RNA (dsRNA), less than 2.7 ng/μg of double stranded RNA (dsRNA), less than 2.6 ng/μg of double stranded RNA (dsRNA), less than 2.5 ng/μg of double stranded RNA (dsRNA), less than 2.4 ng/μg of double stranded RNA (dsRNA), less than 2.3 ng/μg of double stranded RNA (dsRNA), less than 2.2 ng/μg of double stranded RNA (dsRNA), less than 2.1 ng/μg of double stranded RNA (dsRNA), less than 2.0 ng/μg of double stranded RNA (dsRNA), less than 1.9 ng/μg of double stranded RNA (dsRNA), less than 1.8 ng/μg of double stranded RNA (dsRNA), less than 1.7 ng/μg of double stranded RNA (dsRNA), less than 1.6 ng/μg of double stranded RNA (dsRNA), less than 1.5 ng/μg of double stranded RNA (dsRNA), less than 1.4 ng/μg of double stranded RNA (dsRNA), less than 1.3 ng/μg of double stranded RNA (dsRNA), less than 1.2 ng/μg of double stranded RNA (dsRNA), less than 1.1 ng/μg of double stranded RNA (dsRNA), less than 1.0 ng/μg of double stranded RNA (dsRNA), less than 0.9 ng/μg of double stranded RNA (dsRNA), less than 0.8 ng/μg of double stranded RNA (dsRNA), less than 0.7 ng/μg of double stranded RNA (dsRNA), less than 0.6 ng/μg of double stranded RNA (dsRNA), less than 0.5 ng/μg of double stranded RNA (dsRNA), less than 0.4 ng/μg of double stranded RNA (dsRNA), less than 0.3 ng/μg of double stranded RNA (dsRNA), less than 0.2 ng/μg of double stranded RNA (dsRNA), or less than 0.1 ng/μg of double stranded RNA (dsRNA).

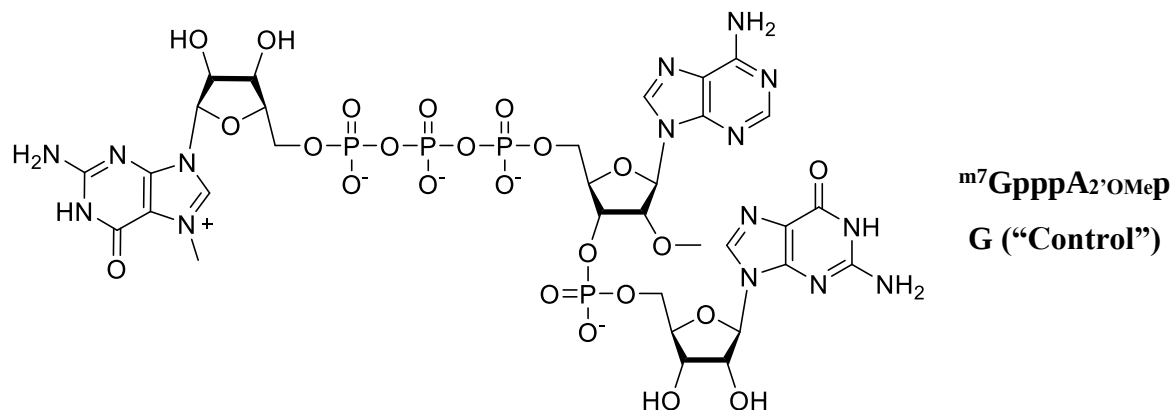
In another aspect, there is provided the use of a mRNA in the manufacture of a medicament for increasing *in vivo* translation of a polypeptide in a subject, comprising a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide to be administered to the subject, wherein the mRNA comprises a 5'-cap having the following formula:



Compound 29

or a stereoisomer thereof,

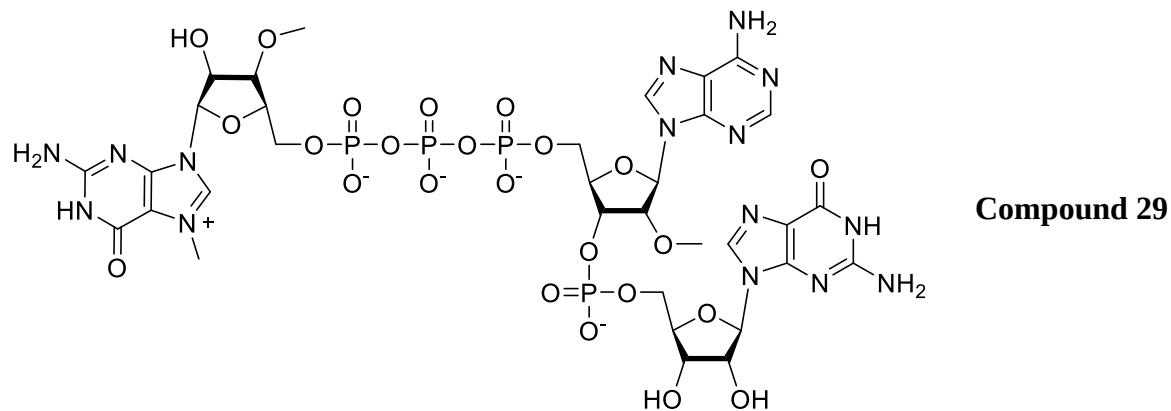
wherein the therapeutic dose unit to be administered is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula:



or a stereoisomer thereof.

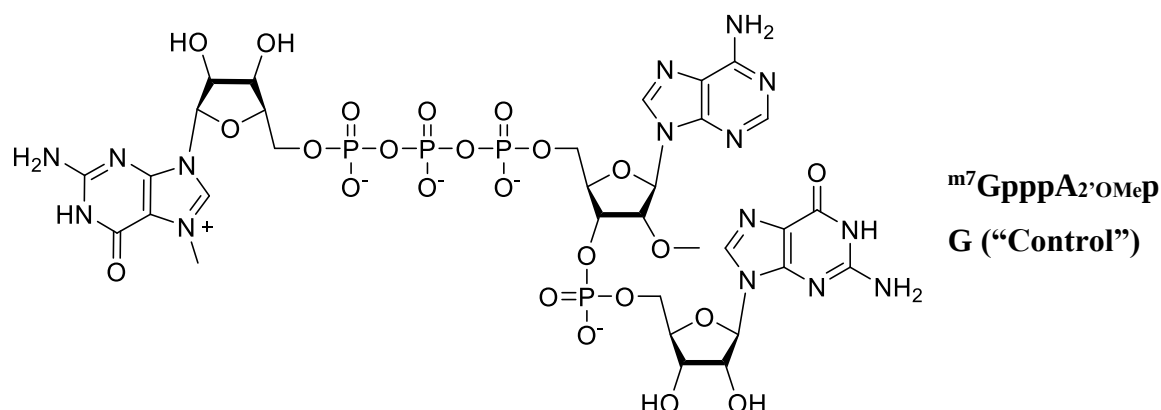
Further provided herein is a method for increasing *in vivo* translation of a polypeptide in a subject, comprising administering to the subject a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide, wherein the mRNA comprises a 5'-

5 cap having the following formula, or any stereoisomer thereof:

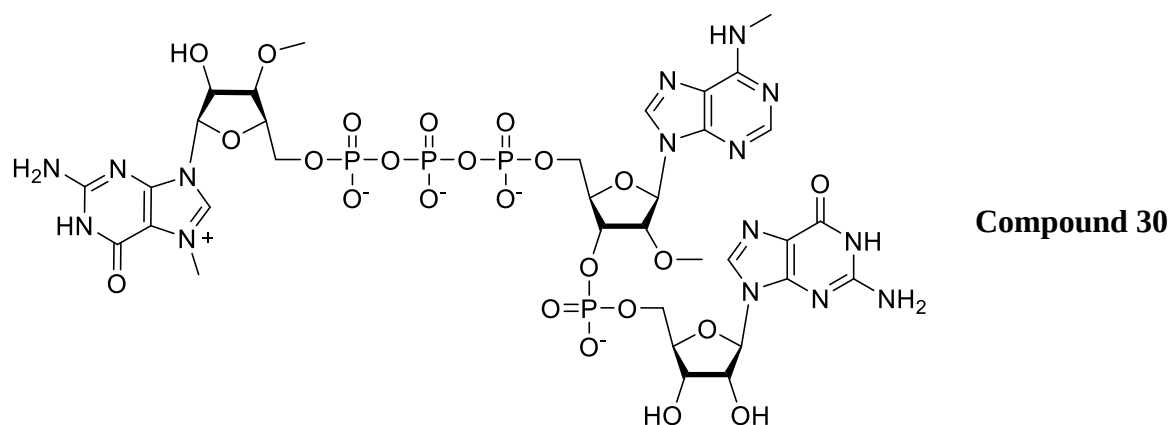


wherein the administered therapeutic dose unit is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative

mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula, or any stereoisomer thereof:

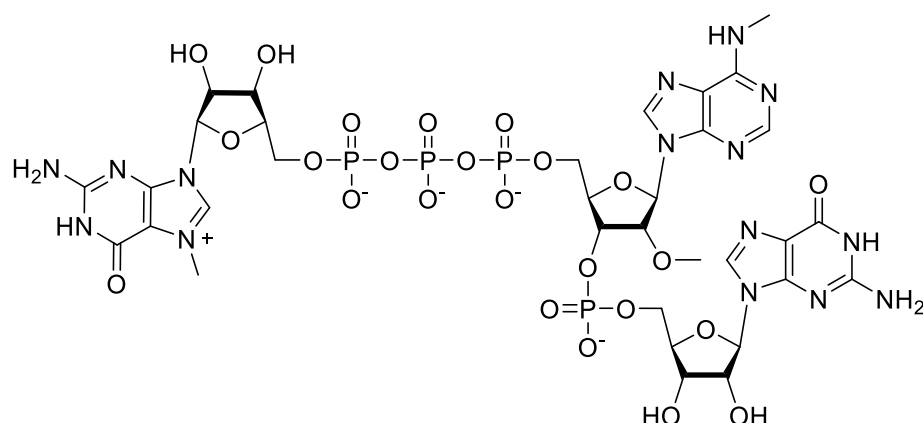


In another aspect, there is provided the use of a mRNA in the manufacture of a medicament for increasing *in vivo* translation of a polypeptide in a subject, comprising a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide to be administered to the subject, wherein the mRNA comprises a 5'-cap having the following formula:



or a stereoisomer thereof,

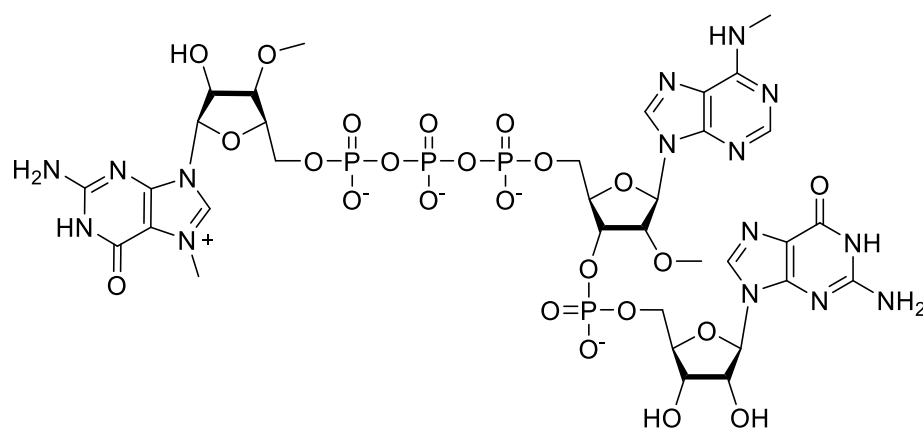
wherein the therapeutic dose unit to be administered is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula:



Compound 31

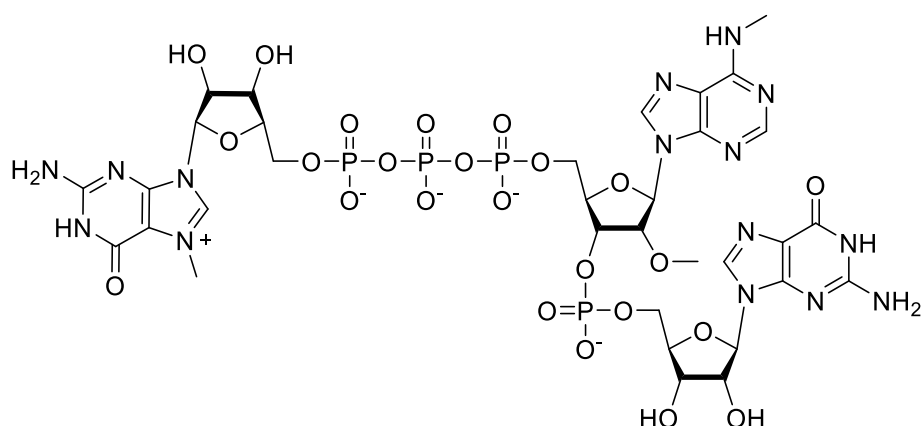
or a stereoisomer thereof.

Also provided herein is a method for increasing *in vivo* translation of a polypeptide in a subject, comprising administering to the subject a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide, wherein the mRNA comprises a 5'-cap having the following formula, or any stereoisomer thereof:



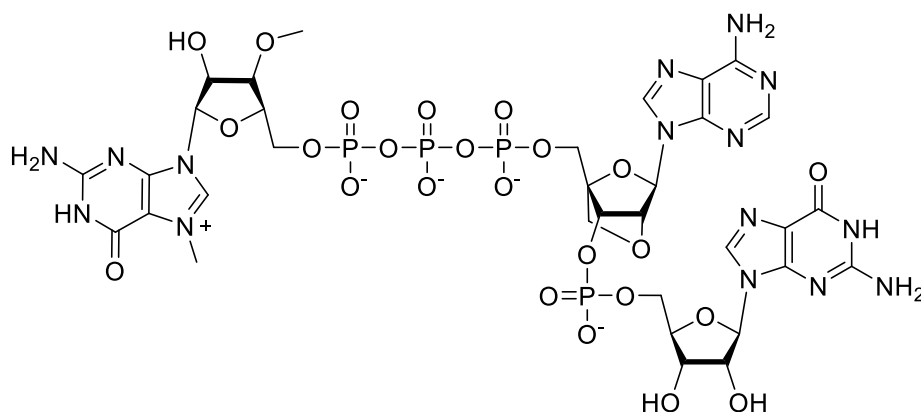
Compound 30

wherein the administered therapeutic dose unit is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula, or any stereoisomer thereof:



Compound 31

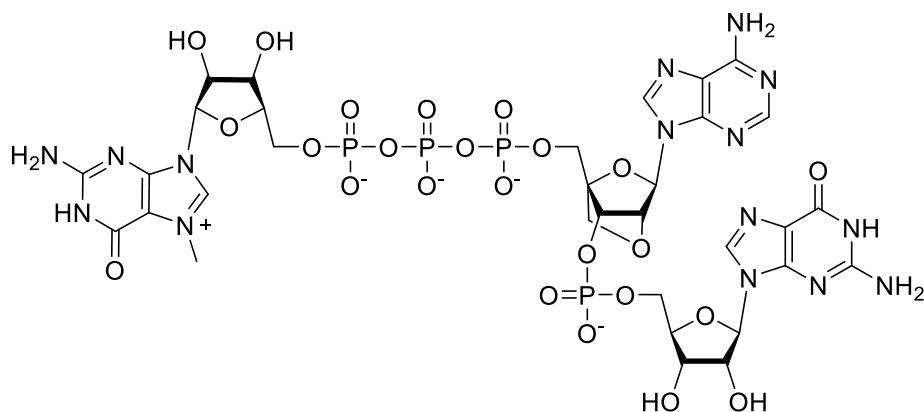
In another aspect, there is provided the use of a mRNA in the manufacture of a medicament for increasing *in vivo* translation of a polypeptide in a subject, comprising a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide to be administered to the subject, wherein the mRNA comprises a 5'-cap having the following formula:



Compound 2

or a stereoisomer thereof,

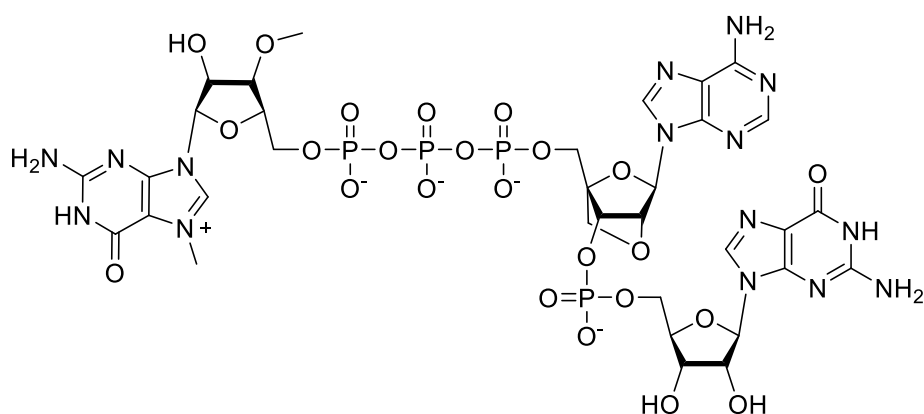
wherein the dose to be administered is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula:



Compound 1

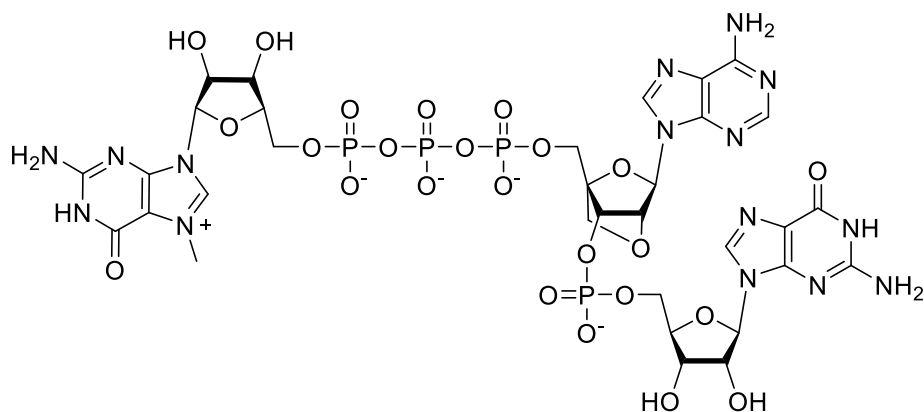
or a stereoisomer thereof.

Further provided herein is a method for increasing *in vivo* translation of a polypeptide in a subject, comprising administering to the subject a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide, wherein the mRNA comprises a 5'-
 5 cap having the following formula, or any stereoisomer thereof:



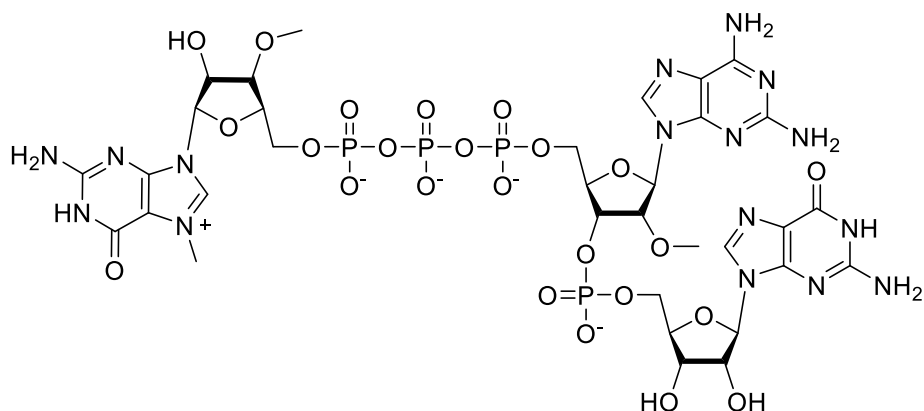
Compound 2

wherein the administered therapeutic dose unit is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having
 10 the following formula, or any stereoisomer thereof:



Compound 1

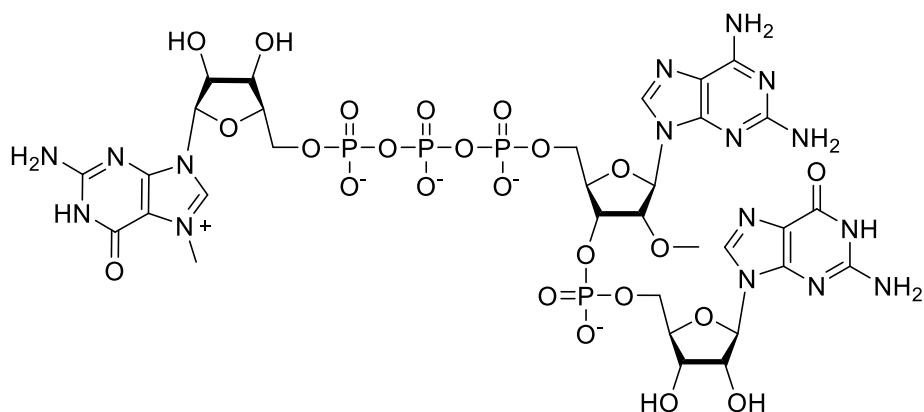
Also in another aspect, there is provided the use of a mRNA in the manufacture of a medicament for increasing *in vivo* translation of a polypeptide in a subject, comprising a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide to be administered to the subject, wherein the mRNA comprises a 5'-cap having the following formula:



Compound 8

or a stereoisomer thereof,

wherein the therapeutic dose unit to be administered is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula:

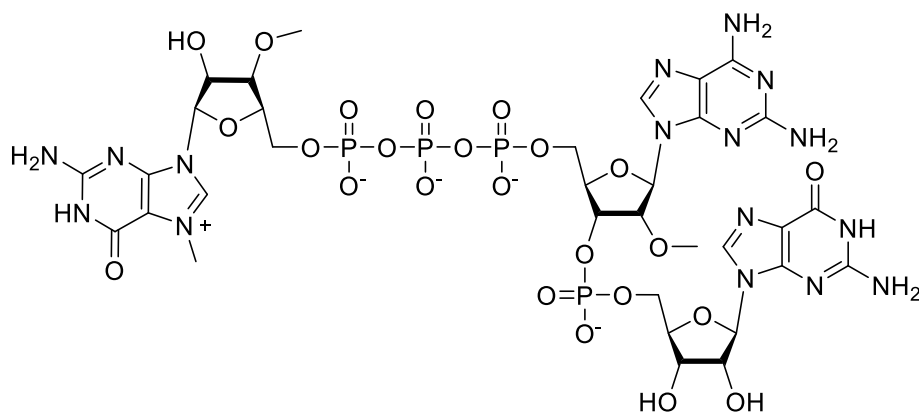


Compound 7

or a stereoisomer thereof.

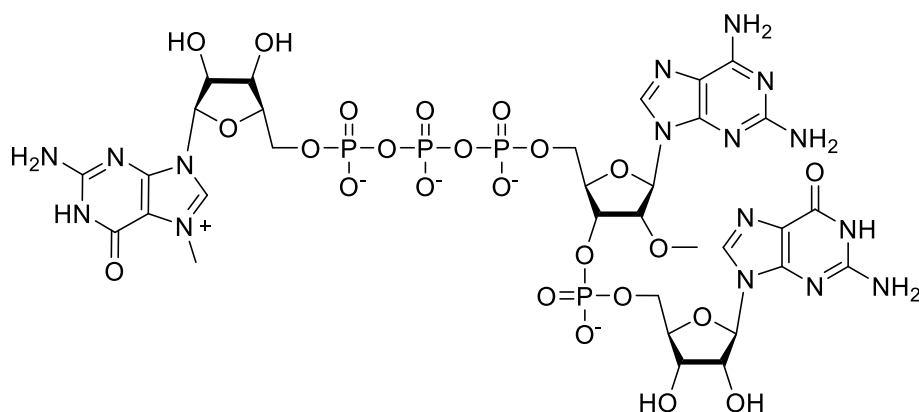
Also provided herein is a method for increasing *in vivo* translation of a polypeptide in a subject, comprising administering to the subject a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide, wherein the mRNA comprises a 5'-cap having the following formula, or any stereoisomer thereof:

5



Compound 8

wherein the administered therapeutic dose unit is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula, or any stereoisomer thereof:



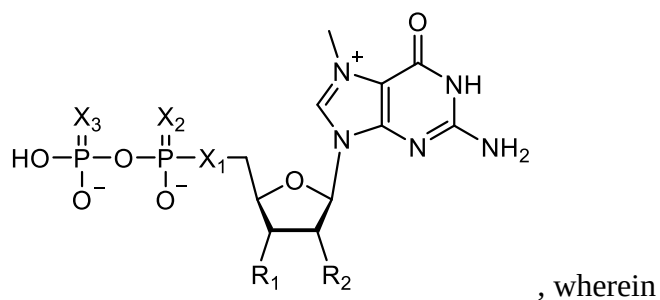
Compound 7

- 5 Optionally, the subject exhibits increased tolerability to the administered therapeutic dose unit as compared to the therapeutic dose unit required to elicit the same response in the subject when administered the comparative mRNA, as measured by testing in a standard *in vivo* assay. In some cases, the increased tolerability is an increase in tolerability of at least 20 % to the administered therapeutic dose unit as measured by testing in a standard *in vivo*
- 10 assay, at least 25 % to the administered therapeutic dose unit as measured by testing in a standard *in vivo* assay, at least 30 % to the administered therapeutic dose unit as measured by testing in a standard *in vivo* assay, at least 35 % to the administered therapeutic dose unit as measured by testing in a standard *in vivo* assay, at least 40 % to the administered therapeutic dose unit as measured by testing in a standard *in vivo* assay, at least 45 % to the administered

RNA (dsRNA), less than 3.5 ng/μg of double stranded RNA (dsRNA), less than 3.4 ng/μg of double stranded RNA (dsRNA), less than 3.3 ng/μg of double stranded RNA (dsRNA), less than 3.2 ng/μg of double stranded RNA (dsRNA), less than 3.1 ng/μg of double stranded RNA (dsRNA), less than 3.0 ng/μg of double stranded RNA (dsRNA), less than 2.9 ng/μg of double stranded RNA (dsRNA), less than 2.8 ng/μg of double stranded RNA (dsRNA), less than 2.7 ng/μg of double stranded RNA (dsRNA), less than 2.6 ng/μg of double stranded RNA (dsRNA), less than 2.5 ng/μg of double stranded RNA (dsRNA), less than 2.4 ng/μg of double stranded RNA (dsRNA), less than 2.3 ng/μg of double stranded RNA (dsRNA), less than 2.2 ng/μg of double stranded RNA (dsRNA), less than 2.1 ng/μg of double stranded RNA (dsRNA), less than 2.0 ng/μg of double stranded RNA (dsRNA), less than 1.9 ng/μg of double stranded RNA (dsRNA), less than 1.8 ng/μg of double stranded RNA (dsRNA), less than 1.7 ng/μg of double stranded RNA (dsRNA), less than 1.6 ng/μg of double stranded RNA (dsRNA), less than 1.5 ng/μg of double stranded RNA (dsRNA), less than 1.4 ng/μg of double stranded RNA (dsRNA), less than 1.3 ng/μg of double stranded RNA (dsRNA), less than 1.2 ng/μg of double stranded RNA (dsRNA), less than 1.1 ng/μg of double stranded RNA (dsRNA), less than 1.0 ng/μg of double stranded RNA (dsRNA), less than 0.9 ng/μg of double stranded RNA (dsRNA), less than 0.8 ng/μg of double stranded RNA (dsRNA), less than 0.7 ng/μg of double stranded RNA (dsRNA), less than 0.6 ng/μg of double stranded RNA (dsRNA), less than 0.5 ng/μg of double stranded RNA (dsRNA), less than 0.4 ng/μg of double stranded RNA (dsRNA), less than 0.3 ng/μg of double stranded RNA (dsRNA), less than 0.2 ng/μg of double stranded RNA (dsRNA), or less than 0.1 ng/μg of double stranded RNA (dsRNA).

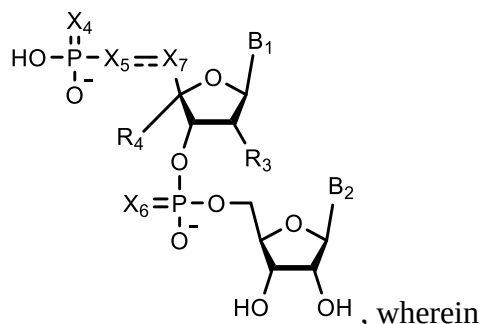
Further described herein are methods of synthesizing a trinucleotide compound. In one aspect, there is provided a method of synthesizing a trinucleotide compound, comprising:

(1) mixing an N7-methyl-guanosine-5'-diphosphate of the following structure:



X_1 and X_2 are each independently selected from the group consisting of O and S;
 X_3 is O;

R₁ and R₂ are each independently selected from H, OH, N₃, F, substituted or unsubstituted alkoxy, and thio, wherein R₁ and R₂ optionally combine to form a heterocycle, with an activating reagent and an imidazole to form an activated intermediate; and
(2) adding a salt reagent and a dinucleotide of the following structure:



== is a single bond or a double bond;

X₄ and X₆ are each independently selected from the group consisting of O and S;

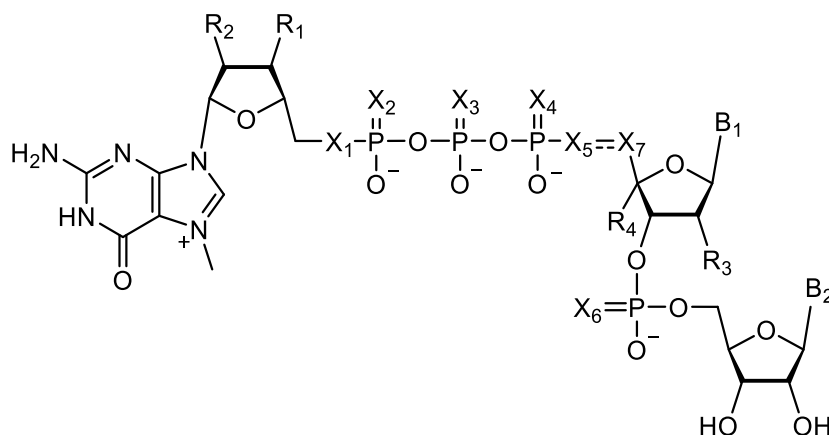
X₅ is O, S, or CH;

X₇ is CH or CH₂;

R₃ is OH, OMe, F and R₄ is H, or wherein R₃ and R₄ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; and

B₁ and B₂ are each independently selected from the group consisting of a purine ring and a pyrimidine ring

to the activated intermediate to form the trinucleotide compound of the following structure:

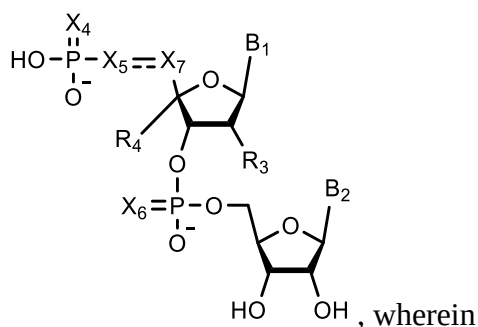


wherein the method is a one-pot synthesis.

In one example, a method of synthesizing a trinucleotide compound comprises mixing an N7-methyl-guanosine-5'-diphosphate with an activating reagent to form a reactive intermediate; reacting the reactive intermediate with an imidazole to form an activated intermediate; and adding a salt reagent (e.g., a chloride salt such as magnesium chloride or zinc chloride) and a dinucleotide to the activated intermediate to form the trinucleotide

compound, wherein the method is a one-pot synthesis. In some examples, the method of synthesizing a trinucleotide compound comprises mixing an N7-methyl-guanosine-5'-diphosphate of the following structure:

(1) mixing a dinucleotide of the following structure:



== is a single bond or a double bond;

X_4 is 0;

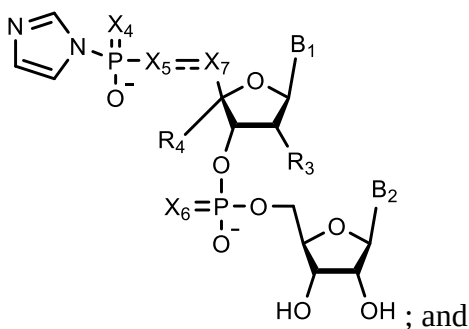
X_6 is O or S;

X₅ is O, S, or CH;

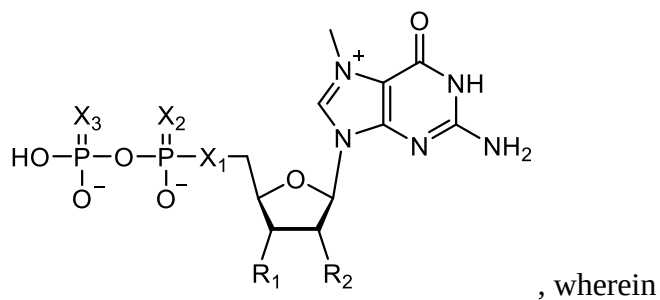
X₇ is CH or CH₂;

R₃ is OH, OMe, F and R₄ is H, or wherein R₃ and R₄ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; and

B₁ and B₂ are each independently selected from the group consisting of a purine ring and a pyrimidine ring,
with an activating reagent and an imidazole to form an activated phosphate imidazolide of the following structure:



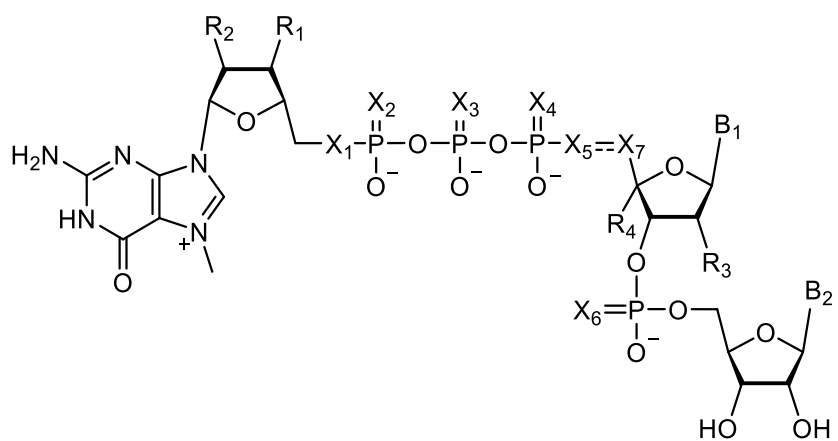
(2) adding a salt reagent and a compound of the following structure:



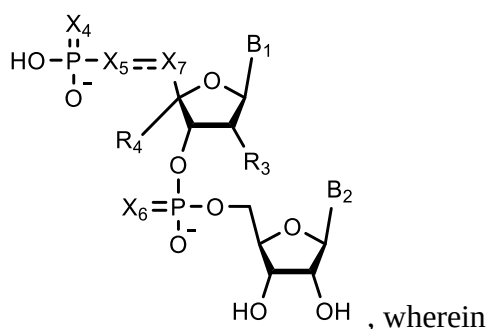
X₁ and X₂ are each independently selected from the group consisting of O and S;

X₃ is S;

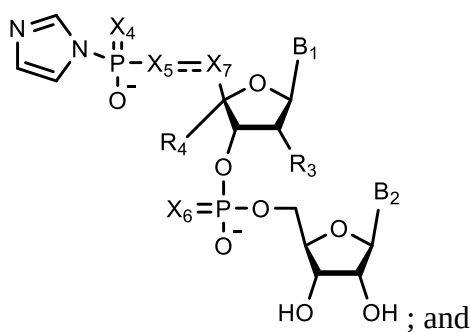
R₁ and R₂ are each independently selected from H, OH, N₃, F, substituted or unsubstituted alkoxy, and thio, wherein R₁ and R₂ optionally combine to form a heterocycle, to the activated phosphate imidazolidine to form the trinucleotide compound of the following structure:



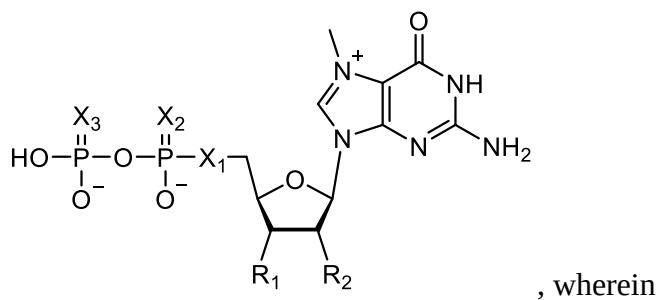
In other examples, the method of synthesizing a trinucleotide compound comprises mixing a dinucleotide of the following structure:



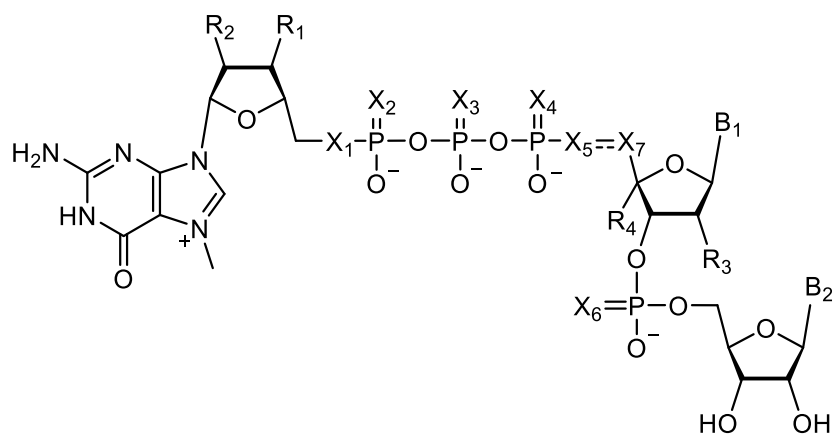
== is a single bond or a double bond; X₄ is O; X₆ is O or S; X₅ is O, S, or CH; X₇ is CH or CH₂; R₃ is OH, OMe, F and R₄ is H, or wherein R₃ and R₄ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; and B₁ and B₂ are each independently selected from the group consisting of a purine ring and a pyrimidine ring, with an activating reagent and an imidazole to form an activated phosphate imidazolidine of the following structure:



adding a salt reagent (e.g., a chloride salt such as magnesium chloride or zinc chloride) and a compound of the following structure:

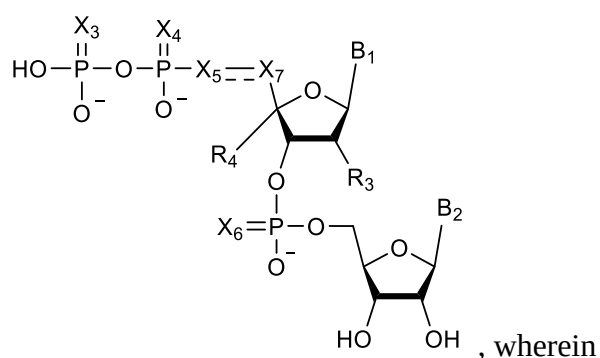


, wherein
 X_1 and X_2 are each independently selected from the group consisting of O and S; X_3 is S; R_1 and R_2 are each independently selected from H, OH, N_3 , F, substituted or unsubstituted alkoxy, and thio, wherein R_1 and R_2 optionally combine to form a heterocycle, to the activated phosphate imidazolidine to form the trinucleotide compound of the following structure:



In another aspect, there is provided a method of synthesizing a trinucleotide compound, comprising:

(1) mixing a diphosphate dinucleotide of the following structure:



== is a single bond or a double bond;

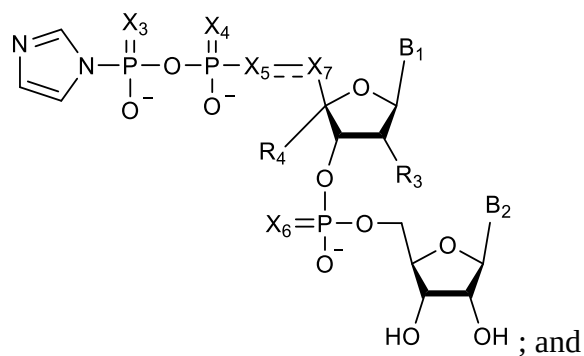
X₃ is O;

X₄ and X₆ are each independently selected from O and S;

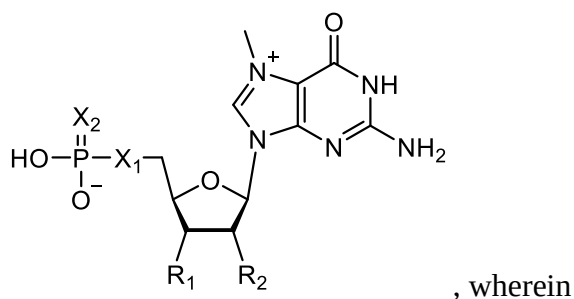
X₅ is O, S, or CH; X₇ is CH or CH₂;

R₃ is OH, OMe, F and R₄ is H, or wherein R₃ and R₄ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; and

B₁ and B₂ are each independently selected from the group consisting of a purine ring and a pyrimidine ring,
with an activating reagent and an imidazole to form an activated phosphate imidazolide of the following structure:

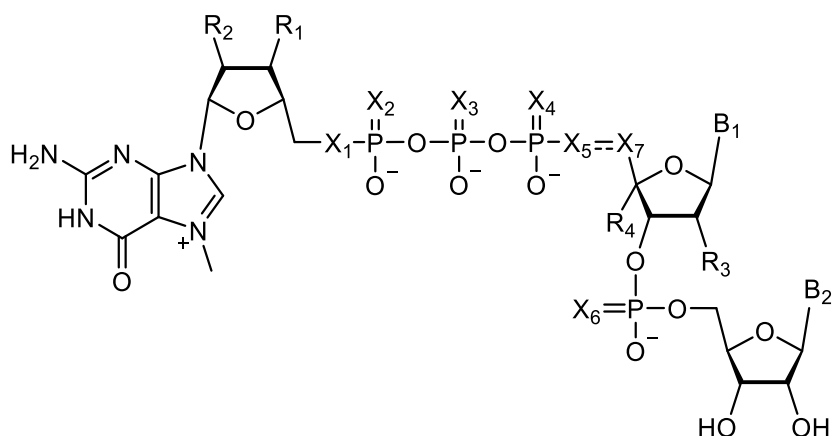


(2) adding a salt reagent and a compound of the following structure:



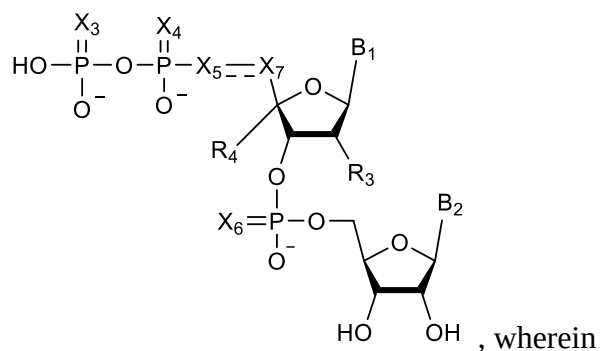
X_1 and X_2 are each independently selected from O and S; and

R_1 and R_2 are each independently selected from H, OH, N_3 , F, substituted or unsubstituted alkoxy, and thio, wherein R_1 and R_2 optionally combine to form a heterocycle, to the activated phosphate imidazolide to form the trinucleotide compound of the following structure:

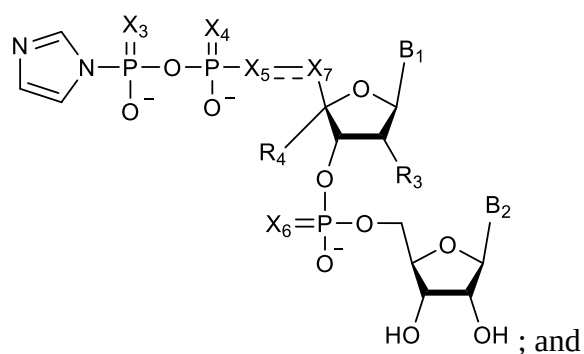


wherein the method is a one-pot synthesis.

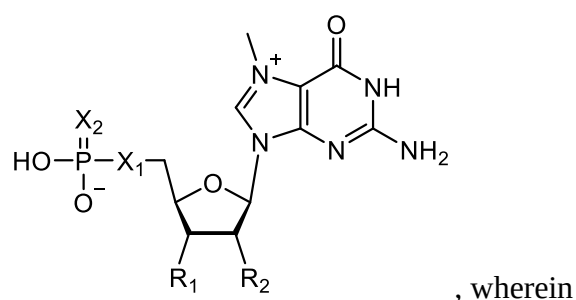
In still other examples, the method of synthesizing a trinucleotide compound comprises mixing a diphosphate dinucleotide of the following structure:



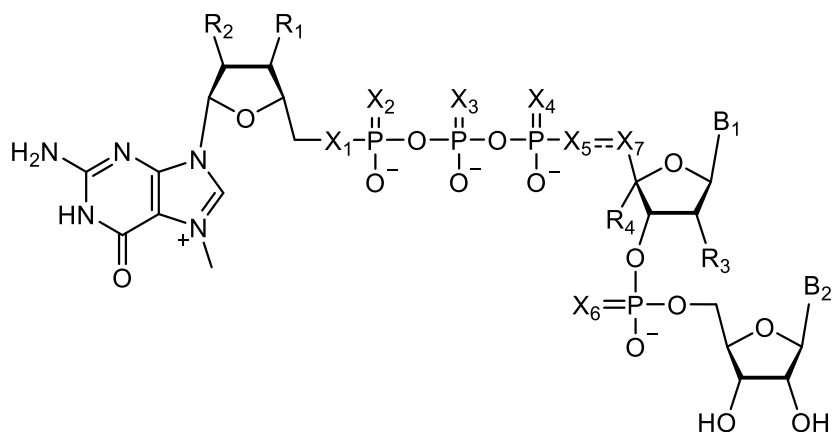
== is a single bond or a double bond; X₃ is O; X₄ and X₆ are each independently selected from O and S; X₅ is O, S, or CH; X₇ is CH or CH₂; R₃ is OH, OMe, F and R₄ is H, or wherein R₃ and R₄ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; and B₁ and B₂ are each independently selected from the group consisting of a purine ring and a pyrimidine ring, with an activating reagent and an imidazole to form an activated phosphate imidazolide of the following structure:



adding a salt reagent (e.g., a chloride salt such as magnesium chloride or zinc chloride) and a compound of the following structure:



X₁ and X₂ are each independently selected from O and S; and R₁ and R₂ are each independently selected from H, OH, N₃, F, substituted or unsubstituted alkoxy, and thio, wherein R₁ and R₂ optionally combine to form a heterocycle, to the activated phosphate imidazolidine to form the trinucleotide compound of the following structure:



wherein the method is a one-pot synthesis.

The details of one or more examples are set forth in the drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

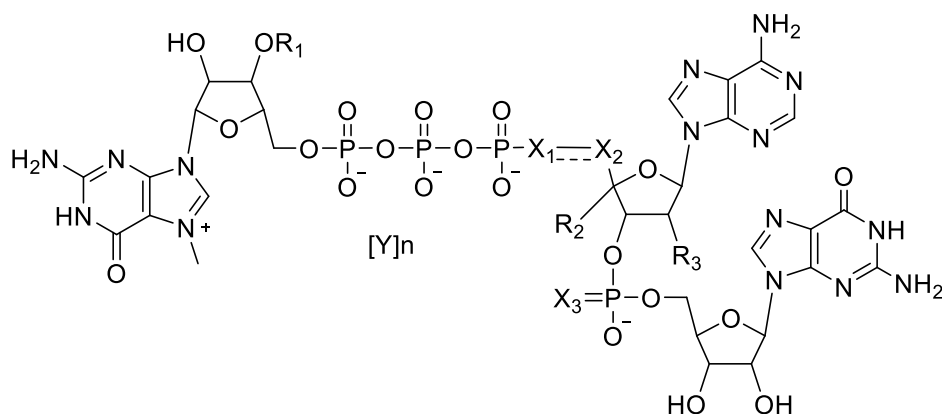
DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing *in vitro* translation using wheat germ extract, a cell-free translation system, for the cap analogs as described herein. The data demonstrate that protein expression for the cap analogs was comparable to or enhanced as compared to mRNA produced from a comparative cap.

Figure 2 is a bar graph showing the *in vivo* luciferase expression in mice injected with LNPs comprising mRNA produced from cap analogs as described herein along with mRNA produced from comparative caps. The data demonstrate that *in vivo* luciferase expression was comparable to or enhanced as compared to mRNA produced from a comparative cap.

WHAT IS CLAIMED IS:

1. A compound of the following formula:



Formula I

or a stereoisomer thereof, wherein:

=== is a single bond or a double bond;

R₁ is H or CH₃;

R₂ is H and R₃ is OCH₃ or F, or R₂ and R₃ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge;

X₁ is O or CH;

X₂ is CH₂ or CH;

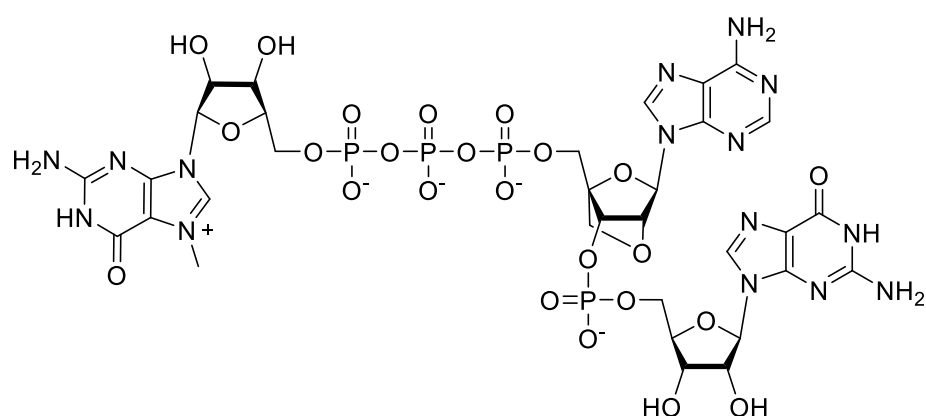
X₃ is O or S;

each independent Y is H⁺ or a cation; and

n is 0, 1, 2, 3, or 4,

wherein when X₁ is CH, X₂ is CH and === is a double bond.

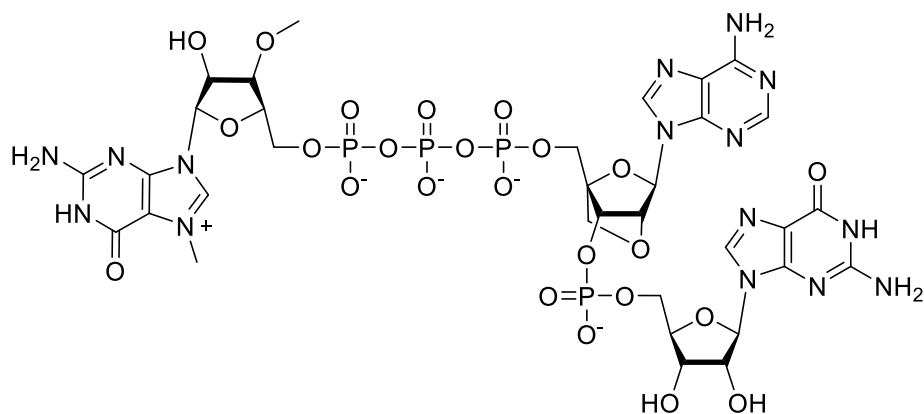
2. The compound of claim 1, wherein the compound has the following structure:



Compound 1

or a stereoisomer thereof.

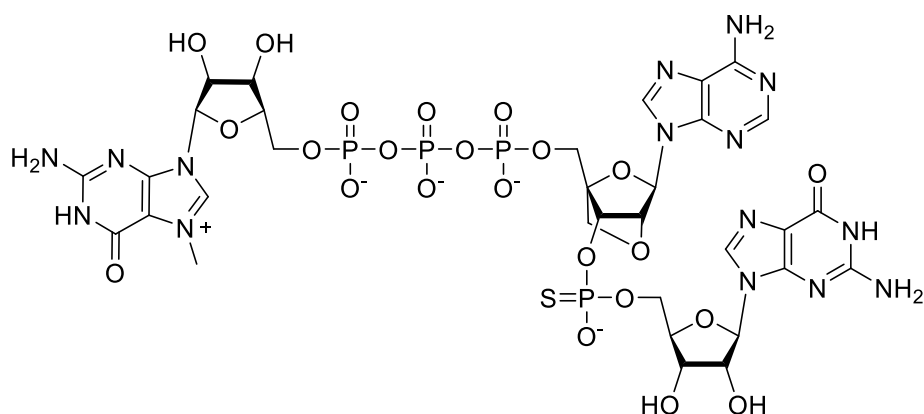
3. The compound of claim 1, wherein the compound has the following structure:



Compound 2

or a stereoisomer thereof.

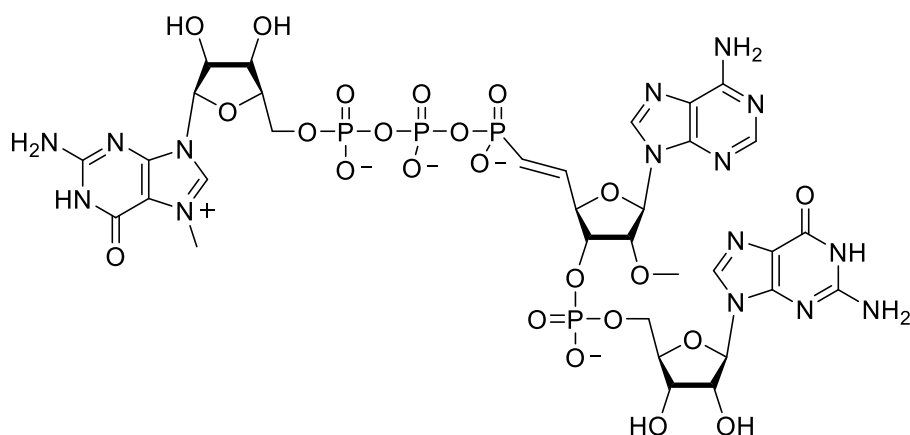
4. The compound of claim 1, wherein the compound has the following structure:



Compound 3

or a stereoisomer thereof.

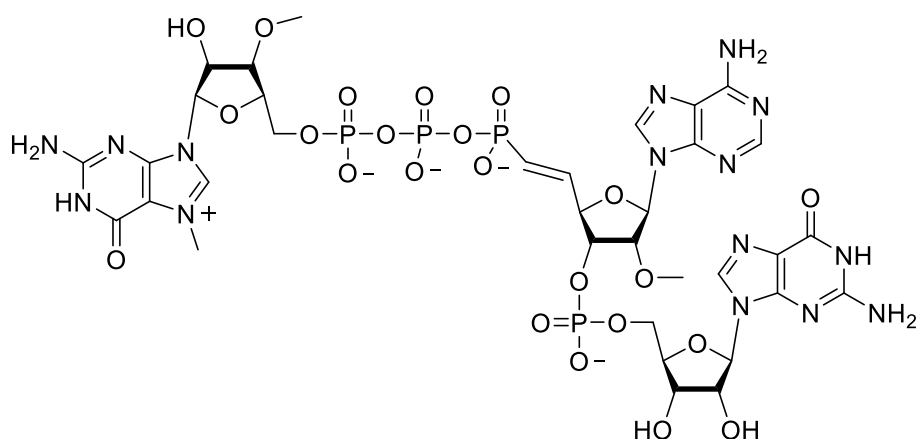
5. The compound of claim 1, wherein the compound has the following structure:



Compound 4

or a stereoisomer thereof.

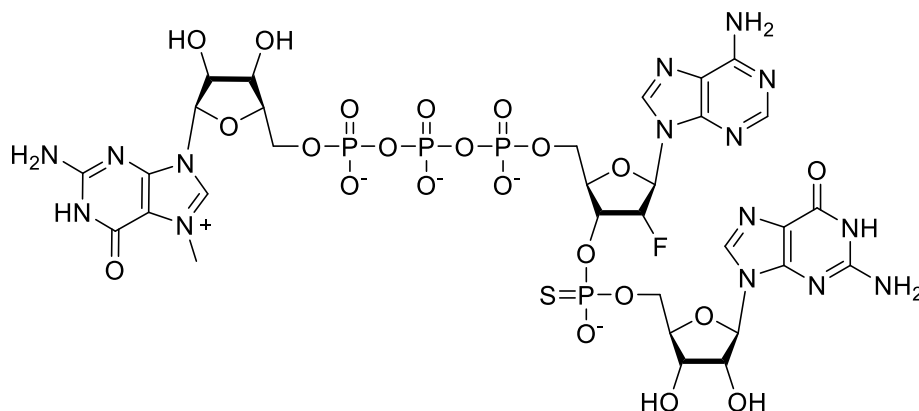
6. The compound of claim 1, wherein the compound has the following structure:



Compound 5

or a stereoisomer thereof.

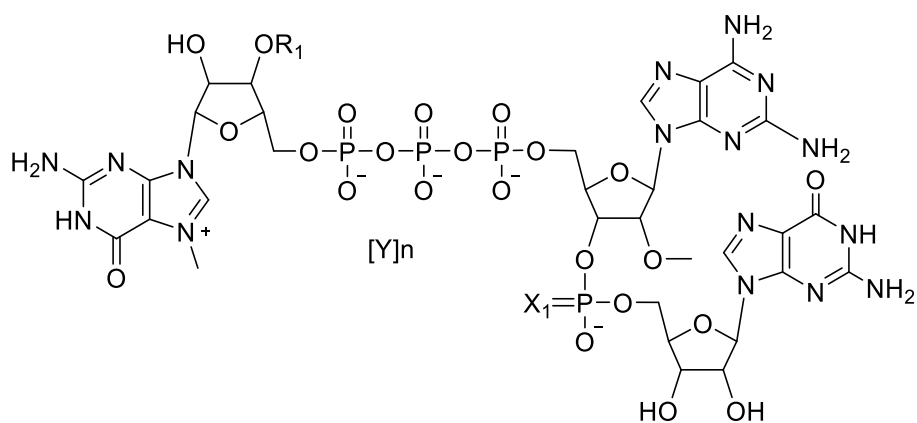
7. The compound of claim 1, wherein the compound has the following structure:



Compound 6

or a stereoisomer thereof.

8. A compound of the following formula:



Formula II

or a stereoisomer thereof, wherein:

R_1 is H or CH_3 ;

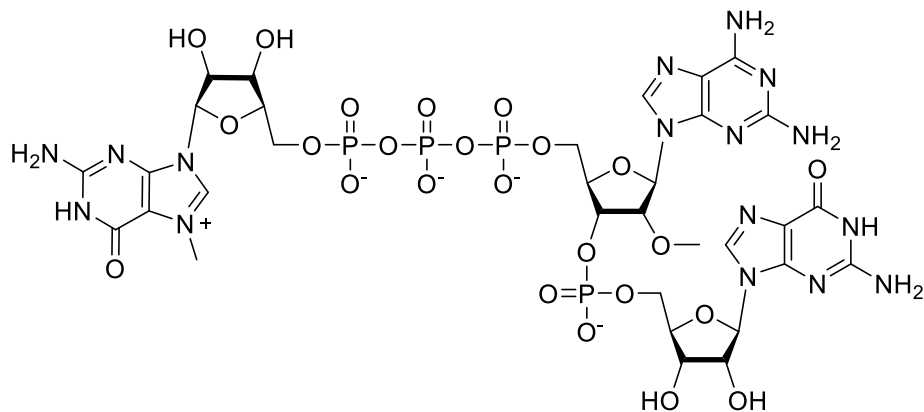
X_1 is O or S;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4,

wherein when X₁ is S, R₁ is CH₃.

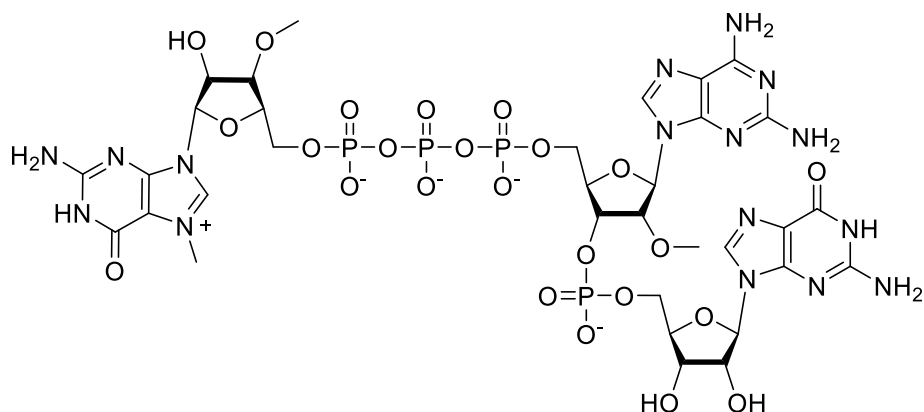
9. The compound of claim 8, wherein the compound has the following structure:



Compound 7

or a stereoisomer thereof.

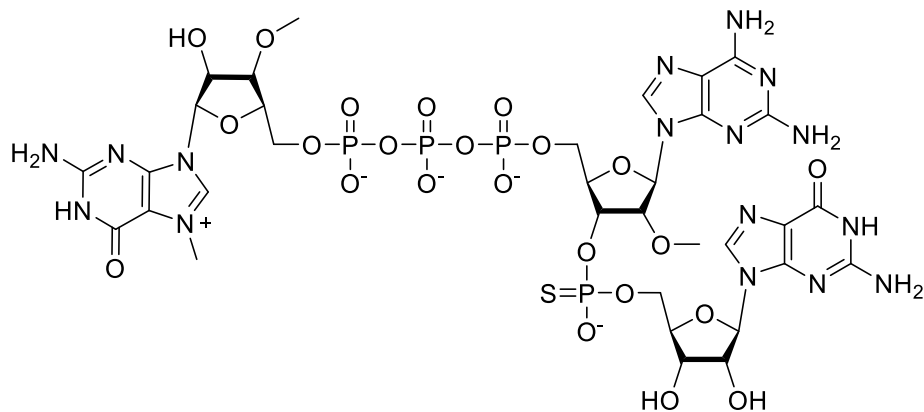
10. The compound of claim 8, wherein the compound has the following structure:



Compound 8

or a stereoisomer thereof.

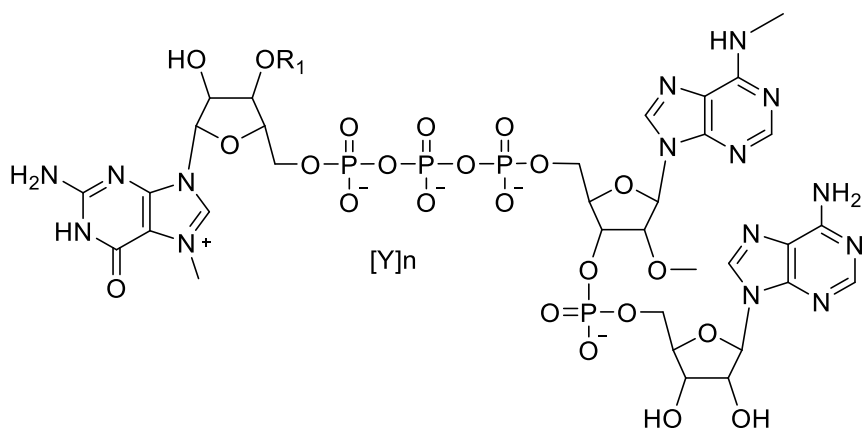
11. The compound of claim 8, wherein the compound has the following structure:



Compound 9

or a stereoisomer thereof.

12. A compound of the following formula:



Formula III

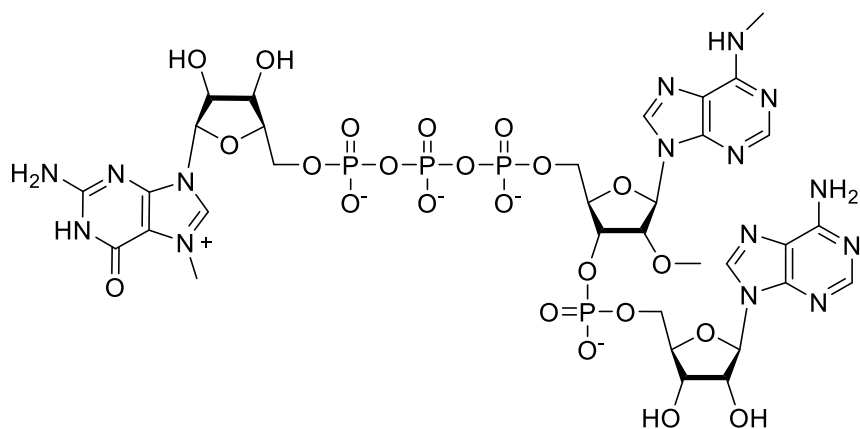
or a stereoisomer thereof, wherein:

R_1 is H or CH_3 ;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4.

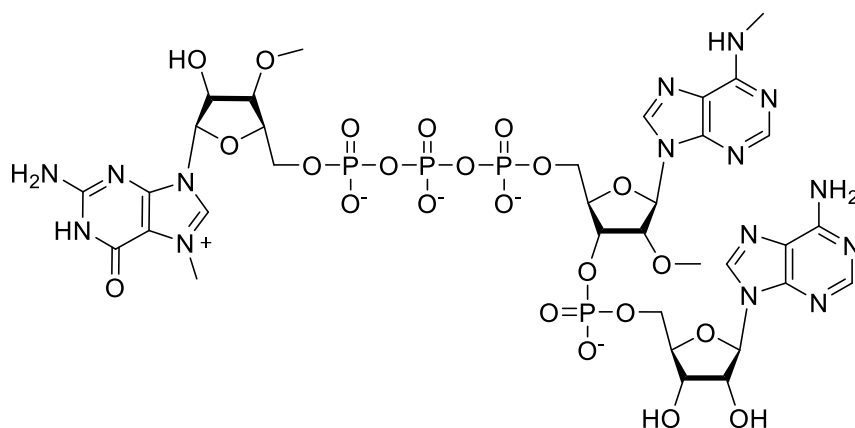
13. The compound of claim 12, wherein the compound has the following structure:



Compound 10

or a stereoisomer thereof.

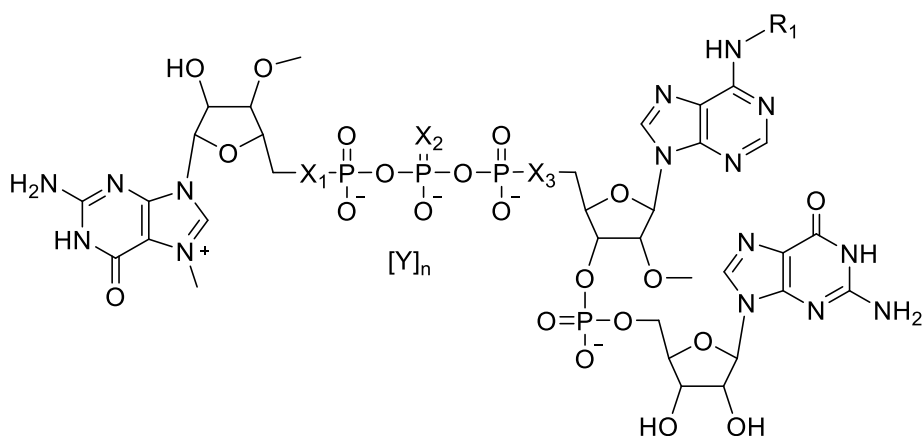
14. The compound of claim 12, wherein the compound has the following structure:



Compound 11

or a stereoisomer thereof.

15. A compound of the following formula:



Formula IV

or a stereoisomer thereof, wherein:

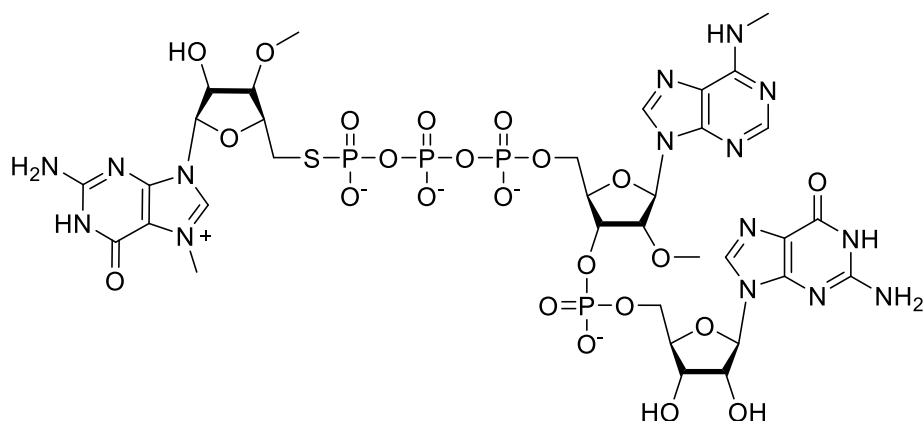
R_1 is H or CH_3 ;

X_1 , X_2 , and X_3 are each independently selected from O and S, wherein when one of X_1 , X_2 , or X_3 is S, the remaining of X_1 , X_2 , and X_3 are O;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4.

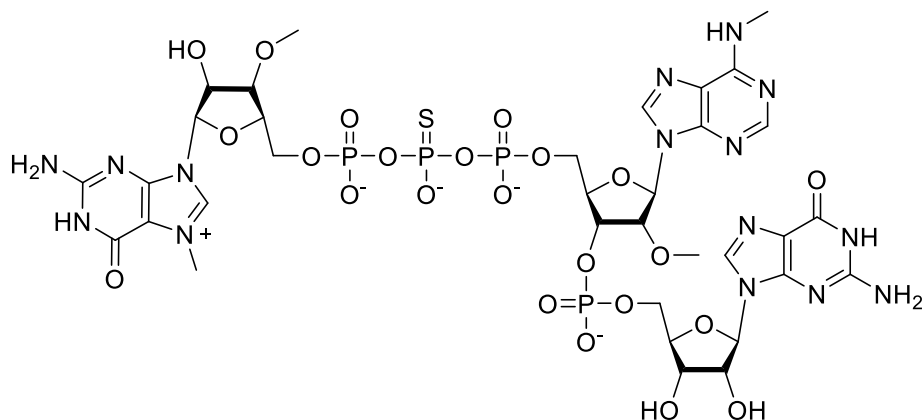
16. The compound of claim 15, wherein the compound has the following structure:



Compound 12

or a stereoisomer thereof.

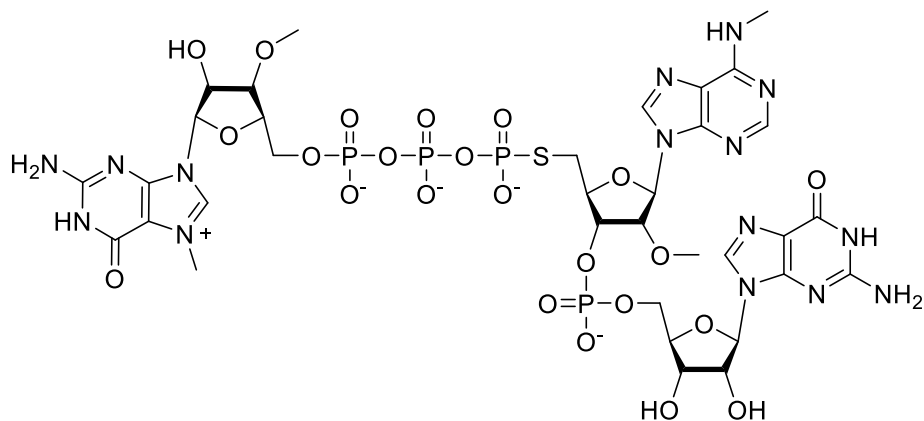
17. The compound of claim 15, wherein the compound has the following structure:



Compound 13

or a stereoisomer thereof.

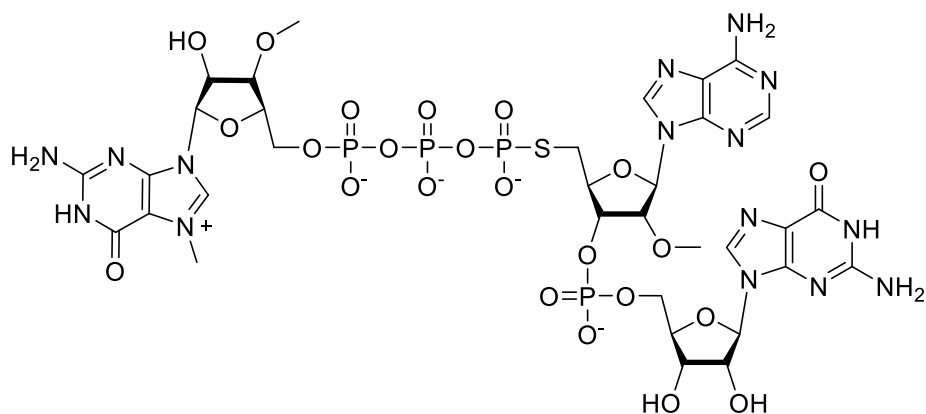
18. The compound of claim 15, wherein the compound has the following structure:



Compound 14

or a stereoisomer thereof.

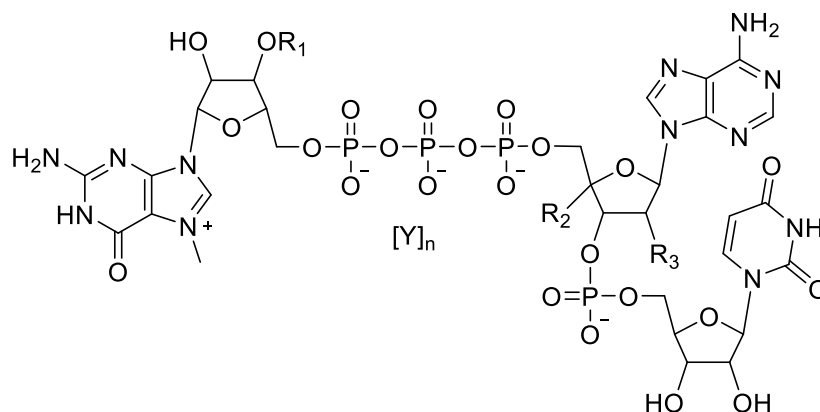
19. The compound of claim 15, wherein the compound has the following structure:



Compound 73

or a stereoisomer thereof.

20. A compound of the following formula:



Formula V

or a stereoisomer thereof, wherein:

R_1 is H or CH_3 ;

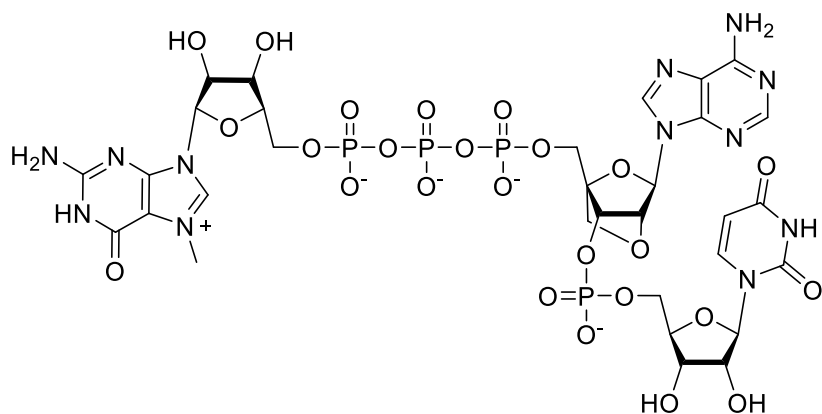
R_2 is H and R_3 is F, or R_2 and R_3 are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4,

wherein when R_2 is H and R_3 is F, R_1 is not H.

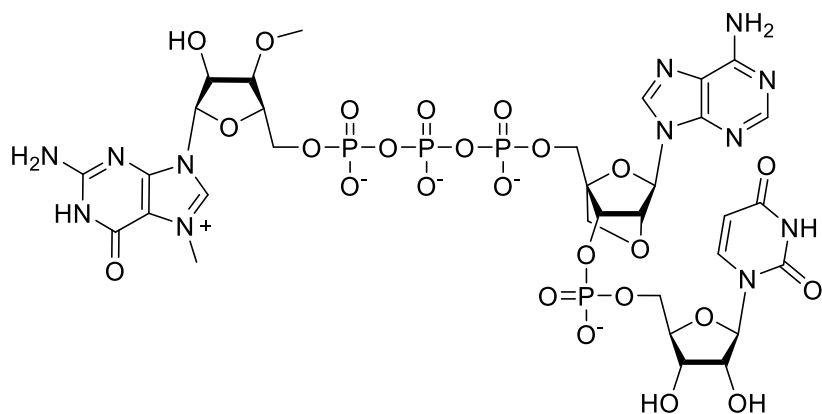
21. The compound of claim 20, wherein the compound has the following structure:



Compound 15

or a stereoisomer thereof.

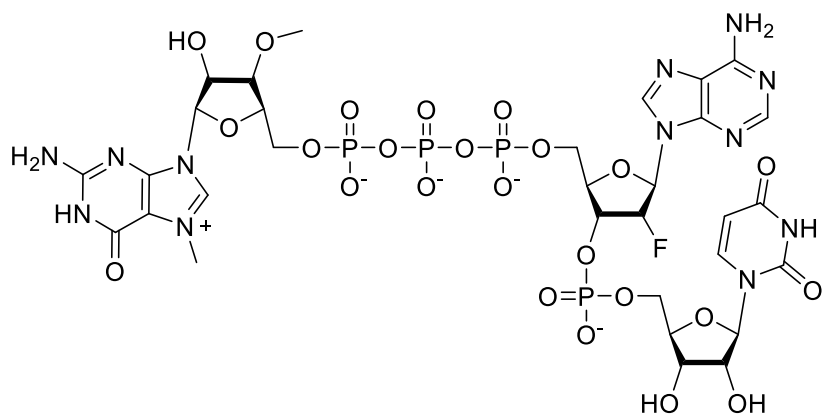
22. The compound of claim 20, wherein the compound has the following structure:



Compound 16

or a stereoisomer thereof.

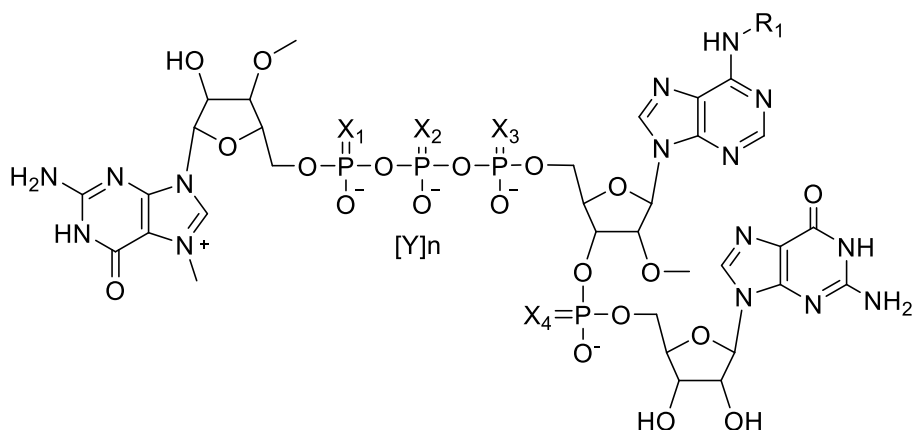
23. The compound of claim 20, wherein the compound has the following structure:



Compound 17

or a stereoisomer thereof.

24. A compound of the following formula:



Formula VI

or a stereoisomer thereof, wherein:

R_1 is H or CH_3 ;

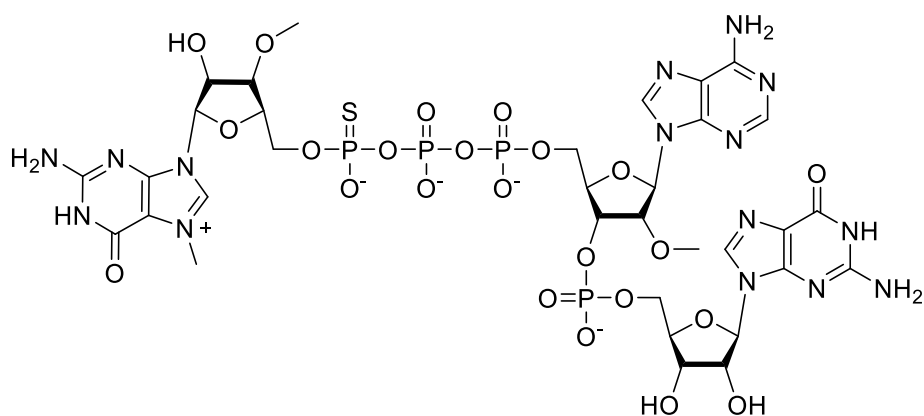
X_1 , X_2 , X_3 , and X_4 are each independently selected from O and S;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4,

wherein one of X_1 , X_2 , X_3 , and X_4 is S.

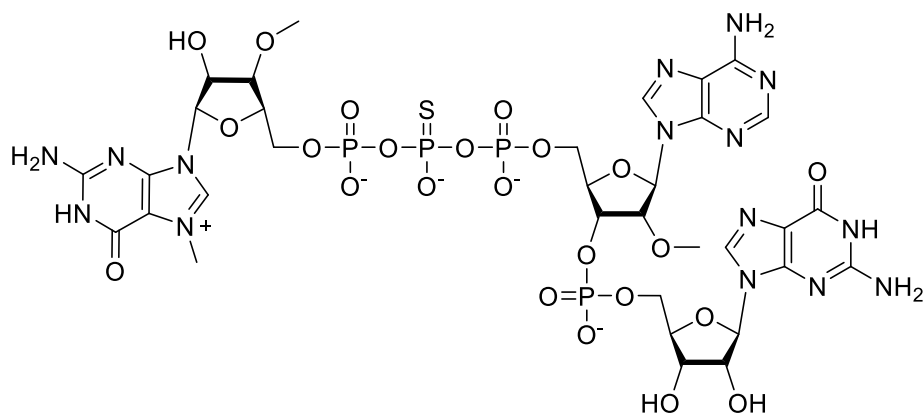
25. The compound of claim 24, wherein the compound has the following structure:



Compound 18

or a stereoisomer thereof.

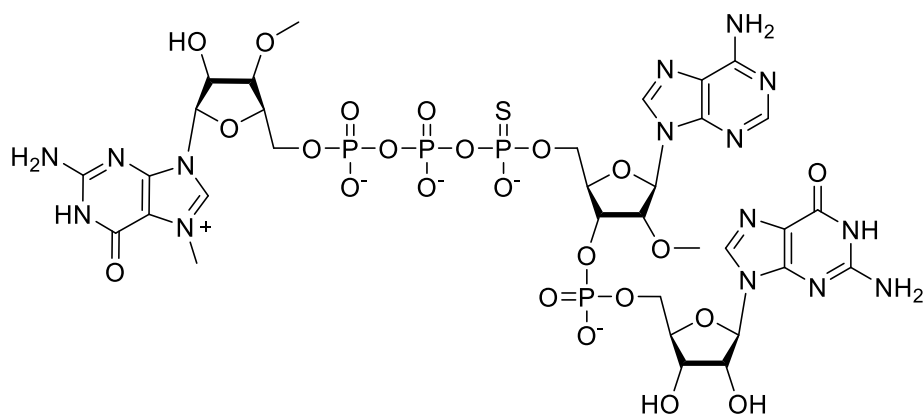
26. The compound of claim 24, wherein the compound has the following structure:



Compound 19

or a stereoisomer thereof.

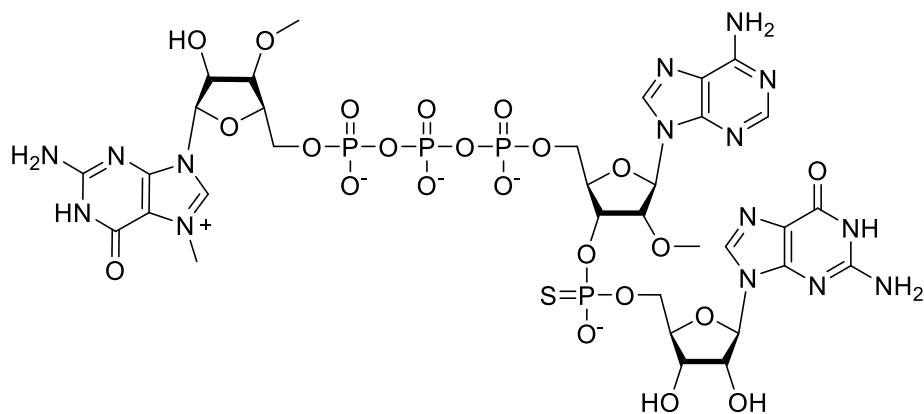
27. The compound of claim 24, wherein the compound has the following structure:



Compound 20

or a stereoisomer thereof.

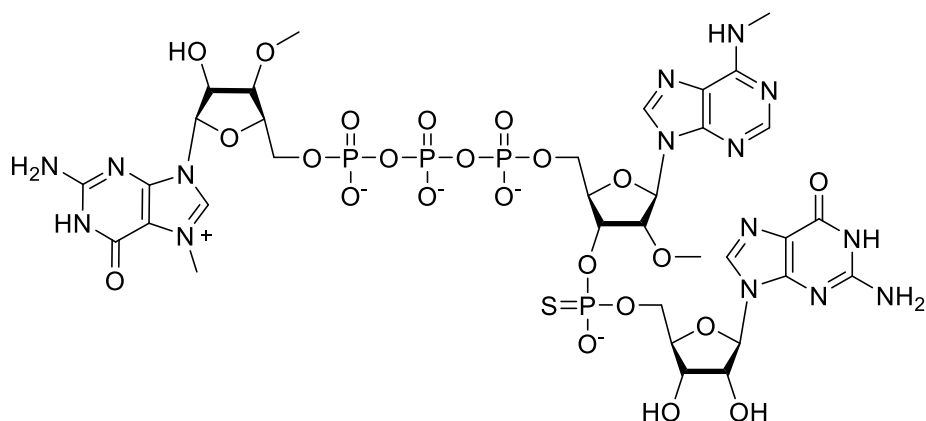
28. The compound of claim 24, wherein the compound has the following structure:



Compound 21

or a stereoisomer thereof.

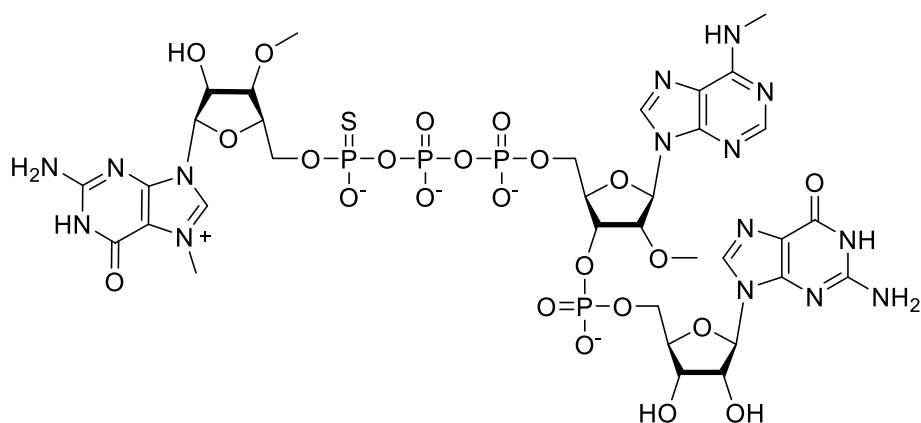
29. The compound of claim 24, wherein the compound has the following structure:



Compound 74

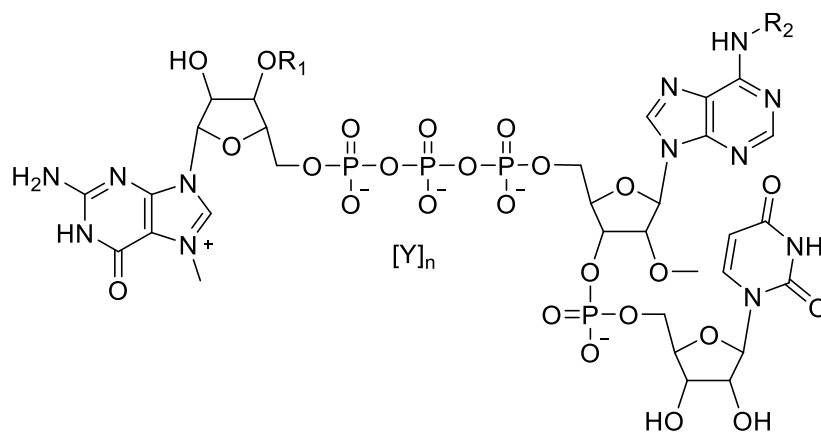
or a stereoisomer thereof.

30. The compound of claim 24, wherein the compound has the following structure:



Compound 81

31. A compound of the following formula:



Formula VII

or a stereoisomer thereof, wherein:

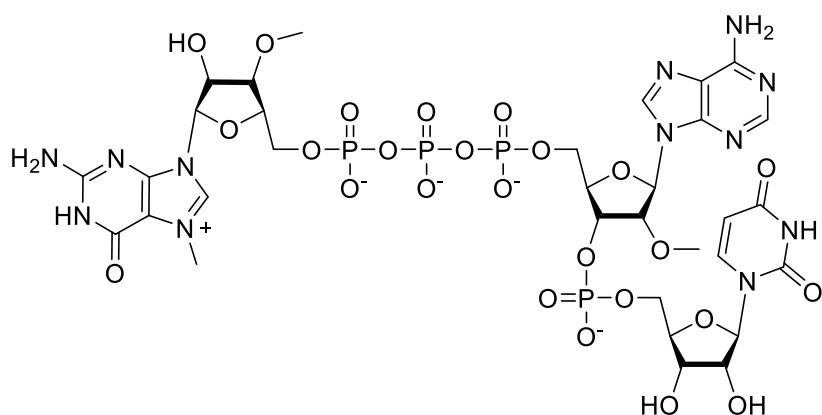
R_1 and R_2 are each independently selected from H and CH_3 ;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4,

wherein at least one of R_1 or R_2 is CH_3 .

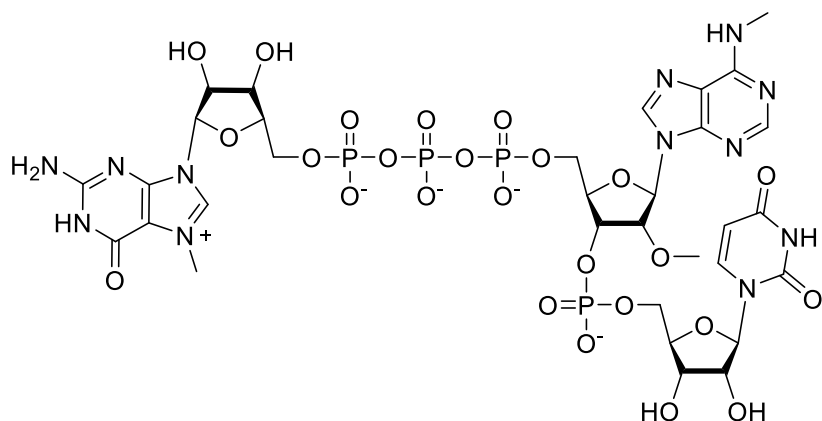
32. The compound of claim 31, wherein the compound has the following structure:



Compound 22

or a stereoisomer thereof.

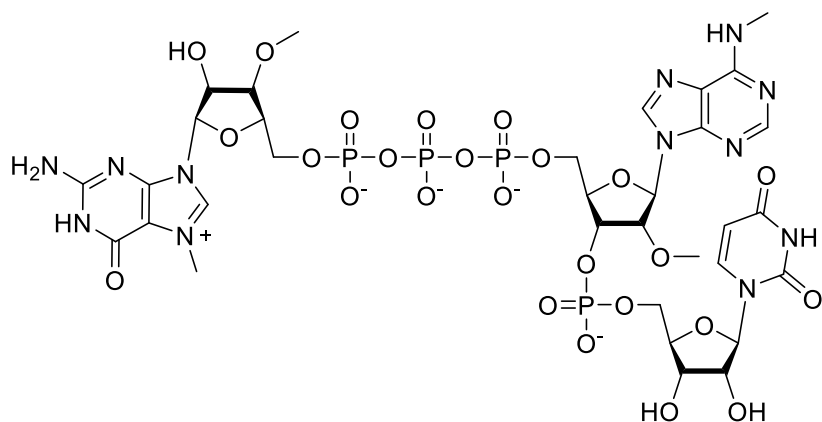
33. The compound of claim 31, wherein the compound has the following structure:



Compound 23

or a stereoisomer thereof.

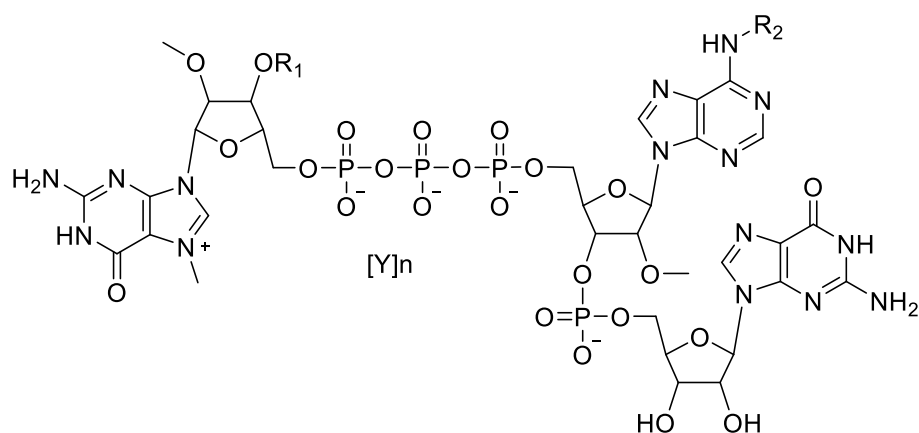
34. The compound of claim 31, wherein the compound has the following structure:



Compound 24

or a stereoisomer thereof.

35. A compound of the following formula:



Formula VIII

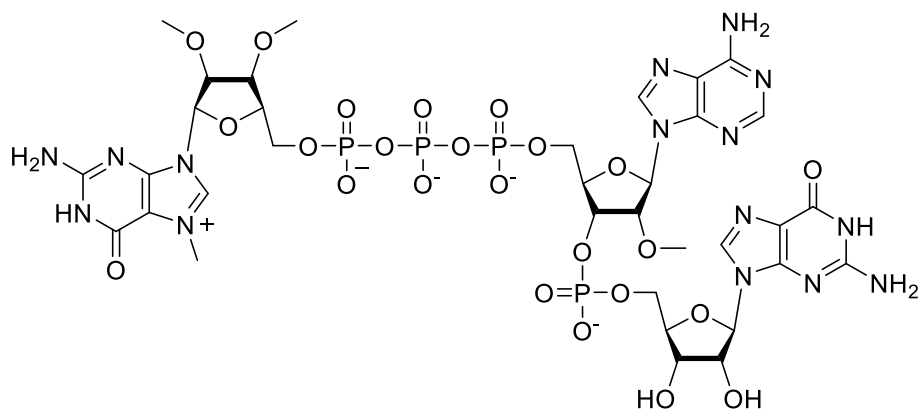
or a stereoisomer thereof, wherein:

R_1 and R_2 are each independently selected from H and CH_3 ;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4.

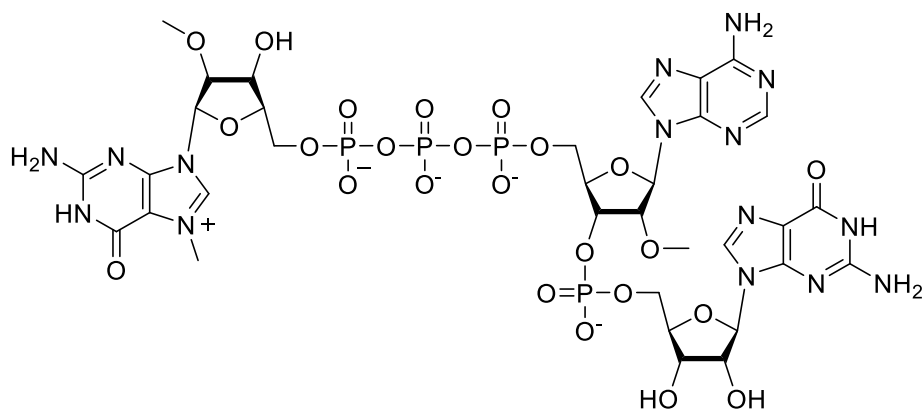
36. The compound of claim 35, wherein the compound has the following structure:



Compound 25

or a stereoisomer thereof.

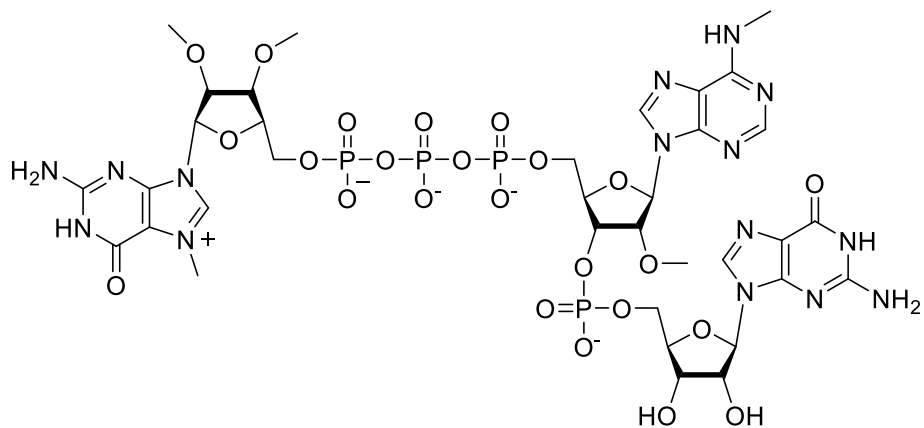
37. The compound of claim 35, wherein the compound has the following structure:



Compound 26

or a stereoisomer thereof.

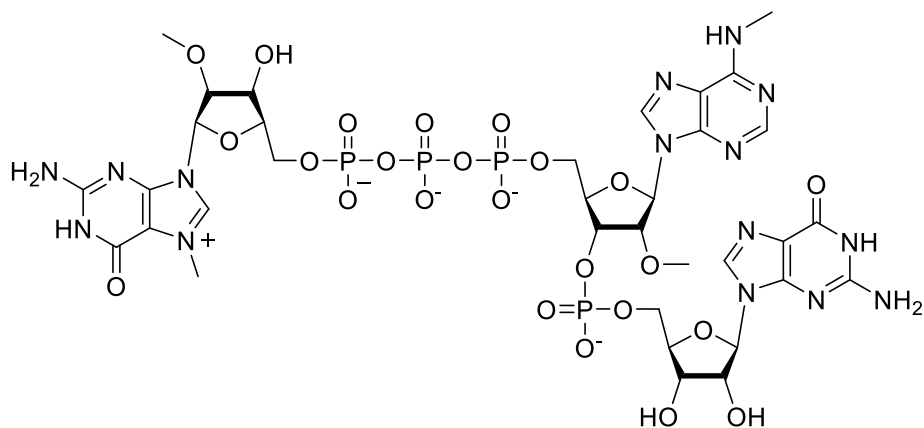
38. The compound of claim 35, wherein the compound has the following structure:



Compound 27

or a stereoisomer thereof.

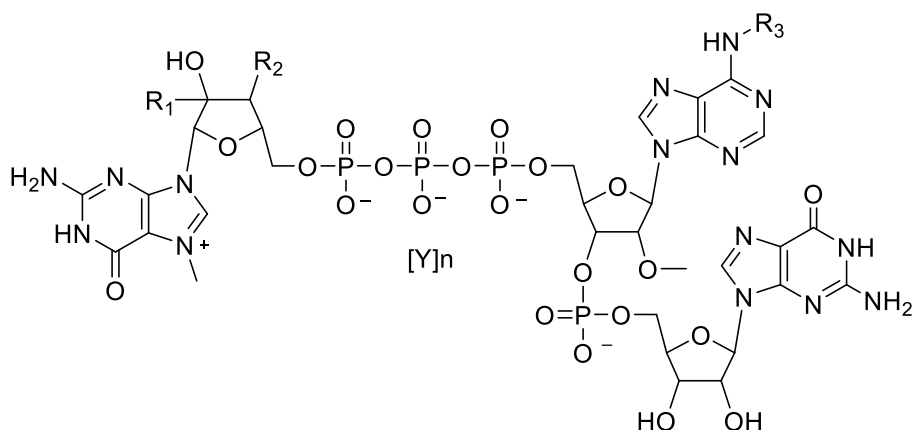
39. The compound of claim 35, wherein the compound has the following structure:



Compound 28

or a stereoisomer thereof.

40. A compound of the following formula:



Formula IX

or a stereoisomer thereof, wherein:

R₁ is H or CH₃;

R₂ is OH, F, substituted or unsubstituted alkoxy, or thio;

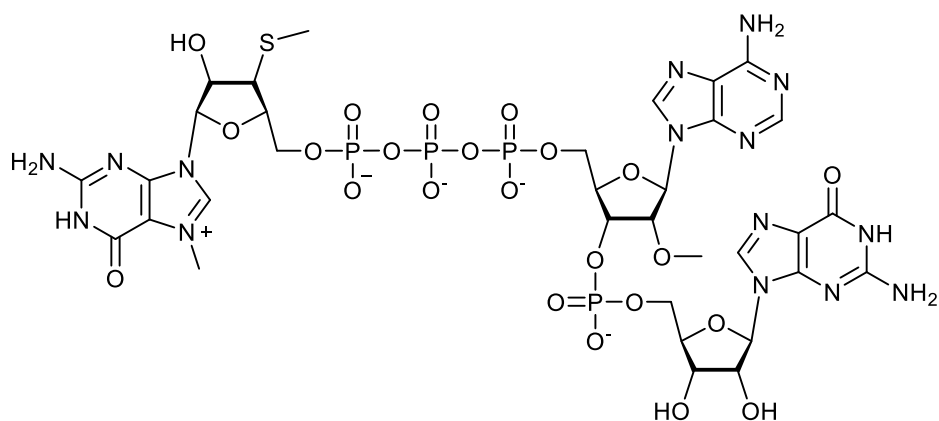
R₃ is H or CH₃;

each independent Y is H⁺ or a cation;

and n is 0, 1, 2, 3, or 4,

wherein when R₂ is OH, R₁ is not H.

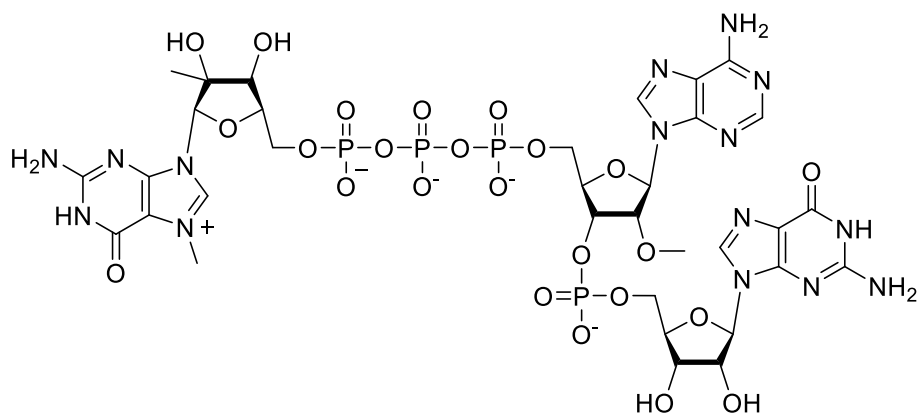
41. The compound of claim 40, wherein the compound has the following structure:



Compound 42

or a stereoisomer thereof.

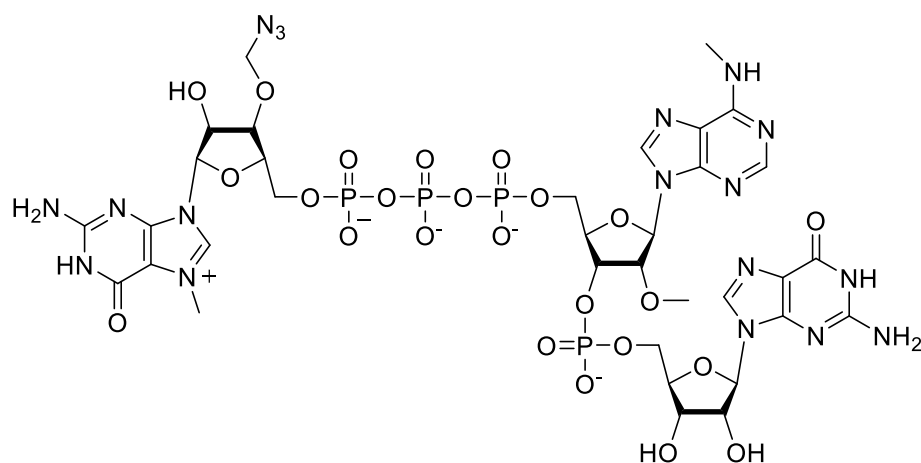
42. The compound of claim 40, wherein the compound has the following structure:



Compound 43

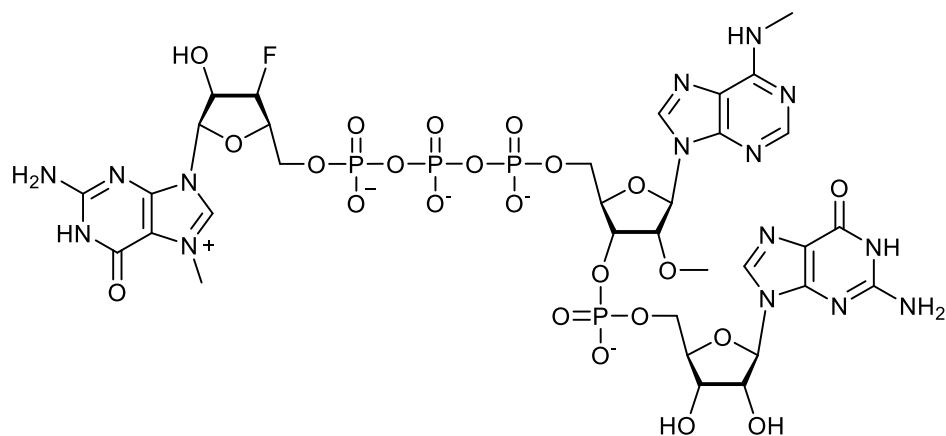
or a stereoisomer thereof.

43. The compound of claim 40, wherein the compound has the following structure:



Compound 45

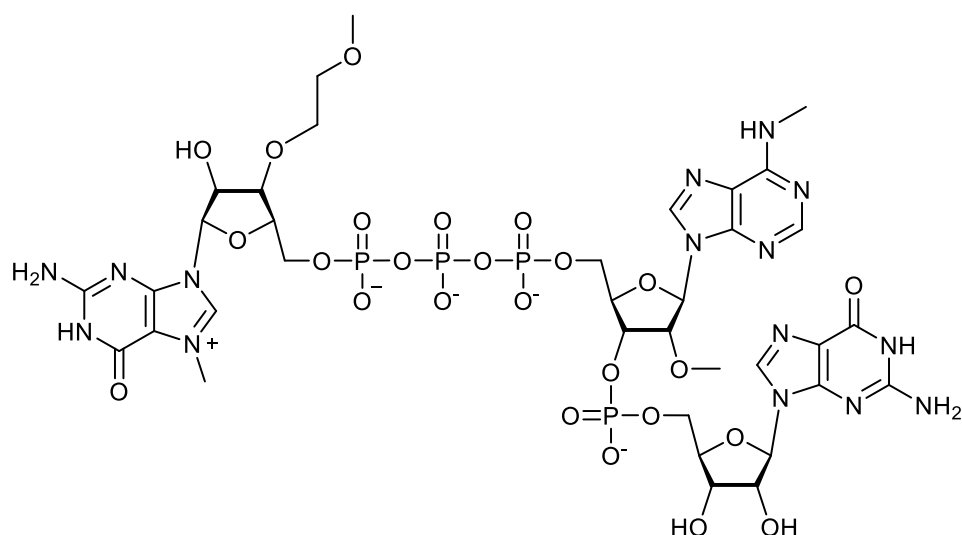
44. The compound of claim 40, wherein the compound has the following structure:



Compound 49

or a stereoisomer thereof.

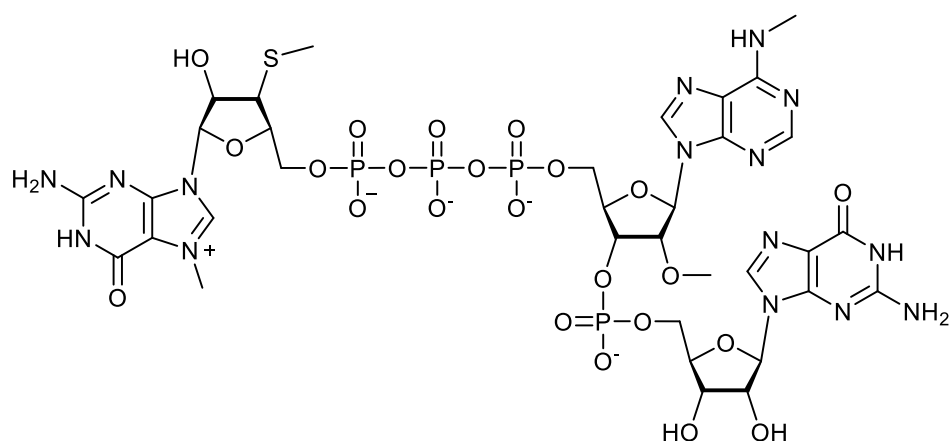
45. The compound of claim 40, wherein the compound has the following structure:



**Compound
50**

or a stereoisomer thereof.

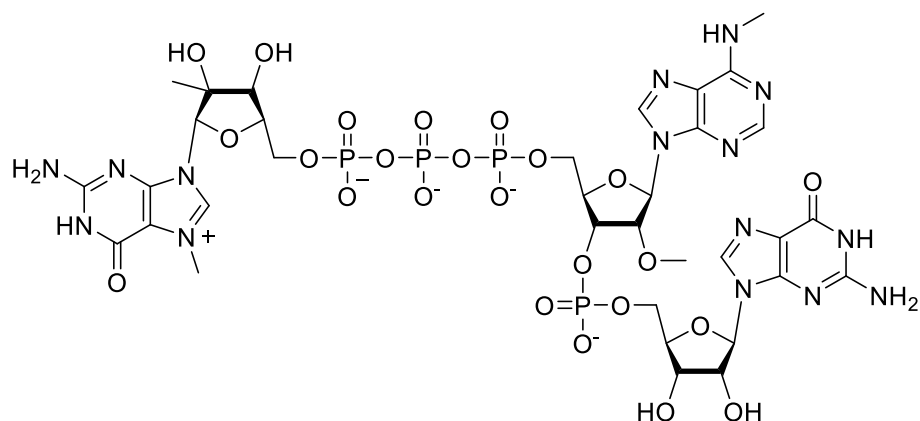
46. The compound of claim 40, wherein the compound has the following structure:



Compound 52

or a stereoisomer thereof.

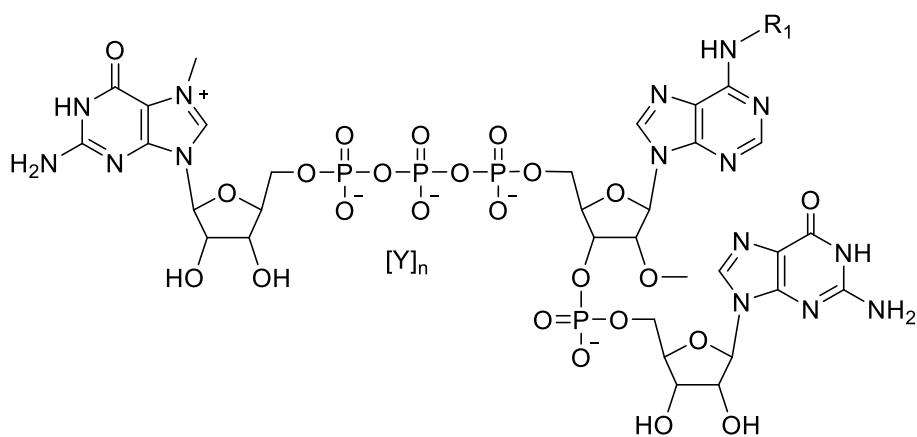
47. The compound of claim 40, wherein the compound has the following structure:



Compound 76

or a stereoisomer thereof.

48. A compound of the following formula:



Formula X

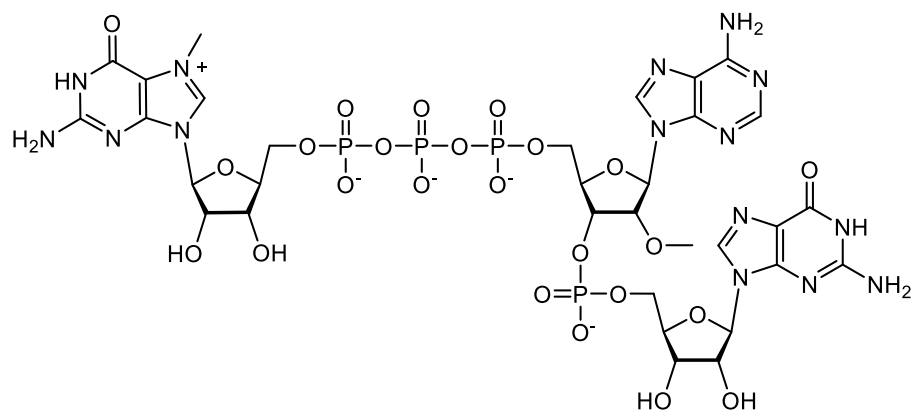
or a stereoisomer thereof, wherein:

R_1 is H and CH_3 ;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4.

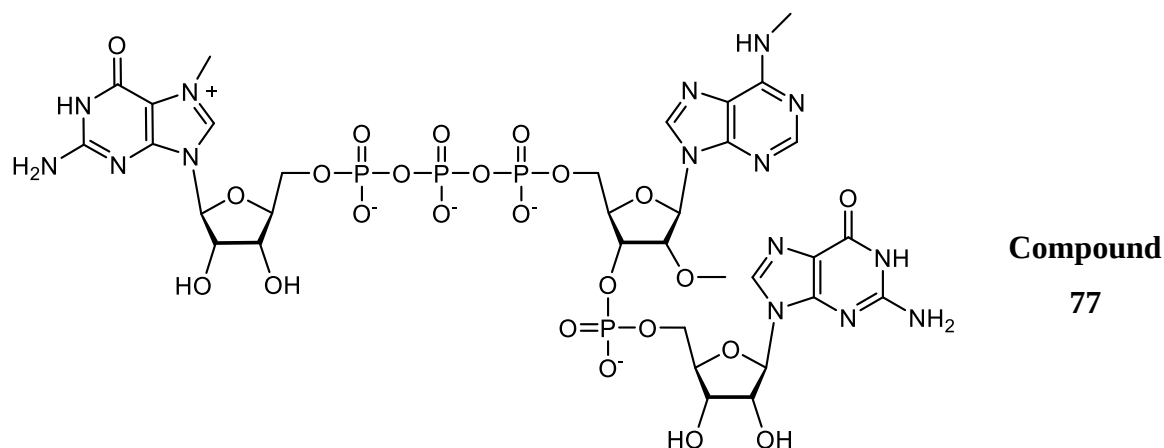
49. The compound of claim 48, wherein the compound has the following structure:



Compound 75

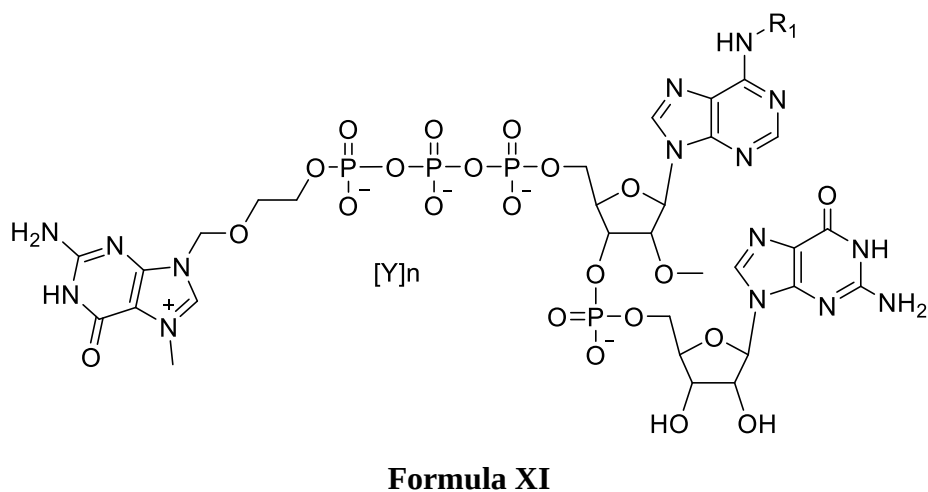
or a stereoisomer thereof.

50. The compound of claim 48, wherein the compound has the following structure:



or a stereoisomer thereof.

51. A compound of the following formula:



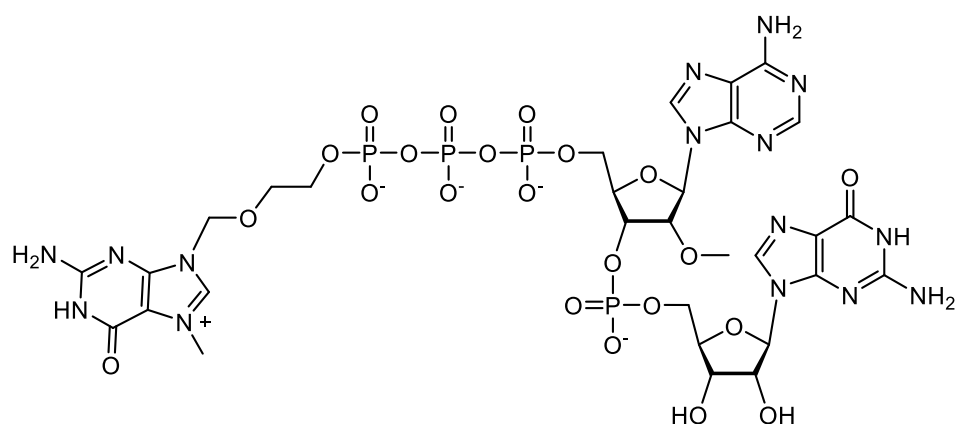
or a stereoisomer thereof, wherein:

R_1 is H and CH_3 ;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, or 4.

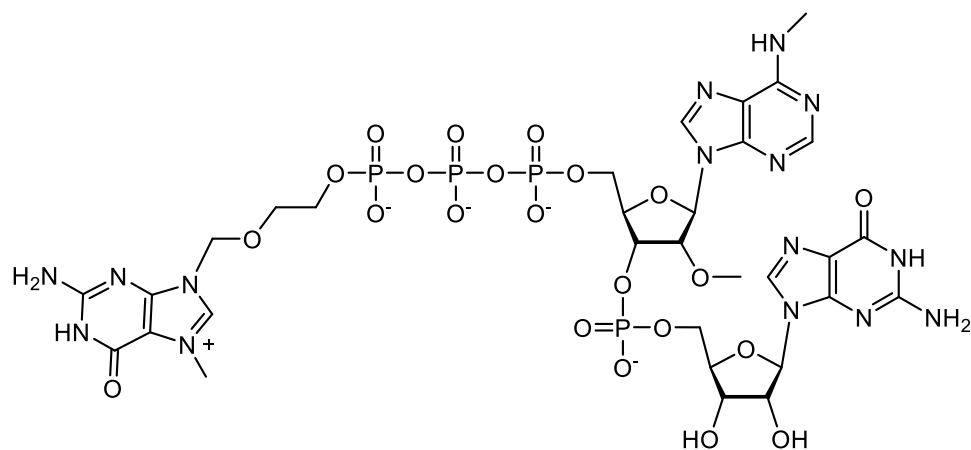
52. The compound of claim 51, wherein the compound has the following structure:



**Compound
82**

or a stereoisomer thereof.

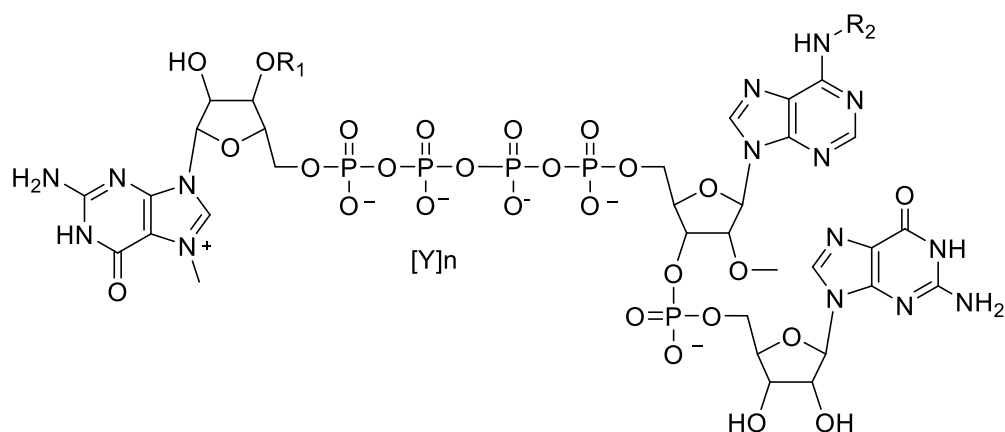
53. The compound of claim 51, wherein the compound has the following structure:



**Compound
78**

or a stereoisomer thereof.

54. A compound of the following formula:



Formula XII

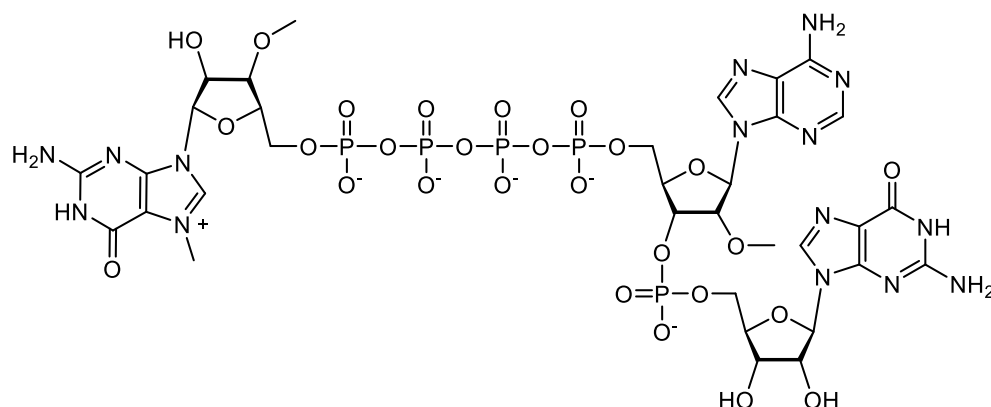
or a stereoisomer thereof, wherein:

R_1 and R_2 are each independently selected from H and CH_3 ;

each independent Y is H^+ or a cation; and

n is 0, 1, 2, 3, 4, or 5.

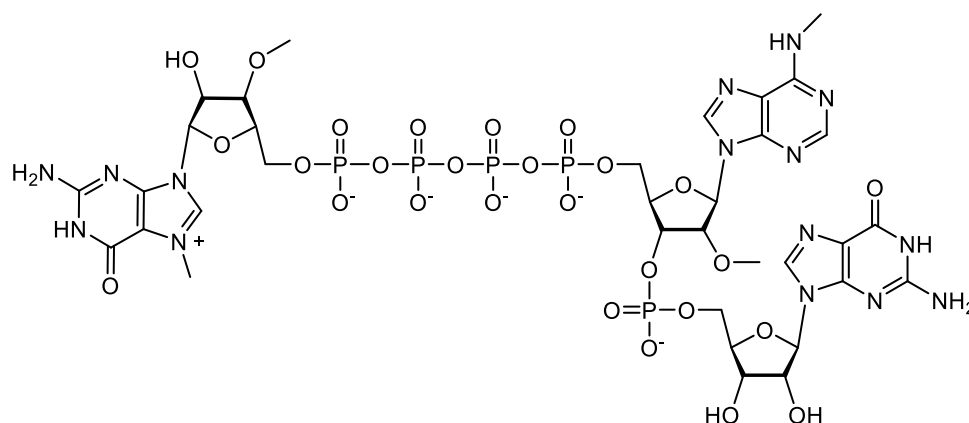
55. The compound of claim 54, wherein the compound has the following structure:



Compound
79

or a stereoisomer thereof.

56. The compound of claim 54, wherein the compound has the following structure:

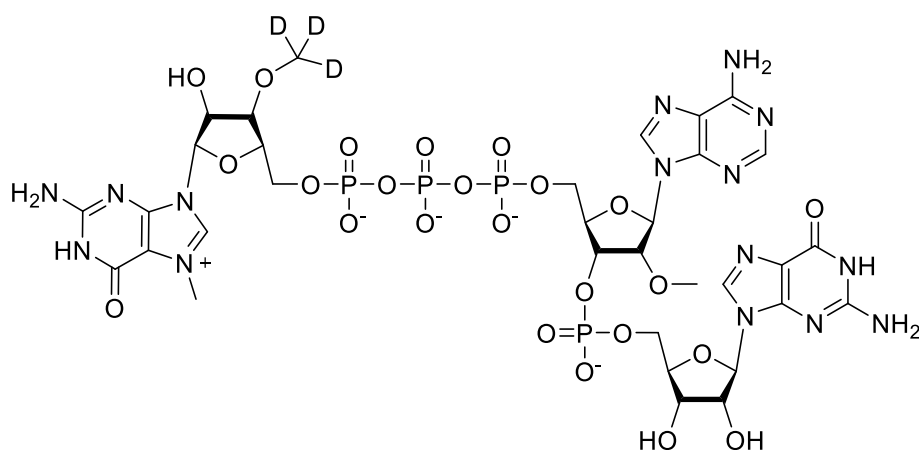


Compound
80

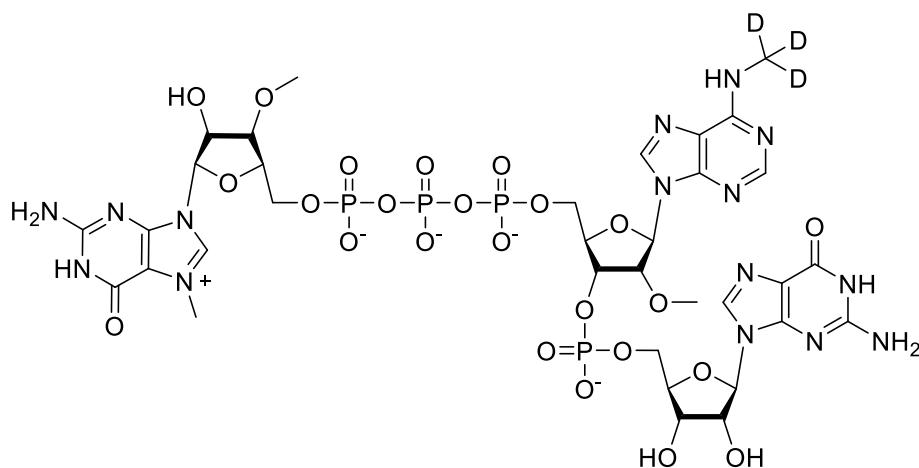
or a stereoisomer thereof.

57. The compound of any one of claims 1-56, wherein the compound is a deuterated form of the compound.

58. A compound selected from the group consisting of:



Compound 83



Compound 84

or a stereoisomer thereof.

59. A pharmaceutical composition, comprising a compound of any one of claims 1-58 and a pharmaceutically acceptable carrier.
60. An RNA molecule comprising a 5'-cap, wherein the 5'-cap comprises a compound of any one of claims 1-58.
61. The RNA molecule of claim 60, wherein the RNA molecule is a messenger RNA (mRNA) molecule or a self-amplifying RNA (saRNA) molecule
62. Use of an RNA molecule according to claim 60 or 61, in the manufacture of a medicament for inducing a therapeutic effect.
63. Use of a therapeutic dose unit of an mRNA molecule comprising a 5'-cap, in the manufacture of a medicament to be administered to a subject, wherein the 5'-cap comprises a compound of any one of claims 1-58.

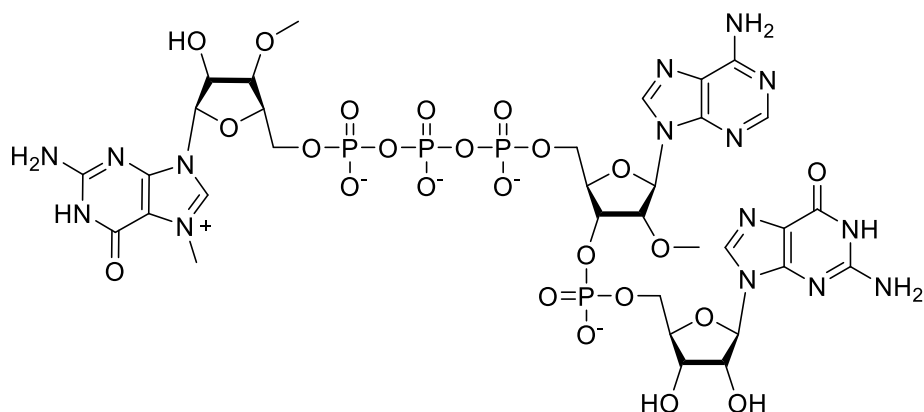
64. The use of claim 63, wherein the therapeutic dose unit of the mRNA molecule comprises less than 7 ng/μg of double stranded RNA (dsRNA).
65. The use of claim 64, wherein the subject exhibits increased tolerability to the therapeutic dose unit of the mRNA molecule to be administered as compared to an equivalent therapeutic dose unit of the mRNA molecule comprising 7 ng/μg or greater dsRNA.
66. The use of claim 65, wherein the increased tolerability is determined by measuring one or more of body weight, organ weight, aspartate aminotransferase (AST) levels, alanine transaminase (ALT) levels, C-reactive protein (CRP) levels, procalcitonin (PCT) levels, interleukin-6 (IL-6) levels, erythrocyte sedimentation rate (ESR), serum amyloid A levels, and serum ferritin levels prior to the administering and a period of time after the administering.
67. The use of claim 65 or 66, wherein the increased tolerability is measured by testing in a standard *in vivo* assay.
68. The use of any one of claims 65-67, wherein the increased tolerability is an increase in tolerability of at least 20 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 25 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 30 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 35 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 40 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 45 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 50 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 55 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 60 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 65 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 70 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 75 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 80 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 85 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 90 % to the dose unit

to be administered as measured by testing in a standard *in vivo* assay, or at least 95 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay.

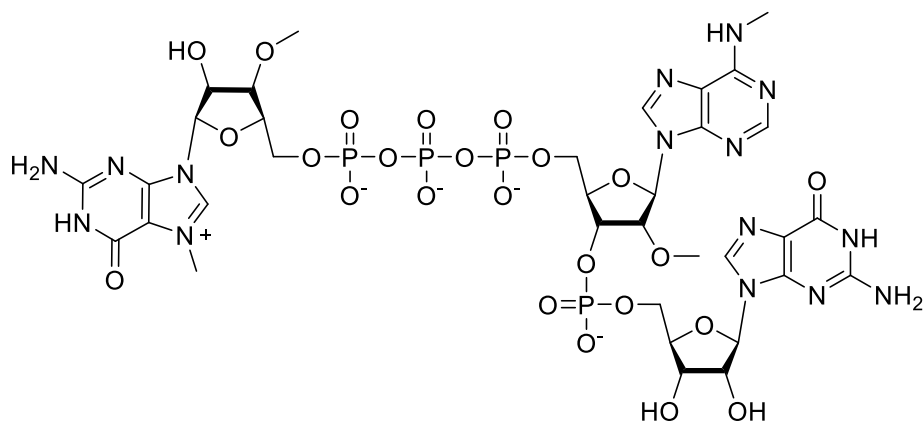
69. The use of any one of claims 65-68, wherein the therapeutic dose unit of the mRNA molecule comprises less than 6.9 ng/μg of double stranded RNA (dsRNA), less than 6.8 ng/μg of double stranded RNA (dsRNA), less than 6.7 ng/μg of double stranded RNA (dsRNA), less than 6.6 ng/μg of double stranded RNA (dsRNA), less than 6.5 ng/μg of double stranded RNA (dsRNA), less than 6.4 ng/μg of double stranded RNA (dsRNA), less than 6.3 ng/μg of double stranded RNA (dsRNA), less than 6.2 ng/μg of double stranded RNA (dsRNA), less than 6.1 ng/μg of double stranded RNA (dsRNA), less than 6.0 ng/μg of double stranded RNA (dsRNA), less than 5.9 ng/μg of double stranded RNA (dsRNA), less than 5.8 ng/μg of double stranded RNA (dsRNA), less than 5.7 ng/μg of double stranded RNA (dsRNA), less than 5.6 ng/μg of double stranded RNA (dsRNA), less than 5.5 ng/μg of double stranded RNA (dsRNA), less than 5.4 ng/μg of double stranded RNA (dsRNA), less than 5.3 ng/μg of double stranded RNA (dsRNA), less than 5.2 ng/μg of double stranded RNA (dsRNA), less than 5.1 ng/μg of double stranded RNA (dsRNA), less than 5.0 ng/μg of double stranded RNA (dsRNA), less than 4.9 ng/μg of double stranded RNA (dsRNA), less than 4.8 ng/μg of double stranded RNA (dsRNA), less than 4.7 ng/μg of double stranded RNA (dsRNA), less than 4.6 ng/μg of double stranded RNA (dsRNA), less than 4.5 ng/μg of double stranded RNA (dsRNA), less than 4.4 ng/μg of double stranded RNA (dsRNA), less than 4.3 ng/μg of double stranded RNA (dsRNA), less than 4.2 ng/μg of double stranded RNA (dsRNA), less than 4.1 ng/μg of double stranded RNA (dsRNA), less than 4.0 ng/μg of double stranded RNA (dsRNA), less than 3.9 ng/μg of double stranded RNA (dsRNA), less than 3.8 ng/μg of double stranded RNA (dsRNA), less than 3.7 ng/μg of double stranded RNA (dsRNA), less than 3.6 ng/μg of double stranded RNA (dsRNA), less than 3.5 ng/μg of double stranded RNA (dsRNA), less than 3.4 ng/μg of double stranded RNA (dsRNA), less than 3.3 ng/μg of double stranded RNA (dsRNA), less than 3.2 ng/μg of double stranded RNA (dsRNA), less than 3.1 ng/μg of double stranded RNA (dsRNA), less than 3.0 ng/μg of double stranded RNA (dsRNA), less than 2.9 ng/μg of double stranded RNA (dsRNA), less than 2.8 ng/μg of double stranded RNA (dsRNA), less than 2.7 ng/μg of double stranded RNA (dsRNA), less than 2.6 ng/μg of double stranded RNA (dsRNA), less than 2.5 ng/μg of double stranded RNA (dsRNA), less than 2.4 ng/μg of double stranded RNA (dsRNA), less than 2.3 ng/μg of double stranded RNA (dsRNA), less than 2.2 ng/μg of double stranded RNA (dsRNA), less than 2.1 ng/μg of double stranded RNA (dsRNA), less than 2.0 ng/μg of

double stranded RNA (dsRNA), less than 1.9 ng/μg of double stranded RNA (dsRNA), less than 1.8 ng/μg of double stranded RNA (dsRNA), less than 1.7 ng/μg of double stranded RNA (dsRNA), less than 1.6 ng/μg of double stranded RNA (dsRNA), less than 1.5 ng/μg of double stranded RNA (dsRNA), less than 1.4 ng/μg of double stranded RNA (dsRNA), less than 1.3 ng/μg of double stranded RNA (dsRNA), less than 1.2 ng/μg of double stranded RNA (dsRNA), less than 1.1 ng/μg of double stranded RNA (dsRNA), less than 1.0 ng/μg of double stranded RNA (dsRNA), less than 0.9 ng/μg of double stranded RNA (dsRNA), less than 0.8 ng/μg of double stranded RNA (dsRNA), less than 0.7 ng/μg of double stranded RNA (dsRNA), less than 0.6 ng/μg of double stranded RNA (dsRNA), less than 0.5 ng/μg of double stranded RNA (dsRNA), less than 0.4 ng/μg of double stranded RNA (dsRNA), less than 0.3 ng/μg of double stranded RNA (dsRNA), less than 0.2 ng/μg of double stranded RNA (dsRNA), or less than 0.1 ng/μg of double stranded RNA (dsRNA).

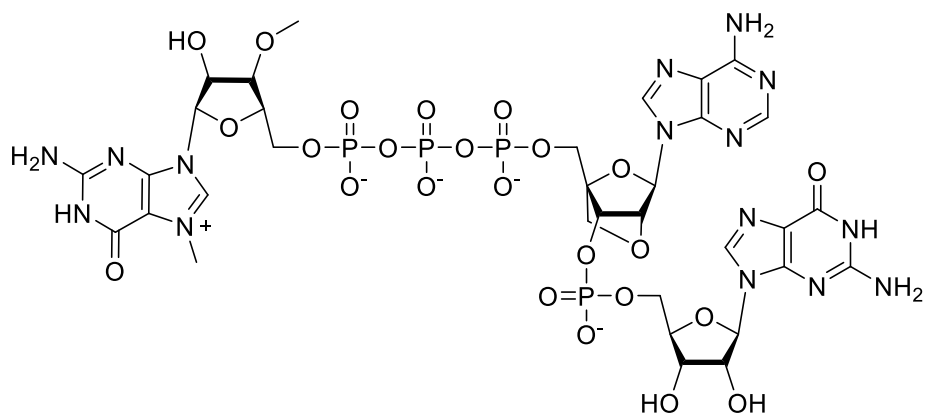
70. Use of a therapeutic dose unit of an mRNA molecule comprising a 5'-cap, in the manufacture of a medicament to be administered to a subject, wherein the 5'-cap comprises a compound selected from the group consisting of:



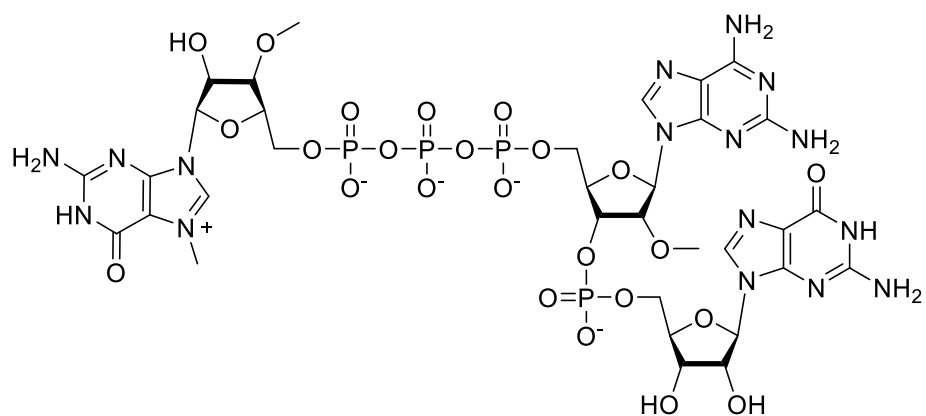
Compound 29



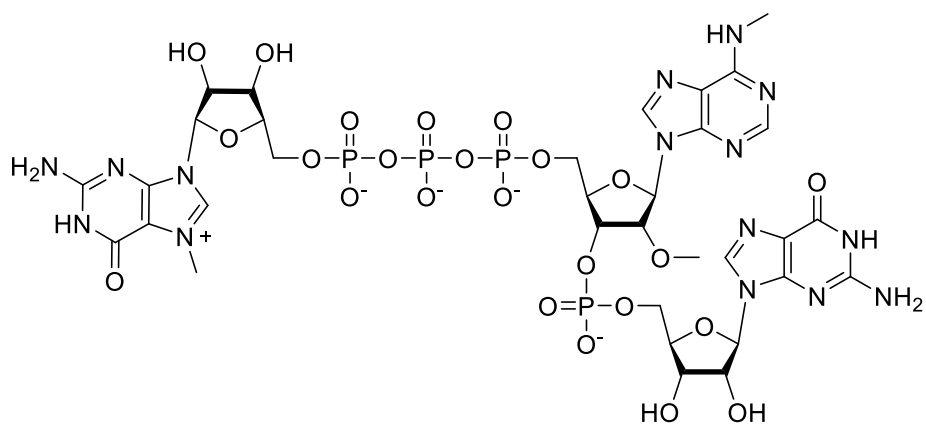
Compound 30



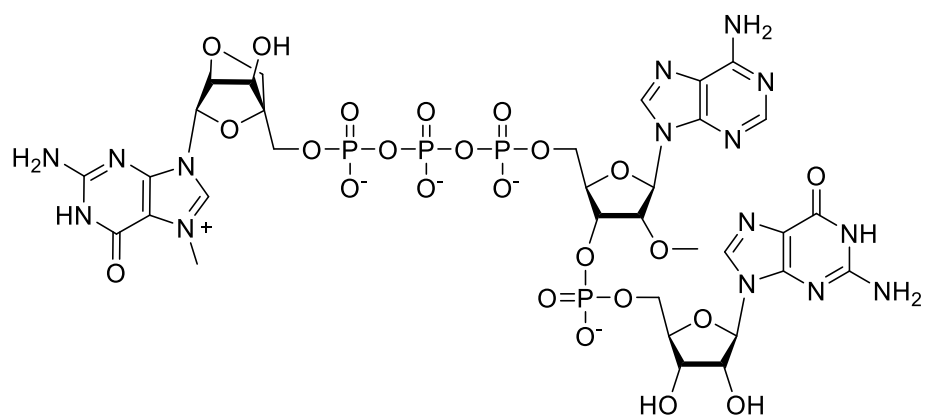
Compound 2



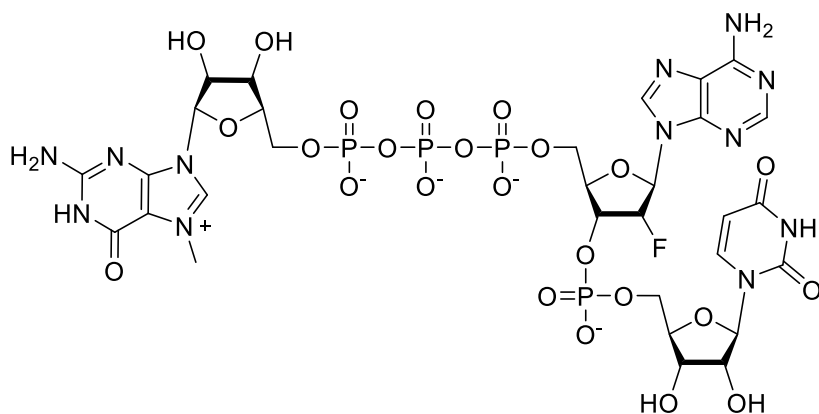
Compound 8



Compound 31



Compound 32



Compound 33

or a stereoisomer thereof,

wherein the therapeutic dose unit of the mRNA molecule comprises less than 7 ng/ μ g of double stranded RNA (dsRNA),

wherein the subject exhibits increased tolerability to the therapeutic dose unit of the mRNA molecule to be administered as compared to an equivalent therapeutic dose unit of the mRNA molecule comprising 7 ng/ μ g or greater dsRNA.

71. The use of claim 70, wherein the increased tolerability is determined by measuring one or more of body weight, organ weight, aspartate aminotransferase (AST) levels, alanine transaminase (ALT) levels, C-reactive protein (CRP) levels, procalcitonin (PCT) levels, interleukin-6 (IL-6) levels, erythrocyte sedimentation rate (ESR), serum amyloid A levels, and serum ferritin levels prior to the administering and a period of time after the administering.

72. The use of claim 70 or 71, wherein the increased tolerability is measured by testing in a standard *in vivo* assay.

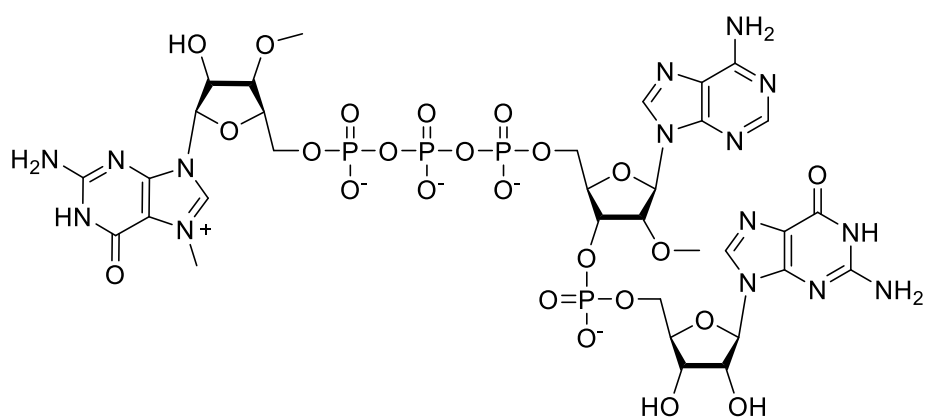
73. The use of any one of claims 70-72, wherein the increased tolerability is an increase in tolerability of at least 20 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 25 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 30 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 35 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 40 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 45 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 50 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least

55 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 60 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 65 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 70 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 75 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 80 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 85 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 90 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay, or at least 95 % to the dose unit to be administered as measured by testing in a standard *in vivo* assay.

74. The use of any one of claims 70-73, wherein the therapeutic dose unit of the mRNA molecule comprises less than 6.9 ng/μg of double stranded RNA (dsRNA), less than 6.8 ng/μg of double stranded RNA (dsRNA), less than 6.7 ng/μg of double stranded RNA (dsRNA), less than 6.6 ng/μg of double stranded RNA (dsRNA), less than 6.5 ng/μg of double stranded RNA (dsRNA), less than 6.4 ng/μg of double stranded RNA (dsRNA), less than 6.3 ng/μg of double stranded RNA (dsRNA), less than 6.2 ng/μg of double stranded RNA (dsRNA), less than 6.1 ng/μg of double stranded RNA (dsRNA), less than 6.0 ng/μg of double stranded RNA (dsRNA), less than 5.9 ng/μg of double stranded RNA (dsRNA), less than 5.8 ng/μg of double stranded RNA (dsRNA), less than 5.7 ng/μg of double stranded RNA (dsRNA), less than 5.6 ng/μg of double stranded RNA (dsRNA), less than 5.5 ng/μg of double stranded RNA (dsRNA), less than 5.4 ng/μg of double stranded RNA (dsRNA), less than 5.3 ng/μg of double stranded RNA (dsRNA), less than 5.2 ng/μg of double stranded RNA (dsRNA), less than 5.1 ng/μg of double stranded RNA (dsRNA), less than 5.0 ng/μg of double stranded RNA (dsRNA), less than 4.9 ng/μg of double stranded RNA (dsRNA), less than 4.8 ng/μg of double stranded RNA (dsRNA), less than 4.7 ng/μg of double stranded RNA (dsRNA), less than 4.6 ng/μg of double stranded RNA (dsRNA), less than 4.5 ng/μg of double stranded RNA (dsRNA), less than 4.4 ng/μg of double stranded RNA (dsRNA), less than 4.3 ng/μg of double stranded RNA (dsRNA), less than 4.2 ng/μg of double stranded RNA (dsRNA), less than 4.1 ng/μg of double stranded RNA (dsRNA), less than 4.0 ng/μg of double stranded RNA (dsRNA), less than 3.9 ng/μg of double stranded RNA (dsRNA), less than 3.8 ng/μg of double stranded RNA (dsRNA), less than 3.7 ng/μg of double stranded RNA (dsRNA), less than 3.6 ng/μg of double stranded RNA (dsRNA), less than 3.5 ng/μg of double stranded RNA (dsRNA), less than 3.4 ng/μg of double stranded RNA (dsRNA), less

than 3.3 ng/μg of double stranded RNA (dsRNA), less than 3.2 ng/μg of double stranded RNA (dsRNA), less than 3.1 ng/μg of double stranded RNA (dsRNA), less than 3.0 ng/μg of double stranded RNA (dsRNA), less than 2.9 ng/μg of double stranded RNA (dsRNA), less than 2.8 ng/μg of double stranded RNA (dsRNA), less than 2.7 ng/μg of double stranded RNA (dsRNA), less than 2.6 ng/μg of double stranded RNA (dsRNA), less than 2.5 ng/μg of double stranded RNA (dsRNA), less than 2.4 ng/μg of double stranded RNA (dsRNA), less than 2.3 ng/μg of double stranded RNA (dsRNA), less than 2.2 ng/μg of double stranded RNA (dsRNA), less than 2.1 ng/μg of double stranded RNA (dsRNA), less than 2.0 ng/μg of double stranded RNA (dsRNA), less than 1.9 ng/μg of double stranded RNA (dsRNA), less than 1.8 ng/μg of double stranded RNA (dsRNA), less than 1.7 ng/μg of double stranded RNA (dsRNA), less than 1.6 ng/μg of double stranded RNA (dsRNA), less than 1.5 ng/μg of double stranded RNA (dsRNA), less than 1.4 ng/μg of double stranded RNA (dsRNA), less than 1.3 ng/μg of double stranded RNA (dsRNA), less than 1.2 ng/μg of double stranded RNA (dsRNA), less than 1.1 ng/μg of double stranded RNA (dsRNA), less than 1.0 ng/μg of double stranded RNA (dsRNA), less than 0.9 ng/μg of double stranded RNA (dsRNA), less than 0.8 ng/μg of double stranded RNA (dsRNA), less than 0.7 ng/μg of double stranded RNA (dsRNA), less than 0.6 ng/μg of double stranded RNA (dsRNA), less than 0.5 ng/μg of double stranded RNA (dsRNA), less than 0.4 ng/μg of double stranded RNA (dsRNA), less than 0.3 ng/μg of double stranded RNA (dsRNA), less than 0.2 ng/μg of double stranded RNA (dsRNA), or less than 0.1 ng/μg of double stranded RNA (dsRNA).

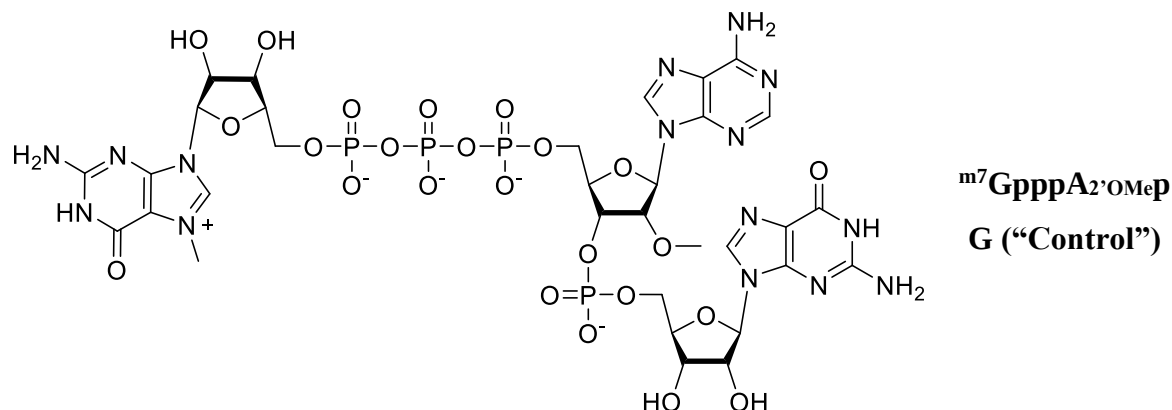
75. Use of a mRNA in the manufacture of a medicament for increasing *in vivo* translation of a polypeptide in a subject, comprising a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide to be administered to the subject, wherein the mRNA comprises a 5'-cap having the following formula:



Compound 29

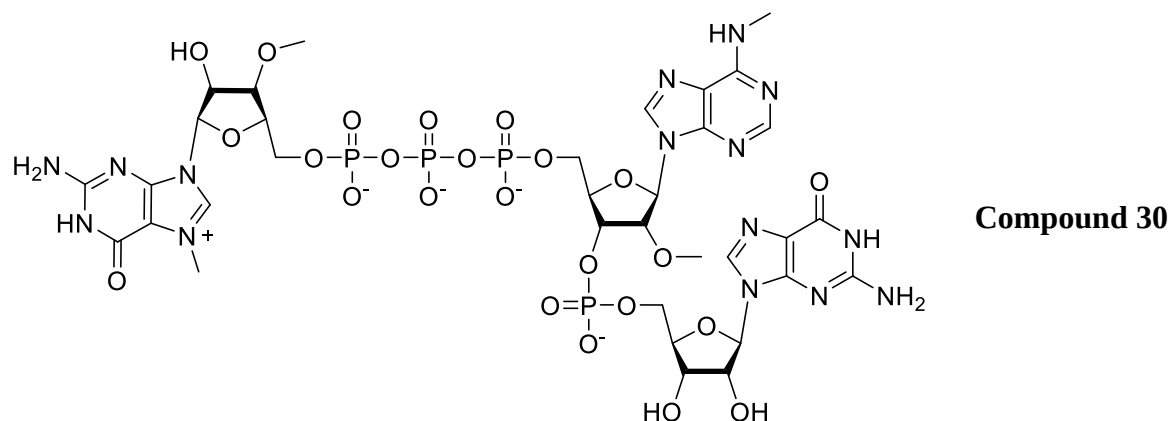
or a stereoisomer thereof,

wherein the therapeutic dose unit to be administered is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula:



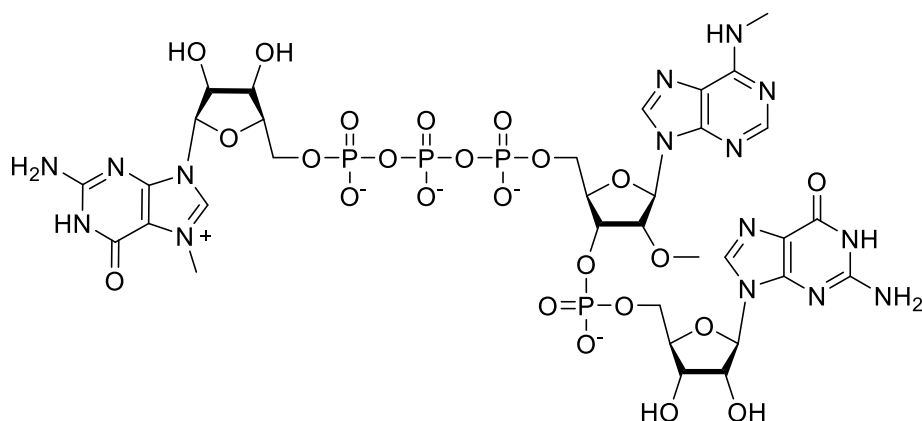
or a stereoisomer thereof.

76. Use of a mRNA in the manufacture of a medicament for increasing *in vivo* translation of a polypeptide in a subject, comprising a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide to be administered to the subject, wherein the mRNA comprises a 5'-cap having the following formula:



or a stereoisomer thereof,

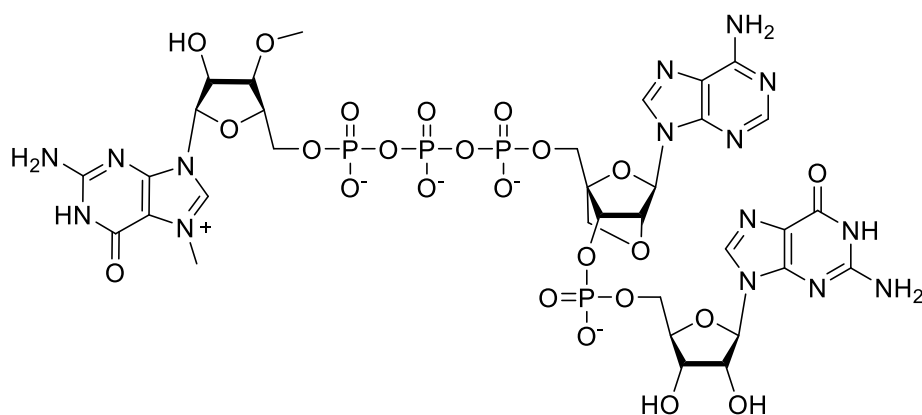
wherein the therapeutic dose unit to be administered is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula:



Compound 31

or a stereoisomer thereof.

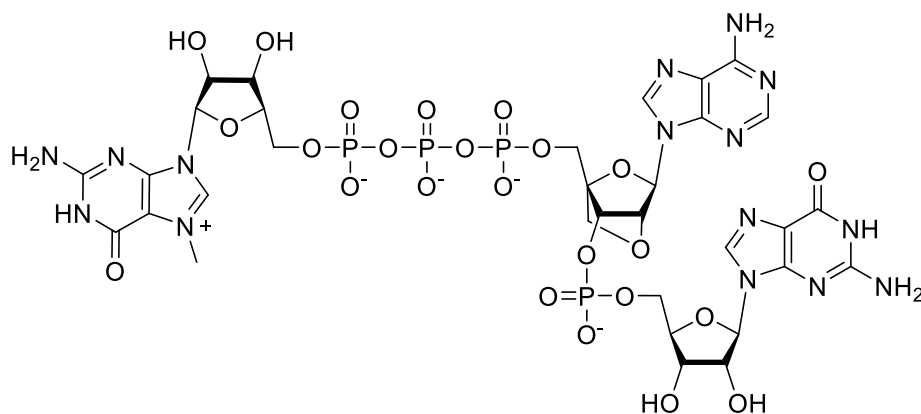
77. Use of a mRNA in the manufacture of a medicament for increasing *in vivo* translation of a polypeptide in a subject, comprising a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide to be administered to the subject, wherein the mRNA comprises a 5'-cap having the following formula:



Compound 2

or a stereoisomer thereof,

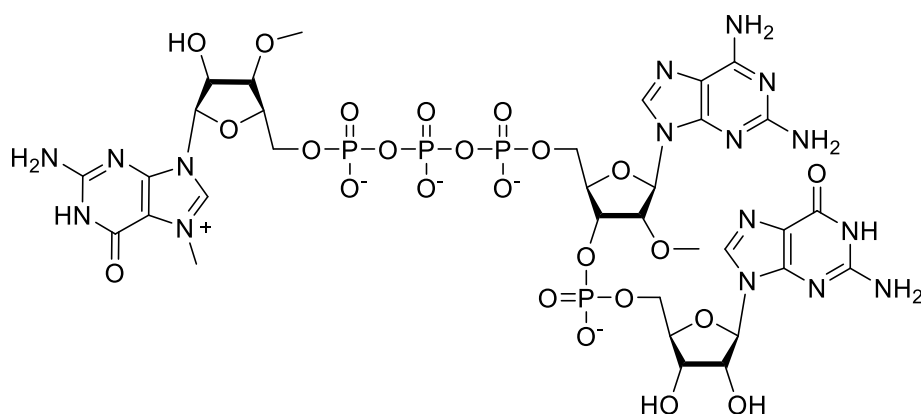
wherein the dose to be administered is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula:



Compound 1

or a stereoisomer thereof.

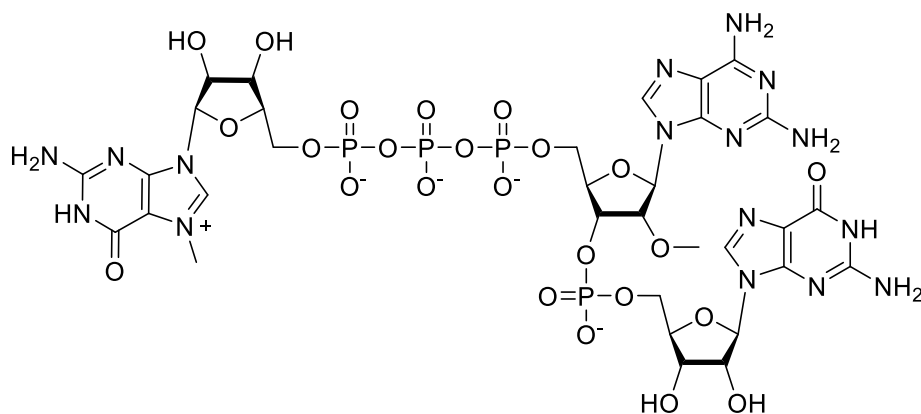
78. Use of a mRNA in the manufacture of a medicament for increasing *in vivo* translation of a polypeptide in a subject, comprising a therapeutic dose unit comprising an effective amount of an mRNA encoding a polypeptide to be administered to the subject, wherein the mRNA comprises a 5'-cap having the following formula:



Compound 8

or a stereoisomer thereof,

wherein the therapeutic dose unit to be administered is at least 20 % lower than the therapeutic dose unit required to elicit the same response in the subject when administered a comparative mRNA encoding the polypeptide, wherein the comparative mRNA comprises a 5'-cap having the following formula:



Compound 7

or a stereoisomer thereof.

79. The use of claim 75-78, wherein the subject exhibits increased tolerability to the therapeutic dose unit to be administered as compared to the therapeutic dose unit required to elicit the same response in the subject when administered the comparative mRNA, as measured by testing in a standard *in vivo* assay.

80. The use of claim 79, wherein the increased tolerability is an increase in tolerability of at least 20 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 25 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 30 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 35 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 40 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 45 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 50 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 55 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 60 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 65 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 70 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 75 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 80 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 85 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay, at least 90 % to the therapeutic dose unit to be

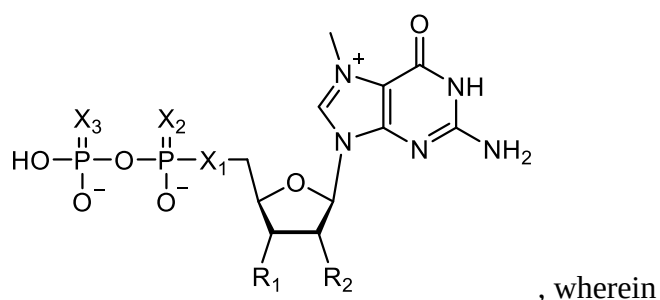
administered as measured by testing in a standard *in vivo* assay, or at least 95 % to the therapeutic dose unit to be administered as measured by testing in a standard *in vivo* assay.

81. The use of any one of claims 75-80, wherein the therapeutic dose unit to be administered comprises less than 6.9 ng/μg of double stranded RNA (dsRNA), less than 6.8 ng/μg of double stranded RNA (dsRNA), less than 6.7 ng/μg of double stranded RNA (dsRNA), less than 6.6 ng/μg of double stranded RNA (dsRNA), less than 6.5 ng/μg of double stranded RNA (dsRNA), less than 6.4 ng/μg of double stranded RNA (dsRNA), less than 6.3 ng/μg of double stranded RNA (dsRNA), less than 6.2 ng/μg of double stranded RNA (dsRNA), less than 6.1 ng/μg of double stranded RNA (dsRNA), less than 6.0 ng/μg of double stranded RNA (dsRNA), less than 5.9 ng/μg of double stranded RNA (dsRNA), less than 5.8 ng/μg of double stranded RNA (dsRNA), less than 5.7 ng/μg of double stranded RNA (dsRNA), less than 5.6 ng/μg of double stranded RNA (dsRNA), less than 5.5 ng/μg of double stranded RNA (dsRNA), less than 5.4 ng/μg of double stranded RNA (dsRNA), less than 5.3 ng/μg of double stranded RNA (dsRNA), less than 5.2 ng/μg of double stranded RNA (dsRNA), less than 5.1 ng/μg of double stranded RNA (dsRNA), less than 5.0 ng/μg of double stranded RNA (dsRNA), less than 4.9 ng/μg of double stranded RNA (dsRNA), less than 4.8 ng/μg of double stranded RNA (dsRNA), less than 4.7 ng/μg of double stranded RNA (dsRNA), less than 4.6 ng/μg of double stranded RNA (dsRNA), less than 4.5 ng/μg of double stranded RNA (dsRNA), less than 4.4 ng/μg of double stranded RNA (dsRNA), less than 4.3 ng/μg of double stranded RNA (dsRNA), less than 4.2 ng/μg of double stranded RNA (dsRNA), less than 4.1 ng/μg of double stranded RNA (dsRNA), less than 4.0 ng/μg of double stranded RNA (dsRNA), less than 3.9 ng/μg of double stranded RNA (dsRNA), less than 3.8 ng/μg of double stranded RNA (dsRNA), less than 3.7 ng/μg of double stranded RNA (dsRNA), less than 3.6 ng/μg of double stranded RNA (dsRNA), less than 3.5 ng/μg of double stranded RNA (dsRNA), less than 3.4 ng/μg of double stranded RNA (dsRNA), less than 3.3 ng/μg of double stranded RNA (dsRNA), less than 3.2 ng/μg of double stranded RNA (dsRNA), less than 3.1 ng/μg of double stranded RNA (dsRNA), less than 3.0 ng/μg of double stranded RNA (dsRNA), less than 2.9 ng/μg of double stranded RNA (dsRNA), less than 2.8 ng/μg of double stranded RNA (dsRNA), less than 2.7 ng/μg of double stranded RNA (dsRNA), less than 2.6 ng/μg of double stranded RNA (dsRNA), less than 2.5 ng/μg of double stranded RNA (dsRNA), less than 2.4 ng/μg of double stranded RNA (dsRNA), less than 2.3 ng/μg of double stranded RNA (dsRNA), less than 2.2 ng/μg of double stranded RNA (dsRNA), less than 2.1 ng/μg of double stranded RNA (dsRNA), less than 2.0 ng/μg of

double stranded RNA (dsRNA), less than 1.9 ng/μg of double stranded RNA (dsRNA), less than 1.8 ng/μg of double stranded RNA (dsRNA), less than 1.7 ng/μg of double stranded RNA (dsRNA), less than 1.6 ng/μg of double stranded RNA (dsRNA), less than 1.5 ng/μg of double stranded RNA (dsRNA), less than 1.4 ng/μg of double stranded RNA (dsRNA), less than 1.3 ng/μg of double stranded RNA (dsRNA), less than 1.2 ng/μg of double stranded RNA (dsRNA), less than 1.1 ng/μg of double stranded RNA (dsRNA), less than 1.0 ng/μg of double stranded RNA (dsRNA), less than 0.9 ng/μg of double stranded RNA (dsRNA), less than 0.8 ng/μg of double stranded RNA (dsRNA), less than 0.7 ng/μg of double stranded RNA (dsRNA), less than 0.6 ng/μg of double stranded RNA (dsRNA), less than 0.5 ng/μg of double stranded RNA (dsRNA), less than 0.4 ng/μg of double stranded RNA (dsRNA), less than 0.3 ng/μg of double stranded RNA (dsRNA), less than 0.2 ng/μg of double stranded RNA (dsRNA), or less than 0.1 ng/μg of double stranded RNA (dsRNA).

82. A method of synthesizing a trinucleotide compound, comprising:

(1) mixing an N7-methyl-guanosine-5'-diphosphate of the following structure:

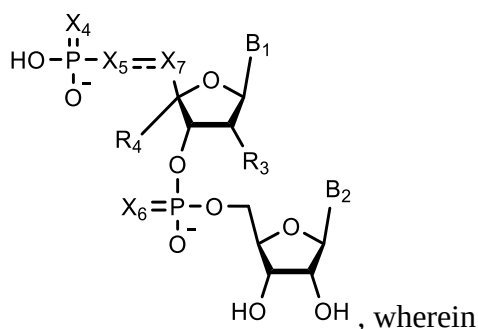


X_1 and X_2 are each independently selected from the group consisting of O and S;

X_3 is O;

R_1 and R_2 are each independently selected from H, OH, N_3 , F, substituted or unsubstituted alkoxy, and thio, wherein R_1 and R_2 optionally combine to form a heterocycle, with an activating reagent and an imidazole to form an activated intermediate; and

(2) adding a salt reagent and a dinucleotide of the following structure:



=== is a single bond or a double bond;

X₄ and X₆ are each independently selected from the group consisting of O and S;

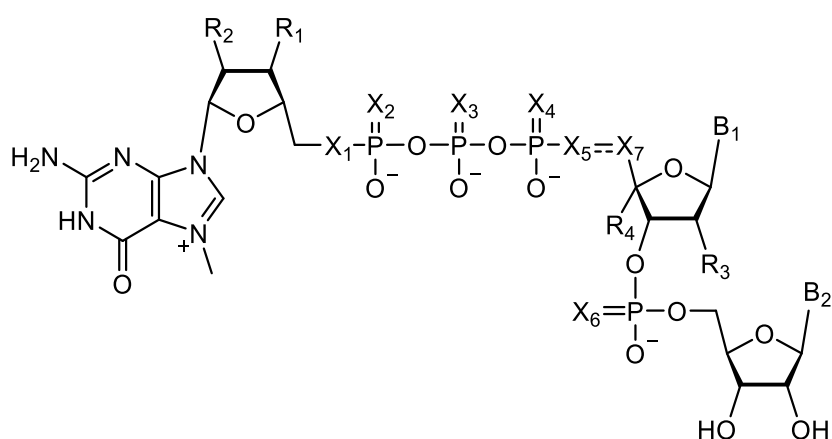
X₅ is O, S, or CH;

X₇ is CH or CH₂;

R₃ is OH, OMe, F and R₄ is H, or wherein R₃ and R₄ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; and

B₁ and B₂ are each independently selected from the group consisting of a purine ring and a pyrimidine ring

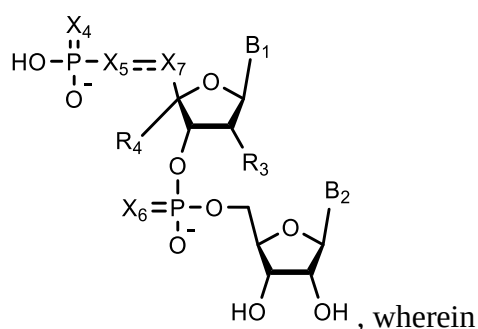
to the activated intermediate to form the trinucleotide compound of the following structure:



wherein the method is a one-pot synthesis.

83. A method of synthesizing a trinucleotide compound, comprising:

(1) mixing a dinucleotide of the following structure:



== is a single bond or a double bond;

X₄ is O;

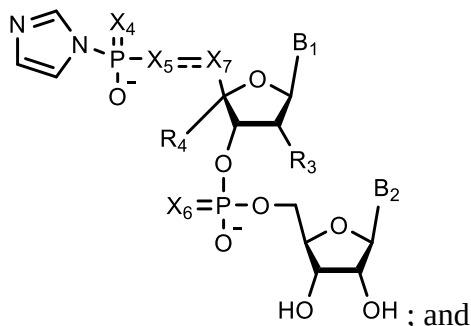
X₆ is O or S;

X₅ is O, S, or CH;

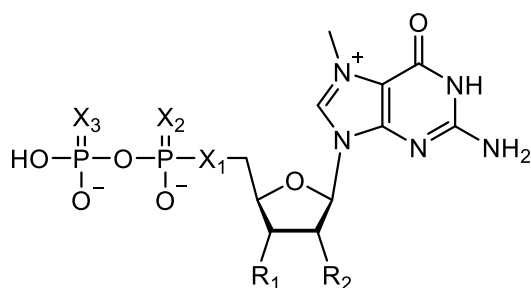
X₇ is CH or CH₂;

R₃ is OH, OMe, F and R₄ is H, or wherein R₃ and R₄ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; and

B₁ and B₂ are each independently selected from the group consisting of a purine ring and a pyrimidine ring,
with an activating reagent and an imidazole to form an activated phosphate imidazolide of the following structure:



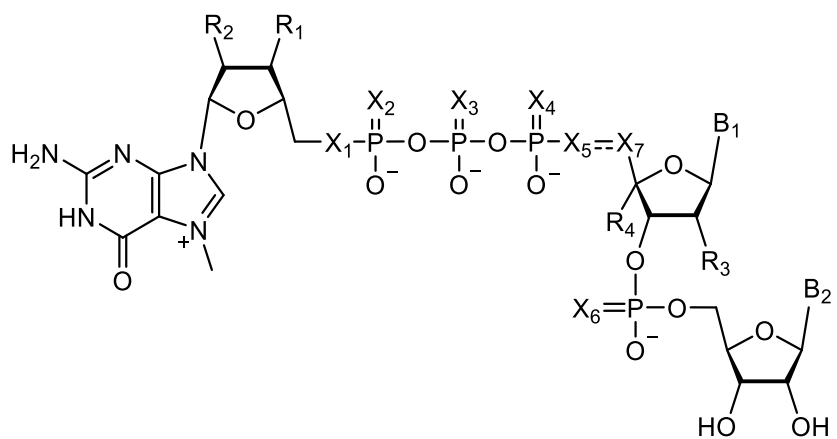
(2) adding a salt reagent and a compound of the following structure:



X₁ and X₂ are each independently selected from the group consisting of O and S;

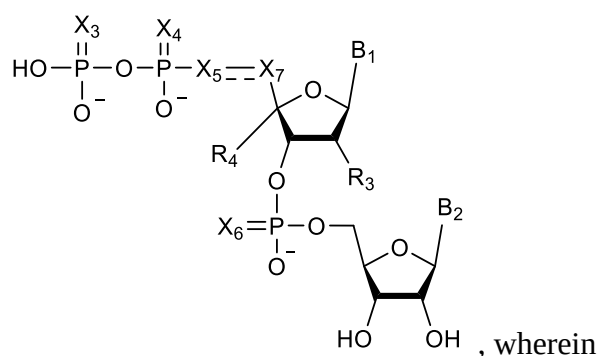
X₃ is S;

R₁ and R₂ are each independently selected from H, OH, N₃, F, substituted or unsubstituted alkoxy, and thio, wherein R₁ and R₂ optionally combine to form a heterocycle, to the activated phosphate imidazolide to form the trinucleotide compound of the following structure:



84. A method of synthesizing a trinucleotide compound, comprising:

(1) mixing a diphosphate dinucleotide of the following structure:



== is a single bond or a double bond;

X₃ is O;

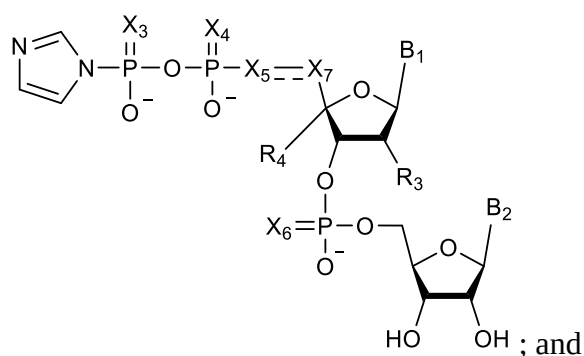
X₄ and X₆ are each independently selected from O and S;

X₅ is O, S, or CH; X₇ is CH or CH₂;

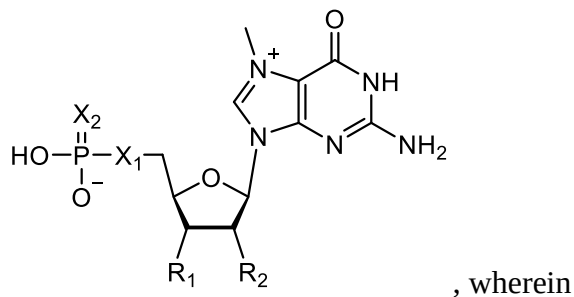
R₃ is OH, OMe, F and R₄ is H, or wherein R₃ and R₄ are covalently bonded together and, together with intermediate atoms, form a 2'-O, 4'-C methylene bridge; and

B₁ and B₂ are each independently selected from the group consisting of a purine ring and a pyrimidine ring,

with an activating reagent and an imidazole to form an activated phosphate imidazolide of the following structure:

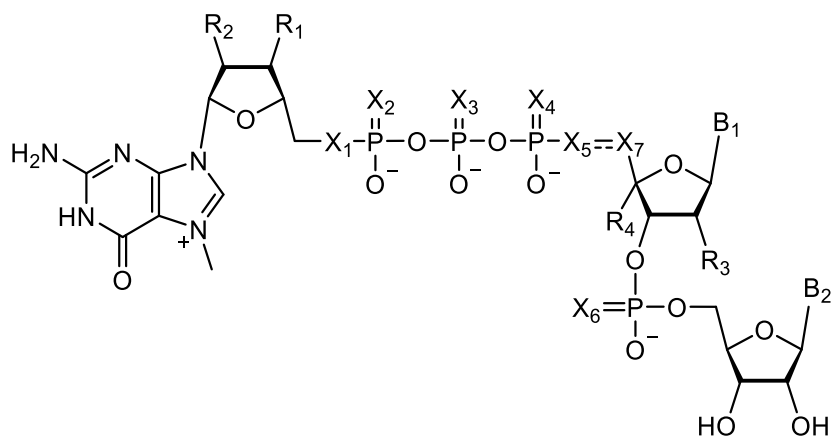


(2) adding a salt reagent and a compound of the following structure:



X₁ and X₂ are each independently selected from O and S; and

R₁ and R₂ are each independently selected from H, OH, N₃, F, substituted or unsubstituted alkoxy, and thio, wherein R₁ and R₂ optionally combine to form a heterocycle, to the activated phosphate imidazolide to form the trinucleotide compound of the following structure:



wherein the method is a one-pot synthesis.