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(71) Applicants: **COMPLETE GENOMICS, INC.** [US/US];
2904 Orchard Parkway, San Jose, California 95134 (US).
BGI SHENZHEN [CN/CN]; Main Building, Beishan Industrial Zone, No. 146 Beishan Road, Yantian District, Shenzhen, 518083 (CN).

(72) Inventors: **LI, Handong**; c/o Complete Genomics, Inc.,
2904 Orchard Parkway, San Jose, California 95134 (US).
DRMANAC, Snezana; c/o Complete Genomics, Inc., 2904
Orchard Parkway, San Jose, California 95134 (US). **DR-**
MANAC, Radoje; c/o Complete Genomics, Inc., 2904
Orchard Parkway, San Jose, California 95134 (US). **XU,**
Xun; c/o BGI Shenzhen, Main Building, Beishan Industrial Zone, No. 146 Beishan Road, Yantian District, Shenzhen, 518083 (CN). **PENG, Lingling**; c/o Complete Genomics, Inc., 2904 Orchard Parkway, San Jose, California 95134 (US). **GABLENZ, Scott**; c/o Complete Genomics, Inc., 2904 Orchard Parkway, San Jose, California 95134 (US).

(74) Agent: **DAVIS, Kimberllynn B.** et al.; Kilpatrick Townsend & Stockton LLP, Mailstop: IP Docketing-22, 1100 Peachtree Street, Suite 2800, Atlanta, Georgia 30309 (US).

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(54) Title: REVERSIBLY BLOCKED NUCLEOSIDE ANALOGUES AND THEIR USE

(57) Abstract: Reversibly blocked nucleoside analogues and methods of using such nucleoside analogues for sequencing of nucleic acids are provided.

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PATENT APPLICATION
REVERSIBLY BLOCKED NUCLEOSIDE ANALOGUES AND THEIR USE

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application Nos. 62/326,620, filed April 22, 2016, and 62/471,547, filed March 15, 2017. The priority applications are incorporated herein in their entireties for all purposes.

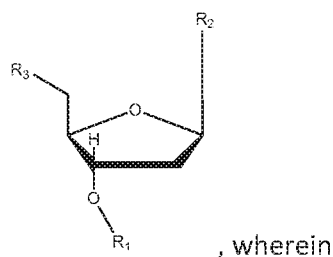
BACKGROUND

[0002] The need for low cost, high-throughput, methods for nucleic acid sequencing and re-sequencing has led to the development of “massively parallel sequencing” (MPS) technologies. Improvements in such sequencing methods are of great value in science, medicine and agriculture.

BRIEF SUMMARY

[0003] The present invention relates to novel nucleoside analogues, and methods of their use for nucleic acid sequencing. In one aspect, the invention relates to nucleoside analogues having a reversible 3'-O blocking group and an affinity tag linked to the nucleobase through a linker.

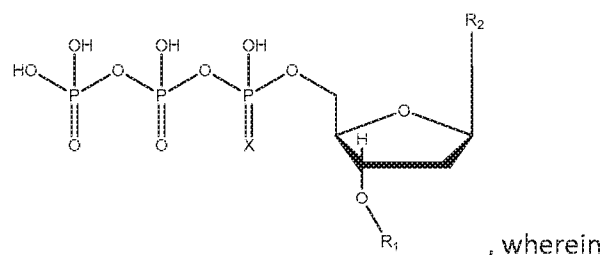
[0004] In some embodiments, the present invention provides a nucleoside analogue of the following formula:



R₁ is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and

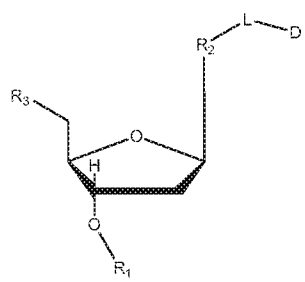
cynoethenaldehyde oximyl; R_2 comprises a nucleobase; and R_3 is a cleavable linking moiety comprising at least three (*e.g.*, 3-10) phosphates, or analogues thereof.

[0005] In some cases, R_3 comprises a 5'-O-1-thio triphosphate. In some cases, the nucleoside has the formula of Formula VIII:



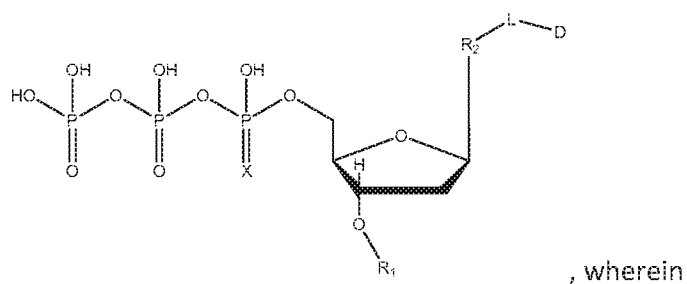
Formula VIII

X is O or S and the nucleoside analogue is a substrate for a DNA polymerase. In some cases, R_2 consists of a nucleobase. In some cases, R_2 and/or R_3 is detectably labeled. In some cases, the nucleoside has the following formula:



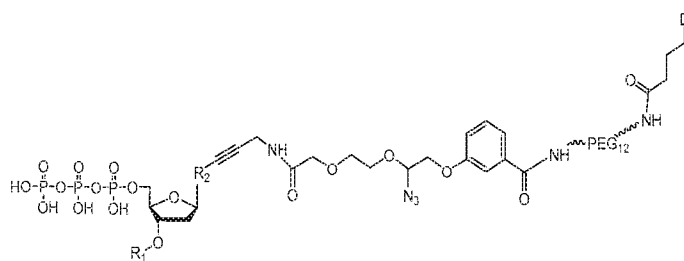
wherein R_2 consists of the nucleobase; L is a linker; and D is a detectable fluorescent label.

[0006] In some cases, the nucleoside analogue has the following formula:



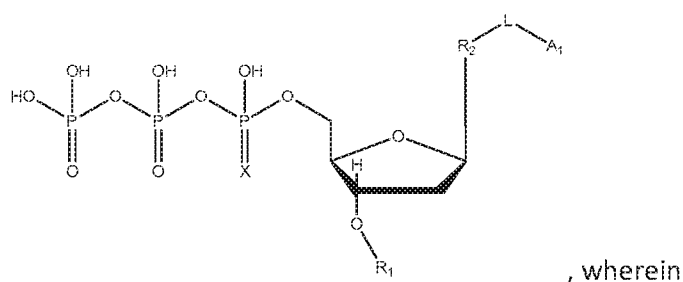
X is selected from the group consisting of O and S.

[0007] In some cases, the nucleoside analogue comprises the following formula:



wherein R₂ is the nucleobase; R₁ is selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; and D comprises a rhodamine dye (*e.g.*, ROX) or a derivative thereof.

[0008] In some embodiments, the present invention provides a nucleoside analogue of Formula VI:



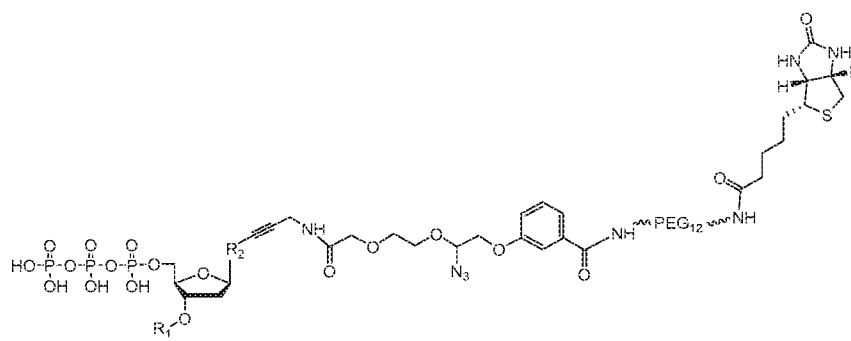
Formula VI

R₁ is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; R₂ is a nucleobase; L is a linker; A₁ comprises an affinity tag; X is selected from the group consisting of O and S; and the nucleoside analogue is a substrate for a DNA polymerase.

[0009] In some cases, L is a cleavable linker. In some cases, R₁ and L can be cleaved from the nucleoside analogue under the same conditions. In some cases, A₁ comprises a nitrilotriacetic acid (NTA) affinity tag, or a peptide affinity tag comprising at least six contiguous histidine amino acids. In some cases, A₁ comprises an affinity tag selected from the group consisting of biotin, a small molecule antigen, and a peptide. In some cases, the small molecule antigen is selected from the group consisting of a fluorophore, an amphetamine, a barbituate, a benzodiazepine, a cocaine metabolite, a cannabinoid, a

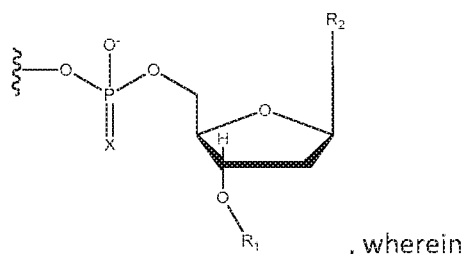
cannabinoid metabolite, tetrahydrocannabinol, methadone, an opiate, propoxyphene, phencyclidine, digoxigenin, and DNP. In some cases, the peptide is selected from the group consisting of a His tag, a Myc tag, a Flag tag, an HA tag, a V5 tag, an AviTag, a calmodulin tag, an E tag, an S tag, an SBP tag, a Softag, a Strep tag, a TC tag, a VSV tag, an Xpress tag, glutathione, an isopeptag, and a SpyTag.

[0010] In some cases, the nucleoside analogue comprises the following formula:



wherein R_2 is a nucleobase and R_1 is selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl. In some cases, the nucleobase is selected from the group consisting of a 7-substituted 7-deaza adenine analogue, a 7 substituted 7-deaza guanine analogue, a 5-substituted thymine, and a 5-substituted cytosine.

[0011] In some embodiments, the present invention provides a composition comprising a nucleoside analogue of the following formula:

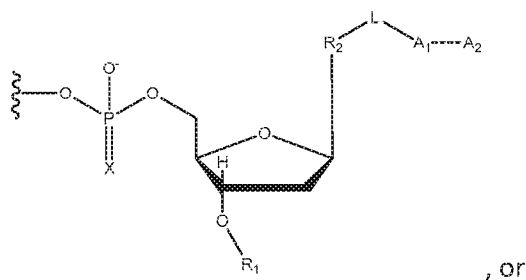


R_1 is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; X is selected from the group consisting of O and S; and R_2 comprises a nucleobase, and optionally a linker and a detectable label or affinity agent, wherein the nucleoside analogue is covalently linked via the 5' phosphate or thiophosphate

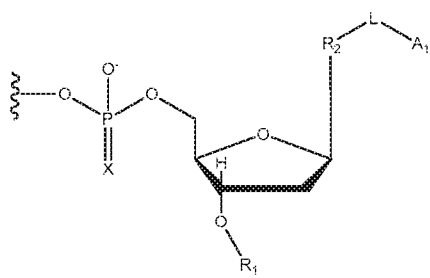
to an oligonucleotide. In some cases, R_2 consists of a nucleobase. In some cases, R_2 comprises a nucleobase, a linker, and a detectable label or affinity agent.

[0012] In some embodiments, the present invention provides a composition comprising

i) a nucleoside analogue of the following formula:



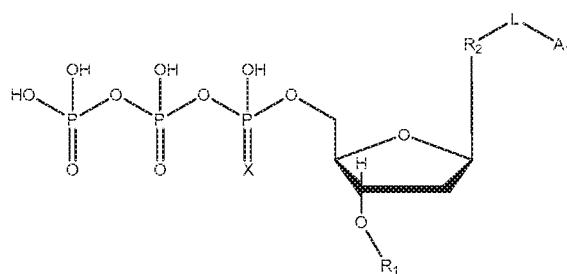
ii) a nucleoside analogue of Formula IX:



Formula IX

wherein R_1 is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; X is selected from the group consisting of O and S; R_2 is a nucleobase; L is a linker; A_1 comprises an affinity tag; and A_2 comprises a detectably labeled affinity agent that forms a specific and non-covalent complex with A_1 , wherein the nucleoside analogue is covalently linked via the 5' phosphate or thiophosphate to an oligonucleotide. In some cases, A_1 comprises a fluorescent dye selected from the group consisting of a fluorone dye, a rhodamine dye, a cyanine dye, a coumarin dye, a phycoerythrin, and an allophycocyanine.

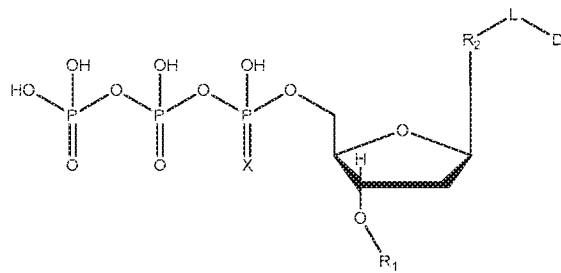
[0013] In some embodiments, the present invention provides a method of sequencing comprising: i) providing a reaction mixture comprising a template nucleic acid, a primer, a polymerase, and a first nucleoside analogue of Formula VI:

**Formula VI**

wherein R_1 is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; X is selected from the group consisting of O and S; R_2 is a nucleobase; L is a linker; and A_1 comprises an affinity tag; ii) extending the primer by incorporating the first nucleoside analogue with the polymerase; iii) contacting the incorporated first nucleoside analogue with a detectably labeled affinity agent that forms a specific and non-covalent complex with A_1 of the incorporated first nucleoside analogue, thereby specifically labeling the incorporated first nucleoside analogue; and iv) detecting the specifically labeled incorporated first nucleoside analogue.

[0014] In some cases, the detectably labeled affinity agent is fluorescently labeled, and the detection comprises detecting a fluorescence emission from the fluorescently labeled affinity agent in complex with A_1 of the incorporated first nucleoside analogue. In some cases, the method further comprises: v) cleaving the reversible blocking group R_1 of the incorporated first nucleoside analogue thereby removing the blocking group from the incorporated first nucleoside analogue; vi) cleaving the linker L thereby removing the affinity tag A_1 of the incorporated first nucleoside analogue, or quenching the label of the detectably labeled affinity agent in complex with A_1 of the incorporated first nucleoside analogue; vii) providing a second nucleoside analogue of Formula VI and a polymerase; viii) extending the primer by incorporating the second nucleoside analogue with the polymerase; ix) contacting the incorporated second nucleoside analogue with a detectably labeled affinity agent that forms a specific and non-covalent complex with A_1 of the incorporated second nucleoside analogue, thereby specifically labeling the incorporated second nucleoside analogue; and x) detecting the specifically labeled incorporated second nucleoside analogue.

[0015] In some embodiments, the present invention provides a method of sequencing comprising: i) providing a reaction mixture comprising template nucleic acid, a primer, a polymerase, and a first nucleoside analogue of Formula VII:

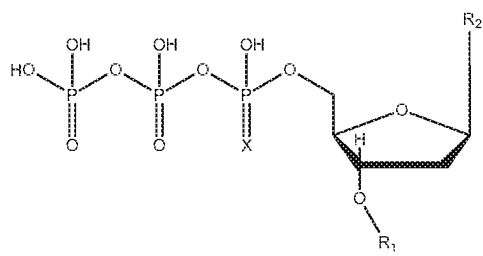


Formula VII

wherein R_1 is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; X is selected from the group consisting of O and S; and R_2 comprises a nucleobase; L is a linker; and D comprises a detectable fluorescent label; ii) extending the primer by incorporating the first nucleoside analogue with the polymerase; and iv) detecting the labeled incorporated first nucleoside analogue by detecting a fluorescence emission from the fluorescent label.

[0016] In some cases, the method further comprises: v) cleaving the reversible blocking group R_1 of the incorporated first nucleoside analogue thereby removing the blocking group from the incorporated first nucleoside analogue; vi) providing a second detectably labeled nucleoside analogue of Formula VII and a polymerase; vii) extending the primer by incorporating the second nucleoside analogue with the polymerase; and viii) detecting incorporation of the second nucleoside analogue by detecting a fluorescence emission from the labeled incorporated second nucleoside analogue.

[0017] In some embodiments, the present invention provides a method of sequencing comprising: i) providing a reaction mixture comprising a template nucleic acid, a primer, a polymerase, and a first nucleoside analogue of Formula VIII:

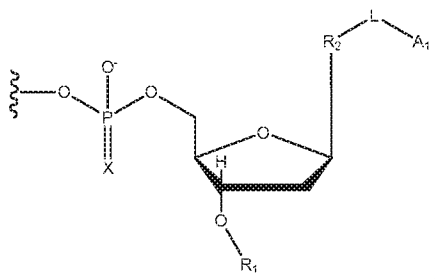


Formula VIII

wherein R_1 is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; X is selected from the group consisting of O and S; and R_2 comprises or consists of a nucleobase; and ii) extending the primer by incorporating the first nucleoside analogue with the polymerase, thereby generating pyrophosphate; and iii) detecting incorporation of the first nucleoside analogue by detecting the pyrophosphate.

[0018] In some cases, the method further comprises: iv) cleaving the reversible blocking group R_1 of the incorporated first nucleoside analogue thereby removing the blocking group from the incorporated first nucleoside analogue; v) providing a second nucleoside analogue of Formula VIII and a polymerase; vi) extending the primer by incorporating the second nucleoside analogue with the polymerase, thereby generating pyrophosphate; and vii) detecting incorporation of the second nucleoside analogue by detecting the pyrophosphate.

[0019] In some embodiments, the present invention provides a method of sequencing comprising: i) providing a reaction mixture comprising a template nucleic acid, a primer, a ligase, and an oligonucleotide comprising a first nucleoside analogue of Formula IX:

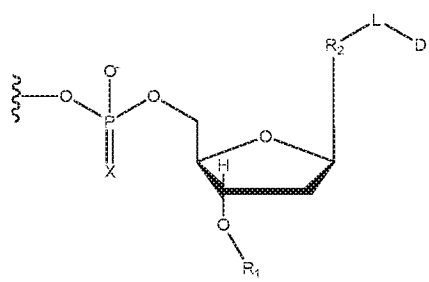
**Formula IX**

wherein R_1 is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; X is selected from the group consisting of O and S; R_2 is a nucleobase; L is a linker; and A_1 comprises an affinity tag; ii) hybridizing the oligonucleotide comprising the first nucleoside analogue of Formula IX to the template nucleic acid at a position 3' of, and adjacent to, the primer; iii) cleaving the reversible blocking group R_1 of the hybridized oligonucleotide comprising the first nucleoside analogue of Formula IX; iv) ligating the hybridized oligonucleotide to the adjacent primer with the ligase, thereby incorporating the first nucleoside analogue of Formula IX into the primer; v)

contacting the incorporated first nucleoside analogue with a detectably labeled affinity agent that forms a specific and non-covalent complex with A of the incorporated first nucleoside analogue, thereby specifically labeling the incorporated first nucleoside analogue; and vi) detecting the specifically labeled incorporated first nucleoside analogue.

[0020] In some cases, the detectably labeled affinity agent is fluorescently labeled, and the detection comprises detecting a fluorescence emission from the fluorescently labeled affinity agent in complex with A of the incorporated first nucleoside analogue. In some cases, the method further comprises: vii) cleaving the linker L thereby removing the affinity tag A₁ of the incorporated first nucleoside analogue, or quenching the label of the detectably labeled affinity agent in complex with A₁ of the incorporated first nucleoside analogue; viii) providing a second nucleoside analogue of Formula IX and a ligase; ix) hybridizing an oligonucleotide comprising a second nucleoside analogue of Formula IX to the template nucleic acid at a position 3' of, and adjacent to, the primer; x) ligating the hybridized oligonucleotide to the adjacent primer with the ligase, thereby incorporating the second nucleoside analogue of Formula IX into the primer; xi) contacting the incorporated second nucleoside analogue with a detectably labeled affinity agent that forms a specific and non-covalent complex with A₁ of the incorporated second nucleoside analogue, thereby specifically labeling the incorporated second nucleoside analogue; and xii) detecting the specifically labeled incorporated second nucleoside analogue.

[0021] In some embodiments, the present invention provides a method of sequencing comprising: i) providing a reaction mixture comprising a template nucleic acid, a primer, a ligase, and an oligonucleotide comprising a first nucleoside analogue of Formula X:



Formula X

wherein R₁ is a reversible blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde

oximyl, and cyanoethenaldehyde oximyl; X is selected from the group consisting of O and S; R₂ is a nucleobase; L is a linker; and D comprises a fluorescent detectable label; ii) hybridizing the oligonucleotide comprising the first nucleoside analogue of Formula X to the template nucleic acid at a position 3' of, and adjacent to, the primer; iii) cleaving the reversible blocking group R₁ of the hybridized oligonucleotide comprising the first nucleoside analogue of Formula X; iv) ligating the hybridized oligonucleotide to the adjacent primer with the ligase, thereby incorporating the first nucleoside analogue of Formula X into the primer; and v) detecting the labeled incorporated first nucleoside analogue.

[0022] In some cases, the method further comprises: vi) cleaving the linker, thereby releasing the label of the labeled incorporated first nucleoside analogue; vii) providing a ligase, and a second oligonucleotide comprising a second nucleoside analogue of Formula X; viii) hybridizing the second oligonucleotide to the template nucleic acid at a position 3' of, and adjacent to, the primer; ix) cleaving the reversible blocking group R₁ of the hybridized second oligonucleotide; x) ligating the hybridized second oligonucleotide to the adjacent primer with the ligase, thereby incorporating the second nucleoside analogue of Formula X into the primer; and xi) detecting the labeled incorporated second nucleoside analogue.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] **Figure 1:** shows polymerase-mediated incorporation of allenyl-dTTP and cyanoethenyl dTTP analogues during a sequencing by synthesis reaction. Both allenyl-dTTP and cyanoethyl-dTTP are as effective polymerase substrates for sequencing as the cold control, 3'-azidomethyl-dTTP.

[0024] **Figure 2:** shows cleavage efficiency in the presence of buffers.

[0025] **Figure 3:** shows cleavage efficiency in the presence of THPP with different buffer formulations.

[0026] **Figure 4:** shows the effect of increasing a cleavage reagent concentration on cleavage efficiency.

[0027] **Figure 5:** shows the effect of increasing the cleavage time on cleavage efficiency.

[0028] **Figure 6:** shows the effect of increasing the cleavage pH on cleavage efficiency.

[0029] **Figure 7:** shows structures of cleavage reagents tested.

DETAILED DESCRIPTION

I. Overview

[0030] In certain aspects, the present invention provides 3'-O reversibly blocked nucleoside analogues for nucleic acid sequencing, as well as polynucleotides containing such nucleoside analogues incorporated therein. Such nucleoside analogues can be incorporated by, *e.g.*, polymerization, ligation, or chemical synthesis. In other aspects, the present invention provides methods of using such nucleoside analogues for nucleic acid sequencing. The 3'-O reversibly blocked nucleoside analogues can be used in any compatible sequencing by synthesis or sequencing by ligation method known in the art.

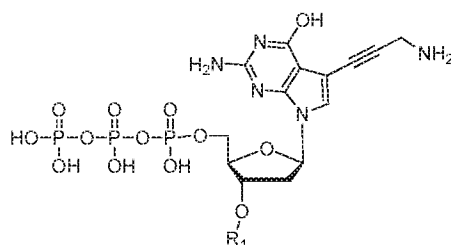
[0031] In one aspect, the nucleoside analogue includes a detectable label (*e.g.*, fluorophore). In some embodiments, the detectable label can be attached to the 3'-O reversibly blocked nucleobase. In some embodiments, the detectable label can be attached to a 5' CH₂O- moiety of the nucleoside. The detectably labeled nucleoside analogue can be used in sequencing-by-synthesis or sequencing-by-ligation methods that include a step of detecting the detectable label. For example, the nucleoside analogue can be incorporated into a primer by a polymerase-mediated primer extension reaction. Alternatively, an oligonucleotide containing an incorporated analogue can be ligated to an anchor primer. The detectable label in the extended primer or ligated oligonucleotide may then be detected.

[0032] In another aspect, the nucleoside analogue includes an affinity tag (*e.g.*, biotin) attached via a cleavable linker to the 3'-O reversibly blocked nucleobase. The affinity tagged nucleoside analogue can be used in sequencing-by-synthesis or sequencing-by-ligation methods that include a step of detecting the affinity tag with a detectably labeled affinity agent. For example, the nucleoside analogue can be incorporated into a primer by a polymerase-mediated primer extension reaction. Alternatively, an oligonucleotide containing an incorporated analogue can be ligated to an anchor primer. The affinity tag in the extended primer or ligated oligonucleotide may then be bound by a detectably labeled affinity agent. The incorporated nucleoside or oligonucleotide containing the nucleoside can then be detected by detecting the bound affinity agent.

II. Definitions

[0033] As used herein, the term “complementary polynucleotide” refers to a polynucleotide complementary to a target nucleic acid. In one approach, the complementary polynucleotide is formed in a sequencing-by-synthesis reaction by sequential addition of nucleosides (*e.g.*, naturally occurring nucleoside monophosphate molecules or analogs thereof) or groups of nucleosides to a primer using the target nucleic acid as a template.

[0034] As used herein, the term “nucleobase” refers to a nitrogenous base that can base-pair with a complementary nitrogenous base of a template nucleic acid. Exemplary nucleobases include adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U), inosine (I) and derivatives of these. In one aspect, the nucleobase is a 7-deaza derivative of adenine, inosine, or guanine. In some cases, the 7-deaza adenine, inosine, and/or guanine derivatives are 7-substituted. In some cases, uracil, cytosine, thymine, and/or derivatives thereof are 5-substituted. Exemplary substitutions at the 7 position for adenine, inosine, or guanine derivatives or at the 5 position for cytosine, uracil, or thymine derivatives include an alkyne substitution (*e.g.*, $-\text{C}\equiv\text{C}-\text{CH}_2-\text{NHR}$). In some cases, the 7-deaza derivative of guanine is a 7-alkyne substituted compound, such as found in compound **11**, wherein R_1 is selected from allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl.



Compound 11

[0035] As used herein, the term “fluorescent dye” refers to a dye fluorophore (a chemical compound that absorbs light energy of a specific wavelength and re-emits light at a longer wavelength). Fluorescent dyes typically have a maximal molar extinction coefficient at a wavelength between about 300 nm to about 1,000 nm or at least about 5,000, more preferably at least about 10,000, and most preferably at least about 50,000

$\text{cm}^{-1} \text{M}^{-1}$, and a quantum yield of at least about 0.05, preferably at least about 0.1, more preferably at least about 0.5, and most preferably from about 0.1 to about 1. Exemplary fluorescent dyes include, without limitation, acridine dyes, cyanine dyes, fluorone dyes, oxazine dyes, phenanthridine dyes, and rhodamine dyes. Exemplary fluorescent dyes include, without limitation, fluorescein, FITC, Texas Red, ROX, Cy3, Alexa Fluor 647, ATTO532, Alexa Fluor 488, ATTO647, and Cy5.

[0036] As used herein, the terms “affinity agent” and “affinity tag” refer to first and second members of a specific binding pair (SBP) or ligand-anti-ligand binding pair, where the members of the pair specifically bind to each other. For convenience, the term “affinity tag” is used to refer to the SBP member that is part of the nucleoside analog structure, and the term “affinity agent” is used to refer to the SBP member that specifically binds the affinity tag. The binding between the members of the binding pair is generally noncovalent, although a covalent disulfide linkage between binding pair members can also be used. Binding between specific binding pairs results in the formation of a binding complex, sometimes referred to as a ligand/antiligand complex or simply as ligand/antiligand. Exemplary binding pairs include, but are not limited to: (a) a haptenic or antigenic compound in combination with a corresponding antibody, or binding portion or fragment thereof; (b) a nucleic acid aptamer and protein; (c) nonimmunological binding pairs (*e.g.*, biotin-avidin, biotin-streptavidin, biotin-Neutravidin, biotin-Tamavidin, streptavidin binding peptide-streptavidin, glutathione-glutathione S-transferase); (d) hormone-hormone binding protein; (e) receptor-receptor agonist or antagonist; (f) lectin-carbohydrate; (g) enzyme-enzyme cofactor; (h) enzyme-enzyme inhibitor; (i) complementary oligonucleotide or polynucleotide pairs capable of forming nucleic acid duplexes; (j) thio (-S-) or thiol (-SH) containing binding member pairs capable of forming an intramolecular disulfide bond; and (k) complementary metal chelating groups and a metal (*e.g.*, metal chelated by the binding pairs nitrilotriacetate(NTA) and a 6x-His tag). Specific binding pair members need not be limited to pairs of single molecules. For example, a single ligand can be bound by the coordinated action of two or more antiligands. Affinity agents can be detectably labeled.

[0037] In the context of the binding of an affinity agent to the affinity tag of a nucleoside analogue, the terms “specific binding,” “specifically binds,” and the like refer to the preferential association of an affinity agent with a nucleoside analogue bearing a particular

target affinity tag in comparison to a nucleoside analogue base lacking the affinity tag or having an alternative affinity tag. Specific binding between an affinity agent and affinity tag generally means an affinity of at least 10^{-6} M^{-1} (*i.e.*, an affinity having a lower numerical value than 10^{-6} M^{-1} as measured by the dissociation constant K_d). Affinities greater than 10^{-8} M^{-1} are preferred. Specific binding can be determined using any assay for antibody binding known in the art, including Western Blot, enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunohistochemistry, and detection of fluorescently labeled affinity agent bound to a nucleoside analogue bearing a target affinity tag in a sequencing reaction.

[0038] As used herein, the term “non-fluorescent affinity tag” refers to an affinity tag that is not a fluorescent dye (*i.e.*, does not comprise a fluorophore). In various exemplary embodiments, “non-fluorescent affinity tag” refers an affinity tag that is not fluorescein or a fluorescein derivative, not a rhodamine fluorescent dye (*e.g.*, Texas Red), not a cyanine fluorescent dye (*e.g.*, Cy 2, Cy 3, Cy 3.5, Cy 5, Cy 5.5, Cy 7, Cy 7.5, and the like), not a boron-dipyrromethene fluorescent dye (*e.g.*, BODIPY 493/503), not a fluorescent coumarin dye, not a phenoxazine fluorescent dye, not an acridine fluorescent dye, not an ALEXA FLUOR® fluorescent dye, not a DYLIGHT® fluorescent dye, not a phycoerythrin fluorescent dye, or is not an allophycocyanine fluorescent dye.

[0039] As used herein, the term “antigen” refers to a compound that can be specifically bound by an antibody. Some antigens are immunogens (*see*, Janeway, *et al.*, Immunobiology, 5th Edition, 2001, Garland Publishing). Exemplary antigens used in the practice of the present invention, include polypeptides, small molecules, or nucleic acids (*e.g.*, aptamers).

[0040] As used herein, the term “small molecule antigen” refers to a small molecule that can specifically bind an antibody. A “small molecule” in the context of a small molecule antigen refers to a molecule having a mass of less than about 1,000 Daltons (*e.g.*, at least 50 Daltons and no more than about 1,000 Daltons). In some cases, the small molecule antigen is a small organic molecule. Exemplary small organic molecules include, but are not limited to, biotin or a derivative thereof (*e.g.*, iminobiotin or biotin carboxylate), fluorescein or a derivative thereof (*e.g.*, carboxyfluorescein or fluorescein isothiocyanate (FITC)), an amphetamine, a barbituate, a benzodiazepine, a cocaine metabolite, a marijuana

metabolites, tetrahydrocannabinol (THC), methadone, an opiate, propoxyphene, phencyclidine (PCP), digoxigenin, digoxin, peptide antigens of less than about 1,000 Daltons, cholesterol or a derivative thereof, and a steroid hormone.

[0041] As used herein, the term "peptide antigen" refers to a primary sequence of amino acids that can specifically bind an antibody. Exemplary peptide antigens include, but are not limited to, a His tag, a Myc tag, a Flag tag, an HA tag, a V5 tag, an AviTag, a calmodulin tag, an E tag, an S tag, an SBP tag, a Softag, a Strep tag, a TC tag, a VSV tag, an Xpress tag, glutathione, an isopeptag, and a SpyTag.

[0042] As used herein, "antibody" refers to an immunoglobulin molecule (*e.g.*, polyclonal and monoclonal antibodies), as well as genetically engineered forms such as chimeric antibodies (*e.g.*, humanized murine antibodies), heteroconjugate antibodies (*e.g.*, bispecific antibodies), and recombinant single chain Fv fragments (scFv), and antigen binding forms of antibody fragments (*e.g.*, Fab, F(ab)₂, VH-VL Fab fragments).

[0043] The term "detectable label," or "detection label," as used herein, refers to any atom or molecule that can be used to provide a detectable and/or quantifiable signal. Suitable labels include radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, and enzyme substrates. In some embodiments, the detection label is a molecule containing a charged group (*e.g.*, a molecule containing a cationic group or a molecule containing an anionic group), a fluorescent molecule (*e.g.*, a fluorescent dye), a fluorogenic molecule, or a metal. Optionally, the detection label is a fluorogenic label. A fluorogenic label can be any label that is capable of emitting light when in an unquenched form (*e.g.*, when not quenched by another agent). The fluorescent moiety emits light energy (*i.e.*, fluoresces) at a specific emission wavelength when excited by an appropriate excitation wavelength. When the fluorescent moiety and a quencher moiety are in close proximity, light energy emitted by the fluorescent moiety is absorbed by the quencher moiety. Optionally, the detection label is a fluorogenic dye. In some embodiments, the fluorogenic dye is a fluorescein, a rhodamine, a phenoxazine, an acridine, a coumarin, or a derivative thereof. In some embodiments, the fluorogenic dye is a carboxyfluorescein. Further examples of suitable fluorogenic dyes include the fluorogenic dyes commercially available under the ALEXA FLUOR product line (Life Technologies;

Carlsbad, CA). Optionally, the label is a redoxgenic label. Optionally, the label is a reduction tag, a thio- or thiol- containing molecule, or a substituted or unsubstituted alkyl.

[0044] As used herein, the term “linker” refers to a chemical moiety that links a nucleoside analogue to an affinity tag and/or detectable label. Generally, linkers useful in the present invention can be up to 30 carbon atoms in length. Preferably, the linkers are 5 - 15 carbon atoms in length. The types of bonds between the linker and the nucleobase, the linker and the affinity tag, and/or the linker and the detectable label include, but are not limited to, amides, amines, esters, carbamates, ureas, thioethers, thiocarbamates, thiocarbonates, and thioureas, and other bonds known by those of ordinary skill in the art.

[0045] As used herein, the term “cleavable linker” refers to a chemical moiety that links a nucleoside analogue to an affinity tag and/or detectable label, and that can be cleaved to remove the affinity tag and/or detectable label from the nucleoside analogue. Cleavage can be performed using chemical or enzymatic methods.

[0046] As used herein, the terms “alkyl,” “alkenyl,” and “alkynyl” include straight- and branched-chain monovalent substituents. Examples include methyl, ethyl, isobutyl, 3-butynyl, and the like. Ranges of these groups useful with the compounds and methods described herein include C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, and C₂-C₁₀ alkynyl. Additional ranges of these groups useful with the compounds and methods described herein include C₁-C₈ alkyl, C₂-C₈ alkenyl, C₂-C₈ alkynyl, C₁-C₆ alkyl, C₂-C₆ alkenyl, C₂-C₆ alkynyl, C₁-C₄ alkyl, C₂-C₄ alkenyl, and C₂-C₄ alkynyl. “Heteroalkyl,” “heteroalkenyl,” and “heteroalkynyl” are defined similarly as alkyl, alkenyl, and alkynyl, but can contain O, S, or N heteroatoms or combinations thereof within the backbone. Ranges of these groups useful with the compounds and methods described herein include C₁-C₁₀ heteroalkyl, C₂-C₁₀ heteroalkenyl, and C₂-C₁₀ heteroalkynyl. Additional ranges of these groups useful with the compounds and methods described herein include C₁-C₈ heteroalkyl, C₂-C₈ heteroalkenyl, C₂-C₈ heteroalkynyl, C₁-C₆ heteroalkyl, C₂-C₆ heteroalkenyl, C₂-C₆ heteroalkynyl, C₁-C₄ heteroalkyl, C₂-C₄ heteroalkenyl, and C₂-C₄ heteroalkynyl. The alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, or heteroalkynyl molecules used herein can be substituted or unsubstituted. As used herein, the term “substituted” includes the addition of an alkoxy, aryloxy, amino, alkyl, alkenyl, alkynyl, aryl, heteroalkyl, heteroalkenyl, heteroalkynyl, heteroaryl, cycloalkyl, or heterocycloalkyl group to a position attached to the main chain of the alkoxy, aryloxy,

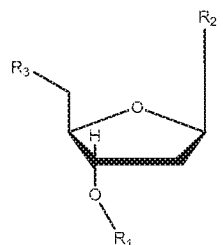
amino, alkyl, alkenyl, alkynyl, aryl, heteroalkyl, heteroalkenyl, heteroalkynyl, heteroaryl, cycloalkyl, or heterocycloalkyl, e.g., the replacement of a hydrogen by one of these molecules. Examples of substitution groups include, but are not limited to, hydroxy, halogen (e.g., F, Br, Cl, or I), and carboxyl groups. Conversely, as used herein, the term “unsubstituted” indicates the alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, or heteroalkynyl has a full complement of hydrogens, i.e., commensurate with its saturation level, with no substitutions, e.g., linear butane ($-(CH_2)_3-CH_3$).

III. Compositions

Nucleoside Analogues

[0047] Described herein are deoxyribose nucleoside analogues having a 3'-O reversible blocking group and a nucleobase. As used herein, the term “reversible blocking group” refers to a group that can be cleaved to provide a hydroxyl group at the 3'-position of the nucleoside analogue. The reversible blocking group can be cleavable by an enzyme, a chemical reaction, heat, and/or light. These 3'-O reversibly blocked deoxyribose nucleoside analogues can be used in a wide variety of nucleic acid sequencing methods, including but not limited to, sequencing by synthesis methods, and sequencing by ligation methods.

[0048] Such nucleoside analogues include but are not limited to those of **Formula I**:



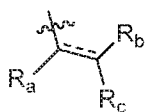
Formula I

In Formula I, R_1 is the 3'-O reversible blocking group (e.g., allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl), R_2 is, or includes, the nucleobase; and R_3 is a cleavable linking moiety comprising at least one (e.g., 1-10), at least two (e.g., 2-10), or at least three (e.g., 3-10), phosphates, or analogues thereof (e.g., a 5' -O-1-thiophosphate). The nucleoside analogue can be suitable as a substrate for an enzyme with DNA polymerase activity. In some cases, R_3 is a cleavable linking moiety comprising at least three (e.g., 3-10)

phosphates, or analogues thereof, and the nucleoside analogue is suitable as a substrate for a DNA polymerase. R_2 can, *e.g.*, be a base selected from adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U), and derivatives thereof. In some cases, the nucleoside analogue is a nucleoside triphosphate (*i.e.*, R_3 consists of three consecutive phosphates). In some cases, R_3 is a cleavable linking moiety comprising at least three (*e.g.*, 3-10) phosphates, or analogues thereof, and a detectable label (*e.g.*, a fluorophore or quenched fluorophore), and the nucleoside analogue is suitable as a substrate for a DNA polymerase.

[0049] Optionally, the reversible blocking group (*i.e.*, R_1) is a substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted heteroalkenyl, or substituted or unsubstituted heteroalkynyl.

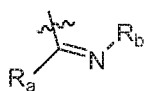
[0050] In some embodiments, the reversible blocking group can be represented by **Structure A**, as shown below:



Structure A

In Structure A, R_a , R_b , and R_c are each individually selected from the group consisting of hydrogen, cyano, halogen (*e.g.*, fluoro, chloro, or bromo), nitro, alkoxy, substituted or unsubstituted amino, substituted or unsubstituted sulfonyl, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, and substituted or unsubstituted carbonyl. Also in Structure A, $=$ represents a single bond or a double bond and “~” indicates where the moiety is connected to the remainder of the molecule.

[0051] In some embodiments, the reversible blocking group can be represented by **Structure B**, as shown below:



Structure B

In Structure B, R_a is hydrogen, cyano, halogen (e.g., fluoro, chloro, or bromo), nitro, alkoxy, substituted or unsubstituted amino, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, or substituted or unsubstituted alkynyl. Also in Structure B, R_b is hydrogen, substituted or unsubstituted alkyl, or substituted or unsubstituted alkoxy. Additionally in Structure B, “~” indicates where the moiety is connected to the remainder of the molecule.

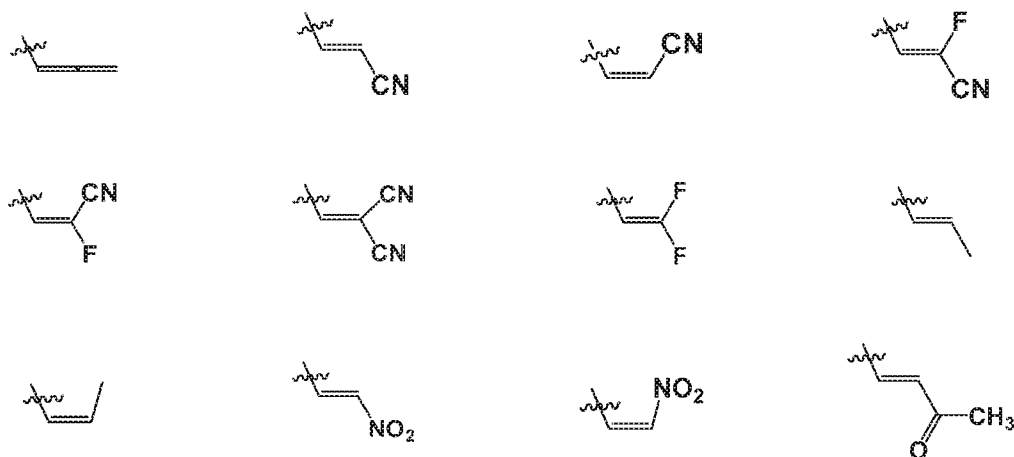
[0052] In some embodiments, the reversible blocking group can be represented by **Structure C**, as shown below:

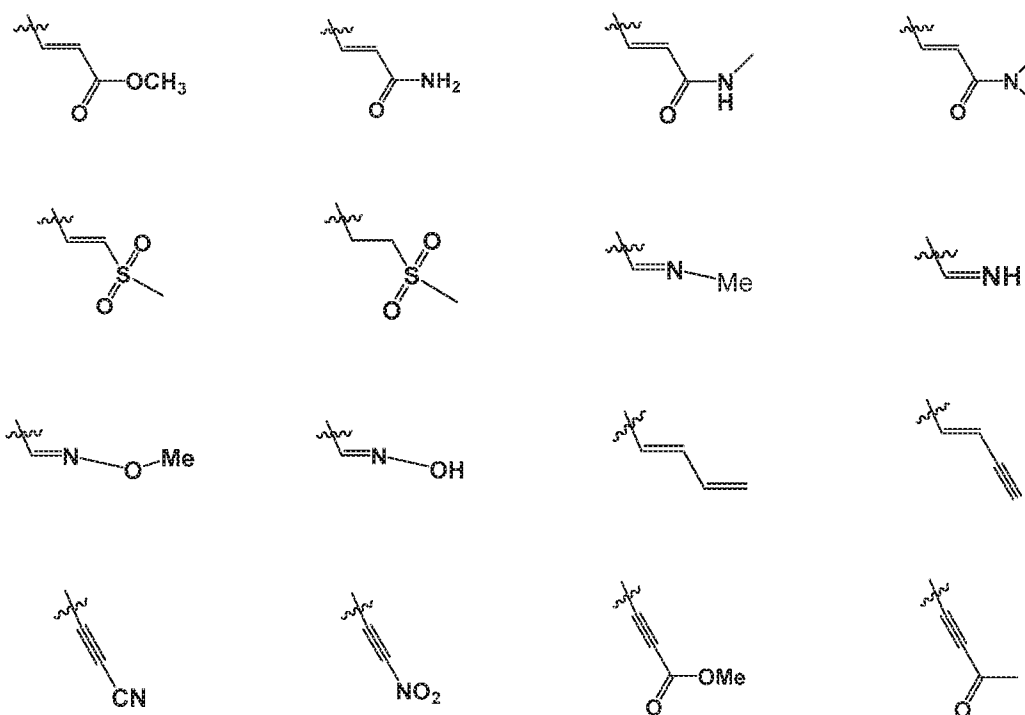


Structure C

In Structure C, R_a is hydrogen, cyano, halogen (e.g., fluoro, chloro, or bromo), nitro, alkoxy, substituted or unsubstituted amino, substituted or unsubstituted sulfonyl, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, or substituted or unsubstituted carbonyl. Also in Structure C, “~” indicates where the moiety is connected to the remainder of the molecule.

[0053] Exemplary reversible blocking groups for use with the nucleoside analogues described herein include the following compounds:





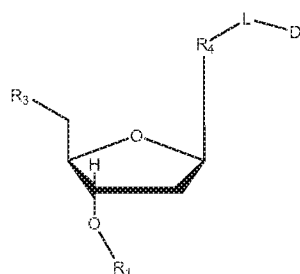
[0054] In some embodiments, the reversible blocking group is or contains allenyl (*e.g.*, $\text{CH}_2=\text{C}=\text{CH}-$). In some embodiments, the reversible blocking group is or contains cyanoethyl (*e.g.*, $\text{N}\equiv\text{CCH}_2\text{CH}_2-$). In some embodiments, the reversible blocking group is or contains cyanoethenyl (*e.g.*, $\text{N}\equiv\text{CCH}=\text{CH}-$). In some embodiments, the reversible blocking group is or contains formaldehyde oximyl (*e.g.*, $\text{CH}_2=\text{N}-$). In some embodiments, the reversible blocking group is or contains acrylaldehyde oximyl (*e.g.*, $\text{CH}_2=\text{CH}-\text{CH}=\text{N}-$). In some embodiments, the reversible blocking group is or contains propionaldehyde oximyl (*e.g.*, $\text{CH}\equiv\text{C}-\text{CH}=\text{N}-$). In some embodiments, the reversible blocking group is or contains cyanoethenaldehyde oximyl (*e.g.*, $\text{N}\equiv\text{C}-\text{CH}=\text{N}-$). In some embodiments, the reversible blocking group is selected from the group consisting of allenyl, cyanoethyl, and cyanoethenyl. In some embodiments, the reversible blocking group is selected from the group consisting of formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl. In some embodiments, the reversible blocking group is selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl.

[0055] In some embodiments, the reversible blocking group is or contains an allenyl, cyanoethyl, or cyanoethenyl moiety and a polyethylene glycol (PEG) moiety. In some

embodiments, the reversible blocking group is or contains a formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl moiety and a polyethylene glycol (PEG) moiety. In some embodiments, the reversible blocking group is or contains an allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl moiety and a polyethylene glycol (PEG) moiety. In some embodiments, the reversible blocking group contains an allenyl, cyanoethyl, or cyanoethenyl moiety and a substituted or unsubstituted alkyl (*i.e.*, a substituted or unsubstituted hydrocarbon). In some embodiments, the reversible blocking group contains a formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl moiety and a substituted or unsubstituted alkyl (*i.e.*, a substituted or unsubstituted hydrocarbon). In some embodiments, the reversible blocking group contains an allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl moiety and a substituted or unsubstituted alkyl (*i.e.*, a substituted or unsubstituted hydrocarbon).

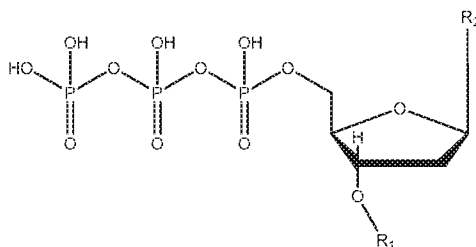
[0056] The nucleoside analogues of Formula I can be labeled with a detectable label (*e.g.*, a fluorescent label) at the R_2 and/or R_3 position. In an exemplary embodiment, the nucleoside analogues of Formula I are labeled with fluorescein, or Texas Red. In an another exemplary embodiment, the nucleoside analogues are labeled with fluorescein, FITC, Texas Red, ROX, Cy3, Alexa Fluor 647, ATTO532, Alexa Fluor 488, ATTO647, or Cy5. Methods and compositions for labeling and detecting a nucleoside analogue with a detectable label such as a fluorophore, or a fluorophore/quencher pair, at the R_2 and/or R_3 position are described, *e.g.*, in co-pending International Patent Application PCT/US2015/057094, entitled "Signal Confinement Sequencing (SCS) and Nucleotide Analogues for Signal Confinement Sequencing," filed on October 23, 2015, the content of which is hereby incorporated by reference in its entirety for all purposes.

[0057] In an exemplary embodiment, the nucleoside analogue of Formula I is a compound of **Formula IA**:

**Formula IA**

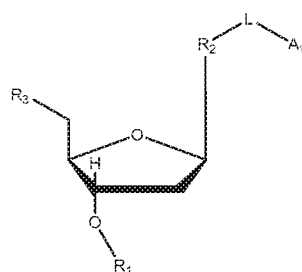
In **Formula IA**, R_1 is or contains a 3'-O reversible blocking group selected from allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl, R_4 is a nucleobase; R_3 is a cleavable linking moiety containing three, or at least three (*e.g.*, 3-10), phosphates, or analogues thereof; L is a linker; and D contains a detectable label (*e.g.*, a fluorescent label). In some cases, R_3 contains at least one thiophosphate, such as a 5' -O-1-thiophosphate. R_4 can, *e.g.*, be a base selected from adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U), and derivatives of these. In some cases, R_3 is a cleavable linking moiety containing three phosphates, or analogues thereof, and the nucleoside analogue of Formula Ia is suitable as a substrate of a DNA polymerase.

[0058] In some cases, the deoxyribose nucleoside analogues include but are not limited to deoxyribose nucleoside triphosphate analogues of **Formula II**:

**Formula II**

where R_1 is the 3'-O reversible blocking group (*e.g.*, allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl), and R_2 is the nucleobase.

[0059] In some cases, the deoxyribose nucleoside analogues include but are not limited to those of **Formula III**:

**Formula III**

In Formula III, R_1 is or contains a 3'-O reversible blocking group selected from allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; R_2 is a nucleobase; R_3 is a cleavable linking moiety containing at least one (*e.g.*, 1-10), at least two (*e.g.*, 2-10), or at least three (*e.g.*, 3-10), phosphates, or analogues thereof; L is a linker; and A_1 is a fluorescent or non-fluorescent affinity tag. In some cases, R_3 is a cleavable linking moiety comprising at least three (*e.g.*, 3-10), phosphates, or analogues thereof, and the nucleoside analogue is suitable as a substrate of a DNA polymerase. In some cases, R_3 comprises at least one thiophosphate, such as a 5'-O-1-thiophosphate.

[0060] The affinity tag can be, *e.g.*, any of the affinity tags, ligands, or antiligands described herein. In some cases, A_1 is an antigen that can be specifically bound by an antibody. In some cases, A_1 is a non-fluorescent affinity tag. In some cases, A_1 is biotin. In some cases, A_1 is not biotin. In some embodiments, the affinity tag is an antibody, an amino acid, cholesterol, FITC, Texas Red, an antigen, a peptide, a peptide antigen, or a small molecule antigen. Optionally, the affinity tag contains an oligonucleotide. In some cases, the affinity tag contains a thio (-S-) or a thiol (-SH) moiety. In some cases, the affinity tag is or contains a metal chelating group (*e.g.*, nitriloacetic acid (NTA), iminodiacetic acid (IDA), or a six-His peptide tag), optionally in complex with a metal (*e.g.*, nickel, zinc, cobalt, copper, etc.). Such metal chelating groups, when in complex with a metal, can be detected with a detectably labeled affinity agent containing a complementary metal chelating group.

Nucleoside Analogue Mixtures

[0061] The nucleoside analogues described herein can be provided or used in the form of a mixture. For example, the mixture can contain two, three, or four structurally different nucleoside analogues. The structurally different nucleoside analogues can differ at the

nucleobase. For example, the mixture can contain four structurally different nucleoside analogues containing the four natural DNA nucleobases (*i.e.*, adenine, cytosine, guanine, and thymine), or derivatives thereof. In some cases, each nucleoside analogue having a structurally different nucleobase can have a distinguishable detectable label or affinity tag. Alternatively, the mixture can contain four different nucleoside analogues but only three different detectable labels or affinity tags, wherein the fourth nucleoside analogue is unlabeled and/or untagged. Alternatively, the mixture can contain four structurally different nucleoside analogues that are used for two-color sequencing, such that a first nucleoside analogue is labeled with a first affinity tag or detectable label, the second nucleoside analogue is labeled with a second affinity tag or detectable label, the third nucleoside analogue is labeled with the first and second affinity tag or detectable label (*e.g.*, is a mixture of third nucleoside analogues labeled with the first affinity tag or label and third nucleotide analogues labeled with the second affinity tag or label), and the fourth nucleoside analogue is unlabeled and/or untagged.

Linkers

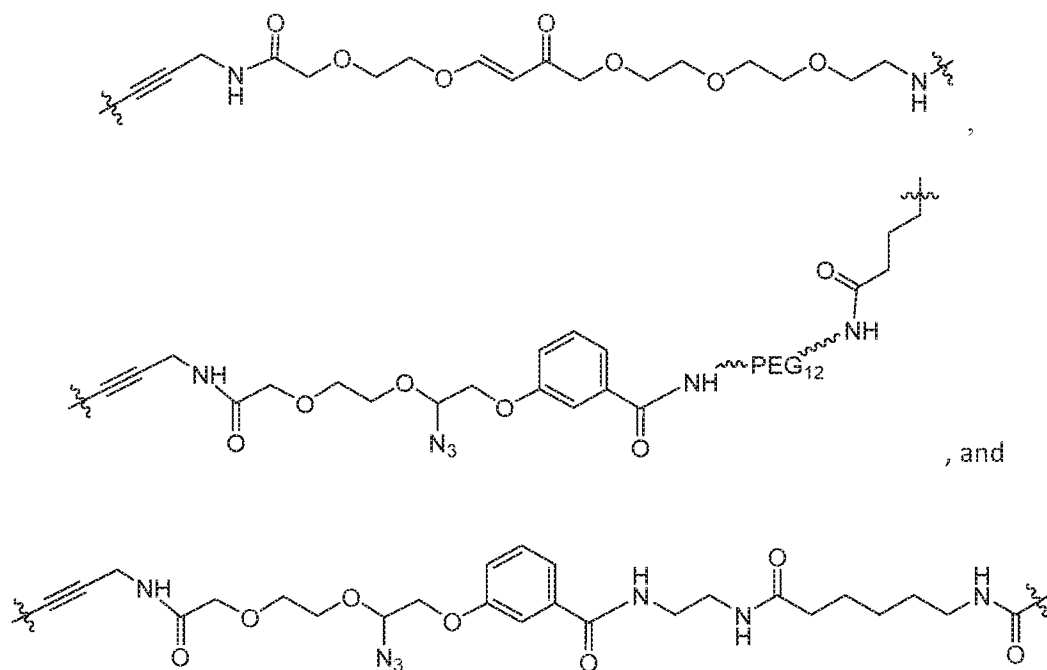
[0062] A nucleoside analogue as described herein can be attached to a label (*e.g.*, an affinity label and/or a detectable label) via a linker. Optionally, the linker used for attaching the nucleoside analogue to the label can be a cleavable linker. The linker can optionally be attached to the nucleobase of the nucleoside analogue. For example, the linker can be attached to the 5-position in a pyrimidine nucleobase or to the 7-position in a purine or deazapurine nucleobase. The linker can optionally be attached to a phosphate group located at the 5'-position of the nucleoside analogue.

[0063] Optionally, the linkers can be cleavable linkers. In nucleic acid sequencing and resequencing methods, the use of cleavable linkers between the nucleoside analogue and the label (*e.g.*, the affinity label and/or the detectable label) allows the removal of the label after incorporation and detection of the nucleoside analogue. Optionally, the cleavable linkers are attached (*e.g.*, covalently bonded) to the nucleoside analogue through the nucleobase of the nucleoside analogue. Optionally, the cleavable linkers are attached (*e.g.*, covalently bonded) to the nucleoside analogue through a phosphate group at the 5' position of the nucleoside analogue. The cleavable linkers as described herein can be cleaved to

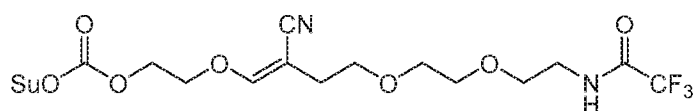
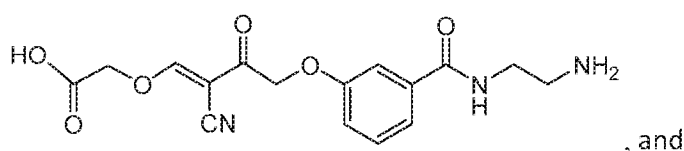
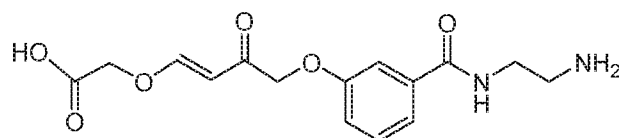
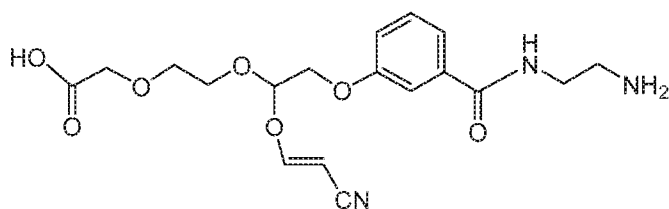
remove the label from the nucleoside analogue without otherwise altering the nucleoside analogue.

[0064] Cleavage can be performed using chemical or enzymatic methods. For example, cleavage can be performed by acid treatment, base treatment, oxidation, reduction, hydrolysis, or by photobleaching. Optionally, cleavage can be performed using phosphine-containing compounds or systems (e.g., phosphine-based transition metal catalysts or water-soluble phosphines). The appropriate cleavage method depends on the nature of the linkage, which can be determined by those of ordinary skill in the art.

[0065] The cleavable linkers can include, for example, an azido group, an allyl group, a disulfide bond, an amide group, or an alkoxy group. Optionally, the cleavable linker contains at least one moiety that is present in the 3'-O reversible blocking group. For example, the cleavable linker can contain at least one of the following groups: allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl groups. Exemplary linkers for use as cleavable linkers in the nucleotide analogues described herein include the following moieties:



[0066] Optionally, an exemplary linker for including in the nucleoside analogues described herein can be prepared from a reagent as shown below:



Detectable Labels

[0067] Nucleobases or nucleoside analogues containing the nucleobase, as described herein, can be covalently linked to a detectable label (*e.g.*, via a linker, as described above). The presence or absence of the detectable label can be detected to determine the sequence of a template nucleic acid. Similarly, affinity agents described herein can be detectably labeled. Detectably labeled affinity agents can be used to detect the presence or absence of a nucleoside analogue, or a reaction product thereof, having a corresponding affinity tag. Thus, the presence or absence of a detectably labeled nucleobase, nucleoside analogue, affinity agent bound to a nucleobase or nucleoside analogue, or reaction product thereof, can be detected to determine the sequence of a template nucleic acid. In some embodiments, the detectable label is a reporter molecule capable of generating a fluorescence signal. Exemplary reporter molecules are fluorescent organic dyes, which may be derivatized for attachment to an affinity agent, nucleobase, or nucleoside analogue.

[0068] There is a great deal of practical guidance available in the literature for selecting appropriate detectable labels for attachment to an affinity agent or nucleoside analogue, as

exemplified by the following references: Grimm *et al.*, 2013, "The chemistry of small-molecule fluorogenic probes," *Prog Mol Biol Transl Sci.* 113:1-34, incorporated herein by reference, and Oushiki *et al.*, 2012, "Near-infrared fluorescence probes for enzymes based on binding affinity modulation of squarylium dye scaffold," *Anal Chem.* 84:4404-10; Medintz & Hildebrandt, editors, 2013, "FRET – Förster Resonance Energy Transfer: from theory to applications," (John Wiley & Sons), and the like. The literature also includes references providing lists of fluorescent molecules, and their relevant optical properties for choosing fluorophores or reporter-quencher pairs, *e.g.*, Haugland, *Handbook of Fluorescent Probes and Research Chemicals (Molecular Probes, Eugene, 2005)*; and the like. Further, there is extensive guidance in the literature for derivatizing reporter molecules for covalent attachment via common reactive groups that can be added to a nucleoside, nucleobase, or affinity agent, as exemplified by the following references: Ullman *et al.*, U.S. Pat. No. 3,996,345; Khanna *et al.*, U.S. Pat. No. 4,351,760; and the like. Each of the aforementioned publications is incorporated herein by reference in its entirety for all purposes.

[0069] Exemplary reporter molecules may be selected from xanthene dyes, including fluoresceins, and rhodamine dyes. Many suitable forms of these compounds are widely available commercially with substituents on their phenyl moieties which can be used as the site for linking to an affinity agent. Another group of fluorescent compounds are the naphthylamines, having an amino group in the alpha or beta position. Included among such naphthylamino compounds are 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate, and 2-p-toluidinyl-6-naphthalene sulfonate. Other dyes include 3-phenyl-7-isocyanatocoumarin; acridines, such as 9-isothiocyanatoacridine and acridine orange; N-(p-(2-benzoxazolyl)phenyl)maleimide; benzoxadiazoles; stilbenes; pyrenes; and the like.

[0070] In some embodiments, reporter molecules are selected from fluorescein and rhodamine dyes. These dyes and appropriate linking methodologies are described in many references, *e.g.*, Khanna *et al.* (cited above); Marshall, *Histochemical J.*, 7:299-303 (1975); Menchen *et al.*, U.S. Pat. No. 5,188,934; Menchen *et al.*, European Patent Application 87310256.0; and Bergot *et al.*, International Application PCT/US90/05565. Fluorophores that can be used as detectable labels for affinity agents or nucleoside analogues include, but are not limited to, rhodamine, cyanine 3 (Cy 3), cyanine 5 (Cy 5), fluorescein, Vic™, Liz™,

Tamra™, 5-Fam™, 6-Fam™, 6-HEX, CAL Fluor Green 520, CAL Fluor Gold 540, CAL Fluor Orange 560, CAL Fluor Red 590, CAL Fluor Red 610, CAL Fluor Red 615, CAL Fluor Red 635, and Texas Red (Molecular Probes).

[0071] By judicious choice of labels, analyses can be conducted in which the different labels are excited and/or detected at different wavelengths in a single reaction. See, *e.g.*, Fluorescence Spectroscopy (Pesce et al., Eds.) Marcel Dekker, New York, (1971); White *et al.*, Fluorescence Analysis: A Practical Approach, Marcel Dekker, New York, (1970); Berlman, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd ed., Academic Press, New York, (1971); Griffiths, Colour and Constitution of Organic Molecules, Academic Press, New York, (1976); Indicators (Bishop, Ed.). Pergamon Press, Oxford, 1972; and Haugland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Eugene (2005). In some embodiments, the presence or absence of nucleoside analogues having distinct affinity tags can be simultaneously and differentially detected with corresponding differentially labeled affinity agents. Such nucleoside analogue/affinity agent pairs can be used in two-, three-, or four-color sequencing.

[0072] Similarly, two-, three-, or four-color sequencing can be performed using differentially labeled nucleoside analogues having an, *e.g.*, fluorescent, detectable label covalently linked (*e.g.*, via a linker) to the nucleobase and/or 5' phosphate. For example, nucleoside analogues comprising a reversible 3'-O blocking group selected from the group consisting of allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl and a detectable label covalently linked (*e.g.*, via a linker) to the nucleobase or 5' phosphate can be used in two-, three-, or four-color sequencing as described below.

Affinity Agents

[0073] Affinity agents can be used to detect the presence or absence of a nucleoside analogue, or a reaction product thereof, having a corresponding affinity tag. For example, the affinity agents can be used to detect incorporation of a nucleoside analogue having a corresponding affinity tag in a polynucleotide generated by a sequencing by synthesis or sequencing by ligation reaction.

[0074] In some embodiments, the affinity agent is a thio- or thiol-containing molecule, a protein, or a dendrimer. In some embodiments, the affinity agent is streptavidin, neutravidin, a tamavidin, glutathione S-transferase, thioredoxin, maltose binding protein, a lectin, or calmodulin binding protein. In some embodiments, the affinity agent is an antibody. In some embodiments, the affinity agent comprises an oligonucleotide complementary to an oligonucleotide affinity tag that is covalently linked to the nucleoside analogue. In some embodiments, the affinity agent comprises an oligonucleotide aptamer that specifically binds to an affinity tag (*e.g.*, a peptide or protein affinity tag) that is covalently linked to the nucleoside analogue. In some embodiments, the affinity agent is a polynucleotide concatemer comprising multiple copies of a complementary affinity agent sequence or multiple copies of an aptamer affinity agent sequence. In some embodiments, the affinity agent contains a metal chelating group (*e.g.*, a six-His tag, or an NTA, or IDA group) capable of forming a ternary metal-chelate complex (chelate-metal-chelate) with a corresponding metal chelating group affinity tag covalently linked to the nucleoside analogue (*e.g.*, a six-His tag, or an NTA, or IDA group).

[0075] One of ordinary skill in the art will recognize that an affinity agent and affinity tag binding pair can be interchanged. Thus the affinity agents described herein (*e.g.*, unlabeled affinity agents) can be used as affinity tags (*e.g.*, covalently linked to a nucleoside analogue). Moreover, the affinity tags described herein (*e.g.*, detectably labeled affinity tags) can be used as affinity agents to detect the presence or absence of a nucleoside analogue.

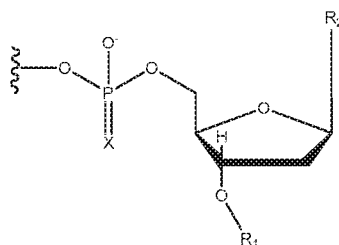
Labeled Oligonucleotides

[0076] Nucleoside analogues described herein can be used to sequence a template nucleic acid by a variety of methods. A variety of different oligonucleotides containing the nucleoside analogue, or a reaction product thereof, can be generated, depending on the sequencing method used and the nucleoside analogue employed. Examples of such oligonucleotides containing a nucleoside analogue of the present invention, or a reaction product thereof, are further described herein. Nucleoside analogues described herein, including those incorporated into an oligonucleotide, can also be useful in a variety of applications other than sequencing, as will be apparent to those of skill in the art. For example, nucleoside analogues described herein that include a fluorescent label covalently linked to a nucleobase can be used in a single nucleotide primer extension assay, such as

described in Synvänen, AC, Nature Reviews Genetics 2, 930-942 (December 2001). As another example, nucleoside analogues described herein that include a fluorescent label covalently linked to an affinity tag can be used to hybridize to and isolate target nucleic acid fragments.

[0077] In one aspect, an oligonucleotide containing a nucleoside analogue, can be hybridized to a template nucleic acid adjacent to an anchor sequence. The oligonucleotide may be ligated to the anchor sequence and the oligonucleotide detected. Detection of the nucleoside analogue identifies the oligonucleotide and thus the sequence of bases complementary to the template nucleic acid, thereby providing the sequence of the template nucleic acid. A 3'-O reversible blocking group of the incorporated nucleoside analogue, and optionally a detectable label or affinity tag, can be removed for further rounds of hybridization, ligation, and detection. In some embodiments, the nucleoside analogue comprises a fluorescent detectable label or affinity tag (*e.g.*, linked to a nucleobase) and is incorporated into an oligonucleotide. In some cases, the nucleoside analogue is incorporated at the 3' end of the oligonucleotide. Such 3' end incorporation can be useful, *e.g.*, for sequencing by ligation in the 5' to 3' direction.

[0078] Such oligonucleotides can comprise a nucleoside analogue of **Formula IV**:



Formula IV

In some cases, R_1 is or contains a reversible blocking group selected from allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; R_2 is or contains a nucleobase; and the nucleoside analogue is covalently linked via the 5' phosphate to an oligonucleotide, wherein:



indicates the location of the 5' phosphodiester bond to the oligonucleotide. X can be selected from O and S.

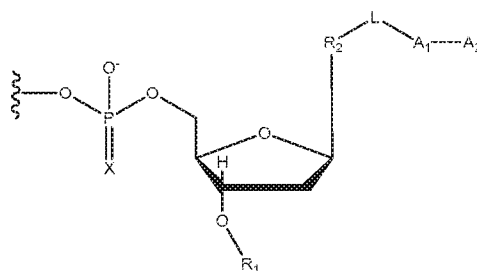
[0079] In an exemplary embodiment, R₂ contains or is a nucleobase, a linker, and a detectable label; or a nucleobase, a linker, and an affinity tag. In some cases, cleavage of the linker between nucleobase and detectable label or affinity tag can be performed under the same conditions as cleavage of the reversible blocking group. In some cases, the linker is not cleavable. In some cases, the linker is cleavable under orthogonal conditions relative to cleavage of the reversible blocking group.

[0080] In another aspect, a nucleoside analogue complementary to a template nucleic acid position and comprising a detectable label or an affinity tag (*e.g.*, linked to a nucleobase) is incorporated into an oligonucleotide during a sequencing by synthesis reaction via a polymerase enzyme. Detection of the nucleoside analogue indicates the sequence of the base complementary to the template nucleic acid, thereby providing the sequence of the template nucleic acid. In some embodiments, the nucleoside analogue contains a fluorescent detectable label or affinity tag (*e.g.*, linked to a nucleobase) and is incorporated into a oligonucleotide.

[0081] Nucleoside analogues of Formula IV, wherein R₂ includes or is a nucleobase (*e.g.*, a nucleobase that is not labeled with an affinity agent or detectable label) can also be utilized for sequencing by synthesis reaction schemes that do not rely on a specific step of detecting a distinguishable label. For example, pyrosequencing reaction schemes—in which nucleoside analogues of each of the four DNA nucleobases (A, G, C, and T) are delivered in succession and detected by a coupled assay for detection of pyrophosphate produced during incorporation of the nucleoside analogue by a polymerase—do not require a labeled or affinity tagged nucleobase. As such, the present invention also includes such nucleoside analogues of Formula IV wherein R₂ is a nucleobase, and their use in “label-free” (*e.g.*, pyrosequencing) sequencing reactions.

[0082] Nucleoside analogues of Formula IV, wherein R₂ includes a nucleobase, a linker, and a detectable label can be covalently (*e.g.*, via disulfide linkage) or non-covalently bound to a detectably labeled affinity agent. The presence or absence of the detectably labeled affinity agent can be determined to identify the sequence of the complementary base of the

template nucleic acid in a sequencing by ligation or sequencing by synthesis reaction. Such oligonucleotides can contain a nucleoside analogue of **Formula V**:



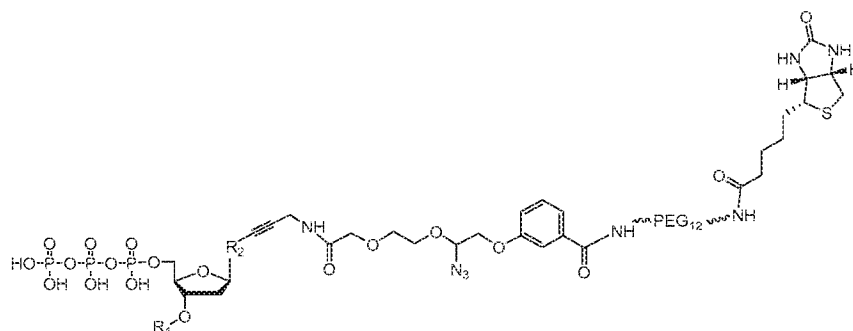
Formula V

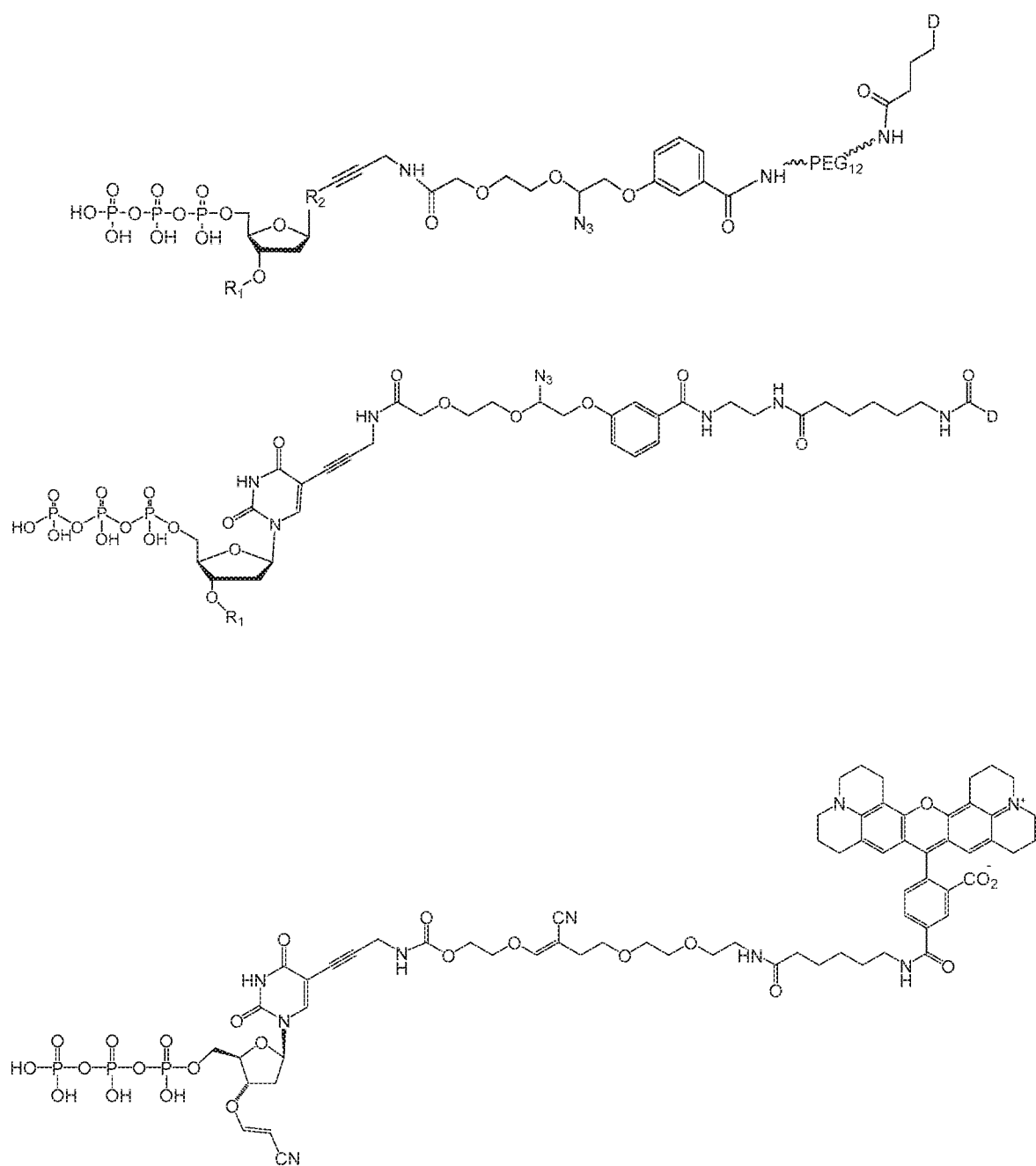
In Formula V, R_1 is or contains a reversible blocking group selected from allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; R_2 is a nucleobase; L is a linker; A_1 is or contains a fluorescent or non-fluorescent affinity tag; and A_2 is or includes a detectably labeled affinity agent that forms a specific complex with A_1 . X can be selected from O and S.

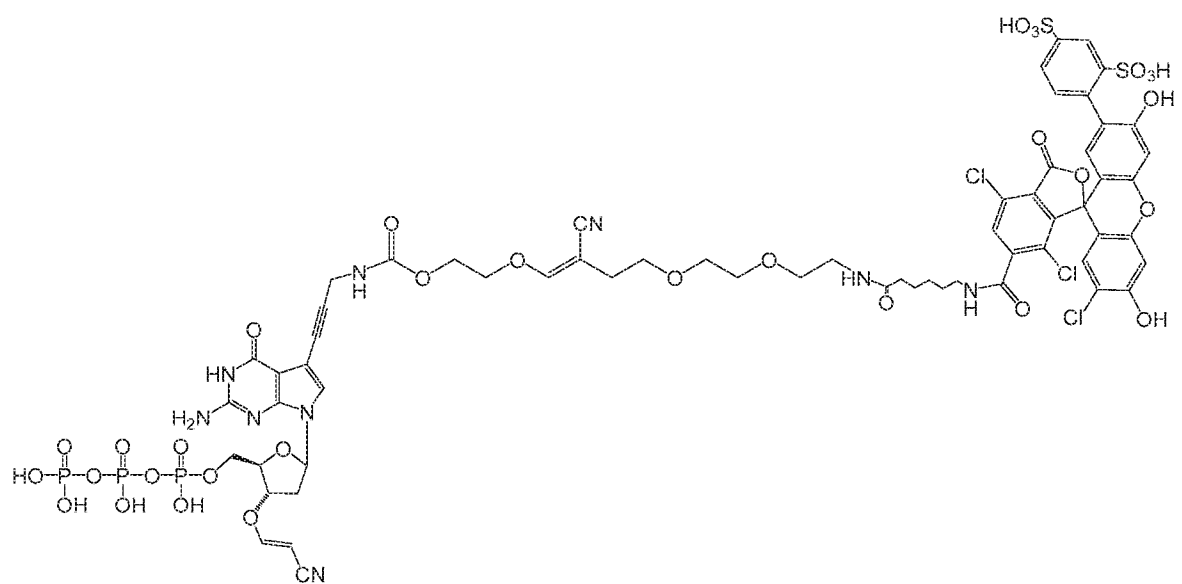
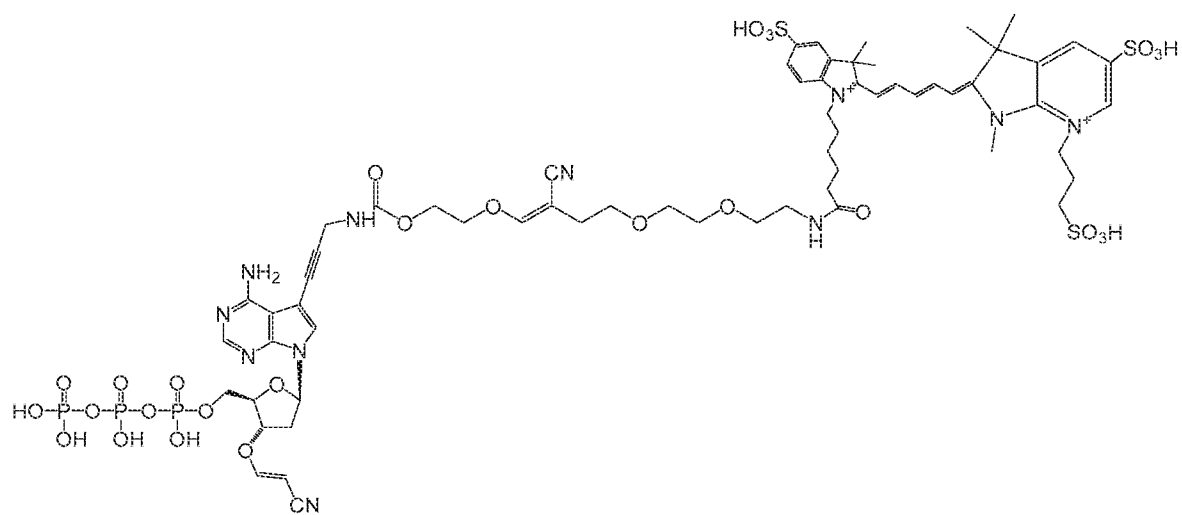
[0083] In some cases, A_2 includes a detectably labeled affinity agent that forms a specific and non-covalent complex with A_1 . In some cases, A_1 includes one or more thio (-S-) or thiol (-SH) groups, A_2 includes a detectably labeled affinity agent containing one or more thio (-S-) or thiol (-SH) groups, and A_2 forms a specific and covalent disulfide-mediated complex with A_1 .

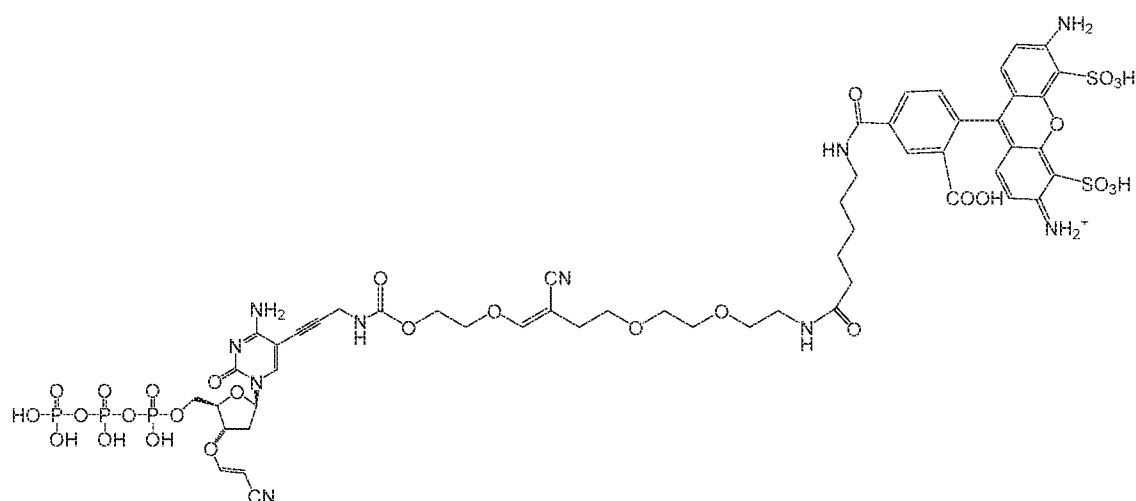
Exemplary Nucleoside Analogues

[0084] Exemplary nucleoside analogues as described herein include the following compounds:









Reaction Mixtures

[0085] Nucleoside analogues and oligonucleotides containing such nucleoside analogues or reaction products thereof can be used as a component of a reaction mixture. For example, such components can be used in reaction mixtures for nucleic acid sequencing (*e.g.*, sequencing by synthesis or by ligation). Exemplary reaction mixtures include, but are not limited to, those containing (a) template nucleic acid; (b) polymerase; (c) oligonucleotide primer; and (d) a 3'-*O* reversibly blocked nucleoside analogue, or a mixture of such 3'-*O* reversibly blocked nucleoside analogues having structurally different nucleobases. The 3'-*O* reversible blocking group can be, or contain, an allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl moiety, or a combination thereof.

[0086] The reaction mixture can further optionally contain one or more of: ATP sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate; and luciferin. In some cases, such a reaction mixture includes (a) template nucleic acid; (b) polymerase; (c) oligonucleotide primer; (d) a 3'-*O* reversibly blocked adenosine nucleoside analogue having an alpha thiophosphate; (e) ATP sulfurylase, (f) luciferase, (g) apyrase, (h) adenosine 5' phosphosulfate; and (i) luciferin. In some cases, the adenosine nucleoside analogue having an alpha thiophosphate is an adenine nucleotide or derivative thereof that is not detectably labeled with a fluorophore or affinity tag, and contains a 3'-*O* reversible blocking group selected from allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl.

[0087] Alternatively, the reaction mixture can contain a 3'-O reversibly blocked nucleoside analogue, where the nucleobase is covalently linked to a linker, and the linker is linked to an affinity tag or detectable label. In some cases, the reaction mixture contains a mixture of nucleoside analogues having different nucleobases, where the nucleobases are covalently linked to a detectable and distinguishable label or affinity tag via a linker. In some cases, the reaction mixture further contains one or more detectably and distinguishably labeled affinity agents. Labeled nucleobases or labeled affinity agents can, *e.g.*, be labeled with detectable fluorescent organic dyes, *e.g.*, detectable and distinguishable fluorescent organic dyes.

Template Nucleic Acids

[0088] In various embodiments, the template polynucleotide is DNA (*e.g.*, cDNA, genomic DNA, or amplification products) or RNA. In various embodiments, the polynucleotide is double stranded or single stranded.

[0089] In some embodiments, the template nucleic acid is immobilized on a solid surface. In some embodiments, the template nucleic acid is immobilized on a substrate (*e.g.*, a bead, flow cell, pad, channel in a microfluidic device and the like). The substrate may comprise silicon, glass, gold, a polymer, PDME, and the like.

[0090] In some embodiments, the template nucleic acid is immobilized or contained within a droplet (optionally immobilized on a bead or other substrate within the droplet).

[0091] In some embodiments, the template nucleic acid is an immobilized DNA concatemer comprising multiple copies of a target sequence. In some embodiments, the template nucleic acid is represented as a DNA concatemer, such as a DNA nanoball (DNB) comprising multiple copies of a target sequence and an "adaptor sequence". See International Patent Publication No. WO 2007133831, the content of which are hereby incorporated by reference in its entirety for all purposes. In some embodiments the template is a single polynucleotide molecule. In some embodiments the template is present as a clonal population of template molecules (*e.g.*, a clonal population produced by bridge amplification or Wildfire amplification).

[0092] It will be understood that the method is not limited to a particular form of template, and the template can be any template such as, for example, a DNA concatemer, a

dendrimer, a clonal population of templates (*e.g.*, as produced by bridge amplification or Wildfire amplification) or a single polynucleotide molecule. Thus, importantly, the specification should be read as if each reference to a template can alternatively refer to a concatemer template, a clonal population of short linear templates, a single molecule template (*e.g.*, in a zero-mode waveguide), and templates in other forms.

[0093] Suitable template nucleic acids, including DNBs, clusters, polonys, and arrays or groups thereof, are further described in U.S. Pat. Nos. 8,440,397; 8,445,194; 8,133,719; 8,445,196; 8,445,197; 7,709,197; 12/335,168, 7,901,891; 7,960,104; 7,910,354; 7,910,302; 8,105,771; 7,910,304; 7,906,285; 8,278,039; 7,901,890; 7,897,344; 8,298,768; 8,415,099; 8,671,811; 7,115,400; 8,236,499, and U.S. Patent Publication Nos. 2015/0353926; 2010/0311602; 2014/0228223; and 2013/0338008, all of which are hereby incorporated by reference in their entirety for all purposes and particularly for all disclosure related to nucleic acid templates, concatemers and arrays according to the present invention.

IV. Methods

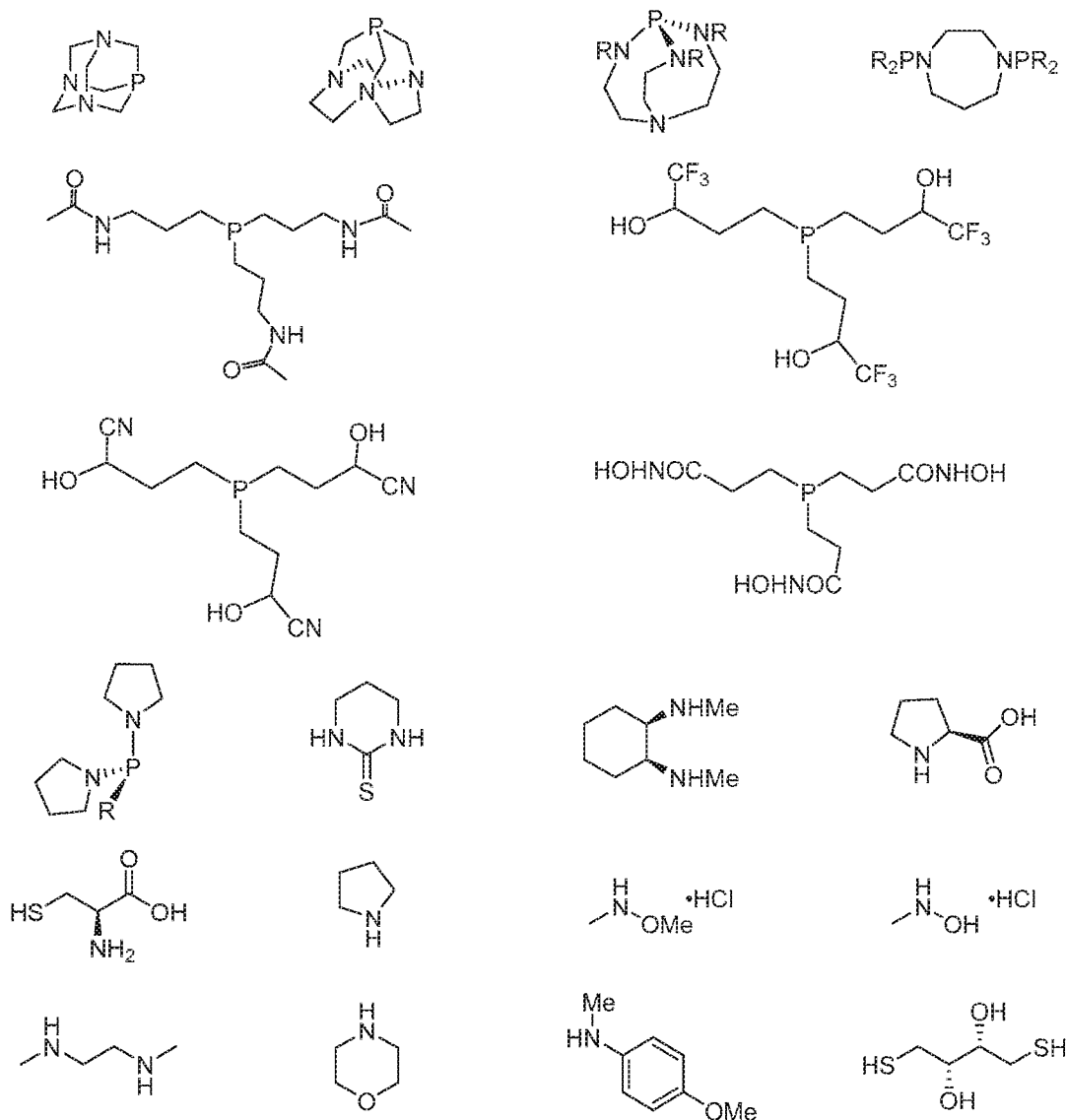
Cleavage of Blocking Groups or Linkers

[0094] Nucleoside analogues described herein are 3'-O reversibly blocked. In some aspects, the blocking group provides for controlled incorporation of a single 3'-O reversibly blocked nucleoside analogue into a sequencing by synthesis primer, *e.g.*, a sequencing by synthesis primer that has been extended in a previous cycle. Similarly, the blocking group provides for controlled incorporation of a single oligonucleotide containing a 3'-O reversibly blocked nucleoside analogue into an adjacent sequencing by ligation anchor primer or previously extended anchor primer. After incorporation and detection, the reversibly blocked nucleoside analogue, or an oligonucleotide containing such an analogue, can be treated to cleave the blocking group and allow further rounds of extension by polymerase or ligase.

[0095] The 3'-O reversible blocking group can be removed by enzymatic cleavage or chemical cleavage (*e.g.*, hydrolysis). The conditions for removal can be selected by one of ordinary skill in the art based on the descriptions provided herein, the chemical identity of the blocking group to be cleaved, and nucleic acid chemistry principles known in the art. In some embodiments, the blocking group is removed by contacting the reversibly blocked

nucleoside with a reducing agent such as dithiothreitol (DTT), or a phosphine reagent such as tris(2-carboxyethyl)phosphine (TCEP), tris(hydroxymethyl)phosphine (THP), tris(3-hydroxypropyl)phosphine (THPP), 1,3,5-triaza-7-phosphaadamantane (PTA), 1,4,7-triaza-9-phosphatricyclo[5.3.2.1]-tridecane (CAP), proazaphosphatrane, trialkylphosphines, and aminophosphines.

[0096] Exemplary reducing agents include $\text{Na}_2\text{S}_2\text{O}_3$, Na_2SO_3 , NaN_3 , EtNO_2 , $\text{CH}_2(\text{CN})_2$, and NEt_3 . Further examples of suitable reducing agents are shown below:



In the structures above, each R group is independently hydrogen or a substituted or unsubstituted alkyl group (e.g., methyl, ethyl, or isopropyl).

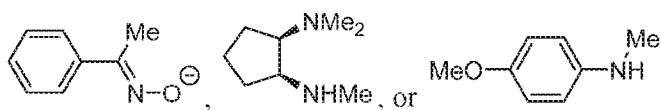
[0097] In some cases, the blocking group is removed by washing the blocking group from the incorporated nucleotide analogue using a reducing agent such as a phosphine reagent. In some cases, the blocking group is photolabile, and the blocking group can be removed by application of, *e.g.*, UV light. In some cases, the blocking group can be removed by contacting the nucleoside analogue with a transition metal catalyzed reaction using, *e.g.*, an aqueous palladium (Pd) solution. In some cases, the blocking group can be removed by contacting the nucleoside analogue with an aqueous nitrite solution. Additionally, or alternatively, the blocking group can be removed by changing the pH of the solution or mixture containing the incorporated nucleotide analogue. For example, in some cases, the blocking group can be removed by contacting the nucleoside analogue with acid or a low pH (*e.g.*, less than 4) buffered aqueous solution.

[0098] In some cases, the blocking group can be removed by use of a strongly electronegative or strongly basic solution. For example, in some cases, the 3'-O reversible blocking groups that can be cleaved by a fluoride or hydroxide containing solution. 3'-O reversible blocking groups that can be cleaved by contacting with a hydroxide or fluoride containing solution include, but are not limited to, allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl blocking groups or blocking groups that contain an allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl moiety.

[0099] In some cases, the 3'-O reversible blocking group (*e.g.*, allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl) can be cleaved by contacting with a nucleophile or base containing solution. In some cases, an allenyl or allenyl containing 3'-O reversible blocking group can be cleaved by contacting with a nucleophile or base containing solution. In some cases, a cyanoethyl or cyanoethyl containing 3'-O reversible blocking group can be cleaved by contacting with a nucleophile or base containing solution. In some cases, a cyanoethenyl or cyanoethenyl containing 3'-O reversible blocking group can be cleaved by contacting with a nucleophile or base containing solution. In some cases, a formaldehyde oximyl or formaldehyde oximyl containing 3'-O reversible blocking group can be cleaved by contacting with a nucleophile or base containing solution. In some cases, an acrylaldehyde oximyl or

acrylaldehyde oximyl containing 3'-O reversible blocking group can be cleaved by contacting with a nucleophile or base containing solution. In some cases, a propionaldehyde oximyl or propionaldehyde oximyl containing 3'-O reversible blocking group can be cleaved by contacting with a nucleophile or base containing solution. In some cases, a cyanoethenaldehyde oximyl or cyanoethenaldehyde oximyl containing 3'-O reversible blocking group can be cleaved by contacting with a nucleophile or base containing solution.

[0100] Exemplary nucleophile or base containing solutions for cleavage include, but are not limited to solutions having one or more of dimethylamine, diethylamine, N,N'-dimethylene diamine, methylhydroxyl amine, morpholine, 4-methoxy-N-methylaniline, dithiothreitol, N,N'-dimethyl-cis-cyclohexane-1,2-diamine, proline (e.g., L-proline), pyrrolidine, cysteine (e.g., L-cysteine), thiosulfate (e.g., sodium thiosulfate), tetrahydropyrimidine-2(1H)-thione, dicyanomethane carbanion, nitro(4-(trifluoromethyl)phenyl)methanide, nitroethane carbanion (e.g., nitroethane), azide (e.g., sodium azide), sulfite (e.g., sodium sulfite), NH_2OH , imidazole, cyclic thiourea, N,O-dimethylhydroxylamine, malononitrile,



. In an exemplary embodiment, 3'-O reversible blocking group (e.g., cyanoethenyl) cleavage conditions are 10-40 equivalents of a nucleophile or base containing solution such as dimethylamine in acetonitrile / 0.1 M TEAB for 5-90 minutes. In some cases, 10 equivalents of dimethylamine in acetonitrile / 0.1 M TEAB provides complete cleavage after about 30 minutes. In some cases, 40 equivalents of dimethylamine in acetonitrile / 0.1 M TEAB provides complete cleavage after about 10 minutes.

[0101] In some aspects, the nucleoside analogue contains a 3'-O reversible blocking group and a linker between the nucleobase and an affinity tag or detectable label. In such cases, it can be advantageous to cleave the linker and thereby remove the affinity tag or detectable label from the nucleobase. In some embodiments, the cleavage is performed under the same conditions as the cleavage of the 3'-O reversible blocking group, providing simultaneous cleavage of blocking group and label or affinity tag. Simultaneous cleavage can be used to reduce the number of processing steps required during multiple rounds of

nucleoside incorporation, detection, and cleavage. Alternatively, the cleavage can be performed under orthogonal conditions. Orthogonal cleavage can be used to control the relative order in which the nucleoside analogue is unblocked and label or labeled affinity agent is removed. The cleavage can be performed according to any of the methods described above for cleavable linkers, including chemical or enzymatic methods. For example, cleavage can be performed by acid treatment, base treatment, oxidation, reduction, hydrolysis, or by photobleaching. Optionally, cleavage can be performed using phosphine-containing compounds or systems (e.g., phosphine-based transition metal catalysts or water-soluble phosphines). In some cases, the label or labeled affinity agent is removed by linker cleavage prior to cleavage of reversible blocking group. In some cases, the label or labeled affinity agent is removed by linker cleavage before cleavage of reversible blocking group.

[0102] Detection is generally performed prior to linker cleavage, if linker cleavage is employed. However, detection can be performed before or after reversible blocking group cleavage. Moreover, in some embodiments, although the nucleoside analogue can contain a group comprising a nucleobase that is covalently linked to a cleavable linker (which in turn is covalently linked to a detectable label or affinity tag), cleavage of the linker is not universally employed or required for performing additional cycles of sequencing-by-synthesis or sequencing-by-ligation. For example, detectable label covalently linked to the linker or detectably labeled affinity agent can be quenched in *lieu* of, or in addition to, linker cleavage. Additionally, or alternatively, detectably labeled affinity agent can be stripped from the affinity agent.

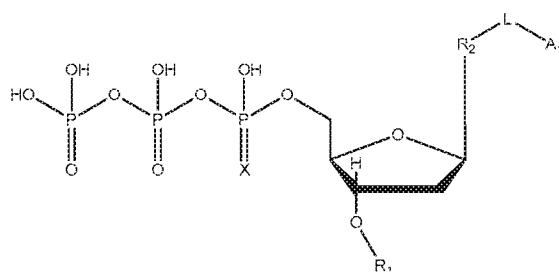
Sequencing

[0103] The nucleoside analogues described herein can be used in a variety of sequencing methods. For example, the analogues can be used in no-label, 2-label, 3-label, or 4-label sequencing methods. Exemplary no-label sequencing methods include, but are not limited to, methods in which nucleoside analogues having different nucleobases (e.g., A, C, G, T) are delivered in succession and incorporation is detected by detecting the presence or absence of the same signal or label for each different nucleobase. Thus, no-label methods are sometimes known as one-label, or one-color methods because the detection signal and/or label is the same for all nucleobases. For example, incorporation of a

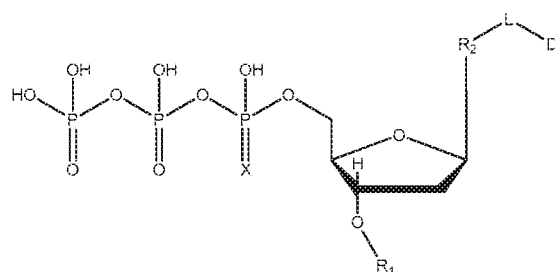
nucleoside into a primer by DNA polymerase mediated template directed polymerization can be detected by detecting a pyrophosphate cleaved from the nucleoside pyrophosphate. Pyrophosphate can be detected using a coupled assay in which ATP sulfurylase converts pyrophosphate to ATP, in the presence of adenosine 5' phosphosulfate, which in turn acts as a substrate for luciferase-mediated conversion of luciferin to oxyluciferin, generating visible light in amounts proportional to ATP generation.

[0104] In an alternative no-label system, an inducer that is released by polymerase-mediated cleavage between alpha and beta phosphate of a nucleoside analogue, and optionally further processed by a second enzyme such as a phosphatase or sulfurylase, then activates a quenched dye on a capture element. This system, method, and compositions for performing the method, is further described, *e.g.*, in co-pending International Patent Application PCT/US2015/057094, entitled "Signal Confinement Sequencing (SCS) and Nucleotide Analogues for Signal Confinement Sequencing," filed on October 23, 2015, (Attorney Docket No. 092171-0961855 (5045-PC)), the contents of which are hereby incorporated by reference in their entirety for all purposes.

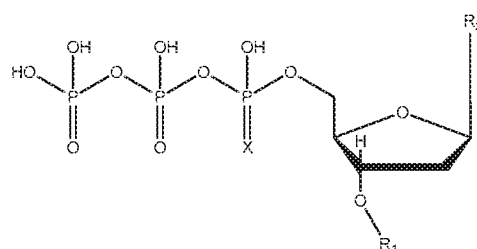
[0105] One of skill in the art will recognize that, although a nucleoside analogue containing a nucleobase-linker moiety attached to a detectable label or affinity agent group is not required for such no-label methods, such nucleoside analogues are compatible with no-label methods in general. As such, no-label sequencing methods can employ any one of the following nucleoside analogues, or mixtures thereof.



Formula VI;



Formula VII; or



Formula VIII, wherein

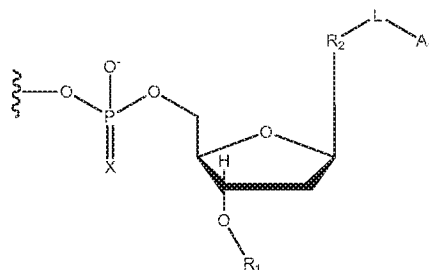
X is selected from O and S; R_1 is or includes a 3'-O reversible blocking group (*e.g.*, allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, or cyanoethenaldehyde oximyl); R_2 is or includes a nucleobase; L is or includes a linker (*e.g.*, a cleavable linker); D is or includes a detectable label (*e.g.*, detectable fluorescent label); and A_1 is or includes an affinity tag.

[0106] Alternatively, 2-label sequencing can be performed with the nucleoside analogues described herein, using two distinguishable signals in a combinatorial fashion to detect incorporation of four different nucleobases. Exemplary 2-label systems, methods, and compositions include, without limitation, those described in U.S. Patent No. 8,617,811, the contents of which are hereby incorporated by reference in the entirety for all purposes and particularly for disclosure related to 2-label sequencing. Briefly, in 2-label sequencing, incorporation of a first nucleobase (*e.g.*, A) is detected by detecting the presence of a first label; and, incorporation of a second nucleobase (*e.g.*, C) is detected by detecting the presence of a second label. Incorporation of a third nucleobase (*e.g.*, T) is detected by detecting the presence of both the first and second label attached to the third nucleobase; and, incorporation of a fourth unlabeled nucleobase (*e.g.*, G) is detected by detecting the absence of both first and second labels. The labels of the nucleoside analogues utilized in a 2-label sequencing method can be attached to affinity agents specifically bound to affinity

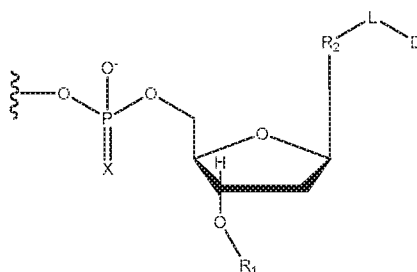
tags linked (*e.g.*, cleavably linked) to a nucleobase, or directly attached to via a covalent linker (*e.g.*, cleavable linker) to the nucleobase.

[0107] Similarly, 3- and 4-label sequencing can be performed with the nucleoside analogues described herein, using three or four distinguishable signals to detect incorporation of four different nucleobases. For example, 3-label sequencing can employ a first nucleobase labeled with a first label, a second nucleobase labeled with a second label, a third nucleobase labeled with a third label, and a fourth nucleobase that is either not labeled or labeled with a combination of first and second, first and third, or second and third labels. The labels of the nucleoside analogues utilized in a 3-label sequencing method can be attached to affinity agents specifically bound to affinity tags that are in turn linked (*e.g.*, cleavably linked) to a nucleobase, or directly attached to via a covalent linker (*e.g.*, cleavable linker) to the nucleobase. Similarly, 4-label sequencing can employ a first nucleobase labeled with a first label, a second nucleobase labeled with a second label, a third nucleobase labeled with a third label, and a fourth nucleobase labeled with a fourth label. The labels of the nucleoside analogues utilized in a 4-label sequencing method can be attached to affinity agents specifically bound to affinity tags that are in turn linked (*e.g.*, cleavably linked) to a nucleobase, or directly attached to via a covalent linker (*e.g.*, cleavable linker) to the nucleobase.

[0108] Such 2-, 3-, and 4-label, also referred to as 2-, 3-, and 4- color, sequencing methods can be used in both sequencing by synthesis and sequencing by ligation. For example, nucleoside analogues of Formulas VI or VII can be utilized for sequencing by synthesis in a 2-, 3-, or 4-label method. Similarly, oligonucleotides containing nucleoside analogues of the following formulas can be used for sequencing by ligation in a 2-, 3-, or 4-label method:



Formula IX; or

**Formula X;**

wherein X is selected from S and O; R₁ is or contains a 3'-O reversible blocking group selected from allenyl, cyanoethyl, cyanoethenyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl; R₂ is or includes a nucleobase; L is or includes a linker; D is or includes a detectable label (*e.g.*, a detectable fluorescent label); and A₁ is or includes an affinity tag.

[0109] Various sequencing by synthesis and sequencing by ligation methods can be used with the nucleoside analogues of the present invention. In some aspects, the sequencing by synthesis methods can be selected from those described in U.S. Pat. Nos. 6,210,891; 6,828,100, 6,833,246; 6,911,345; 6,969,488; 6,897,023; 6,833,246; and 6,787,308; Patent Publication Nos. 2003/0,064,398; and 2003/0,022,207; Margulies *et al.*, 2005, *Nature* 437:376-380; Ronaghi *et al.*, 1996, *Anal. Biochem.* 242:84-89; Constans, A, 2003, *The Scientist* 17(13):36; and Bentley *et al.*, 2008, *Nature* 456(7218): 53-59. In some aspects, sequencing by ligation methods can be selected from those described in International Patent Publication WO 1999/019,341; WO 2005/082,098; WO 2006/073,504; and Shendure *et al.*, 2005, *Science*, 309: 1728-1739. In an exemplary embodiment, the sequencing by synthesis or sequencing by ligation is performed using one or more nucleoside analogues described herein with a method described in co-pending U.S. Provisional Patent Application No. 62/194,741, entitled "DNA Sequencing Using Controlled Strand Displacement," filed on July 20, 2015, the contents of which are hereby incorporated by reference in the entirety for all purposes, and particularly for the template preparation and sequencing methods and compositions described therein.

[0110] For example, a DNA strand for sequencing can be produced by a) providing a template DNA polynucleotide containing a first target DNA sequence interposed between a first adaptor 3' to the first target DNA sequence and a second adaptor 5' to the first target

DNA sequence, and optionally comprising a third adaptor 3' to the first adaptor and a second target DNA sequence interposed between the first adaptor and the third adaptor, wherein the template DNA polynucleotide is immobilized on a substrate; b) combining a first primer with the immobilized template DNA polynucleotide, and hybridizing the first primer to a first primer binding sequence in the first adaptor, wherein the first primer is not immobilized on the substrate when it is combined with the immobilized template DNA polynucleotide; c) extending the first primer using a first DNA polymerase to generate a second strand, wherein the second strand comprises a sequence complementary to the first target DNA sequence and a sequence complementary to at least part of the second adaptor; d) combining a second primer with the immobilized template DNA polynucleotide, hybridizing a second primer to a second primer binding sequence, wherein the second primer binding sequence is 3' to the first primer binding sequence, wherein the second primer is not immobilized on the substrate when it is combined with the immobilized template DNA polynucleotide; e) extending the second primer using a DNA polymerase having strand-displacement activity to generate a third strand, wherein extending the second primer to generate the third strand partially displaces the second strand, thereby producing a partially hybridized second strand having: (i) a hybridized portion that is hybridized to the template DNA polynucleotide; and (ii) an unhybridized overhang portion that contains a sequence that is complementary to the first target DNA sequence and a sequence that is complementary to at least part of the second adaptor, wherein the unhybridized portion is 5' in the second strand to the hybridized portion.

[0111] The prepared template can be then sequenced by, *e.g.*, hybridizing a sequencing oligonucleotide to the sequence in the third strand that is complementary to at least part of the second adaptor. The sequencing oligonucleotide can be an anchor primer for hybridizing to template nucleic acid and ligating to an adjacent hybridized oligonucleotide containing a nucleoside analogue described herein. Thus, the method can be used for sequencing by ligation. Alternatively, the sequencing oligonucleotide can be a polymerase primer that is extended by incorporating a nucleoside analogue described herein. Thus, the method can be used for sequencing by synthesis.

EXAMPLES

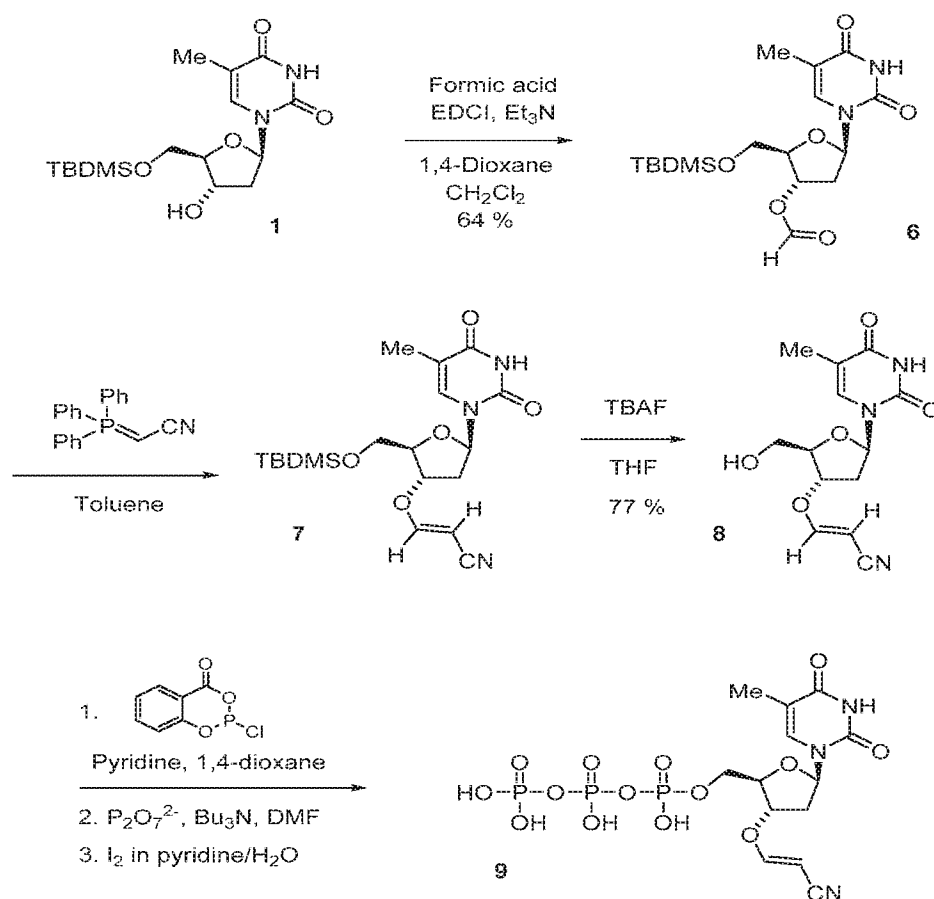
[0112] The present invention may be embodied in other specific forms without departing from its structures, methods, or other essential characteristics as broadly described herein and claimed hereinafter. The described embodiments are to be considered in all respects only as illustrative, and not restrictive. The scope of the invention is, therefore, indicated by the appended claims, rather than by the foregoing description. All changes that come within the meaning and range of equivalency of the claims are to be embraced within their scope.

Example 1: Synthesis of nucleoside analogues***General Procedure for the Preparation of 3'-O-cyanoethenyl-thymidine triphosphate***

[0113] Treatment of 5'-O-TBDMS-thymidine (**1**) with formic acid, using EDCI as the coupling reagent, gave 346 mg of 5'-O-TBDMS-3'-O-formyl-thymidine (**6**) in 64 % yield. Wittig reaction between the formate ester (**6**) and (triphenylphosphoranylidene)acetonitrile in refluxing toluene afforded a 5 to 1 mixture of *trans/cis* 5'-O-TBDMS-3'-O-cyanoethenyl-thymidine (**7**) (*trans* shown for simplicity) in good yield.

[0114] The isomers were readily separated by column chromatography. A 257 mg quantity (58 % yield) of the *trans*-isomer (**7**) was isolated. Deprotection of *trans*-isomer (**7**) with TBAF gave 143 mg of 3'-O-cyanoethenyl-thymidine (**8**) (> 99 % pure by HPLC) in good yield. Synthesis of a second batch of 3'-O-cyanoethenyl-thymidine (**8**) was successfully completed to give a further 90 mg quantity (> 99 % pure by HPLC). Phosphorylation of 3'-O-cyanoethenyl-thymidine (**8**) (50 mg scale) gave impure triphosphate and this was purified to give 14 mg of 3'-O-cyanoethenyl-thymidine triphosphate (**9**) at > 99 % purity by HPLC. The ¹H-NMR, MS and ³¹P-NMR spectral data confirmed the structure.

Scheme:

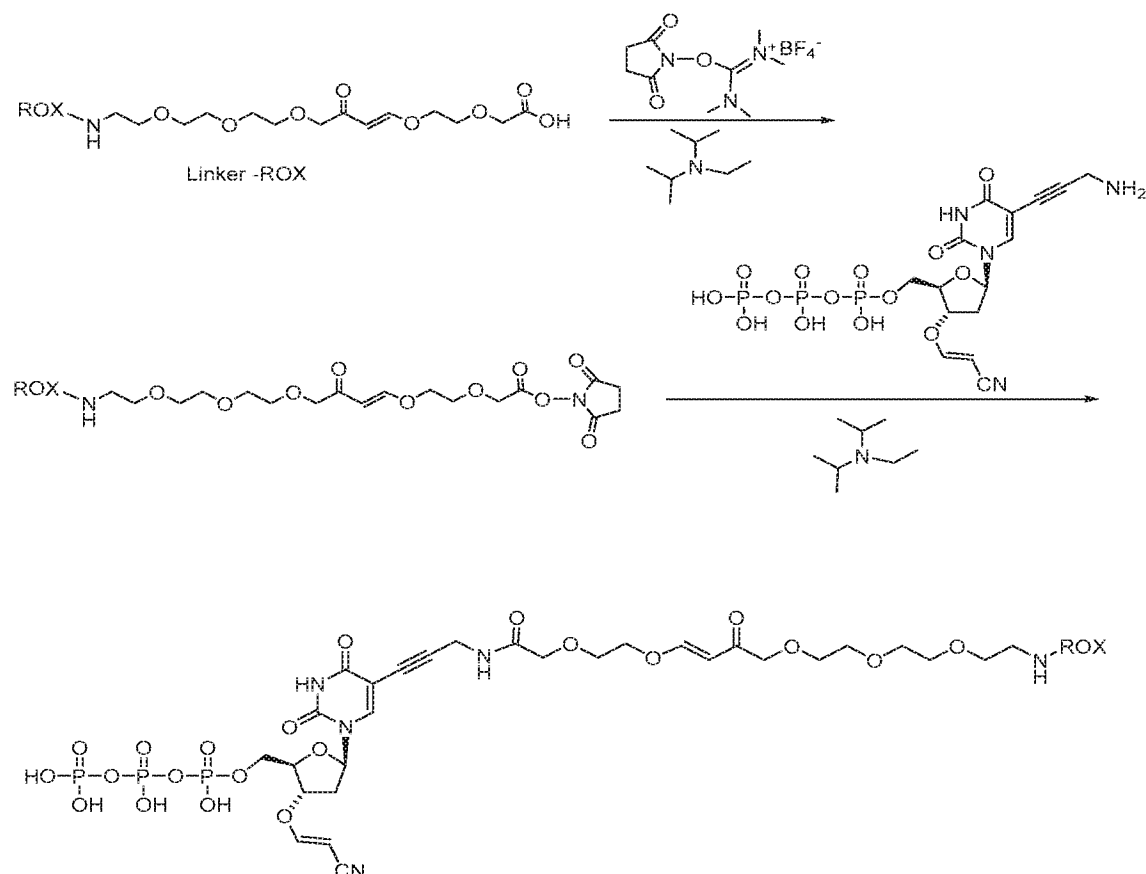


[0115] **General Procedure for the Preparation of 3'-O-cyanoethenyl-5-Rox-1-amino-11,19-dioxo-3,6,9,14,17-pentaoxa-20-azatricos-12-en-22-yn-23-yl)-uridine triphosphate**

[0116] To a solution of linker-ROX (3 μ mol) in DMF (0.5 mL) was added TSTU (6 μ mol) and DIEA (6 μ mol). After 20 minutes, LCMS indicated the complete formation of NHS ester. The reaction mixture in DMF was poured into 10 mL ethyl acetate-isopropanol (4:1 volume ratio) to precipitate the NHS ester. The precipitate was centrifuged, and quickly washed with 2 mL ethyl acetate-isopropanol by centrifuge and decant. The precipitate was dried under vacuum to remove solvent. The precipitate was dissolved in 0.2 mL anhydrous DMF. The resulting product was ready for coupling. To a solution of 3'-O-cyanoethenyl-dTTP-propargylamine (1.5 mg, about 1 μ mol) in NaHCO₃ buffer (0.1 mL, pH 8.7, 50 mM, cold on ice) was added dropwise with stirring the DMF solution of NHS ester of linker-ROX prepared above. The final reaction mixture was stirred on ice and then kept in a fridge overnight with exclusion of light. LCMS monitoring indicated the formation of desired product. The reaction mixture was directly purified by reverse-phase prep-HPLC (C18, solvent A: 20 mM

TEAB in water, solvent B: 20 mM TEAB in MeCN, 5% to 100%) to afford the final product, characterized by LC-MS, H NMR, and P NMR.

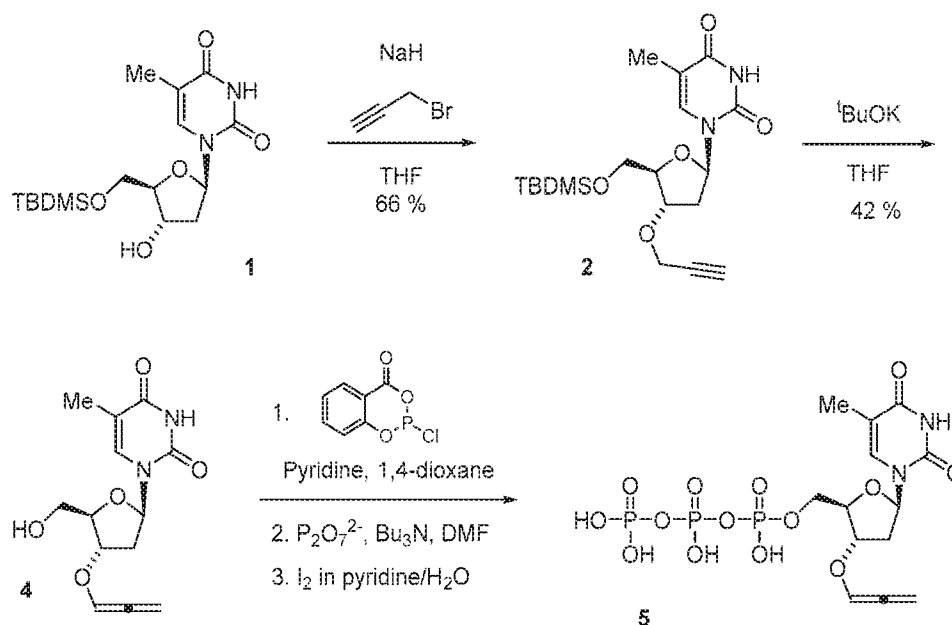
Scheme:



[0117] **General Procedure for the Preparation of 3'-O-allenyl-thymidine triphosphate**

[0118] Reaction of 5'-O-TBDMS-thymidine (**1**) with sodium hydride followed by propargyl bromide gave 365 mg of 5'-O-TBDMS-3'-O-propargyl-thymidine (**2**). Reaction of (**2**) with potassium *tert*-butoxide afforded 3'-O-allenyl-thymidine (**4**) in acceptable yield considering that the TBDMS was also cleaved using these reaction conditions. A total of 111 mg of impure 3'-O-allenyl-thymidine (**4**) was prepared and after two purifications 46 mg of (**4**) was obtained with purity > 95 % by HPLC. Phosphorylation of 3'-O-allenyl-thymidine (**8**) (23 mg scale) was carried out using our standard conditions. After purification by semi-prep HPLC and freeze drying, formation of thymidine triphosphate was observed by HPLC (2.8 %). And after a second freeze-drying, 7 % thymidine triphosphate was observed. A 4 mg quantity of 3'-O-allenyl-thymidine triphosphate (90.1 % purity by HPLC) containing 7 % of thymidine triphosphate was obtained.

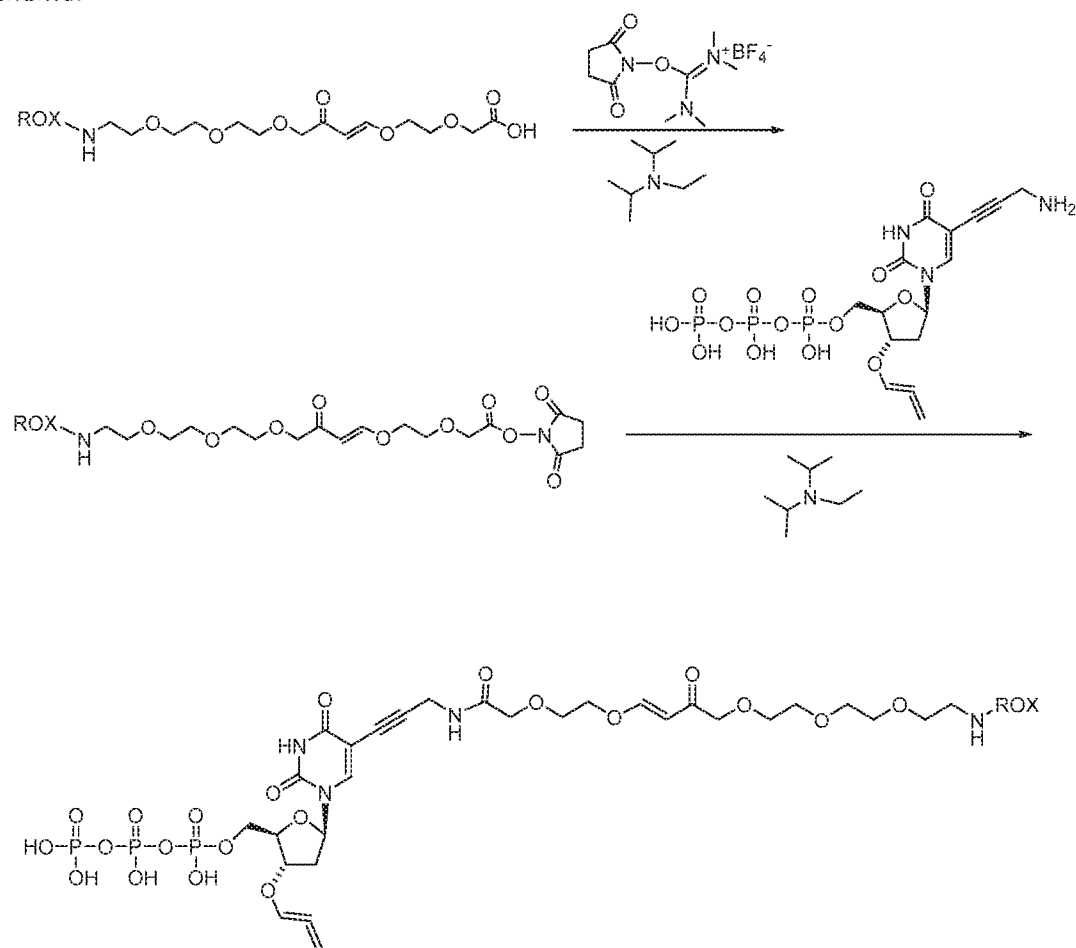
Scheme:



[0119] **General Procedure for the Preparation of 3'-O-allenyl-5-Rox-1-amino-11,19-dioxo-3,6,9,14,17-pentaoxa-20-azatricos-12-en-22-yn-23-yl)-uridine triphosphate**

[0120] To a solution of linker-ROX (3 μmol) in DMF (0.5 mL) was added TSTU (6 μmol) and DIEA (6 μmol). After 20 minutes, LCMS indicated the complete formation of NHS ester. The reaction mixture in DMF was poured into 10 mL ethyl acetate-isopropanol (4:1 volume ratio) to precipitate the NHS ester. The precipitate was centrifuged, and quickly washed with 2 mL ethyl acetate-isopropanol by centrifuge and decant. The precipitate was dried under vacuum to remove solvent. The precipitate was dissolved in 0.2 mL anhydrous DMF. The resulting product was ready for coupling. To a solution of 3'-O-allenyl-dTTP-propargylamine (1.5 mg, about 1 μmol) in NaHCO_3 buffer (0.1 mL, pH 8.7, 50 mM, cold on ice) was added dropwise with stirring the DMF solution of NHS ester of linker-ROX prepared above. The final reaction mixture was stirred on ice and then kept in a fridge overnight with exclusion of light. LCMS monitoring indicated the formation of desired product. The reaction mixture was directly purified by reverse-phase prep-HPLC (C18, solvent A: 20 mM TEAB in water, solvent B: 20 mM TEAB in MeCN, 5% to 100%) to afford the final product, characterized by LC-MS, ^1H NMR, and ^{31}P NMR.

Scheme:

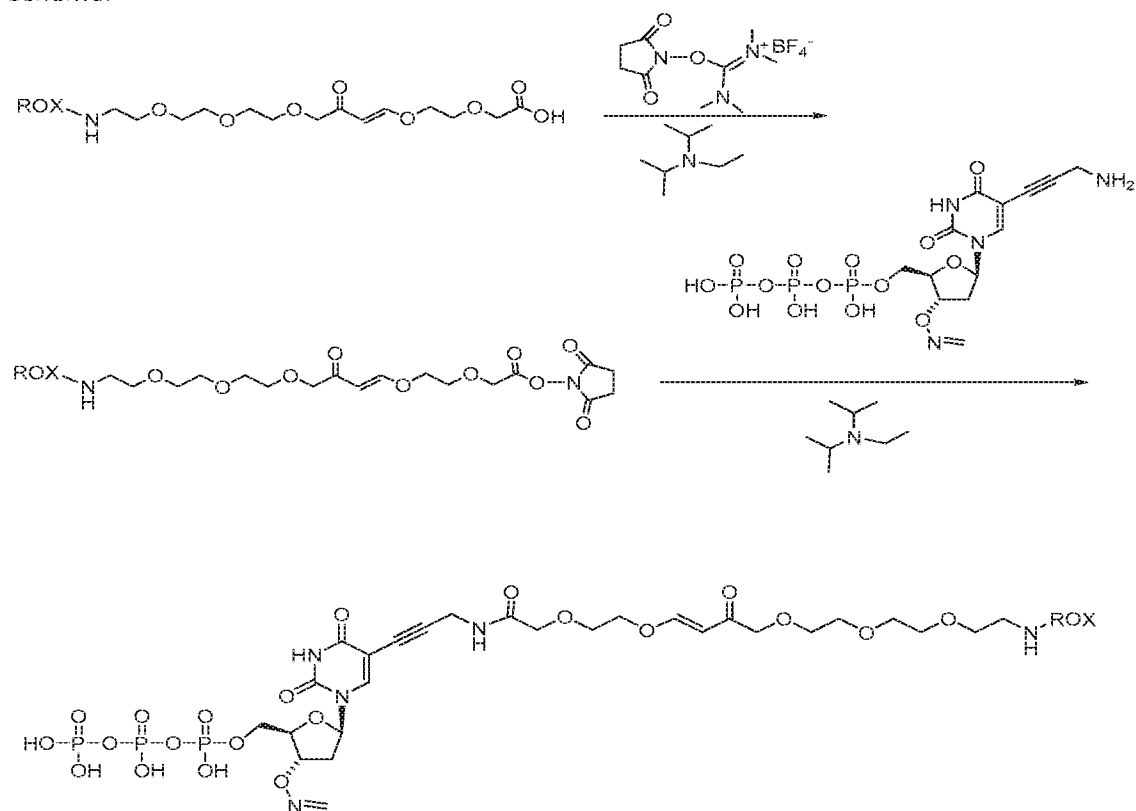


[0121] **General Procedure for the Preparation of 3'-O-formaldehyde oximyl-5-Rox-1-amino-11,19-dioxo-3,6,9,14,17-pentaoxa-20-azatricos-12-en-22-yn-23-yl)-uridine triphosphate**

[0122] To a solution of linker-ROX (3 μ mol) in DMF (0.5 mL) was added TSTU (6 μ mol) and DIEA (6 μ mol). After 20 minutes, LCMS indicated the complete formation of NHS ester. The reaction mixture in DMF was poured into 10 ml ethyl acetate –isopropanol (4:1 volume ratio) to precipitate the NHS ester. The precipitate was centrifuged, and quickly washed with 2 mL ethyl acetate-isopropanol by centrifuge and decant. The precipitate was dried under vacuum to remove solvent. The precipitate was dissolved in 0.2 mL anhydrous DMF. The resulting product was ready for coupling. To a solution of 3'-O-formaldehyde oximyl-dTTP-propargylamine (1.5 mg, about 1 μ mol) in NaHCO₃ buffer (0.1mL, pH 8.7, 50mM, cold on ice) was added dropwise with stirring the DMF solution of NHS ester of linker-ROX prepared above. The final reaction mixture was the stirred on ice and then kept in a fridge

overnight with exclusion of light. LCMS monitoring indicated the formation of desired product. The reaction mixture was directly purified by reverse-phase prep-HPLC (C18, solvent A: 20 mM TEAB in water, solvent B: 20 mM TEAB in MeCN, 5% to 100%) to afford the final product, characterized by LC-MS, ¹H NMR, and ³¹P NMR.

Scheme:



[0123] **Example 2: Monitoring Cleavage of 3'-O reversible blocker**

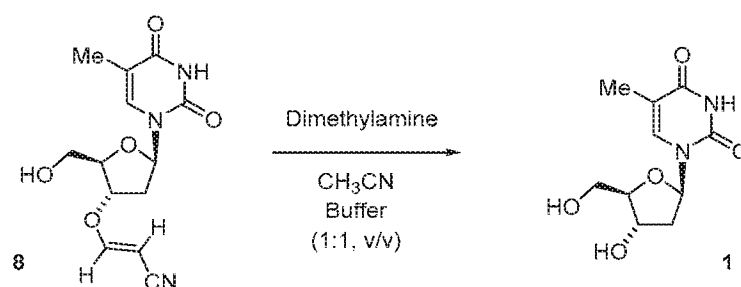
[0124] Cleavage reactions were performed using 5 mg of compound **8** (3'-O-cyanoethynyl-thymidine) in 0.25 mL of acetonitrile and 0.25 mL of buffer. Cleavage was monitored by LC-MS and the results are depicted in the table below.

Entry	Nucleophile (equivalents)	Buffer	Reaction progress (monitored by LCMS) Ratio starting material : Thymidine			
			10 min	20 min	30 min	60 min
1	Dimethylamine (10 equivalents)	0.1 M TEAB (pH = 8.2)	50 : 50	5 : 95	0 : 100	N / A

2	Dimethylamine (40 equivalents)	0.1 M TEAB (pH = 8.2)	0 : 100	N/A	N/A	N/A
3	Dimethylamine (40 equivalents)	1 M Tris-buffer (pH = 8.1)	0 : 100	N/A	N/A	N/A
4	Dimethylamine (40 equivalents)	0.5 M Phosphate buffer (pH = 7.5)	10 : 90	0 : 100	N/A	N/A

[0125] As indicated in the table above, complete cleavage can be achieved in about 20-30 minutes with 10 equivalents of dimethylamine in acetonitrile / 0.1 M TEAB. Complete cleavage can be achieved in a about 10 minutes or less with 40 equivalents of dimethylamine in acetonitrile / 0.1 M TEAB or acetonitrile / 1 M Tris buffer. Complete cleavage can be achieved in about 10-20 minutes with 40 equivalents of dimethylamine in acetonitrile / 0.5 M phosphate buffer.

Scheme:



Example 3: Use of nucleoside analogues for sequencing

[0126] Allenyl-dTTP and cyanoethenyl-dTTP were incubated at room temperature for 24 hours, or 65 °C for 2 hours. Fresh mixes were also made the day of the experiment. Allenyl-dTTP or cyanoethenyl-dTTP were mixed with unlabeled AGC (AGC-Cold) and used as both the hot (labeled) and cold (unlabeled) chase. Nanoball arrays were imaged to obtain a control image. Then, the array was incubated with labeled mixes to check for incorporation on unblocked ends. The results depicted in Figure 1 show that both allenyl-dTTP and cyanoethenyl-dTTP are as effective a polymerase substrate for sequencing by synthesis as the unlabeled control 3'-O-azidomethyl-dTTP. Moreover, both allenyl-dTTP and cyanoethenyl-dTTP are stable at room temperature for 24 hours and 65 °C for 2 hours as

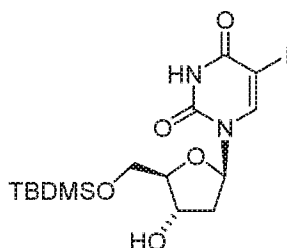
indicated by their ability to efficiently incorporate into polymerization product and thereby block subsequent polymerization and incorporation of the hot chase label.

[0127] **Example 4: Synthesis of 3'-O-Cyanoethynyl-dTTP-linker-ROX**

[0128] **Materials and Methods**

[0129] Chemicals were purchased from Aldrich Chemical Company (Gillingham, Dorset), Alfa Aesar (Heysham, Morecambe, Lancashire) and Carbosynth Limited (Compton, Berkshire), unless otherwise noted, and were used without further purification. Solvents were purchased as anhydrous. Petrol was the fraction boiling between 40 – 60 °C. TLC was carried out using aluminium plates pre-coated with silica gel (Kieselgel 60 F₂₅₄, 0.2 mm, Merck, Darmstadt, Germany). Visualization was by UV light. ¹H NMR spectra were recorded on either a Bruker 300 MHz or 400 MHz spectrometer using the residual proton(s) in the deuterated solvents as internal standards. HPLC analyses were performed on a Shimadzu Prominence instrument with diode array detection. LCMS analyses were performed on a Shimadzu 2010EV instrument operating in positive or negative electrospray (ESI) mode. Automated chromatography was performed on a Biotage Isolera purification system.

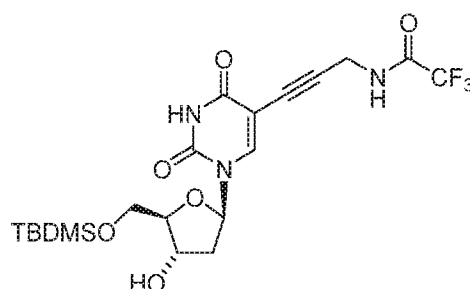
[0130] **5'-O-(*tert*-Butyldimethylsilyl)-2'-deoxy-5-iodouridine**



[0131] To an ice-cold solution of 2'-deoxy-5-iodouridine (1.00 g, 2.82 mmol), imidazole (0.58 g, 8.47 mmol), and 4-(dimethylamino)pyridine (34.5 mg, 0.28 mmol) in anhydrous DMF (13 mL) was added *tert*-butyldimethylsilyl chloride (0.51 g, 3.39 mmol) portionwise and the mixture was stirred for 40 minutes before allowing it to warm slowly to room temperature overnight. The resulting mixture was quenched with saturated aqueous ammonium chloride solution (50 mL) and diluted with ethyl acetate (40 mL). The layers were separated and the aqueous layer was extracted with ethyl acetate (2 × 40 mL). The combined organic extracts were washed with water (5 × 60 mL) and saturated brine (60 mL), dried over MgSO₄, and concentrated to give a colorless oil. This residual material was purified using a Biotage Isolera automated chromatography system under normal phase

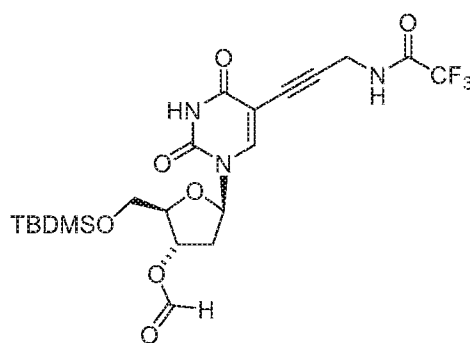
conditions (silica column, gradient of 10 % to 80 % ethyl acetate in dichloromethane) with detection at 254 nm to give 5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxy-5-iodouridine (0.91 g, 69 %), as a white solid. R_f 0.29 (dichloromethane - ethyl acetate, 6 : 4, v/v).

[0132] **5'-*O*-(*tert*-Butyldimethylsilyl)-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)uridine**



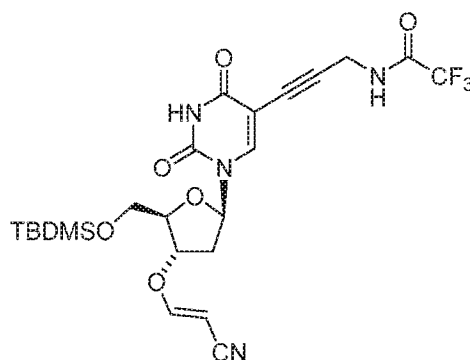
[0133] 5'-*O*-(*tert*-Butyldimethylsilyl)-2'-deoxy-5-iodouridine (0.64 g, 1.36 mmol), copper(I) iodide (52 mg, 0.27 mmol), and tetrakis(triphenylphosphine)palladium(0) (157 mg, 0.14 mmol) were dissolved in anhydrous DMF (7 mL) and the flask was evacuated and purged with nitrogen. Hunig's base (di-isopropylethylamine, 351 mg, 473 μ L, 2.71 mmol) and *N*-trifluoroacetyl-propargylamine (0.62 g, 4.07 mmol) were added and the reaction mixture was stirred at room temperature overnight. The resulting mixture was concentrated under high vacuum and the residue was dissolved in ethyl acetate (30 mL). The organic solution was washed with 5 % aqueous EDTA solution (3 $\times$ 15 mL), water (15 mL), and saturated brine (50 mL), dried over $MgSO_4$, and concentrated under reduced pressure to give an orange oil. This residual oil was purified using a Biotage Isolera automated chromatography system under normal phase conditions (silica column, gradient of 12 % to 100 % ethyl acetate in dichloromethane) with detection at 254 nm to give 5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)uridine (0.65 g, 97 %), as a yellow solid. R_f 0.24 (dichloromethane - ethyl acetate, 1 : 1, v/v).

[0134] **5'-*O*-(*tert*-Butyldimethylsilyl)-2'-deoxy-3'-*O*-formyl-5-(*N*-trifluoroacetyl-3-aminopropynyl) uridine**



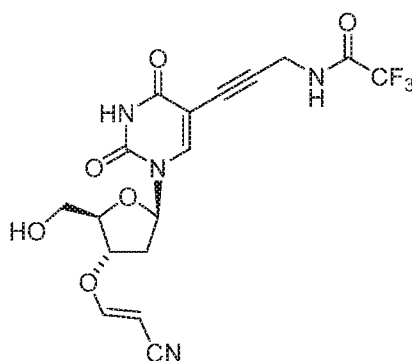
[0135] A solution of 5'-O-(*tert*-butyldimethylsilyl)-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)uridine (1.00 g, 2.03 mmol), formic acid (187 mg, 154 μ L, 4.07 mmol), 4-(dimethylamino)pyridine (25 mg, 0.20 mmol), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (0.78 g, 4.07 mmol) and Hunig's base (1.05 g, 1.42 mL, 8.14 mmol) in a mixture of dichloromethane (18 mL), and 1,4-dioxane (18 mL) was stirred at room temperature overnight. The reaction mixture was concentrated and the residue was partitioned between 1 M hydrochloric acid (50 mL) and dichloromethane (50 mL). The layers were separated and the aqueous layer was extracted with dichloromethane (2 $\times$ 50 mL). The combined organic extracts were washed with saturated aqueous sodium bicarbonate (50 mL) and then with saturated brine (50 mL), dried over MgSO_4 , and concentrated under reduced pressure to give a yellow oil. This material was purified using a Biotage Isolera automated chromatography system under normal phase conditions (silica column, gradient of 12 % to 100 % ethyl acetate in dichloromethane) with detection at 254 nm to give 5'-O-(*tert*-butyldimethylsilyl)-2'-deoxy-3'-O-formyl-5-(*N*-trifluoroacetyl-3-aminopropynyl)uridine (0.49 g, 46 %), as an off-white solid. R_f 0.55 (dichloromethane - ethyl acetate, 1 : 1, v/v).

[0136] **(*E*)-5'-O-(*tert*-Butyldimethylsilyl)-3'-O-cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-amino propynyl)uridine**



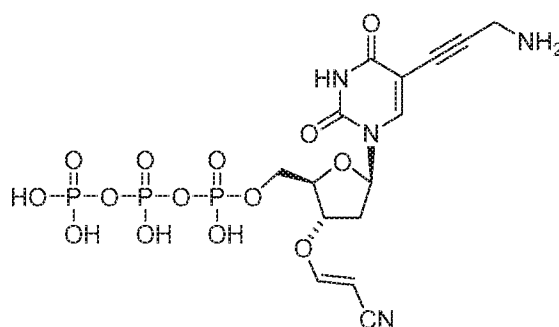
[0137] A suspension of 5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxy-3'-*O*-formyl-5-(*N*-trifluoroacetyl-3-aminopropynyl)uridine (0.44 g, 0.84 mmol) and (triphenylphosphoranylidene)acetonitrile (1.02 g, 3.38 mmol) in toluene (20 mL) in a sealed microwave vial was heated at 115 °C overnight. The orange solution was concentrated under reduced pressure to give a brown solid. This material was purified using a Biotage Isolera automated chromatography system under normal phase conditions (silica column, gradient of 15 → 100 % ethyl acetate in petrol) with detection at 254 nm to give (*E*)-5'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*-cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)uridine (210 mg, 46 %), as an off-white solid. *R*_f 0.55 (petrol - ethyl acetate, 4 : 6, v/v).

[0138] (*E*)-3'-*O*-Cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-amino propynyl)uridine



[0139] To a solution of (*E*)-5'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*-cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)uridine (210 mg, 0.38 mmol) in anhydrous tetrahydrofuran (6.8 mL) was added a solution of 1 M tetrabutylammonium fluoride in tetrahydrofuran (464 µL, 0.46 mmol) and the mixture was stirred at room temperature for 50 minutes. The resulting orange-red solution was concentrated under reduced pressure to give a dark brown oil. This residual material was purified using a Biotage Isolera automated chromatography system under normal phase conditions (silica column, gradient of 2 % to 20 % methanol in dichloromethane) with detection at 254 nm to give (*E*)-3'-*O*-cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-amino propynyl)uridine (98 mg, 59 %), as an off-white solid. *R*_f 0.43 (dichloromethane - methanol, 9 : 1, v/v).

[0140] (*E*)-5-Aminopropargyl-3'-*O*-cyanoethenyl-2'-deoxy-dTTP

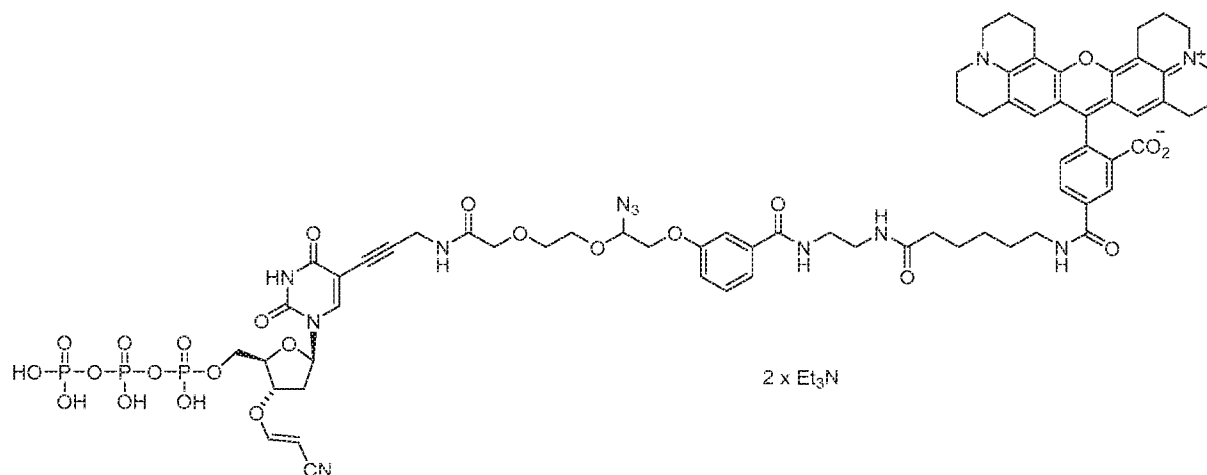


[0141] (*E*)-3'-*O*-Cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)uridine (40 mg, 0.09 mmol) was dissolved in 1,4-dioxane (0.28 mL) and pyridine (0.11 mL) and the flask was evacuated and purged with nitrogen. A 1.0 M salicyl chlorophosphite solution in 1,4-dioxane (103 μ L, 0.10 mmol) was added and the reaction mixture was stirred for 10 min. A 0.5 M tributylammonium pyrophosphate solution in DMF (0.28 mL, 0.14 mmol) and tributylamine (73 mg, 93 μ L, 0.39 mmol) were added and the reaction mixture was stirred for 15 min. A 1 % iodine solution in pyridine and water (1.98 mL, 98 : 2) was added and the solution was stirred for 15 minutes before being quenched with 5 % aqueous sodium thiosulfate solution (0.15 mL) and concentrated under reduced pressure. The residual material obtained was purified using a Biotage Isolera automated chromatography system under reversed-phase conditions (C₁₈ column, gradient of 0 % to 100 % acetonitrile in 0.1 M TEAB at pH 7.0) with detection at 254 nm to afford, after lyophilizing, impure (*E*)-3'-*O*-cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)dTTP (54 mg). This crude material was purified by semi-preparative HPLC injecting 100 μ L portions and collecting the eluent containing the pure substance. The combined fractions were reduced in volume by removing the acetonitrile and most of the water and finally lyophilized to give tri-tributylammonium (*E*)-3'-*O*-cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)dTTP (36 mg), as a white solid. The HPLC conditions were as follows: column: ES Industries Sonoma C18 10 μ , 15 mm $\times$ 250 mm; solvent gradient: 89 % 0.1 M aqueous TEAB (pH 7) to 87.2 % 0.1 M aqueous TEAB (pH 7) over 27 minutes with the balance being acetonitrile; flow rate: 7.8 mL/min; temp: 30 $^{\circ}$ C; and detection: UV at 315 nm. Under these conditions the product had a retention time of $\sim$ 21 min.

[0142] To a solution of di-tributylammonium (*E*)-3'-*O*-cyanoethenyl-2'-deoxy-5-(*N*-trifluoroacetyl-3-aminopropynyl)dTTP (36 mg) in water was added concentrated ammonia (1.5 mL, 35 % in water) and the mixture was stirred at room temperature for 2.5 hours. The

resulting material was purified by semi-preparative HPLC, injecting 100 μ L portions and collecting the eluent containing the pure substance. The combined fractions were reduced in volume by removing the acetonitrile and most of the water and finally lyophilized to give di-triethylammonium (*E*)-5-aminopropargyl-3'-*O*-cyanoethenyl-2'-deoxy-dTTP (17 mg, 24 %), as a white solid. The HPLC conditions were as follows: column: Phenomenex Luna C18(2), 15 mm $\times$ 250 mm; solvent gradient: 94 % 0.1 M aqueous TEAB (pH 7) to 93.1 % 0.1 M aqueous TEAB (pH 7) over 18 min with the balance being acetonitrile; flow rate: 7.8 mL/min; temp: 30 $^{\circ}$ C; and detection: UV at 315 nm. Under these conditions the product had a retention time of $\sim$ 14.5 min.

[0143] **Di-triethylammonium 3'-*O*-cyanoethenyl-dTTP-linker-ROX**

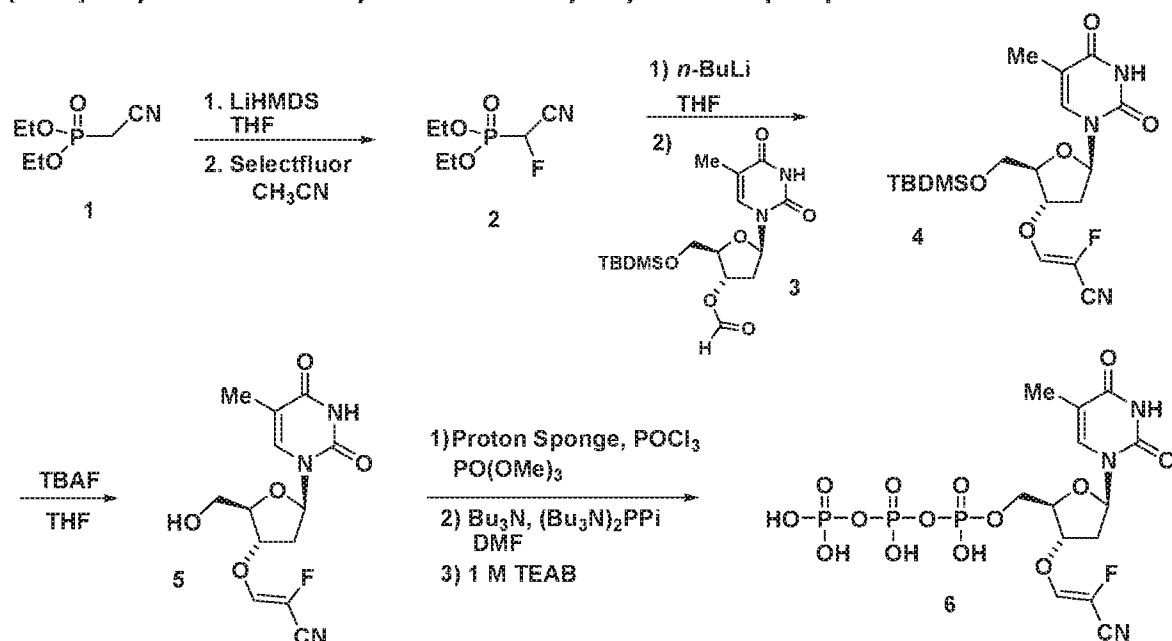


[0144] To a solution of ROX-linker-OH (10 mg, 0.01 mmol) in DMF (1 mL) was added *N,N'*-disuccinimidyl carbonate (3.9 mg, 0.015 mmol) and 4-(dimethylamino)pyridine (1.8 mg, 0.015 mmol) and the mixture was stirred at room temperature for 3 hours. After this time, TLC (dichloromethane - methanol, 75 : 25, v/v) showed the disappearance of the starting material. A solution of di-triethylammonium (*E*)-5-aminopropargyl-3'-*O*-cyanoethenyl-2'-deoxy-dTTP (5.4 mg, 0.007 mmol) in 0.1 M TEAB solution at pH = 8 was added and the mixture was stirred at room temperature for 3 hours. The solution was concentrated under reduced pressure to give a purple solid. This material was purified by semi-preparative HPLC injecting 90 μ L portions and collecting the eluent containing the pure substance. The combined fractions were reduced in volume by removing the acetonitrile and most of the water and finally lyophilized to give di-triethylammonium 3'-*O*-cyanoethenyl-dTTP-linker-ROX (6.5 mg, 50 %), as a purple solid. The HPLC conditions were as follows: column: ES Industries Sonoma C18 10 μ , 15 mm $\times$ 250 mm ;

solvent gradient: 90 % 0.1 M aqueous TEAB (pH 7) to 60 % 0.1 M aqueous TEAB (pH 7) over 24 min with the balance being acetonitrile; flow rate: 7.8 mL/min; temp: 30 °C; and detection: UV at 530 nm. Under these conditions the product had a retention time of ~ 22.5 min

[0145] **Example 5: Synthesis of Analogues**

[0146] **Synthesis of 3'-O-Cyanofluoroethenyl-thymidine Triphosphate 6**



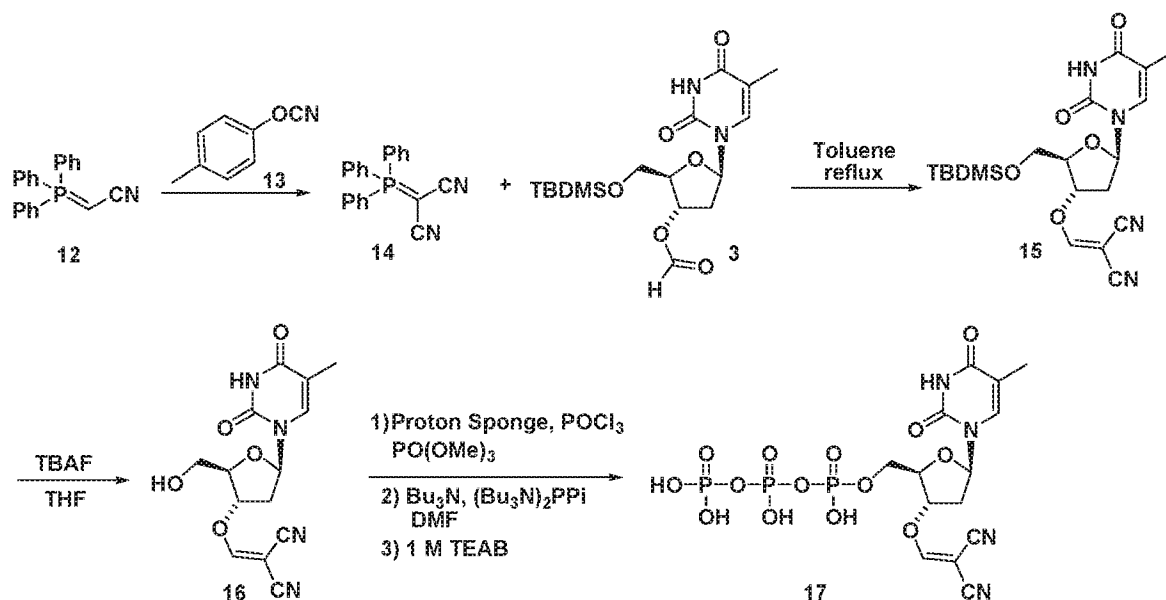
[0147] **1 to 2:** Diethyl (cyanomethyl)phosphonate **1** was dissolved in tetrahydrofuran (THF). LiHMDS (1.2eq) was added and then the slow addition of a solution of Selectfluor (2eq) in acetonitrile gave the fluorinated Horner-Wadsworth Emmons reagent **2** in 22 % yield after column chromatography.

[0148] **2 to 4:** **2** was dissolved in THF and then BuLi (1.2 eq) was added and stirred for 30 minutes at 0 °C. Then, 3'-O-formyl-5'-O-TBDMS-thymidine **3** was added and stirred overnight at room temperature to afford 5'-O-TBDMS-3'-O cyanofluoroethenyl-thymidine **4** in 50 % yield (isolated yield) as a cis (JHF = 4.1 Hz) and trans (JHF = 19.1 Hz) mixture in a 65 to 35 ratio.

[0149] **4 to 5:** To a solution of **4** in anhydrous tetrahydrofuran was added a solution of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (1.2 eq) and the mixture was stirred at room temperature for 50 minutes. The resulting solution was concentrated under reduced pressure and was purified by silica column to give **5** in 92% yield.

[0150] **5 to 6:** Nucleoside **5** and proton sponge (2.0 eq) were dissolved in dry acetonitrile (ACN) and rotovaped to dryness to remove H₂O. This was repeated 3 times. The mixture was further dried under high vacuum overnight. The mixture was then dissolved in 3 mL PO(OMe)₃ under N₂. To this solution was added POCl₃ (1.5 eq) at 0 °C and stirring was continued for 4 hours at 0 °C. In another flask, (Bu₃N)₂PPI (5 eq) was dissolved in 3 mL dry DMF under N₂ and Bu₃N (10 eq) was then added. The nucleoside reaction mixture was taken up in a syringe and added to the solution of (Bu₃N)₂PPI at 0 °C and stirring was continued for 30 minutes at 0 °C. The reaction was quenched with 10 mL TEAB (1 M, pH = 7.5) at 0 °C and stirring was continued for 1 hour at 0 °C. The final reaction mixture was diluted with deionized-H₂O loaded on a DEAE column (GE healthcare DEAE Sephadex A-25). The column was eluted with 100 mL deionized-H₂O then TEAB solution (0.1 M to 0.2 M to 0.4 M to 0.6 M to 0.8 M). The fractions were collected using UV monitor. The combined fractions were rotovaped or lyophilized to remove the TEAB buffer. The residue was further purified with C¹⁸ Prep RP-HPLC.

[0151] **Synthesis of 3'-O-Biscyanoethenyl-thymidine Triphosphate 17**



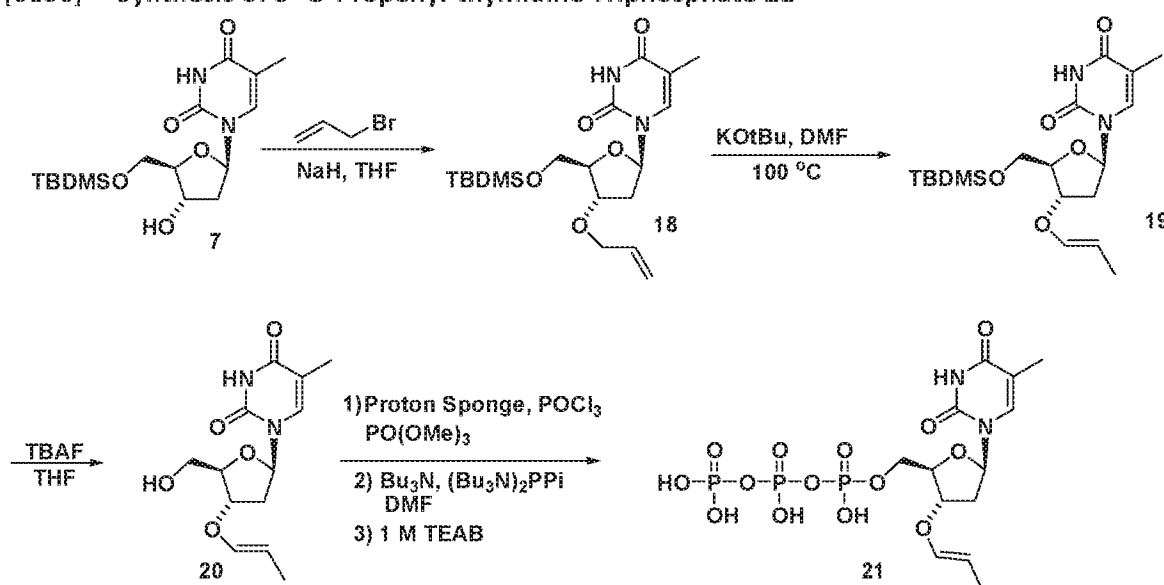
[0152] **12 to 14:** **12** and **13** were mixed to give **14**.

[0153] **14 to 15:** A suspension of **3** and **14** (4 eq) in toluene in a sealed microwave vial was heated at 115 °C overnight. The resulted solution was concentrated under reduced pressure and purified by silica column to give **15**.

[0154] **15 to 16:** To a solution of **15** in anhydrous tetrahydrofuran was added a solution of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (1.2 eq) and the mixture was stirred at room temperature for 50 minutes. The resulting solution was concentrated under reduced pressure and was purified by silica column to give **16**.

[0155] **16 to 17:** The same procedure used above for the conversion of **5** to **6** in the synthesis of 3'-O-Cyanofluoroethenyl-thymidine Triphosphate was used.

[0156] **Synthesis of 3'-O-Propenyl-thymidine Triphosphate 21**



[0157] **7 to 18:** Thymidine **7** was dissolved in THF and was cooled to 0 °C. To the cooled mixture was added NaH (2eq) and the resulting mixture was stirred at room temperature for 0.5 hours. Then allyl bromide **8** was added at 0 °C and stirred overnight at room temperature. The reaction was quenched with NaHCO₃(aq) at 0 °C and extracted with EtOAc. The organic phase was collected and concentrated for purification by silica gel column chromatography to give **18**.

[0158] **18 to 19:** **18** was dissolved in DMF and was cooled to 0 °C. To the resulting mixture was added 0.2 eq KOtBu and the mixture was stirred at 100 °C overnight. The reaction was cooled and quenched with NaHCO₃(aq) at 0 °C, and was extracted with EtOAc. The organic phase was collected and concentrated for purification by silica gel column chromatography to give **19**.

[0159] **19 to 20:** To a solution of **19** in anhydrous tetrahydrofuran was added a solution of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (1.2 eq) and the mixture was

stirred at room temperature for 50 minutes. The resulting solution was concentrated under reduced pressure and was purified by silica gel column chromatography to give **20**.

[0160] **20** to **21**: The same procedure used above for the conversion of **5** to **6** in the synthesis of 3'-O-Cyanofluoroethenyl-thymidine Triphosphate was used.

[0161] **Example 6: Cleavage Studies**

[0162] Cleavage studies were performed according to the scheme shown below using a variety of nucleophiles. The nucleophiles, concentration, pH, and solvent are indicated for each study. The nucleoside analogue containing a 3'-O-blocking group and a linking group were treated with a nucleophile at 50 °C in solvent to form analogues having both the blocking group and linking group cleaved **13** and only the linking group cleaved **15**. The amount of the product obtained after 0.15 hours, 0.5 hours, 1 hour, and 18 hours is indicated in the table. SM indicates starting material.

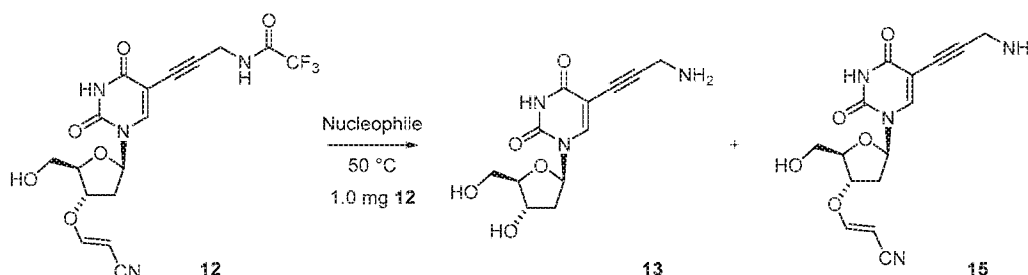


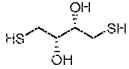
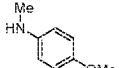
Table of Results

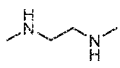
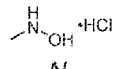
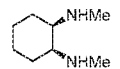
(Experimental Conditions: T = 50 °C; 1.0 mg **12** / mL solvent).

Entry	Nucleophile	Concn /M	pH	Solvent	t = 0.15 h	t = 0.5 h	t = 1 h	t = 18 h
1	L-Proline	1.0	6.7	Water + 20% MeCN	SM	SM	N/A	unidentified species (mass = 299)
2	L-Proline	1.0	9.0	Water + 20% MeCN	SM	SM + 5 % of unidentified species	SM + 30 % of unidentified species	unidentified species as main product (mass = 299)

3	L-Proline	5.0	9.0	Water + 20% MeCN	SM + 5 % of unidentifi ed species	SM + 15 % of unidentifi ed species	30 % SM + multiple peaks	No SM. Two peaks with mass = 299 and 183?
4	L-Proline	10.0	9.0	Water + 20% MeCN	SM + 5 % of unidentifi ed species	SM + 15 % of unidentifi ed species	30 % SM + multiple peaks	No SM. Two peaks with mass = 299 and 183?
5	 Cyclic thiourea	1.0	7.5	Water + 20% MeCN	SM	SM	SM	SM
6	 Cyclic thiourea	1.0	7.5	Water/Me OH 1:1	SM	SM	SM	SM
7	L-Cysteine	1.0	7.0	Water	No SM unidentifi ed species	No SM unidentifi ed species	No SM unidentifi ed species	Product (?) mass = 281
8	L-Cysteine	1.0	9.0	Water	No SM unidentifi ed species	No SM unidentifi ed species	No SM unidentifi ed species	Product (?) mass = 281
9	Pyrrolidine	1.0	7.0	Water	SM	SM	SM + 2 % of unidentifi ed species	15 % SM + unidentifi ed species
10	Pyrrolidine	1.0	9.0	Water	SM	SM + 15 % and 10 % of unidentifi	25 % SM + 20 %, 25 % and 20 % of	No SM + 80 % unidentifi ed

						ed species	unidentifi ed species	species (mass = 299)
11	Dimethylamine (control)	40 eq.	12.0	Water / MeCN (1 : 1)	No SM. 1 to1 mixture of 13 and 14	One peak corresponds to 13 mass = 281	N/A	N/A
12	Na ₂ S ₂ O ₃ Thiosulfate	1.0	7.0	Water + 10% MeCN	SM	SM	N/A	Mixture of SM and 15 (7 : 3)
13	Na ₂ S ₂ O ₃ Thiosulfate	1.0	9.0	Water + 10% MeCN	SM	SM	N/A	Mixture of SM and 15 (8 : 2)
14	Na ₂ SO ₃ Sodium Sulfite	1.0	7.0	Water + 10% MeCN	No SM unidentified species	No SM unidentified species	N/A	No SM unidentified species
15	Na ₂ SO ₃ Sodium Sulfite	1.0	9.0	Water + 10% MeCN	No SM unidentified species	No SM unidentified species	N/A	No SM unidentified species
16	NaN ₃ Sodium azide	1.0	7.0	Water + 10% MeCN	SM	SM	N/A	Mixture of SM and 15 (4 : 6)
17	NaN ₃ Sodium azide	1.0	9.0	Water + 10% MeCN	SM	SM	N/A	Mixture of SM and 15 (1 : 9)
18	EtNO ₂ Nitroethane	1.0	7.0	Water + 10% MeCN	40 % SM + 50 % and 10 % of unidentified species	40 % SM + 50 % and 10 % of unidentified species	N/A	30 % SM + 55 % and 15 % of unidentified species

19	EtNO ₂ Nitroethane	1.0	9.0	Water + 10% MeCN	40 % SM + 5 % and 55 % of unidenti- fied species	40 % SM + 5 % and 55 % of unidenti- fied species	N/A	40 % SM + 5 % and 55 % of unidenti- fied species
20	CH ₂ (CN) ₂ Malononitrile	1.0	7.0	Water + 10% MeCN	35 % SM + 65 % of unidenti- fied species	25 % SM + 75 % of unidenti- fied species	N/A	20 % SM + 80 % of unidenti- fied species
21	CH ₂ (CN) ₂ Malononitrile	1.0	9.0	Water + 10% MeCN	5 % SM + 90 % and 5% of unidenti- fied species	5 % SM + 90 % and 5% of unidenti- fied species	N/A	5 % SM + 90 % and 5% of unidenti- fied species
22	 Morpholine	1.0	9.0	Water + 10% MeCN	N/A	N/A	25 % SM + 40 % of (15) + 35 % of unidenti- fied species	No SM 40 % of (15) + 60 % of unidenti- fied species
23	Diethylamine	1.0	9.0	Water + 10% MeCN	N/A	N/A	90 % SM + 10 % of (15)	20 % SM + 50 % of (15) + 30 % of unidenti- fied species
24	 Cleland's reagent (dithiothreito l)	1.0	9.0	Water + 10% MeCN	N/A	N/A	5 % SM + 95 % of unidenti- fied species	No SM + unidenti- fied species
25	 4-Methoxy- N-	1.0	8.3	Water / MeCN (1:2)	N/A	N/A	95 % SM + 5 % of unidenti- fied	95 % SM + 5 % of unidenti- fied

	methylaniline						species	species
26	 <i>N,N'</i> - Dimethylene diamine	1.0	9.0	Water + 10% MeCN	N/A	N/A	5 % SM + 20 % of (15) + 75 % of unidenti- fied species	No SM + 20 % of (15) + 80 % of unidenti- fied species
27	 <i>N</i> - Methylhydro- xyl amine hydrochlorid- e	1.0	9.0	Water + 10% MeCN	N/A	N/A	16 % Product (13) + 10 % of (15) + 74 % of unidenti- fied species	10 % of (15) + 90 % of unidenti- fied species
28	 <i>N,N'</i> - dimethyl- <i>cis</i> - cyclohexane- 1,2-diamine	1.0	9.0	Water / MeCN (2:1)	N/A	N/A	60 % SM + 35 % of (15) + 5 % of unidenti- fied species	30 % SM + 50 % of (15) + 20 % of unidenti- fied species

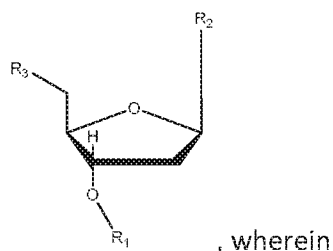
[0163] The 3'-O-Cyanoethyl group could be cleaved in each of the following buffers in solution or on Biochips: (1) 6M Dimethylamine (DMA), pH = 10.4, pH was adjust by adding NaOH, 55-60 °C, 10 minutes; (2) 2. 2.5M MeNHOH, pH = 8, pH was adjust by adding NaOH, 55-60 °C, 10mins; (3) 200 mM THPP, 0.2 M Tris, pH = 9.5, 1.5 M NaCl, 55-60 °C, 10 mins; or (4) 200 mM THPP, 1 M K₂HPO₄, 1M KCl, pH = 9.5, 55-60 °C, 5 mins. K₄PPI and F⁻ also showed an improved effect for cleavage efficiency; however, the reagents resulted in damage on the biochip surface. See Figures 2 and 3. The increase of THPP concentration (Figure 4), cleavage time (Figure 5) and higher pH (no more than 10; Figure 6) resulted in higher cleavage efficiency. Other reagents tested for 3'-O-Cyanoethyl-dNTP cleavage on biochips, as shown in Figure 7, all showed very poor efficiency.

[0164] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will

appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference. Where a conflict exists between the instant application and a reference provided herein, the instant application shall dominate.

WHAT IS CLAIMED IS:

1. A nucleoside analogue of the following formula:



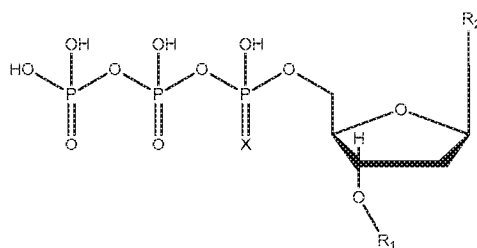
R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

R_2 comprises a nucleobase; and

R_3 is a cleavable linking moiety comprising at least three phosphates, or analogues thereof.

2. The nucleoside analogue of claim 1, wherein R_3 comprises a 5'-O-1-thio triphosphate.

3. The nucleoside analogue of claim 1, wherein the nucleoside has the formula of Formula VIII:



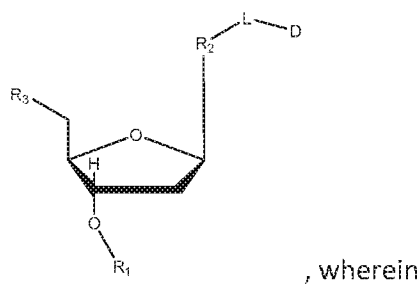
Formula VIII

; wherein

X is O or S and the nucleoside analogue is a substrate for a DNA polymerase.

4. The nucleoside analogue of claim 1, wherein R_2 consists of a nucleobase.
5. The nucleoside analogue of claim 1, wherein R_2 and/or R_3 is detectably labeled.

6. The nucleoside analogue of claim 1, wherein the nucleoside has the following formula:

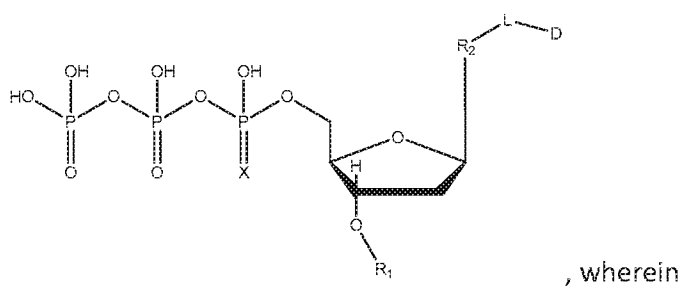


R_2 consists of the nucleobase;

L is a linker; and

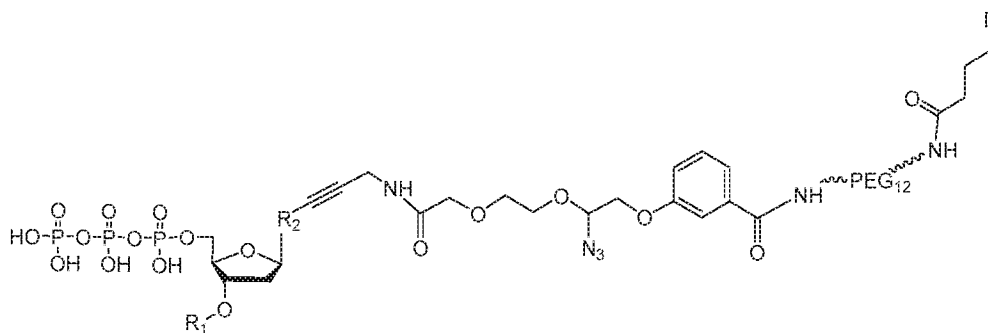
D is a detectable fluorescent label.

7. The nucleoside analogue of claim 6, wherein the nucleoside has the following formula:



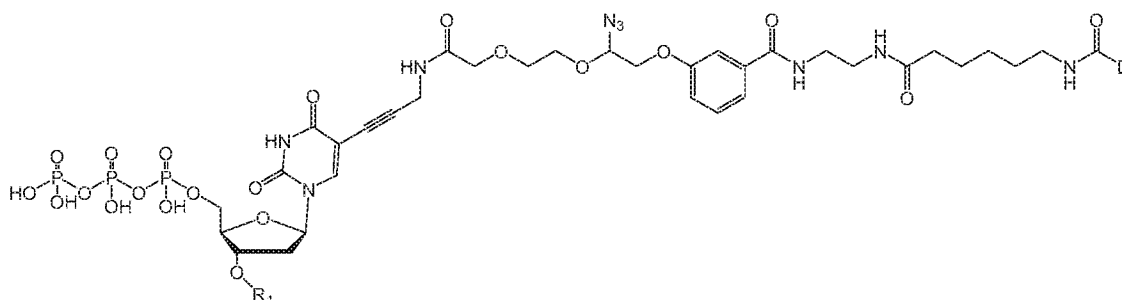
X is selected from the group consisting of O and S.

8. The nucleoside analogue of claim 7, wherein the nucleoside analogue comprises the following formula:

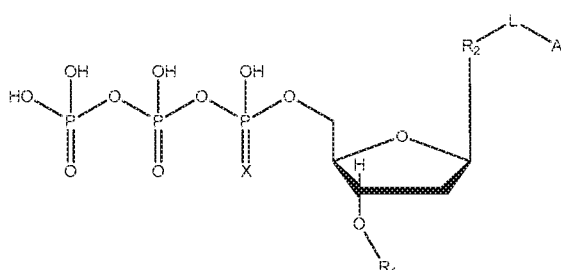


wherein R_2 is the nucleobase and D comprises a rhodamine dye or a derivative thereof.

9. The nucleoside analogue of claim 7, wherein the nucleoside analogue comprises the following formula:



10. A nucleoside analogue of Formula VI:



Formula VI

, wherein

R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

R_2 is a nucleobase;

L is a linker;

A_1 comprises an affinity tag;

X is selected from the group consisting of O and S; and

the nucleoside analogue is a substrate for a DNA polymerase.

11. The nucleoside analogue of claim 10, wherein L is a cleavable linker.

12. The nucleoside analogue of claim 11, wherein R_1 and L can be cleaved from the nucleoside analogue under the same conditions.

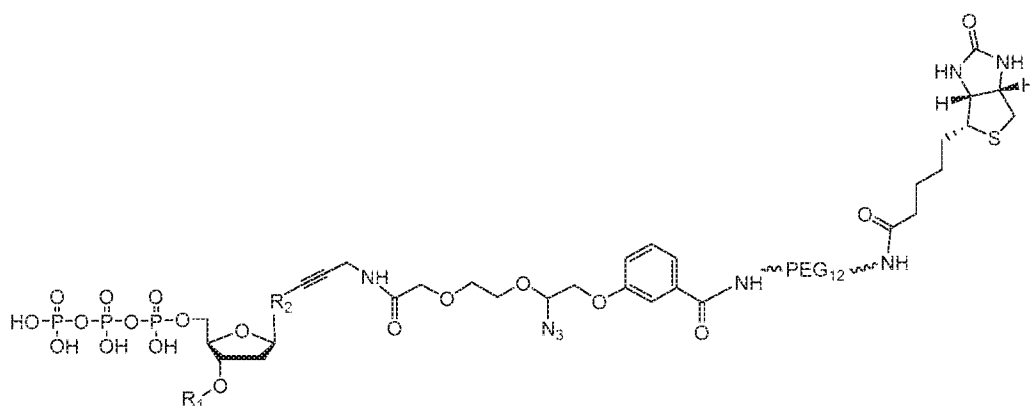
13. The nucleoside analogue of claim 10, wherein A₁ comprises a nitrilotriacetic acid (NTA) affinity tag, or a peptide affinity tag comprising at least six contiguous histidine amino acids.

14. The nucleoside analogue of claim 10, wherein A₁ comprises an affinity tag selected from the group consisting of biotin, a small molecule antigen, and a peptide.

15. The nucleoside analogue of claim 14, wherein the small molecule antigen is selected from the group consisting of a fluorophore, an amphetamine, a barbiturate, a benzodiazepine, a cocaine metabolite, a cannabinoid, a cannabinoid metabolites, tetrahydrocannabinol, methadone, an opiate, propoxyphene, phencyclidine, digoxigenin, and DNP.

16. The nucleoside analogue of claim 14, wherein the peptide is selected from the group consisting of a His tag, a Myc tag, a Flag tag, an HA tag, a V5 tag, an AviTag, a calmodulin tag, an E tag, an S tag, an SBP tag, a Softag, a Strep tag, a TC tag, a VSV tag, an Xpress tag, glutathione, an isopeptag, and a SpyTag.

17. The nucleoside analogue of claim 10, wherein the nucleoside analogue comprises the following formula:

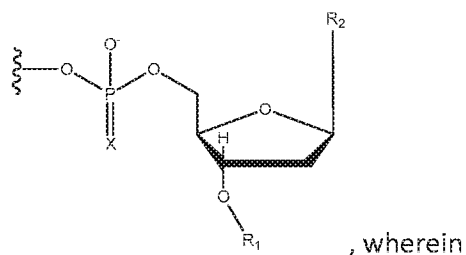


wherein R₂ is the nucleobase.

18. The nucleoside analogue of claim 17, wherein the nucleobase is selected from the group consisting of a 7-substituted 7-deaza adenine analogue, a 7

substituted 7-deaza guanine analogue, a 5-substituted thymine, and a 5-substituted cytosine.

19. A composition comprising a nucleoside analogue of the following formula:



R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

X is selected from the group consisting of O and S; and

R_2 comprises a nucleobase, and optionally a linker and a detectable label or affinity agent,

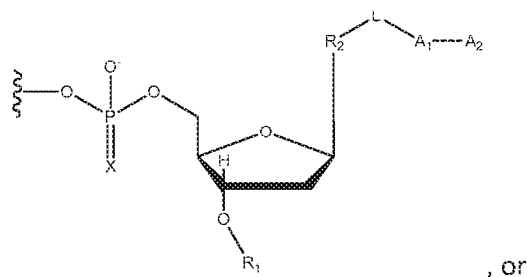
wherein the nucleoside analogue is covalently linked via the 5' phosphate or thiophosphate to an oligonucleotide.

20. The composition of claim 19, wherein R_2 consists of a nucleobase.

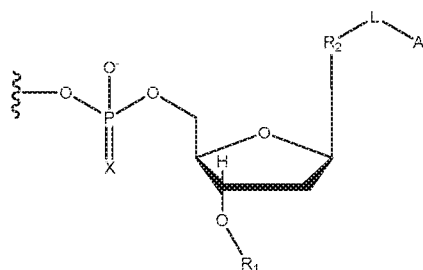
21. The composition of claim 19, wherein R_2 comprises a nucleobase, a linker, and a detectable label or affinity agent.

22. A composition comprising

i) a nucleoside analogue of the following formula:



ii) a nucleoside analogue of Formula IX:

**Formula IX**

; wherein

R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

X is selected from the group consisting of O and S;

R_2 is a nucleobase;

L is a linker;

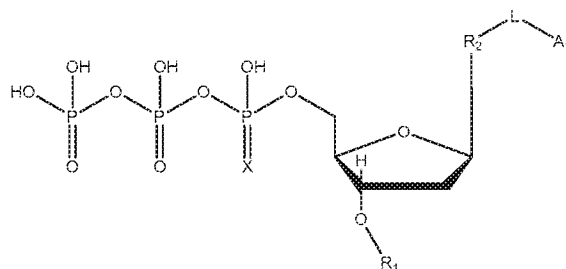
A_1 comprises an affinity tag; and

A_2 comprises a detectably labeled affinity agent that forms a specific and non-covalent complex with A_1 , wherein the nucleoside analogue is covalently linked via the 5' phosphate or thiophosphate to an oligonucleotide.

23. The composition of claim 22, wherein A_1 comprises a fluorescent dye selected from the group consisting of a fluorone dye, a rhodamine dye, a cyanine dye, a coumarin dye, a phycoerythrin, and an allophycocyanine.

24. A method of sequencing comprising:

i) providing a reaction mixture comprising template nucleic acid, a primer, a polymerase, and a first nucleoside analogue of Formula VI:

**Formula VI**

, wherein

R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

X is selected from the group consisting of O and S;

R_2 is a nucleobase;

L is a linker; and

A_1 comprises an affinity tag;

ii) extending the primer by incorporating the first nucleoside analogue with the polymerase;

iii) contacting the incorporated first nucleoside analogue with a detectably labeled affinity agent that forms a specific and non-covalent complex with A_1 of the incorporated first nucleoside analogue, thereby specifically labeling the incorporated first nucleoside analogue; and

iv) detecting the specifically labeled incorporated first nucleoside analogue.

25. The method of claim 24, wherein the detectably labeled affinity agent is fluorescently labeled, and the detection comprises detecting a fluorescence emission from the fluorescently labeled affinity agent in complex with A_1 of the incorporated first nucleoside analogue.

26. The method of claim 24, wherein the method further comprises:

v) cleaving the reversible blocking group R_1 of the incorporated first nucleoside analogue thereby removing the blocking group from the incorporated first nucleoside analogue;

vi) cleaving the linker L thereby removing the affinity tag A_1 of the incorporated first nucleoside analogue, or quenching the label of the detectably labeled affinity agent in complex with A_1 of the incorporated first nucleoside analogue;

vii) providing a second nucleoside analogue of Formula VI and a polymerase;

viii) extending the primer by incorporating the second nucleoside analogue with the polymerase;

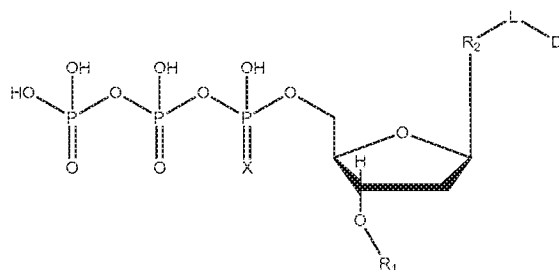
ix) contacting the incorporated second nucleoside analogue with a detectably labeled affinity agent that forms a specific and non-covalent complex with A_1 of the

incorporated second nucleoside analogue, thereby specifically labeling the incorporated second nucleoside analogue; and

x) detecting the specifically labeled incorporated second nucleoside analogue.

27. A method of sequencing comprising:

i) providing a reaction mixture comprising template nucleic acid, a primer, a polymerase, and a first nucleoside analogue of Formula VII:



Formula VII

, wherein

R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

X is selected from the group consisting of O and S; and

R_2 comprises a nucleobase;

L is a linker; and

D comprises a detectable fluorescent label;

ii) extending the primer by incorporating the first nucleoside analogue with the polymerase; and

iii) detecting the labeled incorporated first nucleoside analogue by detecting a fluorescence emission from the fluorescent label.

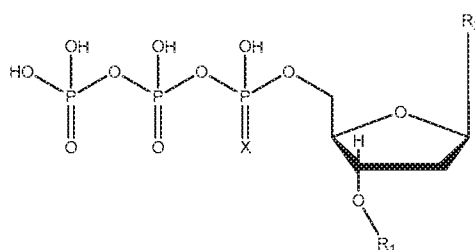
28. The method of claim 27, wherein the method further comprises:

iv) cleaving the reversible blocking group R_1 of the incorporated first nucleoside analogue thereby removing the blocking group from the incorporated first nucleoside analogue;

- v) providing a second detectably labeled nucleoside analogue of Formula VII and a polymerase;
- vi) extending the primer by incorporating the second nucleoside analogue with the polymerase; and
- vii) detecting incorporation of the second nucleoside analogue by detecting a fluorescence emission from the labeled incorporated second nucleoside analogue.

29. A method of sequencing comprising:

- i) providing a reaction mixture comprising template nucleic acid, a primer, a polymerase, and a first nucleoside analogue of Formula VIII:



Formula VIII

, wherein

R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

X is selected from the group consisting of O and S; and

R_2 comprises or consists of a nucleobase; and

- ii) extending the primer by incorporating the first nucleoside analogue with the polymerase, thereby generating pyrophosphate; and
- iii) detecting incorporation of the first nucleoside analogue by detecting the pyrophosphate.

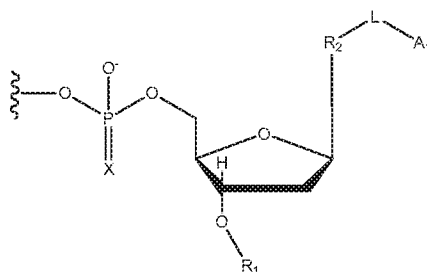
30. The method of claim 29, wherein the method further comprises:

- iv) cleaving the reversible blocking group R_1 of the incorporated first nucleoside analogue thereby removing the blocking group from the incorporated first nucleoside analogue;
- v) providing a second nucleoside analogue of Formula VIII and a polymerase;

vi) extending the primer by incorporating the second nucleoside analogue with the polymerase, thereby generating pyrophosphate; and
 vii) detecting incorporation of the second nucleoside analogue by detecting the pyrophosphate.

31. A method of sequencing comprising:

i) providing a reaction mixture comprising template nucleic acid, a primer, a ligase, and an oligonucleotide comprising a first nucleoside analogue of Formula IX:



Formula IX

, wherein

R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

X is selected from the group consisting of O and S;

R_2 is a nucleobase;

L is a linker; and

A_1 comprises an affinity tag;

ii) hybridizing the oligonucleotide comprising the first nucleoside analogue of Formula IX to the template nucleic acid at a position 3' of, and adjacent to, the primer;

iii) cleaving the reversible blocking group R_1 of the hybridized oligonucleotide comprising the first nucleoside analogue of Formula IX;

iv) ligating the hybridized oligonucleotide to the adjacent primer with the ligase, thereby incorporating the first nucleoside analogue of Formula IX into the primer;

v) contacting the incorporated first nucleoside analogue with a detectably labeled affinity agent that forms a specific and non-covalent complex with A of the incorporated first nucleoside analogue, thereby specifically labeling the incorporated first nucleoside analogue; and

vi) detecting the specifically labeled incorporated first nucleoside analogue.

32. The method of claim 31, wherein the detectably labeled affinity agent is fluorescently labeled, and the detection comprises detecting a fluorescence emission from the fluorescently labeled affinity agent in complex with A of the incorporated first nucleoside analogue.

33. The method of claim 31, wherein the method further comprises:

vii) cleaving the linker L thereby removing the affinity tag A₁ of the incorporated first nucleoside analogue, or quenching the label of the detectably labeled affinity agent in complex with A₁ of the incorporated first nucleoside analogue;

viii) providing a second nucleoside analogue of Formula IX and a ligase;

ix) hybridizing an oligonucleotide comprising a second nucleoside analogue of Formula IX to the template nucleic acid at a position 3' of, and adjacent to, the primer;

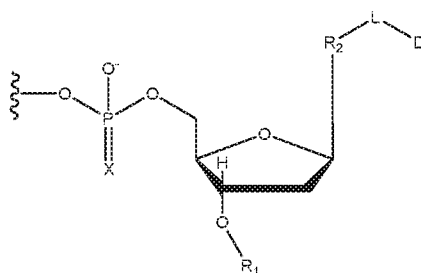
x) ligating the hybridized oligonucleotide to the adjacent primer with the ligase, thereby incorporating the second nucleoside analogue of Formula IX into the primer;

xi) contacting the incorporated second nucleoside analogue with a detectably labeled affinity agent that forms a specific and non-covalent complex with A₁ of the incorporated second nucleoside analogue, thereby specifically labeling the incorporated second nucleoside analogue; and

xii) detecting the specifically labeled incorporated second nucleoside analogue.

34. A method of sequencing comprising:

i) providing a reaction mixture comprising template nucleic acid, a primer, a ligase, and an oligonucleotide comprising a first nucleoside analogue of Formula X:



Formula X

, wherein

R_1 is a reversible blocking group selected from the group consisting of cyanoethenyl, allenyl, cyanoethyl, formaldehyde oximyl, acrylaldehyde oximyl, propionaldehyde oximyl, and cyanoethenaldehyde oximyl;

X is selected from the group consisting of O and S;

R_2 is a nucleobase;

L is a linker; and

D comprises a fluorescent detectable label;

ii) hybridizing the oligonucleotide comprising the first nucleoside analogue of Formula X to the template nucleic acid at a position 3' of, and adjacent to, the primer;

iii) cleaving the reversible blocking group R_1 of the hybridized oligonucleotide comprising the first nucleoside analogue of Formula X;

iv) ligating the hybridized oligonucleotide to the adjacent primer with the ligase, thereby incorporating the first nucleoside analogue of Formula X into the primer; and

v) detecting the labeled incorporated first nucleoside analogue.

35. The method of claim 34, wherein the method further comprises:

vi) cleaving the linker, thereby releasing the label of the labeled incorporated first nucleoside analogue;

vii) providing a ligase, and a second oligonucleotide comprising a second nucleoside analogue of Formula X;

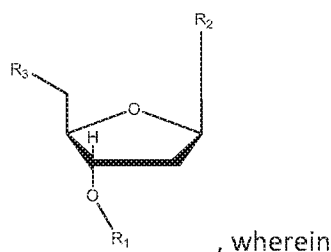
viii) hybridizing the second oligonucleotide to the template nucleic acid at a position 3' of, and adjacent to, the primer;

ix) cleaving the reversible blocking group R_1 of the hybridized second oligonucleotide;

x) ligating the hybridized second oligonucleotide to the adjacent primer with the ligase, thereby incorporating the second nucleoside analogue of Formula X into the primer; and

xi) detecting the labeled incorporated second nucleoside analogue.

36. A nucleoside analogue of the following formula:

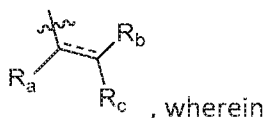


R_1 is a reversible blocking group selected from the group consisting of a substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted heteroalkenyl, or substituted or unsubstituted heteroalkynyl;

R_2 comprises a nucleobase; and

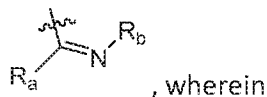
R_3 is a cleavable linking moiety comprising at least three phosphates, or analogues thereof.

37. The nucleoside analogue of claim 36, wherein R_1 is



R_a , R_b , and R_c are each individually selected from the group consisting of hydrogen, cyano, halogen, nitro, alkoxy, substituted or unsubstituted amino, substituted or unsubstituted sulfonyl, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, and substituted or unsubstituted carbonyl; and $\equiv$ is a single bond or a double bond.

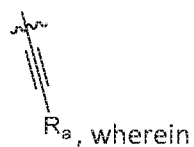
38. The nucleoside analogue of claim 36, wherein R_1 is



R_a is hydrogen, cyano, halogen, nitro, alkoxy, substituted or unsubstituted amino, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, or substituted or unsubstituted alkynyl; and

R_b is hydrogen, substituted or unsubstituted alkyl, or substituted or unsubstituted alkoxy.

39. The nucleoside analogue of claim 36, wherein R_1 is



R_a is hydrogen, cyano, halogen, nitro, alkoxy, substituted or unsubstituted amino, substituted or unsubstituted sulfonyl, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, and substituted or unsubstituted carbonyl.

40. The nucleoside analogue of claim 36, wherein R_1 is selected from the group consisting of cis-cyanoethenyl, trans-cyanoethenyl, allenyl, cis-cyanofluoroethenyl, trans-cyanofluoroethenyl, biscyanoethenyl, bisfluoroethenyl, cis-propenyl, trans-propenyl, nitroethenyl, acetoethenyl, methylcarboethenyl, amidoethenyl, methylsulfoethenyl, methylsulfoethyl, formimidate, formhydroxymate, vinyloethenyl, ethylenoethenyl, cyanoethylenyl, nitroethylenyl, and amidoethylenyl.

Row Labels	Average of Percent BIC	Average of FIT Score	Average of Mean signal TxR	Average of Mean signal FIT	Average of Mean signal Cy6	Average of Mean signal Cy3	Average of Mean Rho C	Average of Mean Rho T	Average of Mean Rho A	Average of Mean Rho G	Average of Mean Local Bgnd. Trd	Average of Mean Local Bgnd. Rho	Average of Mean Local Bgnd. Cy6	Average of Mean Local Bgnd. Cy3	Sum of Registration Status	Lane Condition
1	23.1433	0.21863	4419.803	6780.877	4596.43	5042.498	2701.999	1243.87	1480.09	1238.37	2884	3018.961	2717.56	2751.58	1059	Control Scan
1	92.96	0.87	8901.00	15910.82	9083.36	10151.83	10786.67	4971.76	5874.50	4873.24	3378.23	3875.69	3632.86	3376.33	530.00	Control
2	0.00	0.00	2891.65	3716.15	3107.48	3328.18	0.00	0.00	0.00	0.00	2299.70	2735.63	2436.23	2547.65	0.00	Alienyl dTTP Fresh
3	0.00	0.00	2829.48	3716.04	3029.19	3322.92	0.00	0.00	0.00	0.00	2272.61	2741.99	2388.72	2549.76	0.00	Alienyl dTTP Room Temp
4	0.00	0.00	2863.88	3734.19	3040.61	3334.22	0.00	0.00	0.00	0.00	2297.09	2745.40	2393.76	2558.17	0.00	Alienyl dTTP 65C
5	0.00	0.00	2868.48	3736.33	3082.46	3313.95	0.00	0.00	0.00	0.00	2294.71	2745.68	2422.04	2540.21	0.00	Cyanoethenyl-dTTP Fresh
6	0.00	0.00	3033.43	3715.02	3088.22	3319.66	0.00	0.00	0.00	0.00	2342.75	2725.15	2421.40	2544.51	0.00	Cyanoethenyl-dTTP Room Temp
7	0.00	0.00	2937.55	3747.99	3113.53	3321.36	0.00	0.00	0.00	0.00	2332.05	2743.48	2435.69	2540.56	0.00	Cyanoethenyl-dTTP 65C
8	92.19	0.88	9032.96	15970.48	9226.59	10248.73	10829.32	4977.56	5986.20	5033.73	3454.71	3838.67	3607.80	3355.42	529.00	Control
2	23.0955	0.21615	4441.666	6801.734	4695.86	4855.715	2585.915	1207.21	1428.97	1227.41	2626.33	2994.961	2784.84	2744.71	1059	After hot chase
1	92.88	0.87	8886.14	15473.52	9096.29	9892.74	10388.26	4824.34	5727.10	4910.50	3415.17	3838.61	3659.38	3329.67	530.00	Control
2	0.00	0.00	3007.42	3642.41	3219.38	3308.75	0.00	0.00	0.00	0.00	2380.88	2697.82	2500.34	2553.12	0.00	Alienyl dTTP Fresh
3	0.00	0.00	2949.10	3655.75	3210.08	3301.94	0.00	0.00	0.00	0.00	2349.77	2716.71	2504.75	2552.69	0.00	Alienyl dTTP Room Temp
4	0.00	0.00	2926.77	3624.94	3205.27	3302.74	0.00	0.00	0.00	0.00	2335.16	2680.71	2503.06	2558.94	0.00	Alienyl dTTP 65C
5	0.00	0.00	2957.49	3640.86	3188.97	3271.50	0.00	0.00	0.00	0.00	2348.50	2687.02	2479.68	2522.39	0.00	Cyanoethenyl-dTTP Fresh
6	0.00	0.00	2979.92	3684.04	3200.65	3305.25	0.00	0.00	0.00	0.00	2349.06	2706.94	2480.05	2540.91	0.00	Cyanoethenyl-dTTP Room Temp
7	0.00	0.00	2975.58	3697.34	3217.72	3316.09	0.00	0.00	0.00	0.00	2344.80	2709.44	2489.39	2542.88	0.00	Cyanoethenyl-dTTP 65C
8	91.89	0.86	8836.92	15395.02	9146.49	9946.71	10298.07	4733.30	5704.69	4908.74	3487.33	3842.43	3662.09	3357.04	529.00	Control
Grand Total	23.1194	0.21739	4430.755	6891.315	4641.14	4899.106	2543.957	1225.44	1454.53	1232.89	2605.17	3001.961	2751.2	2748.14	2118	

Figure 1

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P(100 mM)	pH	Buffer	Salt (M)	Temp. (°C)	Time (min)	Cleavage %
THPP	9.49	1M K ₂ HPO ₄	1M KCl	55	2	77
THPP	9.27	1M K ₄ PPi	1M KCl	55	2	95
200 mM TCEP	7.01	1M K ₂ HPO ₄	1M KCl	55	2	12
200 mM TCEP	9.15	1M K ₂ HPO ₄	1M KCl	55	2	22
200 mM THMP	9.06	1M K ₂ HPO ₄	1M KCl	55	2	11

Figure 2

THPP(mM)	pH	New (1 M)	Salt (M)	Tris (mM)	Tween 20	Temp. (°C)	Time (min)	Cleavage %
200	9.04	K ₂ HPO ₄	0	0	0.10%	60	2	85
200	9.85	K ₂ HPO ₄	0	200	0.10%	60	2	82
200	8.97	K ₂ HPO ₄	1M NaCl	0	0.10%	60	2	87
200	9.04	K ₂ HPO ₄	1M KF	0	0.10%	60	2	93
200	9.04	K ₂ HPO ₄	1M CsF	0	0.10%	60	2	101

Figure 3

THPP(mM)	pH	KF (M)	NaCl (M)	Tris (mM)	glycerol	Tween 20	Temp. (°C)	Time (min)	Cleavage %
20	9	1.5	0	200	5%	0.10%	55	10	46
40	9	1.5	0	200	5%	0.10%	55	10	64
60	9	1.5	0	200	5%	0.10%	55	10	77
80	9	1.5	0	200	5%	0.10%	55	10	84
100	9	1.5	0	200	5%	0.10%	55	10	89

Figure 4

THPP(mM)	pH	KF (M)	NaCl (M)	Tris (mM)	glycerol	Tween 20	Temp. (°C)	Time (min)	Cleavage %
100	9	1.5	0	200	5%	0.10%	55	2	44
100	9	1.5	0	200	5%	0.10%	55	4	62
100	9	1.5	0	200	5%	0.10%	55	6	74
100	9	1.5	0	200	5%	0.10%	55	8	82
100	9	1.5	0	200	5%	0.10%	55	10	89

Figure 5

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THPP(m M)	pH	K ₂ HPO ₄ (M)	CsF (M)	Tween 20	Temp. (°C)	Time (min)	Cleavage %
100	8.33	1	1	0.05%	60	1min15s	46
100	8.76	1	1	0.05%	60	1min15s	52
100	9.02	1	1	0.05%	60	1min15s	58
100	9.61	1	1	0.05%	60	1min15s	71
100	10.06	1	1	0.05%	60	1min15s	71

Figure 6

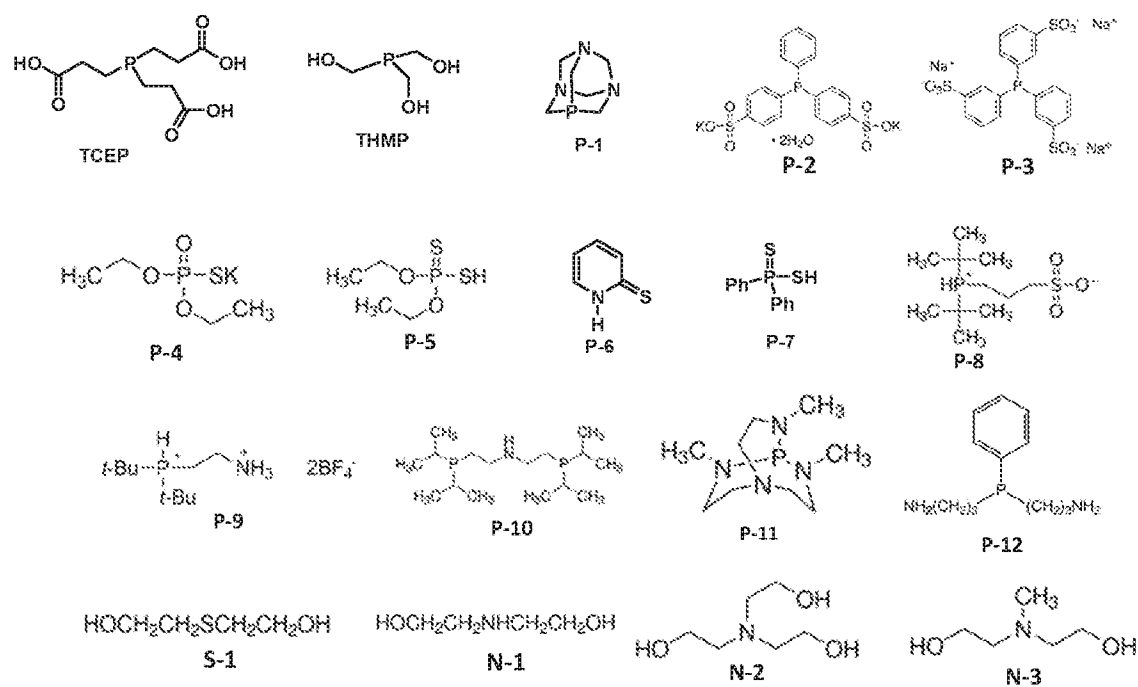


Figure 7

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US2017/028930

A. CLASSIFICATION OF SUBJECT MATTER

C07H 15/04 (2006.01) C07H 21/04 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: Chemical Abstract Registry and CAlplus. Substructure search based on compounds of claimed structures.

Patentscope and Espacenet: Applicant(s)/Inventor(s) name search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 July 2017	Date of mailing of the international search report 20 July 2017
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au	Authorised officer Ben Harris AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832272

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/US2017/028930
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KNAPP, D. C. et al., Fluoride-Cleavable, Fluorescently Labeled Reversible Terminators: Synthesis and Use in Primer Extension, Chemistry - A European Journal, 2011, vol. 17, no. 10, pages 2903-2915 Scheme 2 page 2905, Figure 2 page 2909, Fig. 5 and pages 2910-2911	1, 3, 5-7, 19, 21, 27-30, 36-37
Y	Scheme 2 page 2905, Figure 2 page 2909, Fig. 5 and pages 2910-2911	1-7, 10-16, 19-35 and 37
X	KNAPP, D. C. et al., Fluorescent labeling of (Oligo)nucleotides by a new fluoride cleavable linker capable of versatile attachment modes, Bioconjugate Chemistry, 2010, vol. 21, no. 6, pages 1043-1055 Scheme 6 page 1053 and pages 1052-1053	19 and 21
X	CAS RN 1288999-26-7, STN Entry Date 02 May 2011 Whole entry	1, 3, 5-7 and 36-37
X	CAS RN 1288999-24-5, STN Entry Date 02 May 2011 Whole entry	1, 3, 5-7 and 36-37
X	CAS RN 1288998-62-8, STN Entry Date 02 May 2011 Whole entry	1, 3, 5-7 and 36-37
X	CAS RN 1288998-56-0, STN Entry Date 02 May 2011 Whole entry	1, 3, 5-7 and 36-37
X	WO 2013/044018 A1 (ILLUMINA, INC.) 28 March 2013 pages 8-9 and examples on 50-60	36
Y	pages 8-9, examples on 50-60, and page 36 lines 21-26	1-7, 10-16, 19-35 and 37
X	WO 2010/110775 A1 (BENNER, STEVEN, ALBERT et al.) 30 September 2010 Figures 8-15 and pages 6-7	36
X	WO 2004/018497 A2 (SOLEXA LIMITED) 04 March 2004 example pages 46-112, and claim 1	36
X	WO 2007/053719 A2 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 10 May 2007 Figures 1-3, and claim 19	36
X	BI, L. et al., Design and Synthesis of a Chemically Cleavable Fluorescent Nucleotide, 3'-O-Allyl-dGTP-allyl-Bodipy-FL-510, as a Reversible Terminator for DNA Sequencing by Synthesis, Journal of the American Chemical Society, 2006, vol. 128, no. 8, pages 2542-2543 Scheme 1 page 2542 and abstract	36

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/US2017/028930	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2013/044018 A1	28 March 2013	WO 2013044018 A1	28 Mar 2013
		CA 2859660 A1	28 Mar 2013
		EP 2768972 A1	27 Aug 2014
		EP 2768972 B1	12 Jul 2017
		HK 1201079 A1	21 Aug 2015
		US 2013079232 A1	28 Mar 2013
		US 9453258 B2	27 Sep 2016
		US 2017022559 A1	26 Jan 2017
WO 2010/110775 A1	30 September 2010	WO 2010110775 A1	30 Sep 2010
		CN 102971335 A	13 Mar 2013
		CN 102971335 B	29 Jun 2016
		US 7544794 B1	09 Jun 2009
		US 2011275124 A1	10 Nov 2011
		US 8212020 B2	03 Jul 2012
WO 2004/018497 A2	04 March 2004	WO 2004018497 A2	04 Mar 2004
		AU 2002349155 A1	17 Jun 2003
		AU 2003259350 A1	11 Mar 2004
		AU 2003259352 A1	11 Mar 2004
		BR 0214683 A	23 Nov 2004
		CA 2469066 A1	12 Jun 2003
		CN 1617937 A	18 May 2005
		EP 1451351 A2	01 Sep 2004
		EP 1451351 B1	01 Feb 2017
		EP 1530578 A2	18 May 2005
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			
Form PCT/ISA/210 (Family Annex)(July 2009)			

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/US2017/028930	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
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