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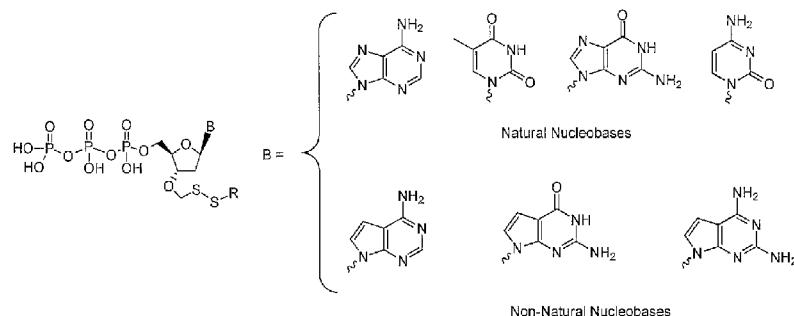
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(54) Title: NUCLEOTIDE ANALOGUES

FIGURE 1



(57) Abstract: The present invention provides methods, compositions, mixtures and kits utilizing deoxynucleoside triphosphates comprising a 3'-O position capped by a group comprising methylenedisulfide as a cleavable protecting group and a detectable label reversibly connected to the nucleobase of said deoxynucleoside. Such compounds provide new possibilities for future sequencing technologies, including but not limited to Sequencing by Synthesis.

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NUCLEOTIDE ANALOGUES

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Patent Applications No. 62/251,884 filed November 6, 2015 and 62/327,555 filed April 26, 2016, which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention provides methods, compositions, mixtures and kits utilizing deoxynucleoside triphosphates comprising a 3'-O position capped by group comprising methylenedisulfide as a cleavable protecting group and a detectable label reversibly connected to the nucleobase of said deoxynucleoside. Such compounds provide new possibilities for future sequencing technologies, including but not limited to Sequencing by Synthesis.

BACKGROUND OF THE INVENTION

DNA sequencing is one of the most important analytical methods in modern biotechnology. Detailed reviews on current sequencing technologies are provided in M. L. Metzker, *Nature Reviews* 2010, 11, 31 [1], and C. W. Fuller et al., *Nature Biotechnology* 2009, 27, 1013 [2].

A well-known sequencing method is the Sequencing-by-synthesis (SBS) method. According to this method, the nucleoside triphosphates are reversibly blocked by a 3'OH-protecting group, in particular esters and ethers. Examples for esters are alkanoic esters like acetyl, phosphates and carbonates. The nucleoside triphosphate usually comprises a label at

the base.

A method of enzymatically synthesizing a polynucleotide of a predetermined sequence in a stepwise manner using reversibly 3'OH-blocked nucleoside triphosphates was described by Hiatt and Rose (U.S. Patent No. 5,990,300) [3]. They disclose besides esters, ethers, carbonitriles, phosphates, phosphoramides, carbonates, carbamates, borates, sugars, phosphoramidates, phenylsulfenates, sulfates and sulfones also nitrates as cleavable 3'OH-protecting group. The deprotection may be carried out by chemical or enzymatic means. There are neither synthesis procedures nor deprotection conditions and enzymatic incorporation data disclosed for the nitrate group. The claimed deblocking solution preferably contains divalent cations like Co²⁺ and a biological buffer like Tris. 3'OH-blocked nucleoside triphosphates containing a label are not disclosed.

Buzby (US 2007-0117104) [4] discloses nucleoside triphosphates for SBS which are reversibly protected at the 3'-hydroxyl group and carry a label at the base. The label is connected via a cleavable linker such as a disulfide linker or a photocleavable linker. The linker consists of up to about 25 atoms. The 3'OH-protection group can be besides hydroxylamines, aldehydes, allylamines, alkenes, alkynes, alcohols, amines, aryls, esters, ethers, carbonitriles, phosphates, carbonates, carbamates, borates, sugars, phosphoramidates, phenylsulfanates, sulfates, sulfones and heterocycles also nitrates.

What is needed in order to achieve longer read length and better accuracy in nucleic acid sequencing is a nucleotide analogue with a cleavable protecting group and a cleavable linker which do not leave reactive residues after cleavage [5].

SUMMARY OF THE INVENTION

The present invention provides methods, compositions, mixtures and kits utilizing

deoxynucleoside triphosphates comprising a 3'-O position capped by a group comprising methylenedisulfide as a cleavable protecting group and a detectable label reversibly connected to the nucleobase of said deoxynucleoside. In one embodiment, the present invention contemplates a nucleotide analogue with a reversible protecting group comprising methylenedisulfide and a cleavable oxymethylenedisulfide linker between the label and nucleobase. Such compounds provide new possibilities for future sequencing technologies, including but not limited to Sequencing by Synthesis.

In terms of mixtures, the present invention in one embodiment contemplates deoxynucleoside triphosphates comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase and a 3'-O position capped by a group comprising methylenedisulfide as a cleavable protecting group in mixtures with one or more additional sequencing reagents, including but not limited to buffers, polymerases, primers, template and the like. In terms of kits, the present invention contemplates in one embodiment a sequencing kit where sequencing reagents are provided together in separate containers (or in mixtures), including deoxynucleoside triphosphates comprising a 3'-O position capped by a group comprising methylenedisulfide as a cleavable protecting group, along with (optionally) instructions for using such reagents in sequencing. It is not intended that the present invention be limited by the number or nature of sequencing reagents in the kit. In one embodiment, the kit comprises one or more additional sequencing reagents, including but not limited to buffers, polymerases, primers and the like.

It is not intended that the present invention be limited to any particular polymerase. The present invention contemplates engineered (e.g. mutated) polymerases with enhanced incorporation of nucleotide derivatives. For example, Tabor, S. and Richardson, C.C. ((1995) Proc. Natl. Acad. Sci (USA) 92:6339 [6]) describe the replacement of phenylalanine 667 with tyrosine in *T. aquaticus* DNA polymerase and the effects this has on discrimination of

dideoxynucleotides by the DNA polymerase. In one embodiment, the present invention contemplates polymerases that lack 3'-5' exonuclease activity (designated exo-). For example, an exo- variant of 9°N polymerase is described by Perler et al., 1998 US 5756334 [7] and by Southworth et al., 1996 Proc. Natl Acad. Sci USA 93:5281 [8]. Another polymerase example is an A486Y variant of Pfu DNA polymerase (Evans *et al.*, 2000. Nucl. Acids. Res. 28:1059 [9]). Another example is an A485T variant of Tsp JDF-3 DNA polymerase (Arezi *et al.*, 2002. J. Mol. Biol. 322:719 [10]). WO 2005/024010 A1 relates to the modification of the motif A region and to the 9°N DNA polymerase, hereby incorporated by reference [11].

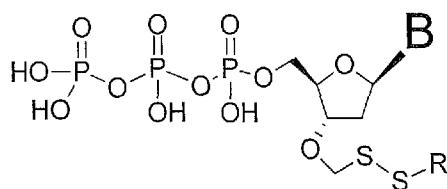
In terms of methods, the present invention contemplates both methods to synthesize deoxynucleoside triphosphates comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase and a 3'-O position capped by a group comprising methylenedisulfide as a cleavable protecting group, as well as methods to utilize deoxynucleoside triphosphates comprising a 3'-O position capped by a group comprising methylenedisulfide as a cleavable protecting group.

In one embodiment, the invention relates to (a) nucleoside triphosphates with 3'-O capped by a group comprising methylenedisulfide (e.g. of the general formula $-\text{CH}_2\text{-SS-}R$) as cleavable protecting group; and (b) their labeled analogs, where labels are attached to the nucleobases via cleavable oxymethylenedisulfide linker ($-\text{OCH}_2\text{-SS-}$) (although the linker may contain additional groups). Such nucleotides can be used in nucleic acid sequencing by synthesis (SBS) technologies. In one embodiment, the invention relates to the synthesis of nucleotides 3'-O capped by a group comprising methylenedisulfide (e.g. $-\text{CH}_2\text{-SS-}R$) as cleavable protecting group, the deprotection conditions or enzymatic incorporation.

In one embodiment, the invention relates to a deoxynucleoside triphosphate comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase and a 3'-O capped by a

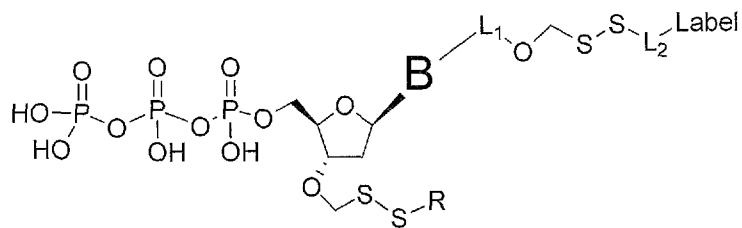
group comprising methylenedisulfide as a cleavable protecting group. In one embodiment, the nucleobase of said nucleoside is non-natural. In one embodiment, the non-natural nucleobase of said nucleoside is selected from the group comprising 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine. In one embodiment, said group comprising methylenedisulfide is $-\text{CH}_2\text{-SS-}R$, wherein R is selected from the group comprising alkyl and substituted alkyl groups. In one embodiment, said detectable label is attached to said nucleobase via cleavable oxymethylenedisulfide linker (e.g. of the formula $-\text{OCH}_2\text{-SS-}$). In one embodiment, said detectable label is a fluorescent label. In one embodiment, R in the formula $(-\text{CH}_2\text{-S-S-}R)$ could be alkyl or allyl.

In one embodiment, the invention relates to a deoxynucleoside triphosphate according to the following structure:



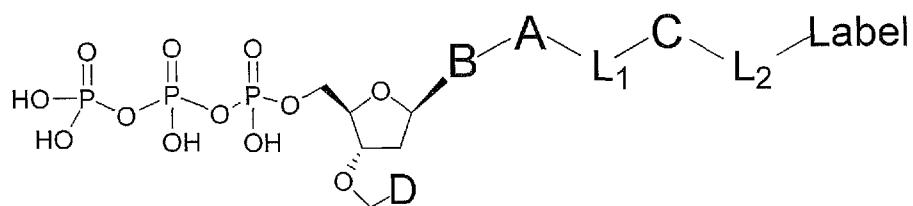
wherein B is a nucleobase and R is selected from the group comprising alkyl and substituted alkyl groups. In one embodiment, said nucleobase is a natural nucleobase (cytosine, guanine, adenine, thymine and uracil). In one embodiment, said nucleobase is a non-natural nucleobase selected from the group comprising 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine. In the case of analogs, the detectable label may also include a linker section between the nucleobase and said detectable label.

In one embodiment, the invention relates to a labeled deoxynucleoside triphosphate according to the following structure:



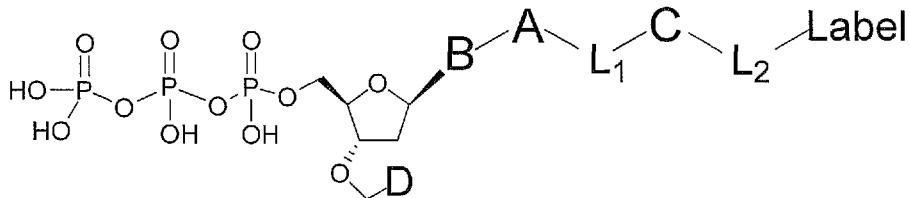
wherein B is a nucleobase, R is selected from the group comprising alkyl and substituted alkyl groups, and L₁ and L₂ are connecting groups. In one embodiment, said nucleobase is a natural nucleobase analog. In one embodiment, said nucleobase is a non-natural nucleobase analog selected from the group comprising 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine. In the case of analogs, the detectable label may also include a linker section between the nucleobase and said detectable label. In one embodiment, L₁ and L₂ are independently selected from the group comprising -CO-, -CONH-, -NHCONH-, -O-, -S-, -ON, and -N=N-, alkyl, aryl, branched alkyl, branched aryl or combinations thereof. It is preferred that L₂ not be “-S-.” In one embodiment, the present invention contemplates L₁ to be either an amine on the base or a hydroxyl on the base. In one embodiment, said label is selected from the group consisting of fluorophore dyes, energy transfer dyes, mass-tags, biotin, and haptens. In one embodiment, said label is a detectable label.

In one embodiment, the invention relates to a labeled deoxynucleoside triphosphate according to the following structure:



wherein D is selected from the group consisting of disulfide allyl, and disulfide substituted allyl groups; B is a nucleobase; A is an attachment group; C is a cleavable site core; L₁ and L₂ are connecting groups; and Label is a label (e.g. a detectable moiety).

In one embodiment, the invention relates to a labeled deoxynucleoside triphosphate according to the following structure:

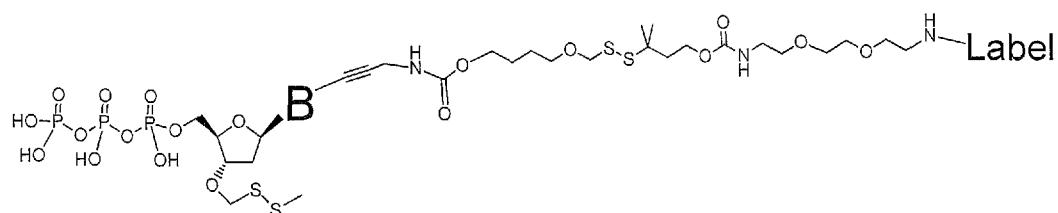


wherein D is

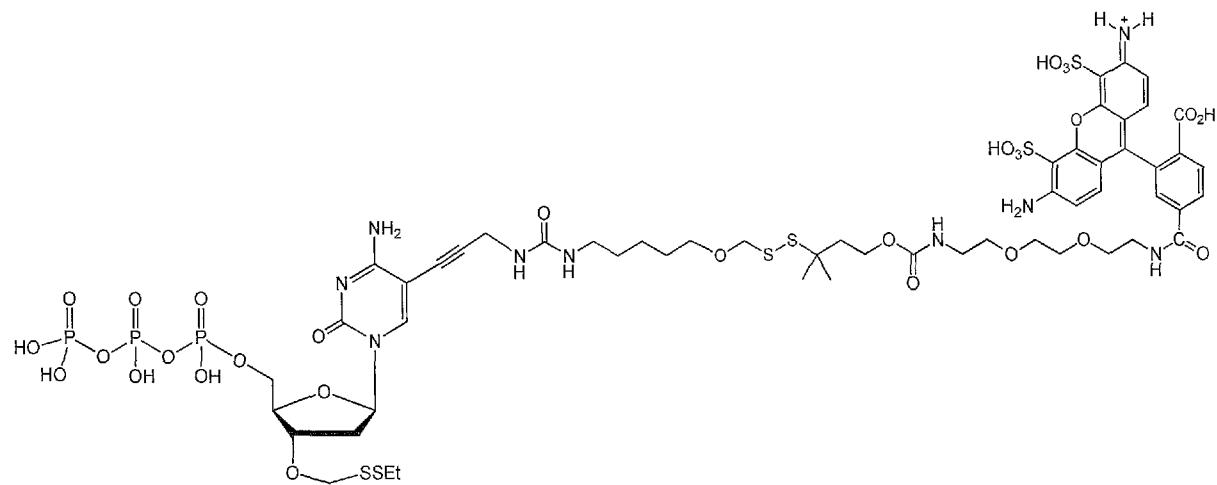
selected from the group consisting of an azide, disulfide alkyl, disulfide substituted alkyl groups; B is a nucleobase; A is an attachment group; C is a cleavable site core; L₁ and L₂ are connecting groups; and Label is a label. In one embodiment, said nucleobase is a non-natural nucleobase analog selected from the group consisting of 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine. In one embodiment, said attachment group A is chemical group selected from the group consisting of propargyl, hydroxymethyl, exocyclic amine, propargyl amine, and propargyl hydroxyl. In one embodiment, said cleavable site core is selected from the

group consisting of , , and , wherein R₁ and R₂ are independently selected alkyl groups.

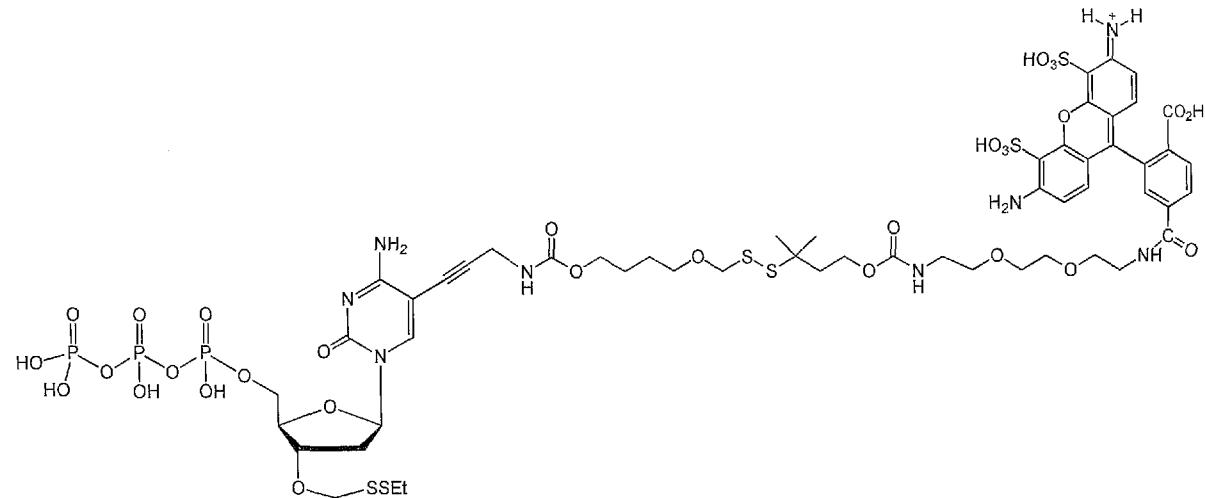
In one embodiment, L₁ is selected from the group consisting of $-\text{CONH}(\text{CH}_2)_x-$, $-\text{CO-O}(\text{CH}_2)_x-$, $-\text{CONH}-(\text{OCH}_2\text{CH}_2\text{O})_x-$, $-\text{CO-O}(\text{CH}_2\text{CH}_2\text{O})_x-$, and $-\text{CO}(\text{CH}_2)_x-$, wherein x is 0-10, but more preferably from 1-6. In one embodiment, L₂ is selected from the group consisting of $-\text{NH}-$, $-(\text{CH}_2)_x\text{NH}-$, $-\text{C}(\text{Me})_2(\text{CH}_2)_x\text{NH}-$, $-\text{CH}(\text{Me})(\text{CH}_2)_x\text{NH}-$, $-\text{C}(\text{Me})_2(\text{CH}_2)_x\text{CO}-$, $-\text{CH}(\text{Me})(\text{CH}_2)_x\text{CO}-$, $-(\text{CH}_2)_x\text{OCONH}(\text{CH}_2)_y\text{O}(\text{CH}_2)_z\text{NH}-$, $-(\text{CH}_2)_x\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_y(\text{CH}_2)_z\text{NH}-$, and $-\text{CONH}(\text{CH}_2)_x-$, $-\text{CO}(\text{CH}_2)_x-$, wherein x, y, and z are each independently selected from 0-10, but more preferably from 1-6. In one embodiment, said label is selected from the group consisting of fluorophore dyes, energy transfer dyes, mass-tags, biotin, and haptene. In one embodiment, the compound has the structure:



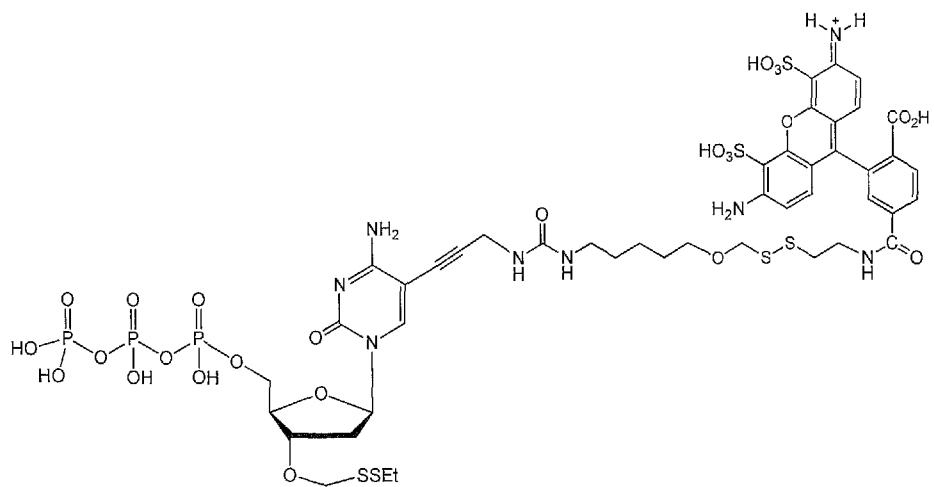
, wherein said label is a dye. In one embodiment, the compound has the structure:



In one embodiment, the compound has the structure:

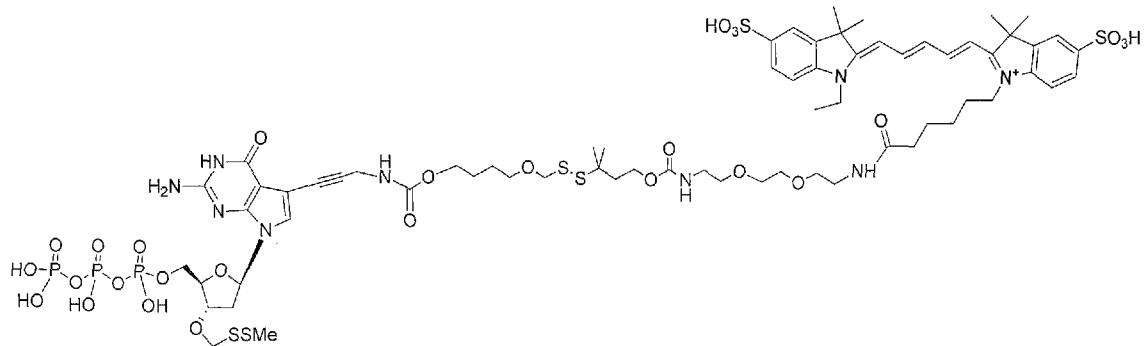


In one embodiment, the compound has the structure:



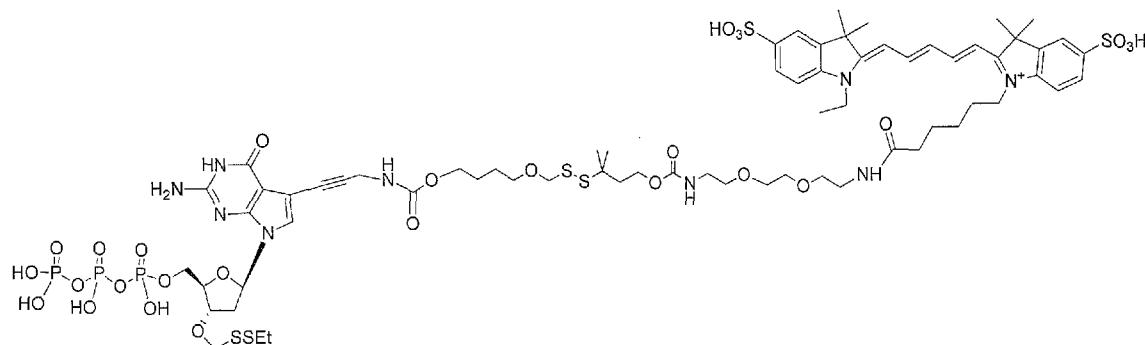
. In one embodiment,

the compound has the structure:



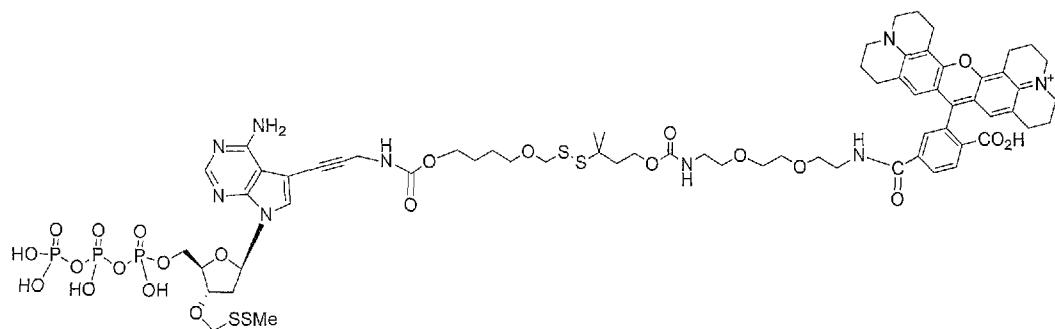
In

one embodiment, the compound has the structure:

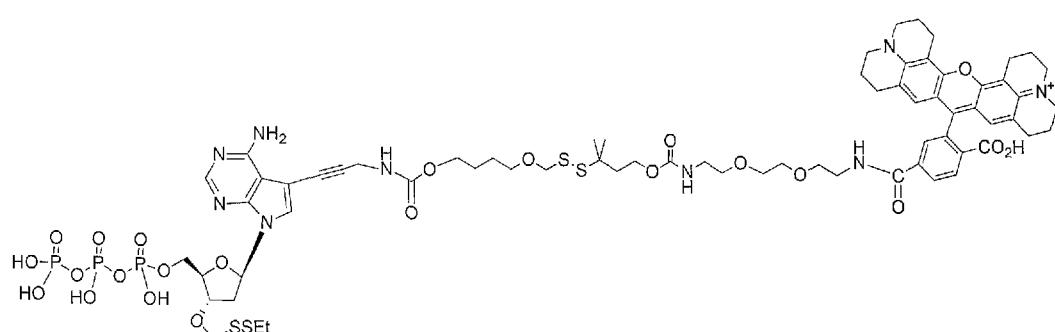


In

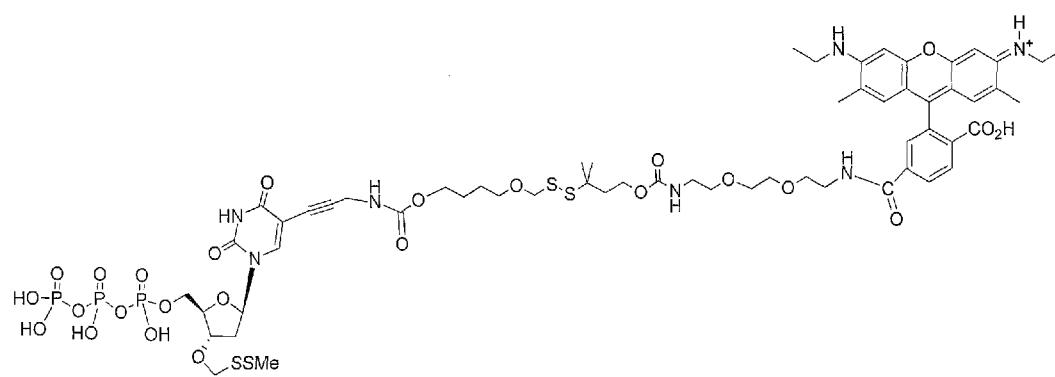
one embodiment. the compound has the structure:



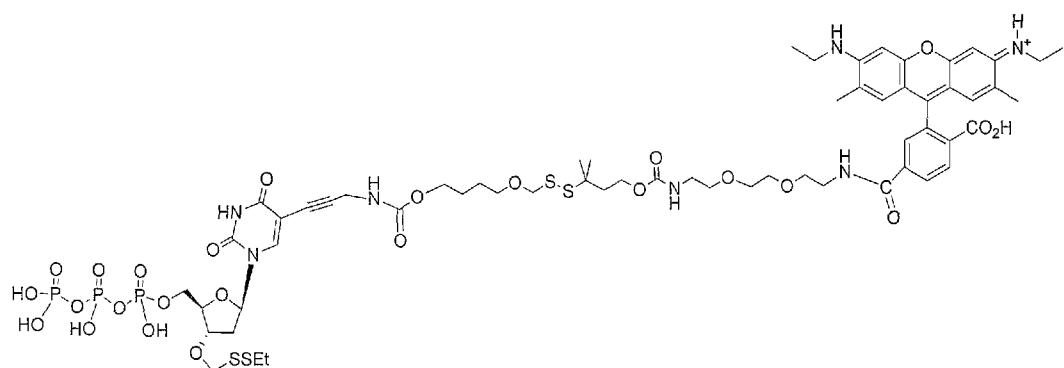
embodiment, the compound has the structure:



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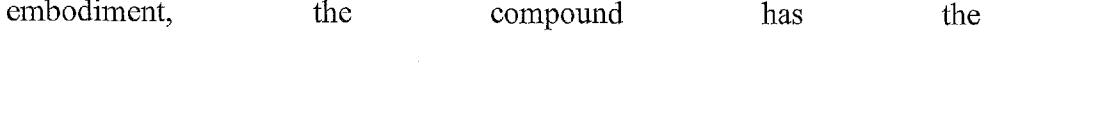
embodiment, the compound has the structure:



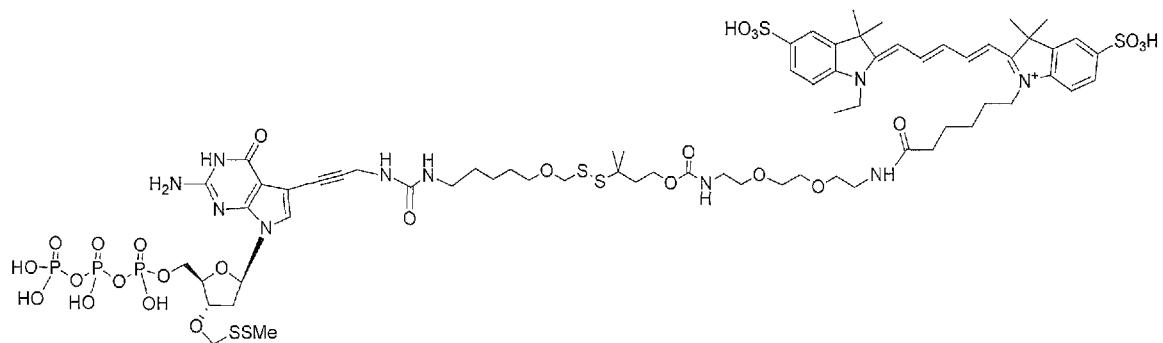
embodiment, the compound has the structure:



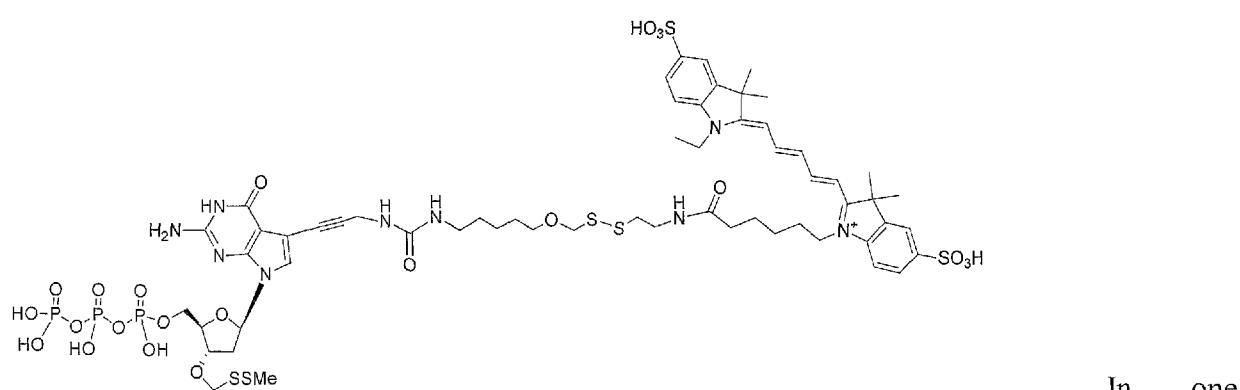
embodiment, the compound has the structure:



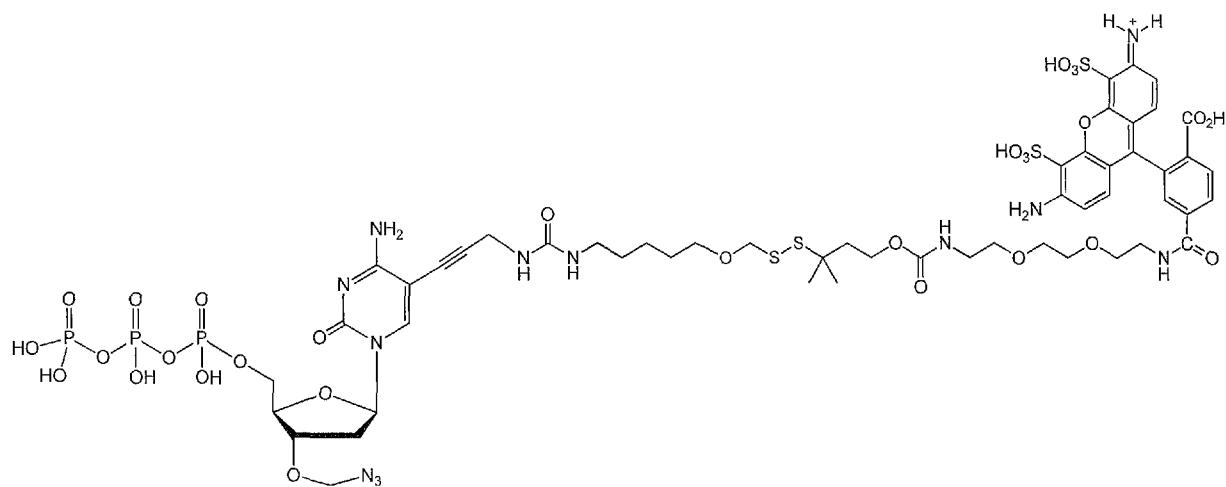
embodiment, the compound has the structure:



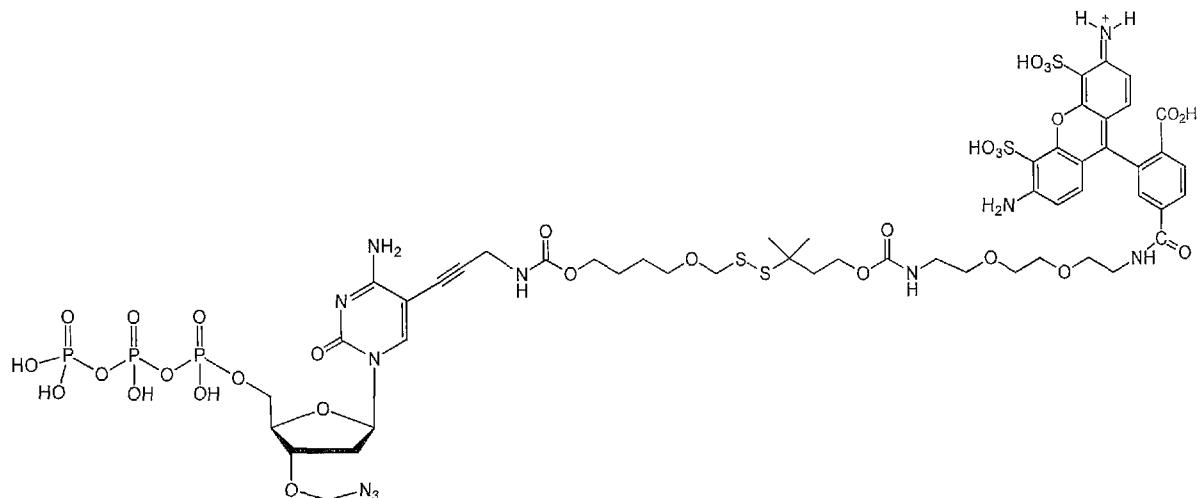
In one embodiment, the compound has the structure:



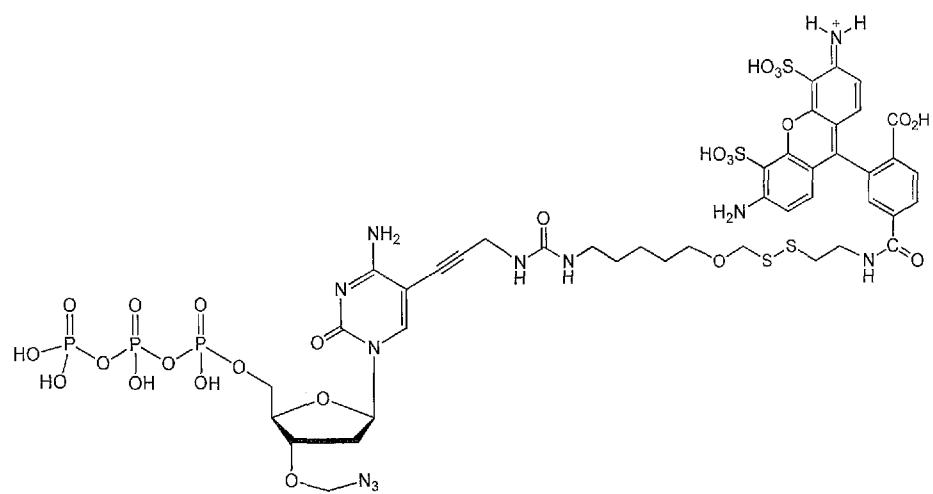
In one embodiment, the compound has the structure:



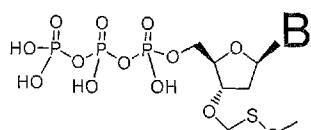
In one embodiment, the compound has the structure:



In one embodiment, the compound has the structure:



In one embodiment, the invention relates to a deoxynucleoside triphosphate according to



the following structure: wherein B is a nucleobase.

In one embodiment, the invention relates to a kit comprising one or more sequencing reagents (e.g. a DNA polymerase) and at least one deoxynucleoside triphosphate comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase, a 3'-O capped by a group comprising methylenedisulfide as a cleavable protecting group. In one embodiment, said

nucleobase is a natural nucleobase analog. In one embodiment, the nucleobase of said nucleoside is non-natural. In one embodiment, the non-natural nucleobase of said nucleoside is selected from the group comprising 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine.

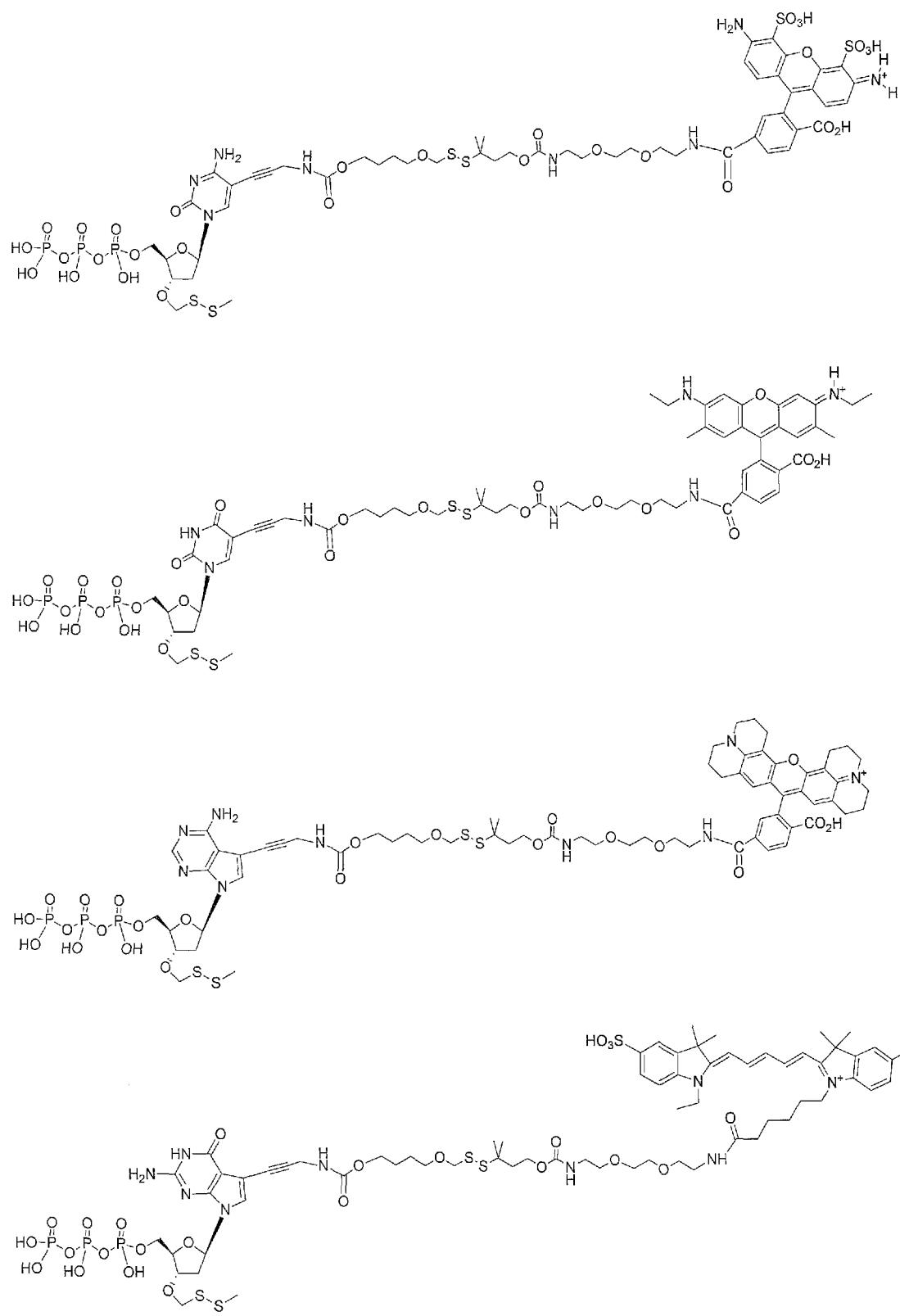
The present invention also contemplates mixtures, i.e. at least one deoxynucleoside triphosphate comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase, a 3'-O capped by a group comprising methylenedisulfide as a cleavable protecting group in a mixture with one or more additional reagents (whether dry or in solution). In one embodiment, the invention relates to a reaction mixture comprising a nucleic acid template with a primer hybridized to said template, a DNA polymerase, and at least one deoxynucleoside triphosphate comprising a nucleobase, a label and a sugar, a cleavable oxymethylenedisulfide linker between the label and nucleobase, said sugar comprising a 3'-O capped by a group comprising methylenedisulfide as a cleavable protecting group, wherein said nucleoside further comprises a detectable label covalently bound to the nucleobase of said nucleoside.

In one embodiment, the invention relates to a method of performing a DNA synthesis reaction comprising the steps of a) providing a reaction mixture comprising a nucleic acid template with a primer hybridized to said template, a DNA polymerase, at least one deoxynucleoside triphosphate comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase, with a 3'-O capped by a group comprising methylenedisulfide as a cleavable protecting group, and b) subjecting said reaction mixture to conditions which enable a DNA polymerase catalyzed primer extension reaction. This permits incorporation of at least one deoxynucleoside triphosphate (comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase, with a 3'-O capped by a group comprising methylenedisulfide as a cleavable protecting group) into the bound primer. In one embodiment, said DNA polymerase

catalyzed primer extension reaction is part of a sequencing reaction (e.g. SBS). In one embodiment, said detectable label is removed from said nucleobase by exposure to a reducing agent. It is not intended that the invention is limited to one type of reducing agent. Any suitable reducing agent capable of reducing disulfide bonds can be used to practice the present invention. In one embodiment the reducing agent is phosphine [12], for example, triphenylphosphine, tributylphosphine, trihydroxymethyl phosphine, trihydroxypropyl phosphine, tris carboethoxy-phosphine (TCEP) [13, 14]. In one embodiment, said reducing agent is TCEP. In one embodiment, said detectable label and 3'-OCH₂-SS-R group are removed from said nucleobase by exposure to compounds carrying a thiol group [15] so as to perform cleavage of dithio-based linkers and terminating (protecting) groups, such thiol-containing compounds including (but not limited to) cysteine, cysteamine, dithio-succinic acid, dithiothreitol, 2,3-Dimercapto-1-propanesulfonic acid sodium salt, dithiobutyamine [16] , meso-2,5-dimercapto-N,N,N',N'-tetramethyladipamide, 2-mercapto-ethane sulfonate, and N,N'-dimethyl, N,N'-bis(mercaptoacetyl)-hydrazine [17]. Reactions can be further catalyzed by inclusion of selenols [18]. In addition borohydrides, such as sodium borohydrides can also be used for this purpose [19] (as well as ascorbic acid [20]. In addition, enzymatic methods for cleavage of disulfide bonds are also known such as disulfide and thiooxidoreductase and can be used with compounds of the present invention [21].

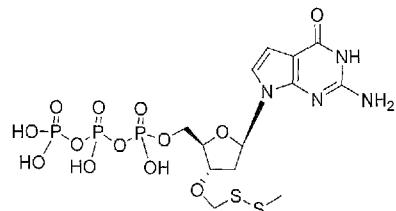
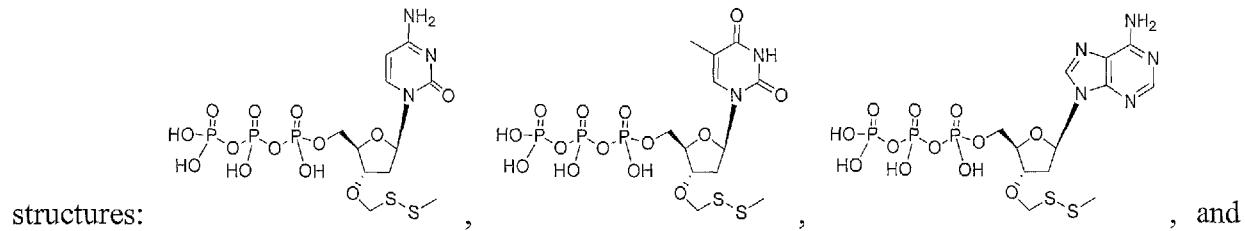
In one embodiment, the invention relates to a method for analyzing a DNA sequence comprising the steps of a) providing a reaction mixture comprising nucleic acid template with a primer hybridized to said template forming a primer/template hybridization complex, b) adding DNA polymerase, and a first deoxynucleoside triphosphate comprising a nucleobase, a cleavable oxymethylenedisulfide linker between the label and nucleobase, with a 3'-O capped by a group comprising methylenedisulfide as cleavable protecting group, c) subjecting said reaction mixture

to conditions which enable a DNA polymerase catalyzed primer extension reaction so as to create a modified primer/template hybridization complex, and d) detecting said first detectable label of said deoxynucleoside triphosphate in said modified primer/template hybridization complex. In one embodiment, the detecting allows one to determine which type of analogue (A, T, G, C or U) has been incorporated. In one embodiment, the method further comprises the steps of e) removing said cleavable protecting group and optionally said detectable label from said modified primer/template hybridization complex, and f) repeating steps b) to e) at least once (and typically repeating these steps many times, e.g. 10-200 times). In one embodiment, the cleavable oxymethylenedisulfide-containing linker is hydrophobic and has a logP value of greater than 0. In one embodiment, the cleavable oxymethylenedisulfide-containing linker is hydrophobic and has a logP value of greater than 0.1. In one embodiment, the cleavable oxymethylenedisulfide-containing linker is hydrophobic and has a logP value of greater than 1.0. In one embodiment, the method further comprises adding a second deoxynucleoside triphosphate is added during repeat of step b), wherein said second deoxynucleoside triphosphate comprises a second detectable label, wherein said second detectable label is different from said first detectable label. In one embodiment, the nucleobase of said second deoxynucleoside triphosphate is different from the nucleobase of said first deoxynucleoside triphosphate. In one embodiment, a mixture of at least 4 differently labeled, 3'-O methylenedisulfide capped deoxynucleoside triphosphate compounds representing analogs of A, G, C and T or U are used in step b). In one embodiment, said mixture of at least 4 differently labeled, 3'-O methylenedisulfide capped deoxynucleoside triphosphate compounds with the structures:



used in step b). In one embodiment, said mixture further comprises unlabeled 3'-O

methylenedisulfide capped deoxynucleoside triphosphate compounds such as those with the



also used in step b). In one embodiment, step e) is performed by exposing said modified primer/template hybridization complex to a reducing agent. In one embodiment, said reducing agent is TCEP. In one embodiment, said detectable label is removed from said nucleobase by exposure to compounds carrying a thiol group so as to perform cleavage of dithio-based linkers and terminating (protecting) groups, such thiol-containing compounds including (but not limited to) cysteine, cysteamine, dithio-succinic acid, dithiothreitol, 2,3-Dimercapto-1-propanesulfonic acid sodium salt, dithiobutyramine, meso-2,5-dimercapto-N,N,N',N'-tetramethyladipamide, 2-mercaptop-ethane sulfonate, and N,N'-dimethyl, N,N'-bis(mercaptoproacetyl)-hydrazine.

It is not intended that the present invention be limited to a particular sequencing platform. However, a preferred instrument is QIAGEN's GeneReader DNA sequencing system (GR). In one embodiment, a DNA sequence is determined by a method of sequencing by synthesis (SBS). In one embodiment, each cycle of sequencing consists of eight steps: extension 1, extension 2, wash 1, addition imaging solution, imaging, wash 2, cleave, and wash 3. Data collected during imaging cycles is processed by analysis software yielding error rates, throughput values, and applied phasing correction values.

It is contemplated that the same or similar method could improve the performance of

other SBS platforms in general (i.e. any sequencing-by-synthesis methods that operate under similar conditions), as well as specific SBS platforms, such as HiSeq and miSeq platforms from Illumina; Roche 454; the Ion Torrent PGM and Proton platforms; and the PacificBio platform.

It is not intended that the present invention be limited to only one type of sequencing. In one embodiment, said deoxynucleoside triphosphate (comprising a nucleobase, a label and a sugar, a cleavable oxymethylenedisulfide linker between the label and nucleobase, said sugar comprising a 3'-O capped by a group comprising methylenedisulfide as cleavable protecting group) may be used in pyrosequencing.

In one embodiment, the invention relates to a deoxynucleoside triphosphate comprising a nucleobase and a sugar, said nucleobase comprising a detectable label attached via a cleavable oxymethylenedisulfide linker, said sugar comprising a 3'-O capped by a group comprising a methylenedisulfide group as a cleavable protecting group. In one embodiment, said nucleoside is in a mixture with a polymerase (or some other sequencing reagent). In one embodiment, the nucleobase of said nucleoside is non-natural. In one embodiment, the non-natural nucleobase of said nucleoside is selected from the group comprising 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine. In one embodiment, said group comprising a methylenedisulfide group is of the formula $-\text{CH}_2\text{-SS-}R$, wherein R is selected from the group comprising alkyl and substituted alkyl groups. In one embodiment, said mixture further comprises a primer. In one embodiment, said primer is hybridized to nucleic acid template. In one embodiment, said detectable label is a fluorescent label. In one embodiment, said nucleic acid template is immobilized (e.g. in a well, channel or other structure, or alternatively on a bead).

In one embodiment, the invention relates to a method of preparing a 3'-*O*-(methylthiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside, comprising: a)

providing a 5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside, wherein said 5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside comprises a nucleobase and a sugar, and ii) a methylthiomethyl donor ; and b) treating said 5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside under conditions so as to create a 3'-O-(methylthiomethyl)-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside. In one embodiment, said methylthiomethyl donor is DMSO. In one embodiment, said conditions comprise acidic conditions. In one embodiment, said 5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside comprises a protecting group on the nucleobase of said nucleoside. In one embodiment, said 3'-O-(methylthiomethyl)-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside is purified with column chromatography.

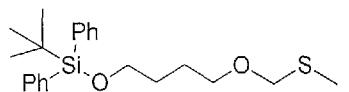
In one embodiment, the invention relates to a method of preparing a 3'-O-(R-substituted-dithiomethyl)-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside, comprising: a) providing i) a 3'-O-(methylthiomethyl)-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside, and ii) R-SH, wherein R comprises alkyl or substituted alkyl; and b) treating said 3'-O-(methylthiomethyl)-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside under conditions so as to create a 3'-O-(R-substituted-dithiomethyl)-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside. In one embodiment, said R-SH is ethanethiol. In one embodiment, said conditions comprise basic conditions.

In one embodiment, the invention relates to a method of preparing a 3'-O-(R-substituted-dithiomethyl)-2'-deoxynucleoside, comprising: a) providing a 3'-O-(R-substituted-dithiomethyl)-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside; and b) treating said 3'-O-(R-substituted-dithiomethyl)-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside under conditions so as to create a 3'-O-(R-substituted-dithiomethyl)-2'-deoxynucleoside. In one embodiment, said conditions comprise exposing said

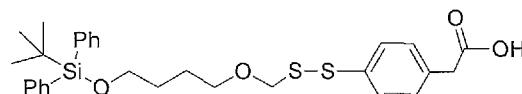
3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside to NH₄F.

In one embodiment, the invention relates to a method of preparing a triphosphate of 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside, comprising: a) providing a 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside; and b) treating said 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside under conditions so as to create a triphosphate of 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside. In one embodiment, said conditions comprises exposing said 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside to (MeO)₃PO with POCl₃ and Bu₃N. In one embodiment, said method further comprises step c) removal of said nucleobase protecting group. In one embodiment, said protecting group comprises a N-trifluoroacetyl-aminopropargyl protecting group. In one embodiment, said N-trifluoroacetyl-aminopropargyl protecting group is removed by solvolysis to produce a 5'-*O*-(triphosphate)-3'-*O*-(R-substituted-dithiomethyl)-5-(aminopropargyl)-2'-deoxynucleoside.

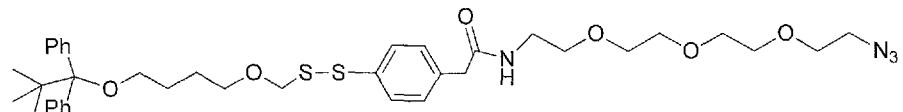
In one embodiment, the invention relates to a compound wherein the structure is:



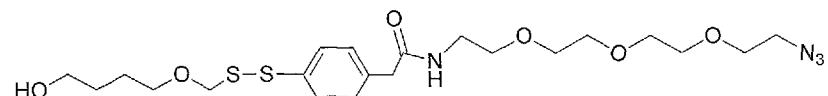
In one embodiment, the invention relates to a compound wherein the structure is:



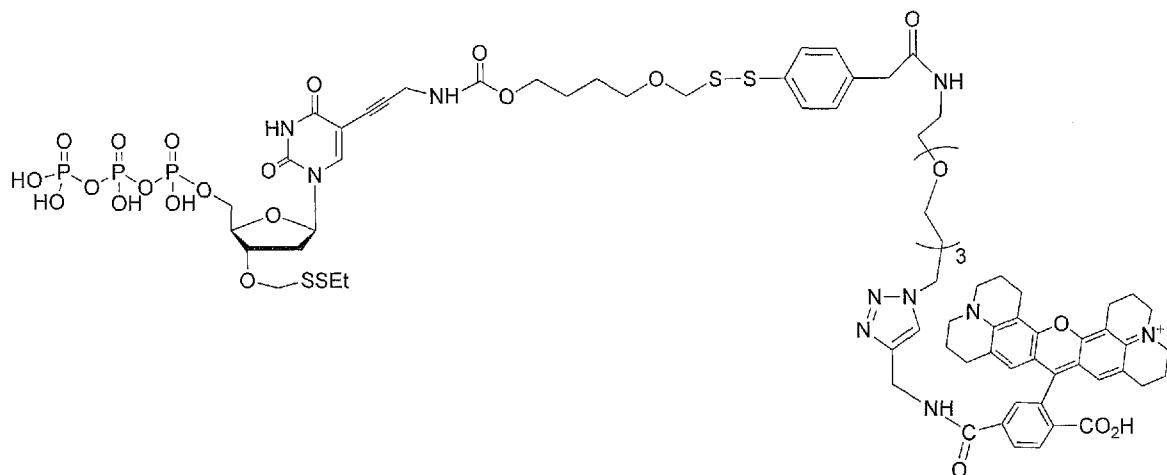
In one embodiment, the invention relates to a compound wherein the structure is:



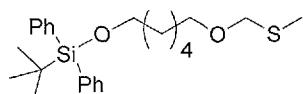
In one embodiment, the invention relates to a compound wherein the structure is:



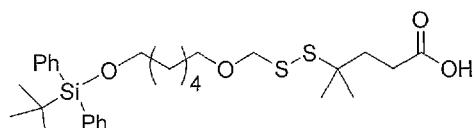
In one embodiment, the invention relates to a compound wherein the structure is:



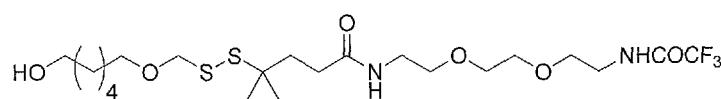
In one embodiment, the invention relates to a compound wherein the structure is:



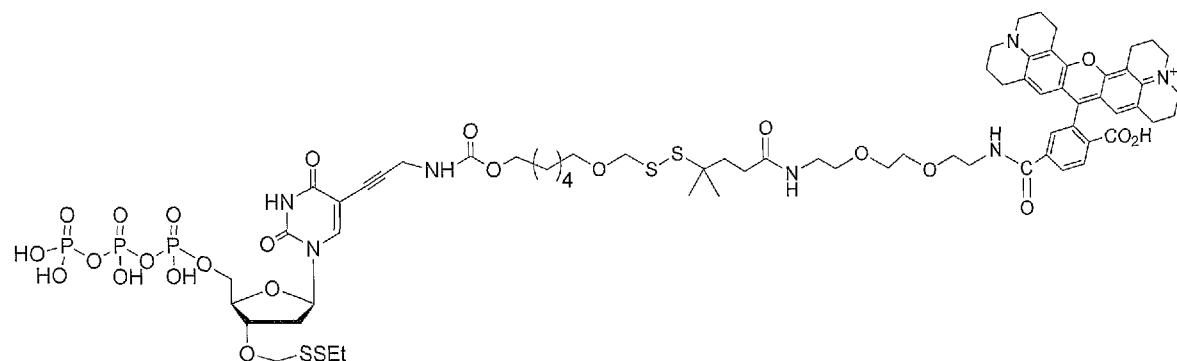
In one embodiment, the invention relates to a compound wherein the structure is:



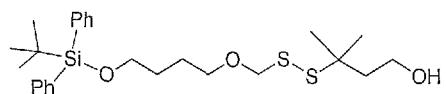
In one embodiment, the invention relates to a compound wherein the structure is:



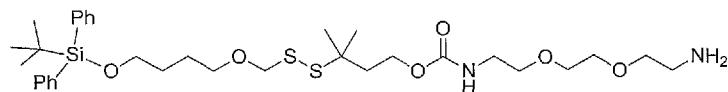
In one embodiment, the invention relates to a compound wherein the structure is:



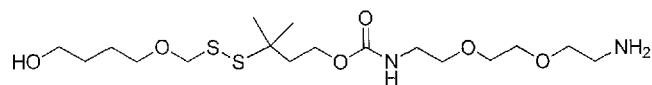
In one embodiment, the invention relates to a compound wherein the structure is:



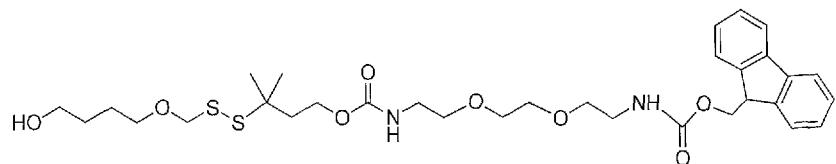
In one embodiment, the invention relates to a compound wherein the structure is:



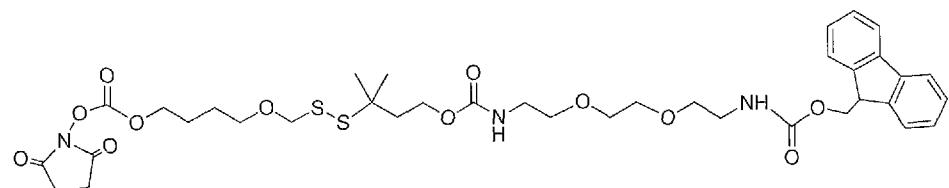
In one embodiment, the invention relates to a compound wherein the structure is:



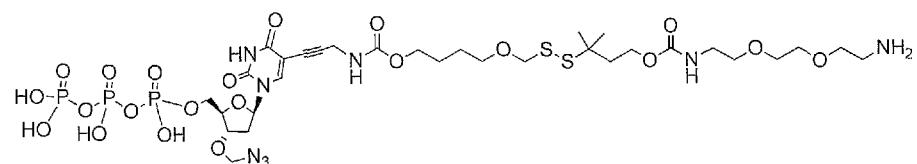
In one embodiment, the invention relates to a compound wherein the structure is:



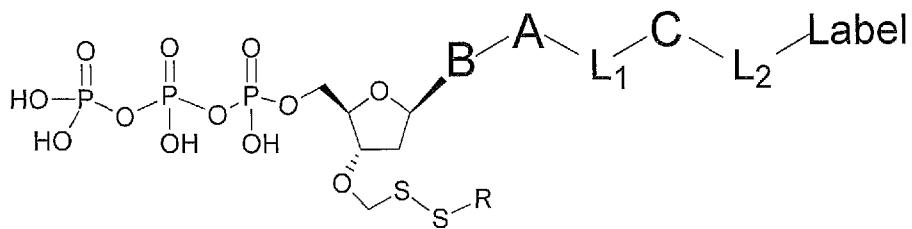
In one embodiment, the invention relates to a compound wherein the structure is:



In one embodiment, the invention relates to a compound wherein the structure is:



In one embodiment, the invention relates to a labeled deoxynucleoside triphosphate according to the following structure:



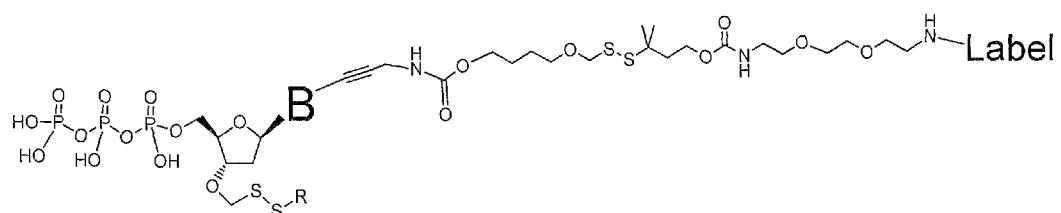
wherein R is selected

from the group consisting of alkyl, substituted alkyl groups, allyl, substituted allyl; B is a nucleobase; A is an attachment group; C is a cleavable site core; L₁ and L₂ are connecting groups; and Label is a label. In one embodiment, said nucleobase is a non-natural nucleobase analog selected from the group consisting of 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine. In one embodiment, said attachment group A is chemical group selected from the group consisting of propargyl, hydroxymethyl, exocyclic amine, propargyl amine, and propargyl hydroxyl. In one embodiment, said cleavable site core selected from the

group consisting of:

, and

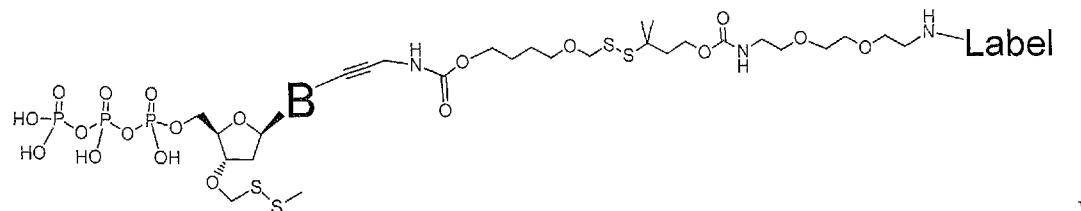
wherein R₁ and R₂ are independently selected alkyl groups. In one embodiment, wherein L₁ is selected from the group consisting of $-\text{CONH}(\text{CH}_2)_x-$, $-\text{COO}(\text{CH}_2)_x-$, $-\text{CO}(\text{CH}_2)_x-$, wherein x is 0-10, but more preferably from 1-6. In one embodiment, wherein L₂ is selected from the group consisting of $-\text{NH}-$, $-(\text{CH}_2)_x\text{OCONH}(\text{CH}_2)_y\text{O}(\text{CH}_2)_z\text{NH}-$, $-(\text{CH}_2)_x\text{OCONH}(\text{CH}_2)_y\text{O}(\text{CH}_2)_z\text{NH}-$, $-\text{CONH}(\text{CH}_2)_x-$, $-\text{CO}(\text{CH}_2)_x-$, wherein x, y, and z are each independently selected from 0-10, but more preferably from 1-6. In one embodiment, said label is selected from the group consisting of fluorophore dyes, energy transfer dyes, mass-tags, biotin, and haptene. In one embodiment, the compound has the structure:



, wherein said

label is a dye and wherein R is selected from the group consisting of alkyl, substituted alkyl

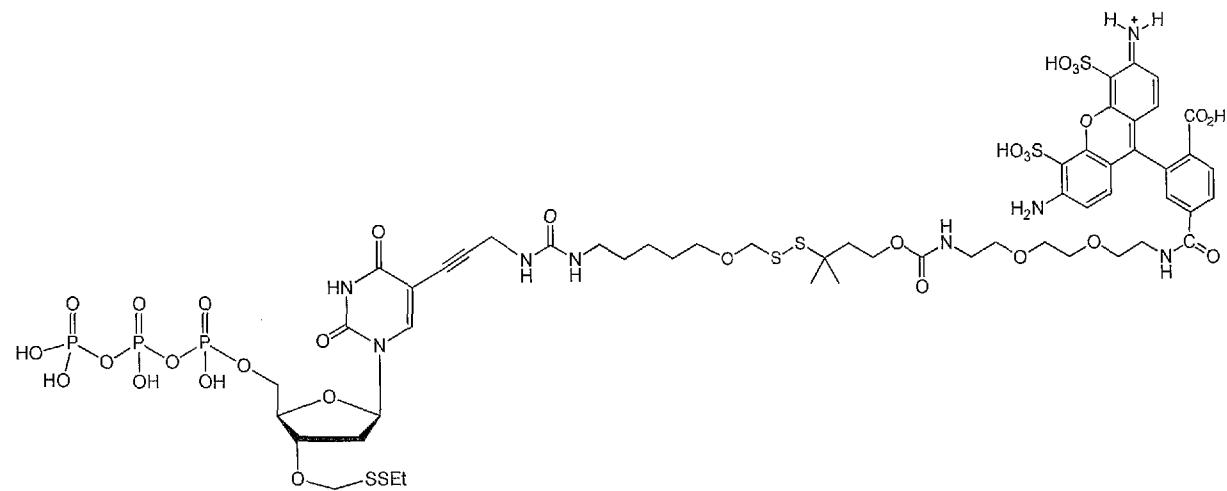
groups, allyl, substituted allyl. In one embodiment, the compound has the structure:



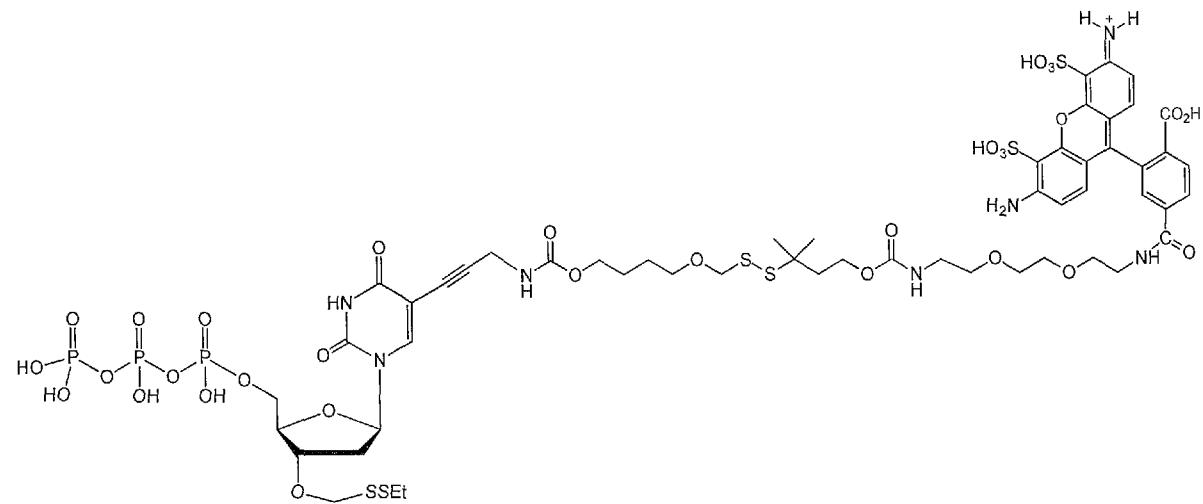
, wherein said

label is a dye.

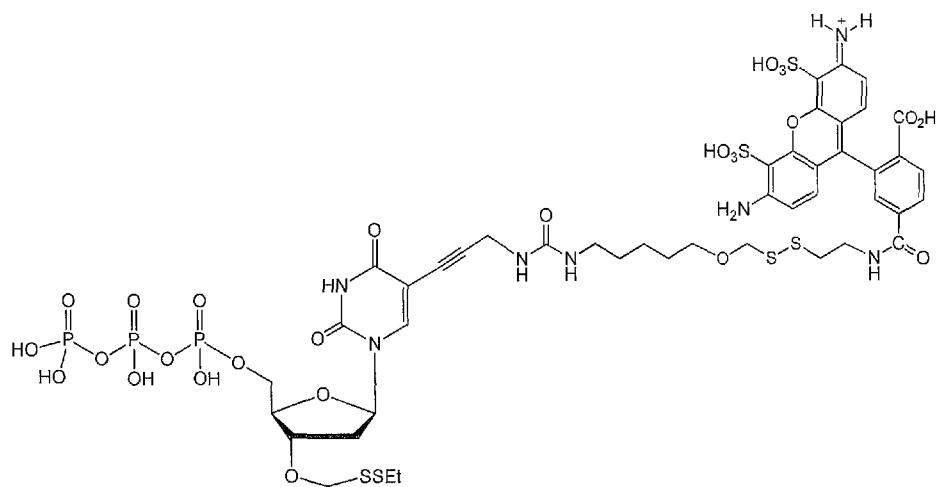
In one embodiment, the compound has the structure:



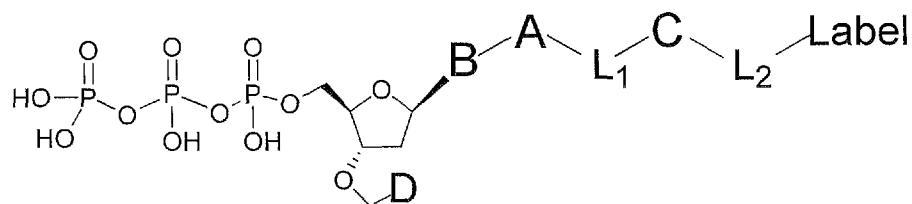
In one embodiment, the compound has the structure:



In one embodiment, the compound has the structure:

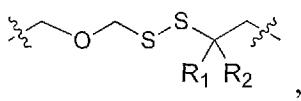
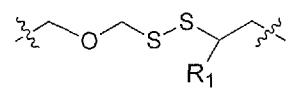
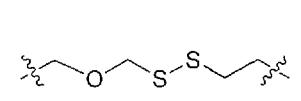
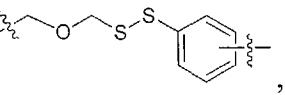


In one embodiment, the invention relates to a labeled deoxynucleoside triphosphate according to the following structure:

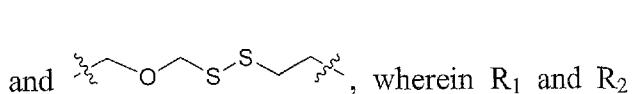
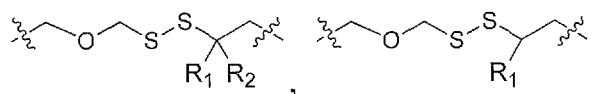


wherein D is selected

from the group consisting of an azide (-N₃), disulfide alkyl (-SS-R) and disulfide substituted alkyl groups, B is a nucleobase, A is an attachment group, C is a cleavable site core, L₁ and L₂ are connecting groups, and Label is a label. In one embodiment, said nucleobase is a natural nucleobase. In one embodiment, said nucleobase is a non-natural nucleobase analog selected from the group consisting of 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine. In one embodiment, said attachment group (A) is chemical group selected from the group consisting of propargyl, hydroxymethyl, exocyclic amine, propargyl amine, and propargyl hydroxyl. In one embodiment, said cleavable (C) site core selected from the group

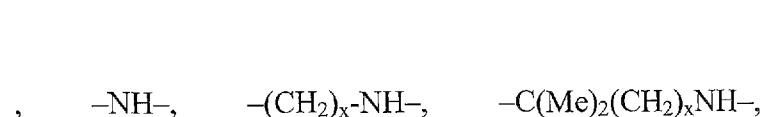
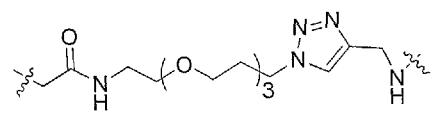
consisting of:    , and  , wherein R₁ and R₂ are independently selected alkyl groups. In one

embodiment, said cleavable site core selected from the group consisting of:



are independently selected alkyl groups. In one embodiment, L_1 is selected from the group consisting of $-\text{CONH}(\text{CH}_2)_x-$, $-\text{CO-O}(\text{CH}_2)_x-$, $-\text{CONH}-(\text{OCH}_2\text{CH}_2\text{O})_x-$, $-\text{CO-O}(\text{CH}_2\text{CH}_2\text{O})_x-$, and $-\text{CO}(\text{CH}_2)_x-$, wherein x is 0-100. In some embodiments, x is 0-10, but more preferably from 1-6.

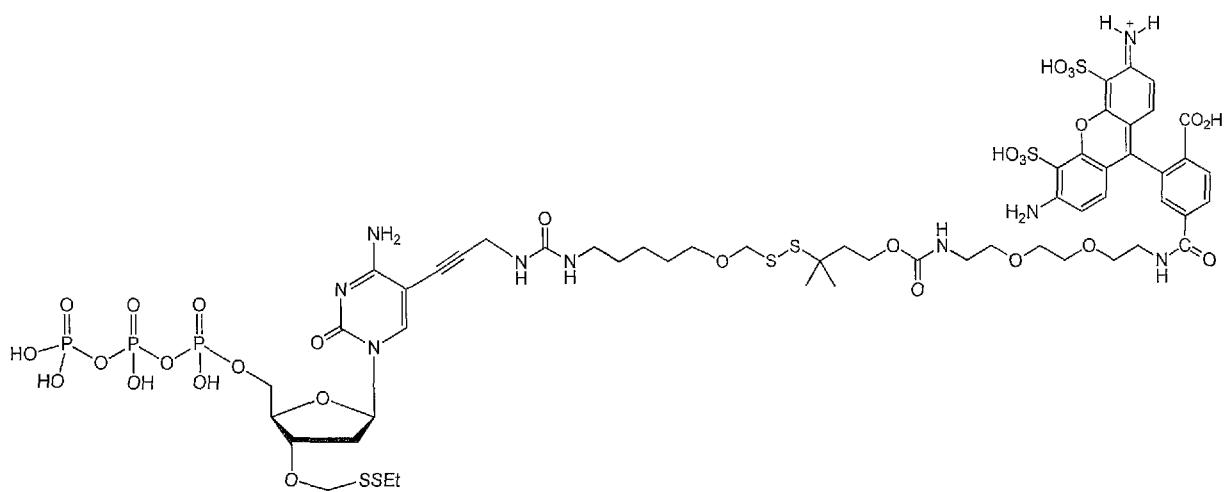
In one embodiment, L_2 is selected from the group consisting of



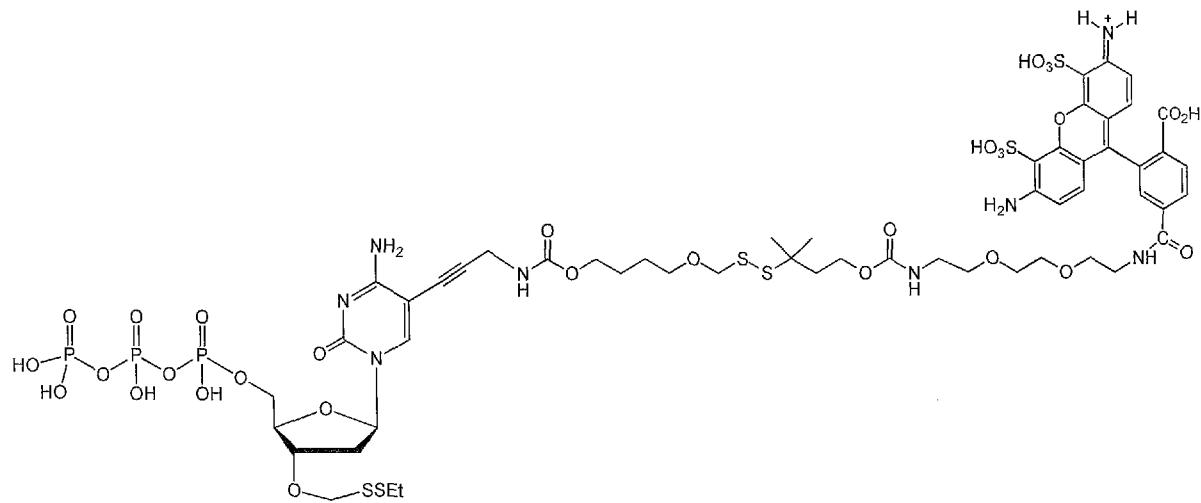
and $-\text{CH}(\text{Me})(\text{CH}_2)_x\text{NH}-$, $-\text{C}(\text{Me})_2(\text{CH}_2)_x\text{CO}-$, $-\text{CH}(\text{Me})(\text{CH}_2)_x\text{CO}-$, $-(\text{CH}_2)_x\text{OCONH}(\text{CH}_2)_y\text{O}(\text{CH}_2)_z\text{NH}-$, $-(\text{CH}_2)_x\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_y(\text{CH}_2)_z\text{NH}-$, and $-\text{CONH}(\text{CH}_2)_x-$, $-\text{CO}(\text{CH}_2)_x-$, wherein x , y , and z are each independently selected from 0-10, but more preferably from 1-6. In one embodiment, L_2 is selected from the group consisting of

$-\text{NH}-$, $-(\text{CH}_2)_x\text{NH}-$, $-\text{C}(\text{Me})_2(\text{CH}_2)_x\text{NH}-$, $-\text{CH}(\text{Me})(\text{CH}_2)_x\text{NH}-$, $-\text{C}(\text{Me})_2(\text{CH}_2)_x\text{CO}-$,

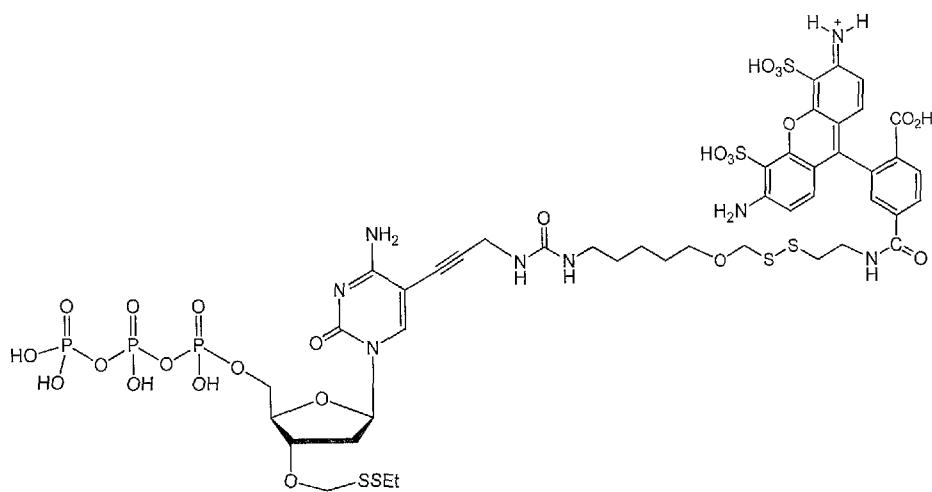
$-\text{CH}(\text{Me})(\text{CH}_2)_x\text{CO}-$, $-(\text{CH}_2)_x\text{OCONH}(\text{CH}_2)_y\text{O}(\text{CH}_2)_z\text{NH}-$, and $-\text{CONH}(\text{CH}_2)_x-$, $-\text{CO}(\text{CH}_2)_x-$, wherein x , y , and z are each independently selected from 0-100. In one embodiment, x , y , and z are each independently selected from 0-10, but more preferably from 1-6. In one embodiment, said label is selected from the group consisting of fluorophore dyes, energy transfer dyes, mass-tags, biotin, and haptene. In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):



In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):

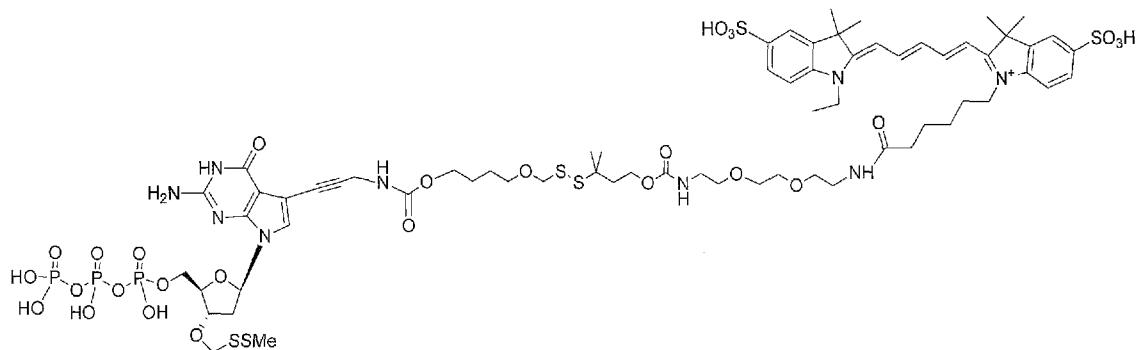


In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):

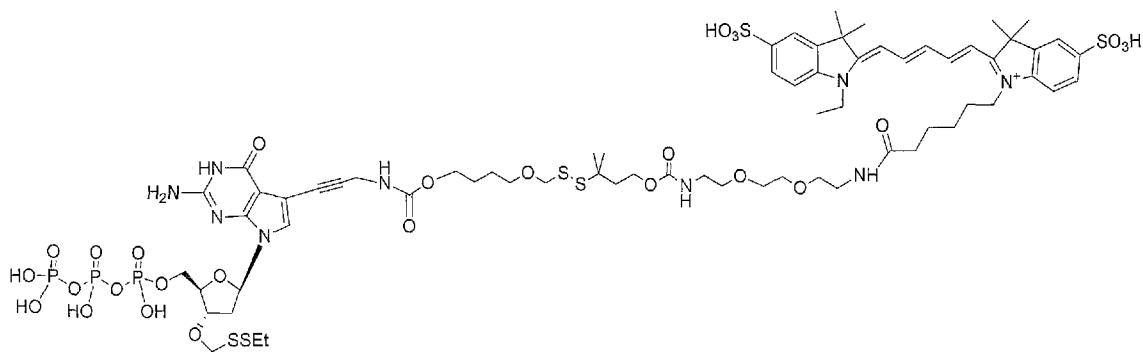


In one

embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):

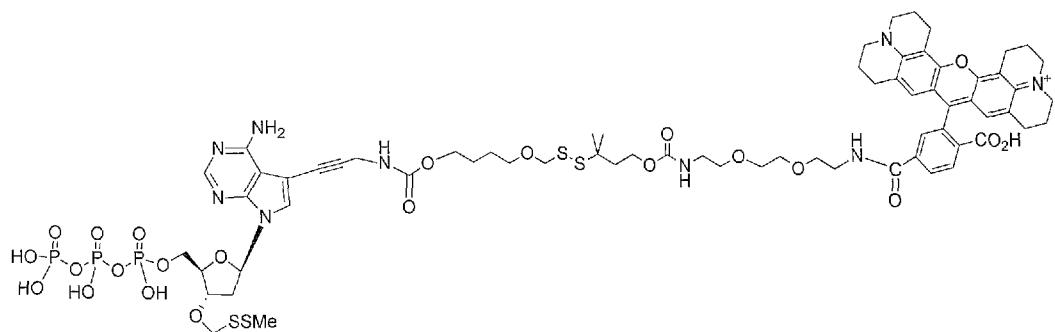


In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):



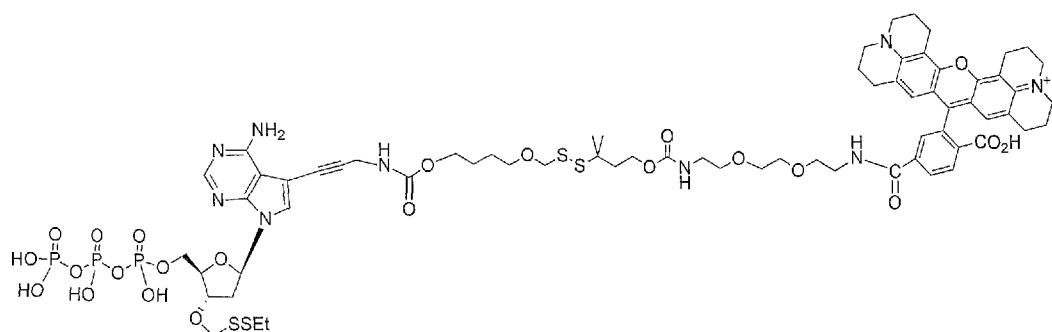
. In

one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):



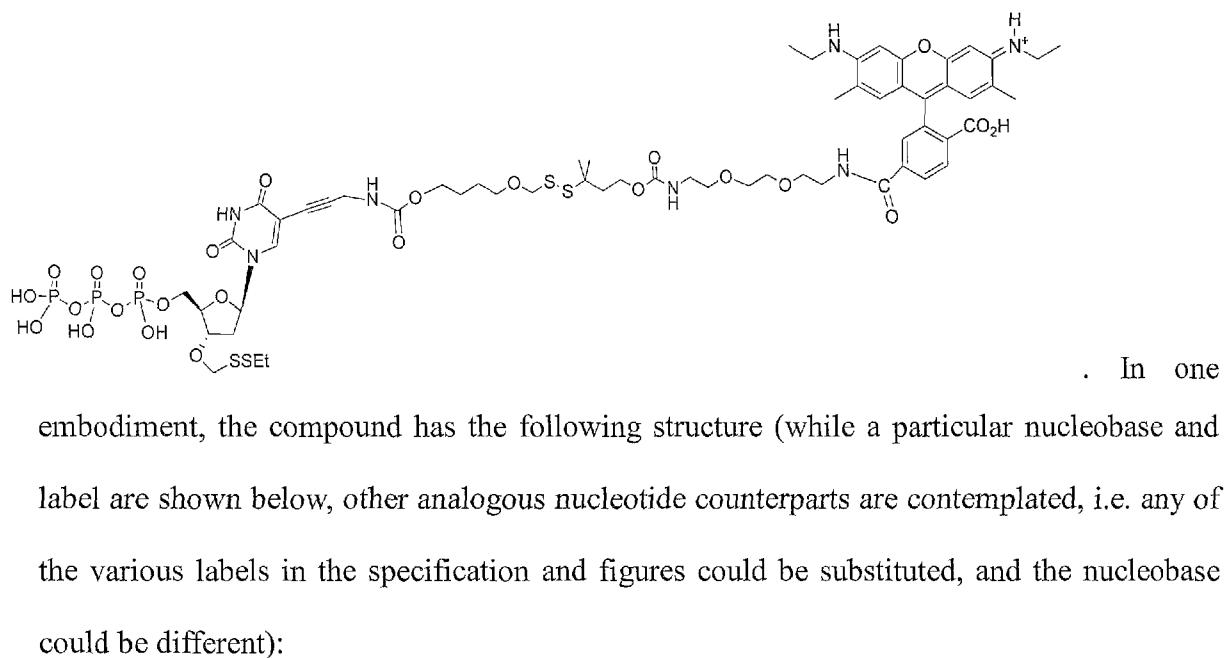
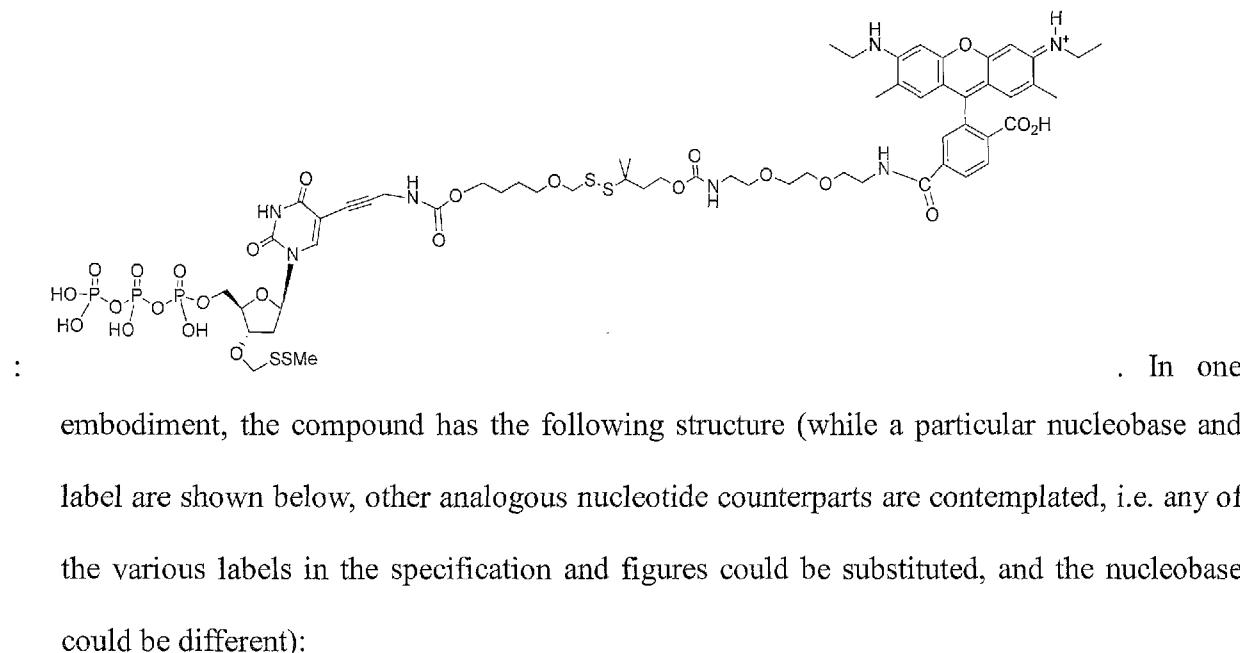
. In one

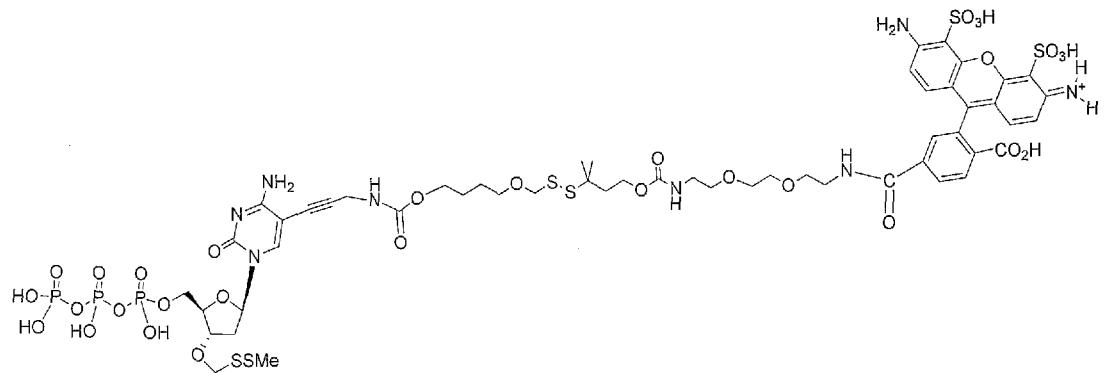
embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):



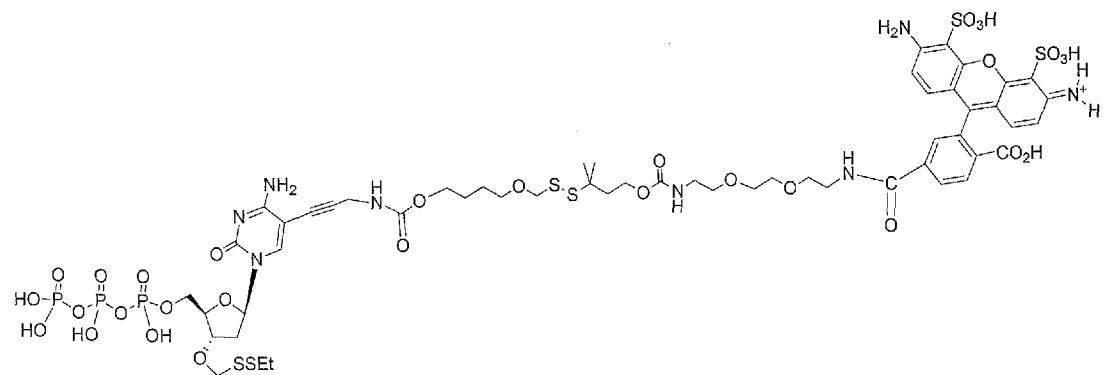
. In one

embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):

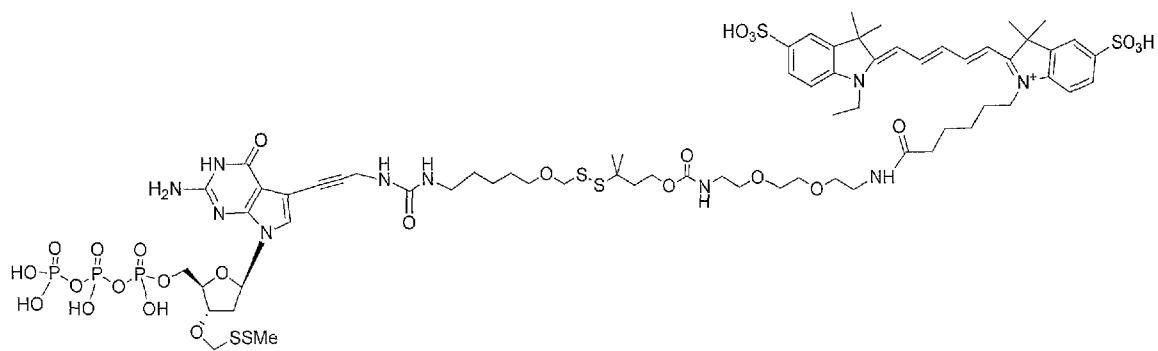




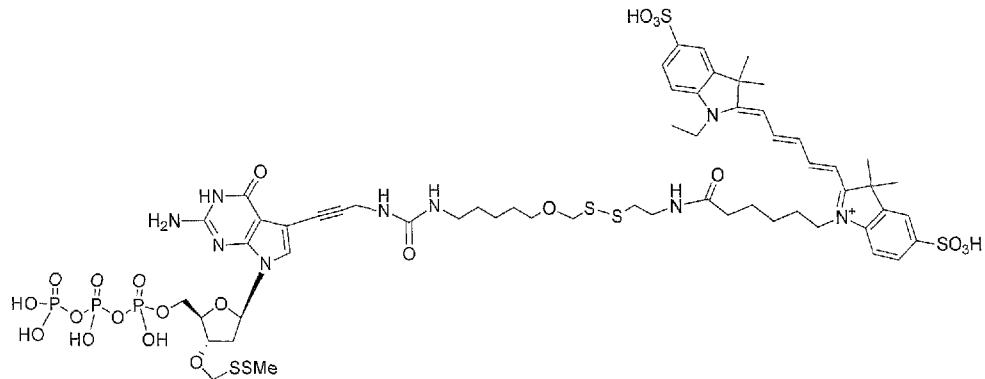
In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):



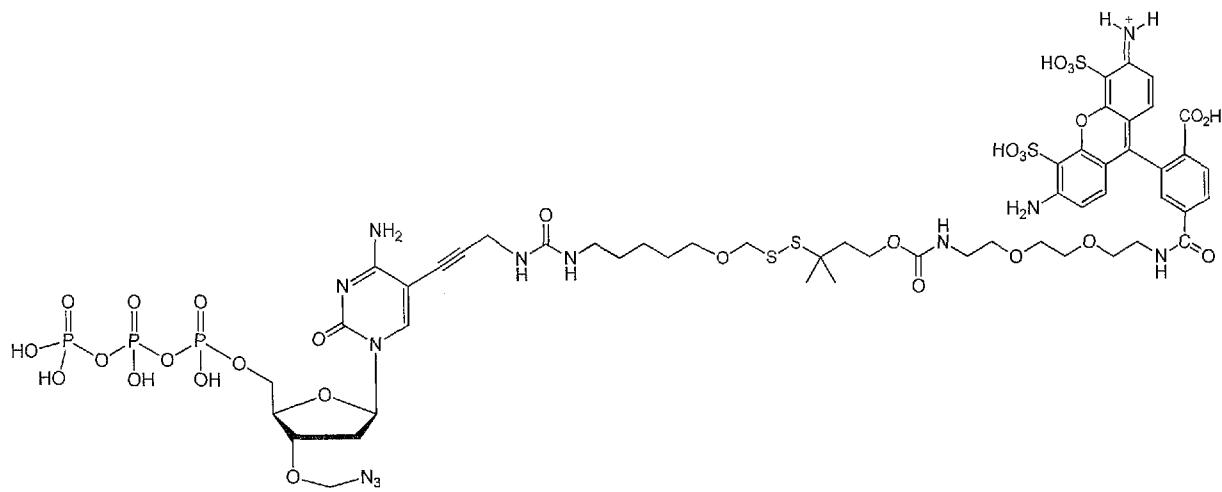
In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):



In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):

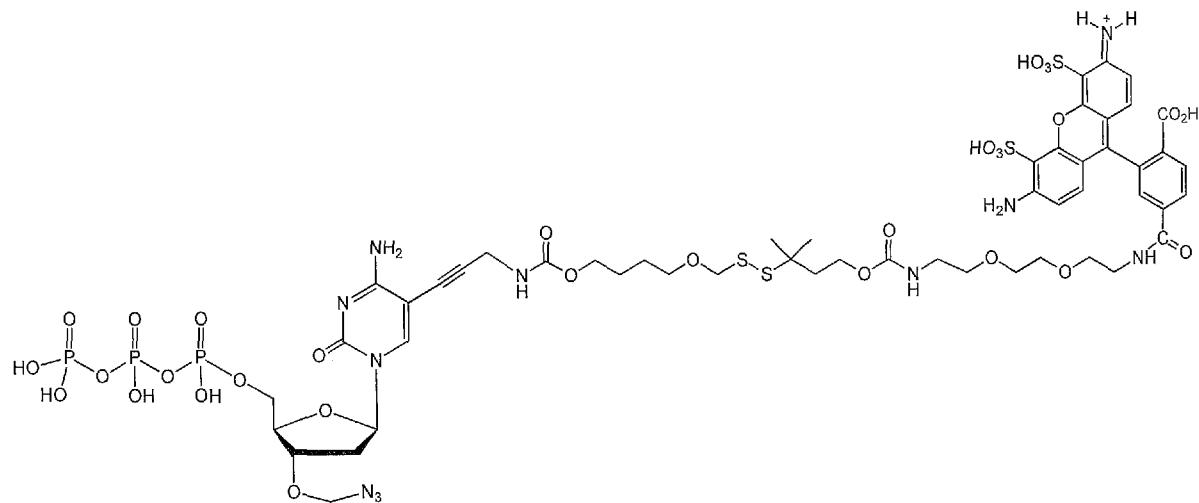


In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):

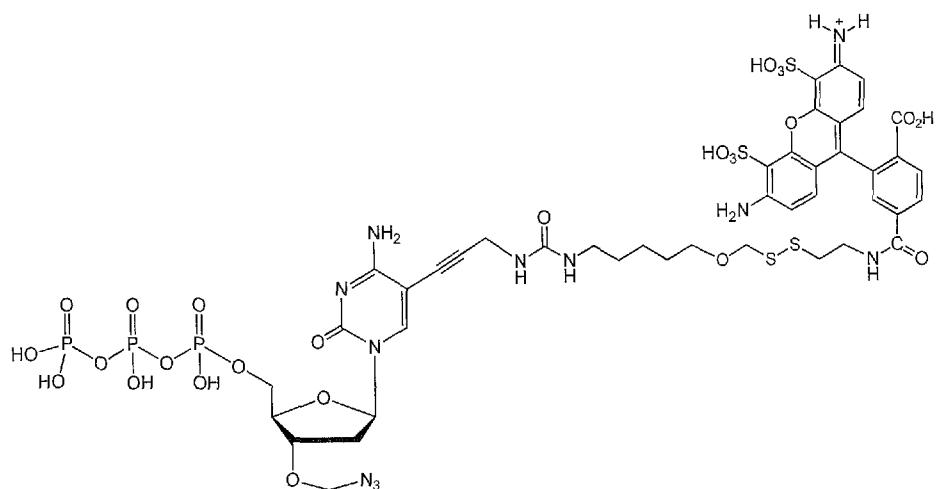


In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the

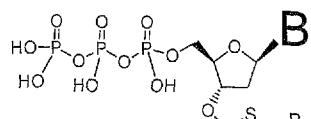
various labels in the specification and figures could be substituted, and the nucleobase could be different):

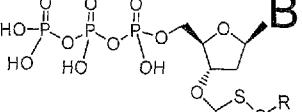


In one embodiment, the compound has the following structure (while a particular nucleobase and label are shown below, other analogous nucleotide counterparts are contemplated, i.e. any of the various labels in the specification and figures could be substituted, and the nucleobase could be different):

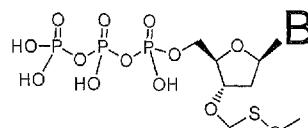


In one embodiment, the present invention contemplates unlabeled compounds. In one

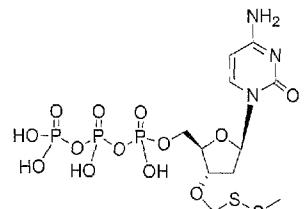


embodiment, the compound has the structure:  , wherein R is selected

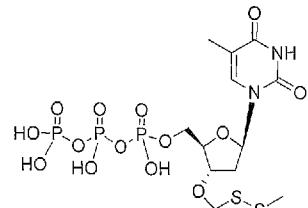
from the group consisting of alkyl, substituted alkyl groups, allyl, substituted allyl; and B is a nucleobase. In one embodiment, the compound has the structure (again B is a nucleobase):



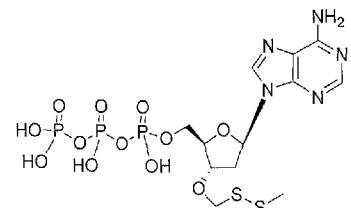
. In one embodiment, the compound has the structure:



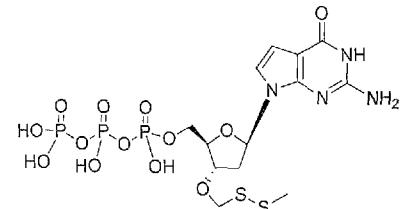
. In one embodiment, the compound has the structure:



. In one embodiment, the compound has the structure:



. In one embodiment, the compound has the structure:



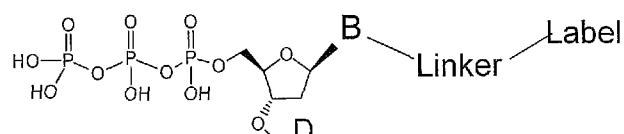
. In one embodiment, said nucleobase is a non-natural nucleobase

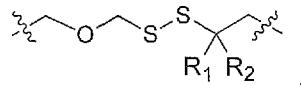
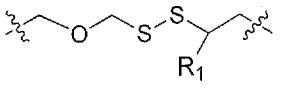
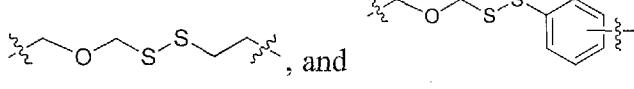
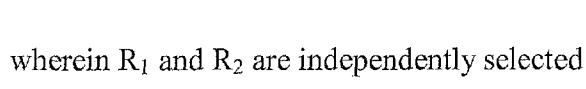
analog selected from the group consisting of 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine.

In one embodiment, the invention relates to a method of synthesizing 3'-OCH₂-SSMe

nucleotide analogs using 3'-(2,4,6-trimethoxyphenyl)methanethiol nucleoside as intermediate, and DMTSF and dimethyldisulfide as sulfur source shown in Figure 43.

In one embodiment, the invention relates to a labeled deoxynucleoside triphosphate according to the following structure:



, wherein D is selected from the group consisting of an azide, disulfide alkyl, disulfide substituted alkyl groups, disulfide allyl, and disulfide substituted allyl groups; B is a nucleobase; Linker comprises a cleavable oxymethylenedisulfide-containing site core. In one embodiment, said cleavable site core is selected from the group consisting of:   ,  , and  , wherein R₁ and R₂ are independently selected alkyl groups; and Label is a label. In one embodiment, said Linker is hydrophobic. In one embodiment, said Linker has a logP value of greater than 0. In one embodiment, said Linker has a logP value of greater than 0.1. In one embodiment, said Linker has a logP value of greater than 0.5. In one embodiment, said Linker has a logP value of greater than 1.0.

DEFINITIONS

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the

invention, but their usage does not delimit the invention, except as outlined in the claims.

As used herein, “hydrogen” means $-H$; “hydroxy” means $-OH$; “oxo” means $=O$; “halo” means independently $-F$, $-Cl$, $-Br$ or $-I$; “amino” means $-NH_2$ (see below for definitions of groups containing the term amino, *e.g.*, alkylamino); “hydroxyamino” means $-NHOH$; “nitro” means $-NO_2$; “imino” means $=NH$ (see below for definitions of groups containing the term imino, *e.g.*, alkylamino); “cyano” means $-CN$; “azido” means $-N_3$; “mercapto” means $-SH$; “thio” means $=S$; “sulfonamido” means $-NHS(O)_2-$ (see below for definitions of groups containing the term sulfonamido, *e.g.*, alkylsulfonamido); “sulfonyl” means $-S(O)_2-$ (see below for definitions of groups containing the term sulfonyl, *e.g.*, alkylsulfonyl); and “silyl” means $-SiH_3$ (see below for definitions of group(s) containing the term silyl, *e.g.*, alkylsilyl).

As used herein, “methylene” means a chemical species in which a carbon atom is bonded to two hydrogen atoms. The $-CH_2-$ group is considered to be the standard methylene group. Methylene groups in a chain or ring contribute to its size and lipophilicity. In this context dideoxy also refers the methylene groups. In particular a 2,3-dideoxy compound is the same as 2,3-methylene (2,3-methylene- glycoside =2,3-dideoxy- glycoside).

For the groups below, the following parenthetical subscripts further define the groups as follows: “ (C_n) ” defines the exact number (n) of carbon atoms in the group; “ $(C \leq n)$ ” defines the maximum number (n) of carbon atoms that can be in the group; $(C_{n-n'})$ defines both the minimum (n) and maximum number (n') of carbon atoms in the group. For example, “ $alkoxy_{(C \leq 10)}$ ” designates those alkoxy groups having from 1 to 10 carbon atoms (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, or any range derivable therein (*e.g.*, 3-10 carbon atoms)). Similarly, “ $alkyl_{(C_{2-10})}$ ” designates those alkyl groups having from 2 to 10 carbon atoms (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10, or any range derivable therein (*e.g.*, 3-10 carbon atoms)).

The term “alkyl” when used without the “substituted” modifier refers to a non-aromatic monovalent group with a saturated carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, no carbon-carbon double or triple bonds, and no atoms other than carbon and hydrogen. The groups, $-\text{CH}_3$ (Me), $-\text{CH}_2\text{CH}_3$ (Et), $-\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Pr), $-\text{CH}(\text{CH}_3)_2$ (*iso*-Pr or *i*-Pr), $-\text{CH}(\text{CH}_2)_2$ (cyclopropyl), $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Bu), $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ (*sec*-butyl or *sec*-Bu), $-\text{CH}_2\text{CH}(\text{CH}_3)_2$ (*iso*-butyl or *i*-Bu), $-\text{C}(\text{CH}_3)_3$ (*tert*-butyl or *t*-Bu), $-\text{CH}_2\text{C}(\text{CH}_3)_3$ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl are non-limiting examples of alkyl groups. The term “substituted alkyl” refers to a non-aromatic monovalent group with a saturated carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, no carbon-carbon double or triple bonds, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The following groups are non-limiting examples of substituted alkyl groups: $-\text{CH}_2\text{OH}$, $-\text{CH}_2\text{Cl}$, $-\text{CH}_2\text{Br}$, $-\text{CH}_2\text{SH}$, $-\text{CF}_3$, $-\text{CH}_2\text{CN}$, $-\text{CH}_2\text{C}(\text{O})\text{H}$, $-\text{CH}_2\text{C}(\text{O})\text{OH}$, $-\text{CH}_2\text{C}(\text{O})\text{OCH}_3$, $-\text{CH}_2\text{C}(\text{O})\text{NH}_2$, $-\text{CH}_2\text{C}(\text{O})\text{NHCH}_3$, $-\text{CH}_2\text{C}(\text{O})\text{CH}_3$, $-\text{CH}_2\text{OCH}_3$, $-\text{CH}_2\text{OCH}_2\text{CF}_3$, $-\text{CH}_2\text{OC}(\text{O})\text{CH}_3$, $-\text{CH}_2\text{NH}_2$, $-\text{CH}_2\text{NHCH}_3$, $-\text{CH}_2\text{N}(\text{CH}_3)_2$, $-\text{CH}_2\text{CH}_2\text{Cl}$, $-\text{CH}_2\text{CH}_2\text{OH}$, $-\text{CH}_2\text{CF}_3$, $-\text{CH}_2\text{CH}_2\text{OC}(\text{O})\text{CH}_3$, $-\text{CH}_2\text{CH}_2\text{NHCO}_2\text{C}(\text{CH}_3)_3$, and $-\text{CH}_2\text{Si}(\text{CH}_3)_3$.

The terms “cleavable oxymethylenedisulfide linker” and “cleavable oxymethylenedisulfide-containing linker” are meant to indicate that the linker comprises an oxymethylenedisulfide group, and are not to be considered limited to only an oxymethylenedisulfide group, but rather linkers that may contain more than just that group, for example as seen in the compounds in Figure 25. Similarly, the terms “oxymethylenedisulfide site core” and “oxymethylenedisulfide-containing site core” are meant to indicate that the site core comprises an oxymethylenedisulfide group, and are not to be considered limited to only an oxymethylenedisulfide group, but rather site cores that may contain more than just that group.

The term “nucleic acid” generally refers to both DNA or RNA, whether it is a product of amplification, synthetically created, products of reverse transcription of RNA or naturally occurring. Typically, nucleic acids are single- or double-stranded molecules and are composed of naturally occurring nucleotides. Double-stranded nucleic acid molecules can have 3' - or 5' -overhangs and as such are not required or assumed to be completely double-stranded over their entire length. Furthermore, the nucleic acid can be composed of non-naturally occurring nucleotides and/or modifications to naturally occurring nucleotides. Examples are listed herein, but are not limited to: phosphorylation of 5' or 3' nucleotides to allow for ligation or prevention of exonuclease degradation/polymerase extension, respectively; amino, thiol, alkyne, or biotinyl modifications for covalent and near covalent attachments; fluorophores and quenchers; phosphorothioate, methylphosphonates, phosphoroamidates and phosphotriester linkages between nucleotides to prevent degradation; methylation; and modified bases or nucleosides such as deoxy-inosine, 5-bromo-dU, 2' -deoxy-uridine, 2-aminopurine, 2' ,3' -dideoxy-cytidine, 5-methyl-dC, locked nucleic acids (LNA's), iso-dC and -dG bases, 2' -O-methyl RNA bases and fluorine modified nucleosides.

In some of the methods contemplated herein, primers are at least partially complementary to at least a portion of template to be sequenced. The term “complementary” generally refers to the ability to form favorable thermodynamic stability and specific pairing between the bases of two nucleotides (e.g. A with T) at an appropriate temperature and ionic buffer conditions. This pairing is dependent on the hydrogen bonding properties of each nucleotide. The most fundamental examples of this are the hydrogen bond pairs between thymine/adenine and cytosine/guanine bases. In the present invention, primers for amplification of target nucleic acids can be both fully complementary over their entire length with a target nucleic acid molecule or “semi-complementary” wherein the primer contains an additional, non-complementary sequence

minimally capable or incapable of hybridization to the target nucleic acid.

The term “hybridize” generally refers to the base-pairing between different nucleic acid molecules consistent with their nucleotide sequences. The terms “hybridize” and “anneal” can be used interchangeably.

The term “oligonucleotide” generally refers to a nucleic acid sequence typically designed to be single-stranded DNA and less than 75 nucleotides in length.

The term “primer” generally refers to an oligonucleotide that is able to anneal, or hybridize, to a nucleic acid sequence and allow for extension under sufficient conditions (buffer, dNTP's, polymerase, mono- and divalent salts, temperature, etc. . . .) of the nucleic acid to which the primer is complementary.

The terms “template nucleic acid”, “template molecule”, “target nucleic acid”, and “target molecule” can be used interchangeably and refer to a nucleic acid molecule that is the subject of an amplification reaction that may optionally be interrogated by a sequencing reaction in order to derive its sequence information. The template nucleic acid may be a nucleic acid which has been generated by a clonal amplification method and which may be immobilized on a solid surface, i.e. immobilized on beads or an array.

The term “nucleoside” refers to a compound consisting of a base linked to the C-1' carbon of a sugar, for example, ribose or deoxyribose. The base portion of the nucleoside is usually a heterocyclic base, e.g., a purine or pyrimidine.

The term “nucleotide” refers to a phosphate ester of a nucleoside, as a monomer unit or within a polynucleotide. “Nucleoside 5'-triphosphate” refers to a nucleotide with a triphosphate ester group attached to the sugar 5'-carbon position, and is sometimes denoted as “NTP”, “dNTP” (2'-deoxynucleoside triphosphate or deoxynucleoside triphosphate) and “ddNTP” (2',3'-dideoxynucleoside triphosphate or dideoxynucleoside triphosphate). “Nucleoside

“5'-tetraphosphate” refers to an alternative activated nucleotide with a tetraphosphate ester group attached to the sugar 5'-carbon position. PA-nucleotide refers to a propargyl analogue.

The term “protecting group,” as that term is used in the specification and/or claims, is used in the conventional chemical sense as a group, which reversibly renders unreactive a functional group under certain conditions of a desired reaction and is understood not to be H. After the desired reaction, protecting groups may be removed to deprotect the protected functional group. In a preferred embodiment, all protecting groups should be removable (and hence, labile) under conditions which do not degrade a substantial proportion of the molecules being synthesized. A protecting group may also be referred to as a “capping group” or a “blocking group” or a “cleavable protecting group.” It should be noted that, for convenience, the functionality protected by the protecting group may also be shown or referred to as part of the protecting group. In the context of the nucleotide derivatives described herein, a protecting group is used on the 3' position. It is not intended that the present invention be limited by the nature or chemistry of this protecting group on the reversibly terminating nucleotides used in sequencing. A variety of protecting groups is contemplated for this purpose, including but not limited to: 3'-O-azidomethyl nucleotides, 3'-O-aminoxy nucleotides, 3'-O-allyl nucleotides; and disulfide nucleotides, 3'-O-azidoalkyl, 3'-O-dithiomethyl alkyl, 3'-O-dithiomethyl aryl, 3'-O-acetyl, 3'-O-carbazate, 3'-O-alkyl ether, 3'-O-alkyl ester, 3'-O-aldoxime (-O-N=CH-R), 3'-O-ketoxime (-O-N=C(R, R')).

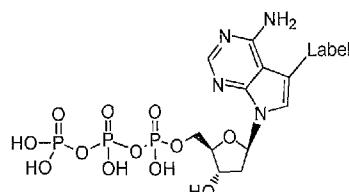
One embodiment of the present invention contemplates attaching markers directly on the 3'-OH function of the nucleotide via functionalization of the protective groups.

The term “label” or “detectable label” in its broadest sense refers to any moiety or property that is detectable, or allows the detection of that which is associated with it. For example, a nucleotide, oligo- or polynucleotide that comprises a label is detectable. Ideally, a

labeled oligo- or polynucleotide permits the detection of a hybridization complex, particularly after a labeled nucleotide has been incorporated by enzymatic means into said hybridization complex of a primer and a template nucleic acid. A label may be attached covalently or non-covalently to a nucleotide, oligo- or polynucleotide. In various aspects, a label can, alternatively or in combination: (i) provide a detectable signal; (ii) interact with a second label to modify the detectable signal provided by the second label, e.g., FRET; (iii) stabilize hybridization, e.g., duplex formation; (iv) confer a capture function, e.g., hydrophobic affinity, antibody/antigen, ionic complexation, or (v) change a physical property, such as electrophoretic mobility, hydrophobicity, hydrophilicity, solubility, or chromatographic behavior. Labels vary widely in their structures and their mechanisms of action. Examples of labels include, but are not limited to, fluorescent labels, non-fluorescent labels, colorimetric labels, chemiluminescent labels, bioluminescent labels, radioactive labels, mass-modifying groups, antibodies, antigens, biotin, haptens, enzymes (including, e.g., peroxidase, phosphatase, etc.), and the like. To further illustrate, fluorescent labels may include dyes of the fluorescein family, dyes of the rhodamine family, dyes of the cyanine family, or a coumarine, an oxazine, a boradiazaindacene or any derivative thereof. Dyes of the fluorescein family include, e.g., FAM, HEX, TET, JOE, NAN and ZOE. Dyes of the rhodamine family include, e.g., Texas Red, ROX, R110, R6G, and TAMRA. FAM, HEX, TET, JOE, NAN, ZOE, ROX, R110, R6G, and TAMRA are commercially available from, e.g., Perkin-Elmer, Inc. (Wellesley, Mass., USA), Texas Red is commercially available from, e.g., Life Technologies (Molecular Probes, Inc.) (Grand Island, N.Y.). Dyes of the cyanine family include, e.g., CY2, CY3, CY5, CY5.5 and CY7, and are commercially available from, e.g., GE Healthcare Life Sciences (Piscataway, N.J., USA).

The term “differently labeled,” as used herein, refers to the detectable label being a different label, rather than the label being found in a different position upon the labeled nucleoside nucleobase.

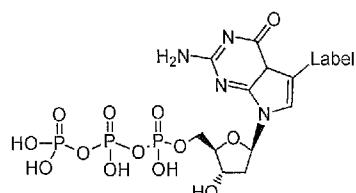
The term “analogs of A, G, C and T or U” refers to modified deoxynucleoside triphosphate compounds, wherein the nucleobase of said deoxynucleoside closely resembles the corresponding nucleoside Deoxyadenosine, Deoxyguanosine, Deoxycytidine, and Thymidine or Deoxyuridine. In the case of detectable labeled deoxynucleoside triphosphate compounds an analog of A or



Deoxyadenosine would be represented as

although it is preferred that

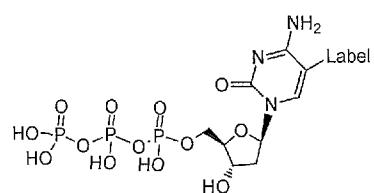
there be a linker between the nucleobase and the label. In the case of detectable labeled deoxynucleoside triphosphate compounds an analog of G or Deoxyguanosine would be



represented as

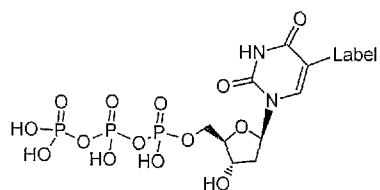
although it is preferred that there be a linker between

the nucleobase and the label. In the case of detectable labeled deoxynucleoside triphosphate compounds an analog of C or Deoxycytidine would be represented as



although it is preferred that there be a linker between the nucleobase

and the label. In the case of detectable labeled deoxynucleoside triphosphate compounds an analog of T or U or Thymidine or Deoxyuridine would be represented as



although it is preferred that there be a linker between the nucleobase and the label. Additional nucleobase may include: non-natural nucleobase selected from the group consisting of 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine. In the case of analogs, the detectable label may also include a linker section between the nucleobase and said detectable label.

The term “TCEP” or “tris(2-carboxyethyl)phosphine)” refers to a reducing agent frequently used in biochemistry and molecular biology applications. It is often prepared and used as a hydrochloride salt (TCEP-HCl) with a molecular weight of 286.65 gram/mol. It is soluble in water and available as a stabilized solution at neutral pH and immobilized onto an agarose support to facilitate removal of the reducing agent. It is not intended that the invention is limited to one type of reducing agent. Any suitable reducing agent capable of reducing disulfide bonds can be used to practice the present invention. In one embodiment the reducing agent is phosphine [12], for example, triphenylphosphine, tributylphosphine, trihydroxymethyl phosphine, trihydroxypropyl phosphine, tris carboethoxy-phosphine (TCEP) [13, 14]. It is not intended that the present invention be limited to the use of TCEP. In one embodiment, said detectable label and 3'-OCH₂-SS-R group are removed from said nucleobase by exposure to compounds carrying a thiol group so as to perform cleavage of dithio-based linkers and terminating (protecting) groups, such thiol-containing compounds including (but not limited to) cysteine, cysteamine, dithio-succinic acid, dithiothreitol, 2,3-Dimercapto-1-propanesulfonic acid sodium salt, dithiobutylamine, meso-2,5-dimercapto-N,N,N',N'-tetramethyladipamide, 2-mercaptop-ethane sulfonate, and N,N'-dimethyl, N,N'-bis(mercaptoproacetyl)-hydrazine [17]. Reactions can be further

catalyzed by inclusion of selenols [18]. In addition borohydrides, such as sodium borohydrides can also be used for this purpose [19] (as well as ascorbic acid [20]. In addition, enzymatic methods for cleavage of disulfide bonds are also well known such as disulfide and thiooxidase and can be used with compounds of the present invention [21].

DESCRIPTION OF THE FIGURES

The accompanying figures, which are incorporated into and form a part of the specification, illustrate several embodiments of the present invention and, together with the description, serve to explain the principles of the invention. The figures are only for the purpose of illustrating a preferred embodiment of the invention and are not to be construed as limiting the invention.

Figure 1 shows examples of nucleoside triphosphates with 3'-O capped by a group comprising methylenedisulfide, where the R represents alkyl group such as methyl, ethyl, isopropyl, t-butyl, n-butyl, or their analogs with substituent group containing hetero-atoms such as O, N, S etc.

Figure 2 shows labeled analogs of nucleoside triphosphates with 3'-O methylenedisulfide-containing protecting group, where labels are attached to the nucleobase via cleavable oxymethylenedisulfide linker (-OCH₂-SS-). The analogs are (clockwise from the top left) for Deoxyadenosine, Thymidine or Deoxyuridine, Deoxycytidine and Deoxyguanosine.

Figure 3 shows a step-wise mechanism of deprotection of the 3'-O protection group with a reducing agent, such as TCEP.

Figure 4 shows the cleavage reactions products a traditional sulfide and oxymethylene sulfide linked labeled nucleotides.

Figure 5 shows an example of the labeled nucleotides where the spacer of the cleavable

linker includes the propargyl ether linker. The analogs are (clockwise from the top left) for Deoxyadenosine, Thymidine or Deoxyuridine, Deoxycytidine and Deoxyguanosine.

Figure 6 shows an example of the labeled nucleotides where the spacer of the cleavable linker includes the propargylamine linker. The analogs are (clockwise from the top left) for Deoxyadenosine, Thymidine or Deoxyuridine, Deoxycytidine and Deoxyguanosine.

Figure 7 shows an example of the labeled nucleotides where the spacer of the cleavable linker includes the methylene $-(\text{CH}_2)_n-$ directly attached to the nucleobases at 5- position for pyrimidine, and at 7- de-aza-carbon for purines. This linker may be methylene ($n=1$) or polymethylene ($n>1$) where after cleavage, the linker generates $-(\text{CH}_2)_n\text{OH}$ group at the point of attachment on the nucleobases, and where the L_1 and L_2 represent spacers, and substituents R_1 , R_2 , R_3 and R_4 are group of atoms that provide stability to the cleavable linker as described earlier. The analogs are (clockwise from the top left) for Deoxyadenosine, Thymidine or Deoxyuridine, Deoxycytidine and Deoxyguanosine.

Figure 8 shows a synthesis of the unlabeled dT analog (compound **5**).

Figure 9 shows the synthesis of 3'-O-(ethyldithiomethyl)-dCTP (**10**).

Figure 10 shows a synthetic route of the labeled nucleotides specific for labeled dT intermediate.

Figure 11 shows a cleavable linker synthesis starting from an 1,4-dutanediol.

Figure 12 shows another variant of cleavable linker, where the stabilizing gem-dimethyl group is attached to α -carbon of the cleavable linker.

Figure 13 shows the synthesis of a cleavable linker, where the disulfide is flanked by gem-dimethyl groups and attached to a flexible ethylene glycol linker (PEG). The linker is attached to the PA-nucleotide via carbamate group ($-\text{NH}-\text{C}(=\text{O})\text{O}-$). The resulting nucleotide analogue in such case can be as in compound **35** (dUTP analogue).

Figure 14 shows the synthesis of a cleavable linker for dATP analogue where the cleavable disulfide is flanked by gem-dimethyl group and the linker is attached to PA-nucleotide via urea group (-NH(C=O)NH-). For other nucleotide analogues (e.g. for analogues of dCTP, dGTP, dUTP) can be synthesized similarly replacing **42** by appropriate PA-analogues at the last step of the reaction sequence.

Figure 15 shows the synthesis of a cleavable linker compound **45**, where the linker is tethered to PA-nucleotides *via* urea functionality and the disulfide is connected to the dye by a two carbon linker. The resulting nucleotide analogue in such case can be as in compound **49** (dGTP analogue). Other nucleotide analogues (e.g. analogues of dATP, dUTP, dCTP) can be synthesized similarly by replacing nucleotide **46** with appropriate PA-nucleotide analogues in the third step of the reaction sequence.

Figure 16 shows that when labeled nucleotide **50** was exposed to 10 eq of TCEP at 65 °C, it generated a number of side products including compound **52** along with the expected product **51**.

Figure 17 shows an LC-MS trace of the TCEP exposed product of compound **50**, extracted at 292 nm (bottom) and 524 nm (top), analyzed after 5 minutes exposure, where peak at 11.08min corresponds to compound **51**, peak at 10.88 min to compound **52** and other peaks to side products.

Figure 18 shows an LC-MS trace of the TCEP exposed product of compound **50**, extracted at 292 nm (bottom) and 524 nm (top), analyzed after 15 minutes exposure; where peak at 11.32min corresponds to compound **51** and other peaks to side products.

Figure 19 shows that under identical cleavage conditions, the oxymethylenedisulfide linked nucleotide **35** cleanly produced the desired cleavage products, compounds **53** and **54**. The methylene thiol segment (-CH₂SH) of the linker was fully eliminated from the nucleotide

upon cleavage of the disulfide group

Figure 20 shows an LC-MS trace of the TCEP exposed product of compound **35**, extracted at 292 nm (bottom) and 524 nm (top), analyzed after 5 minutes exposure, where peak at 11.24min corresponds to compound **53** and peak at 34.70 min to compound **54**.

Figure 21 shows LC-MS trace of the TCEP exposed product of compound **35**, extracted at 292 nm (bottom) and 524 nm (top), analyzed after 15 minutes exposure, where peak at 11.25min corresponds to compound **53** and peak at 34.70 min to compound **54**.

Figure 22 shows the synthesis of 3'-OCH₂-SS-Me analogues with the replacement of mercaptoethanol (EtSH) by methanethiol or sodium thiomethoxide at the appropriate step, different from that of 3'-OCH₂-SS-Et (Figure 10).

Figure 23 shows the coupling of PA-nucleotide (e.g. **57**) to the appropriate cleavable -OCH₂-SS- linkers, and finally to fluorophore dye using the activated linker **32**.

Figure 24 shows nucleotide analogues with different linker achieved, compounds **60** and **61**.

Figure 25 shows the structure of 4-nucleotide analogues labeled by different fluorophore reporting groups, where R = Me- or Et-.

Figure 26 shows the structure of 4-nucleotide analogues labeled by different fluorophore reporting groups, where R = Me- or Et- group.

Figure 27 shows the structure of 4-nucleotide analogues labeled by different fluorophore reporting groups, where R = Me- or Et- group.

Figure 28 shows Generic universal building blocks structures comprising new cleavable linkers of present invention. PG = Protective Group, L1, L2 – linkers (aliphatic, aromatic, mixed polarity straight chain or branched). RG = Reactive Group. In one embodiment of present invention such building blocks carry an Fmoc protective group on one end of the linker and

reactive NHS carbonate or carbamate on the other end. This preferred combination is particularly useful in modified nucleotides synthesis comprising new cleavable linkers. A protective group should be removable under conditions compatible with nucleic acid/nucleotides chemistry and the reactive group should be selective. After reaction of the active NHS group on the linker with amine terminating nucleotide, an Fmoc group can be easily removed using base such as piperidine or ammonia, therefore exposing amine group at the terminal end of the linker for the attachment of cleavable marker. A library of compounds comprising variety of markers can be constructed this way very quickly.

Figure 29 shows generic structure of nucleotides carrying cleavable marker attached via novel linker of present invention. S = sugar (i.e., ribose, deoxyribose), B = nucleobase, R = H or reversibly terminating group (protective group). Preferred reversibly terminating groups include but are not limited to: Azidomethyl (-CH₂N₃), Dithio -alkyl (-CH₂-SS-R), aminoxy (-ONH₂).

Figure 30 shows another generic structure for nucleotides carrying cleavable marker attached via the cleavable linker of present invention, wherein D is selected from the group comprising an azide, disulfide alkyl and disulfide substituted alkyl groups, B is a nucleobase, A is an attachment group, C is a cleavable site core, L₁ and L₂ are connecting groups, and Label is a label (in the compounds with a label).

Figure 31 shows the chemical structures of compounds (L-series (96), B-series (97), (A-series, (98), and (G-series (99) family) tested in Figure 32A-C.

Figure 32A shows a time course of incorporation of 3'-O-azidomethyl Alexa488 labeled nucleotide analogs with various disulfide based cleavable linkers: L-Series (96), B-series (97), A-series (98), and G-series (99) family.

Figure 32B shows reaction rates of incorporation for 3'-O-azidomethyl Alexa488 labeled nucleotide analogs with various disulfide based cleavable linkers: L-series (96), B-series (97),

A-series (98), and G-series (99) family.

Figure 32C shows reaction rates of incorporation for 3'-O-azidomethyl Alexa488 labeled nucleotide analogs with various disulfide based cleavable linkers: L-series (96), B-series (97), A-series (98), and G-series (99) family vs concentration of nucleotides.

Figure 33 shows incorporation kinetics for the dA 3'- reversibly terminating nucleotides: -CH₂-N₃, -CH₂-SS-Et, -CH₂-SS-Me.

Figure 34 shows incorporation kinetics of dC 3'-reversibly terminating nucleotide with 3'-O-CH₂-SS-Et terminating group with 3 different DNA polymerases: T9, J5 and J8.

Figure 35 shows optimized concentrations of nucleotides used in Extend A reactions on GR sequencer [nM].

Figure 36 shows sequencing performance of A-series (98) nucleotides as measured by raw error rate.

Figure 37 shows sequencing performance of A-series (98) nucleotides as measured by percentage of perfect (error free) reads.

Figure 38 shows sequencing performance of A-series (98) nucleotides as measured by variety of sequencing metrics.

Figure 39 shows sequencing performance of G-series (99) nucleotides as measured by raw error rate.

Figure 40 shows sequencing performance of G-series (99) nucleotides as measured by percentage of perfect (error free) reads.

Figure 41 shows identification of multiplex barcodes from sequencing runs containing 3'-O-CH₂-SS-Et nucleotides in ExtB and in both ExtB and A.

Figure 42 shows a comparison of stability at elevated temperature in Extend A buffer of labeled, reversibly terminating dC with various cleavable linkers: B = B-series (97, 116, 117, and

118), G = G-series (99, 103, 104, and 105), A = A-series (98, 100, 101, and 102), and SS = L-series (96, 50, 106, and 115).

Figure 43 shows a synthetic scheme illustrating the synthesis of compounds 63-67 from compound 62. The synthesis is described in Example 33, Example 34 and Example 35.

Figure 44 shows a synthetic scheme illustrating the synthesis of compounds 69-71 and 119-120 from compound 68. The synthesis is described in Example 36, Example 37, and Example 38.

Figure 45 shows complete chemical structures of four labeled nucleotides corresponding to dCTP, dTTP, dATP and dGTP from top to bottom (A-series, 98, 100, 101, and 102).

Figure 46 shows complete chemical structures of four labeled nucleotides corresponding to dCTP, dTTP, dATP and dGTP from top to bottom (G-series, 99, 103, 104, and 105).

Figure 47 shows complete chemical structures of four labeled nucleotides corresponding to dCTP, dTTP, dATP and dGTP from top to bottom (L-series, 96, 50, 106, and 115).

Figure 48 shows complete chemical structures of four labeled nucleotides corresponding to dCTP, dTTP, dATP and dGTP from top to bottom (B-series: compounds 97, 116, 117, and 118).

Figure 49 shows example concentrations of nucleotides used in sequencing on GR instrument (labeled, compounds 72, 74, 76, 78) and non-labeled (compounds 120, 126, 132, 138), all carrying the -CH₂-SS-Me on their 3' as reversibly terminating group.

Figure 50 shows example of intensities generated in sequencing run on GR using novel nucleotides (labeled and non-labeled as in described for Figure 49), all carrying the -CH₂-SS-Me).

Figure 51 shows a series of non-linker examples of nucleoside triphosphates with 3'-O capped by a group comprising methylenedisulfide methyl.

Figure 52 shows the structure of 4-nucleotide analogues labeled by different fluorophore reporting groups with 3'-O capped by a group comprising methylenedisulfide methyl.

Figure 53 is a schematic that shows one embodiment of a synthesis of NHS activated form of common linker.

Figure 54 is a schematic that shows one embodiment of the synthesis of MeSSdATP.

Figure 55 is a schematic that shows one embodiment of the synthesis of MeSSdCTP.

Figure 56 is a schematic that shows one embodiment of the synthesis of MeSSdGTP.

Figure 57 is a schematic that shows one embodiment of the synthesis of MeSSdTTP.

Figure 58 is a schematic that shows one embodiment of the synthesis of MeSSdATP-PA.

Figure 59 is a schematic that shows one embodiment of the synthesis of **76**.

Figure 60 is a schematic that shows one embodiment of the synthesis of MeSSdCTP-PA.

Figure 61 is a schematic that shows one embodiment of the synthesis of **72**.

Figure 62 is a schematic that shows one embodiment of the synthesis of MeSSdGTP-PA.

Figure 63 is a schematic that shows one embodiment of the synthesis of MeSSdGTP-ARA-Cy5.

Figure 64 is a schematic that shows one embodiment of the synthesis of MeSSdUTP-PA.

Figure 65 is a schematic that shows one embodiment of the synthesis of **74**.

Figure 66 is a schematic that shows the structures of 3'-OCH₂S-(2,4,6-trimethoxyphenyl)methane-dNTPs.

Figure 67 is a schematic that shows one embodiment of the synthesis of 3'-(OCH₂SSMe)-dNTPs from 3'-OCH₂S-(2,4,6-trimethoxyphenyl)methane-dNTPs.

Figure 68 shows the key intermediates for the synthesis of 3'-(OCH₂SSMe)-dNTP-PAs.

Figure 69 is a schematic that shows one embodiment of the synthesis of 3'-(OCH₂SSMe)-dNTP-PA from 3'-OCH₂S-(2,4,6-trimethoxyphenyl)methane-dNTP-PAs.

Figure 70 is a schematic that shows linker installation and conjugation of fluorescent dye.

Figure 71 shows structures of hydroxymethyl derivatives nucleobases derivatives that could be used to attach linkers and terminating groups of the present invention. R = reversibly terminating group, CL = cleavable linker of the present invention.

Figure 72 shows structures of hydroxymethyl derivatives nucleobases derivatives after cleavage has been performed.

Figure 73 shows examples of compounds carrying thiol, function that could be used to perform cleavage of dithio-based linkers and terminating groups of the present invention: A) – cysteamine, B) – dithio-succinic acid, C) – cysteamine, D) – dithiothreitol, E) – 2,3-Dimercapto-1-propanesulfonic acid sodium salt, F) – dithiobutylamine, G) – meso-2,5-dimercapto-N,N,N',N'-tetramethyladipamide, H) 2-mercaptopropane sulfonate, I) – N,N'-dimethyl, N,N'-bis(mercaptopropanoyl)-hydrazine.

Figure 74 shows an example of selective and stepwise cleavage of linker and 3'-protective group – chemical structures and reaction scheme.

Figure 75 shows an example of selective and stepwise cleavage of linker – chromatograms associated with each step of the cleavage.

Figure 76 shows an example of selective and stepwise cleavage of linker – absorption spectra extracted from peaks corresponding to all steps of selective cleavage reactions.

Figure 77 shows cleavage reaction scheme for nucleotide bearing dithio protecting group on the 3' and dithio based linker.

Figure 78A shows chromatograms of starting material and cleavage reaction mixtures analyzed by RP-HPLC after 10 minutes of incubation with cleavage reagents: dithiosuccinic acid, L-cysteine, DTT and cysteamine.

Figure 78B shows compositions of reaction mixtures as analyzed by RP-HPLC.

DESCRIPTION OF THE INVENTION

The present invention provides methods, compositions, mixtures and kits utilizing deoxynucleoside triphosphates comprising a 3'-O position capped by group comprising methylenedisulfide as a cleavable protecting group and a detectable label reversibly connected to the nucleobase of said deoxynucleoside. Such compounds provide new possibilities for future sequencing technologies, including but not limited to Sequencing by Synthesis. The present invention contemplates, as compositions of matter, the various structures shown in the body of the specification and the figures. These compositions can be used in reactions, including but not limited to primer extension reactions. These compositions can be in mixtures. For example, one or more of the labeled nucleotides (e.g. such as those shown in Figure 25) can be in a mixture (and used in a mixture) with one or more unlabeled nucleotides (e.g. such as those shown in Figure 51). They can be in kits with other reagents (e.g. buffers, polymerases, primers, etc.)

In one embodiment, the labeled nucleotides of the present invention require several steps of synthesis and involve linking variety of dyes to different bases. It is desirable to be able to perform linker and dye attachment in a modular fashion rather than step by step process. The modular approach involves pre-building of the linker moiety with protecting group on one end and activated group on the other. Such pre-built linker can then be used to couple to apropargylamine nucleotide; one can then, deprotect the masked amine group and then couple the activated dye. This has the advantage of fewer steps and higher yield as compare to step-by-step synthesis.

In one embodiment, the labeled nucleotides of the present invention are used in DNA sequencing. DNA sequencing is a fundamental tool in biology. It is a widely used method in

basic research, biomedical, diagnostic, and forensic applications, and in many other areas of basic and applied research. New generation DNA sequencing technologies are changing the way research in biology is routinely conducted. It is poised to play a critical role in the coming years in the field of precision medicines, companion diagnostics, etc.

Sequencing by synthesis (SBS) is a revolutionary next-generation sequencing (NGS) technology, where millions of DNA molecules, single or cluster thereof can be sequenced simultaneously. The basis of this technology is the use of modified nucleotides known as cleavable nucleotide terminators that allow just a single base extension and detection of the DNA molecules on solid surface allowing massive parallelism in DNA sequencing (for comprehensive reviews: Cheng-Yao, Chen, *Frontiers in Microbiology*, 2014, 5, 1 [22]; Fei Chen, et al, *Genomics Proteomics Bioinformatics*, 2013, 11, 34-40 [5]; C.W. Fuller et al, *Nature Biotechnology*, 2009, 27, 1013 [2]; M.L. Metzker, *Nature Reviews*, 2010, 11, 31 [1]) – all of which are hereby incorporated by reference.

Modified nucleotides, with 3'-OH positions blocked by a cleavable protecting group, which after incorporation into DNA primers and subsequent detection, can be removed by chemical reaction, are the key to the success of the SBS chemistry (Ju et al, US 7,883,869, 2011 [23]; Ju et al, US 8,088,575, 2012 [24]; Ju et al, US 8,796,432, 2014 [25]; Balasubramanian, US 6,833,246, 2004 [26]; Balasubramanian et al, US 7785796B2, 2010 [27]; Milton et al, US 7,414,116 B2, 2008 [28]; Metzker, M. L., et al, *Nucleic Acids Res*, 1994, 22:4259-4267 [29]; Ju et al, *Proc. Nat. Acad. Sci. USA*, 103 (52), 19635, 2006 [30]; Ruparel et. al, *Proc. Nat. Acad. Sci. USA*, 102 (17), 5932, 2005 [31]; Bergmann et al, US 2015/0140561 A1 [32]; Kwiatkowski, US 2002/0015961 A1 [33]) – all of which are hereby incorporated by reference.

There have also been attempts to develop nucleotide analogs, known as virtual terminators, where the 3'-OH is unprotected but the bases are modified in such a manner that the

modifying group prevents further extension after a single base incorporation to the DNA templates, forcing chain termination event to occur (Andrew F. Gardner et al., NucleicAcidsRes 40(15), 7404-7415 2012 [34], Litosh et al, Nuc. Acids, Res., 2011, vol 39, No. 6, e39 [35], Bowers et al, Nat. Methods, 2009, 6, 593 [36]) – all of which are hereby incorporated by reference.

Also disclosed were ribo-nucleotide analogs, where the 2'-OH is protected by removable group, which prevents the adjacent 3'-OH group from participating in chain extension reactions, thereby stopping after a single base extension (Zhao et al, US 8,399,188 B2, 2013 [37]), incorporated by reference.

On the other hand, Zon proposed the use of dinucleotide terminators containing one of the nucleotides with the 3'-OH blocked by removable group (Gerald Zon, US 8,017,338 B2, 2011 [38]), incorporated by reference.

Previously a cleavable disulfide linker (-SS-) has been used to attach fluorescent dye in the labeled nucleotides for use in the GeneReader sequencing. It is believed that the -SH scars left behind on the growing DNA strand after cleaving step, causes a number of side reactions which limit achieving a longer read-length.

It is known that -SH residues can undergo free radical reactions in the presence of TCEP used in cleaving step, creating undesired functional group, and it potentially can damage DNA molecules (Desulfurization of Cysteine-Containing Peptides Resulting from Sample Preparation for Protein Characterization by MS, Zhouxi Wang et all, Rapid Commun Mass Spectrom, 2010, 24(3), 267-275 [39]).

The -SH scars can also interact with the incoming nucleotides inside the flow-cell cleaving the 3' OH protecting group prematurely causing further chain elongation and thereby it can cause signal de-phasing.

The end result of the detrimental side reactions of -SH is the reduction of the read-length and increased error rates in the sequencing run.

DETAILED DESCRIPTON OF THE INVENTION

The present invention provides methods, compositions, mixtures and kits utilizing deoxynucleoside triphosphates comprising a 3'-O position capped by group comprising methylenedisulfide as a cleavable protecting group and a detectable label reversibly connected to the nucleobase of said deoxynucleoside. Such compounds provide new possibilities for future sequencing technologies, including but not limited to Sequencing by Synthesis.

The present invention, in one embodiment involves the synthesis and use of a labeled nucleoside triphosphates comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase, with a 3'-O group comprising methylenedisulfide as a protecting group, having the formula -CH₂-SS-R, in DNA sequencing (e.g. sequencing by synthesis), where the R represents alkyl group such as methyl, ethyl, isopropyl, t-butyl, n-butyl, or their analogs with substituent group containing hetero-atoms such as O, N, S etc (see Figure 1). In one embodiment, the R group may contain a functional group that could modulate the stability and cleavability of the 3'-O capping group, while being acceptable to DNA polymerase enzymes.

In another aspect, the invention relates to a labeled nucleoside triphosphates comprising a cleavable oxymethylenedisulfide linker between the label and nucleobase, with 3'-O positions capped by a group comprising methylenedisulfide wherein the nucleobases can be natural, or non-natural bases which can form DNA duplex by hydrogen bond interactions with natural nucleobases of the DNA templates, and that can be 7-deaza analog of dG and dA, and 2-amino-dA. 7-deaza analogs of dA and dG can reduce the formation of DNA tertiary structures due to the lack of 7-N atom. It is envisioned that in one embodiments, such nucleosides could

potentially improve DNA sequencing read-length by enhancing DNA templates and polymerase interaction. It may also be possible that the 2-amino-dA can increase DNA duplex stability due to its ability to form more stable 3 hydrogen bonds with its complementary base (rather than 2 bond in natural state), therefore, it can reduce the risk of losing DNA primers during sequencing run (A Jung et all, Mol. Pathol., 2002, 55 (1), 55-57 [40]; 2-amino-dATP: Igor V. Kutyavin, Biochemistry, 2008, 47(51), 13666-73 [41]).

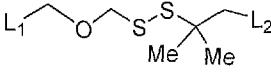
In another embodiment, said nucleotides may have detectable reporter molecules, such as fluorescent dyes linked to nucleobases via cleavable linker $-\text{OCH}_2\text{SS}-$. Labeled nucleotides, where the $-\text{OCH}_2\text{SS}-$ group is directly attached to the nucleobases and the use thereof as cleavable linker are not known in prior-art. Contrary to the traditional, widely used disulfide linkers ($-\text{SS}-$), this class of cleavable linker ($-\text{OCH}_2\text{SS}-$) leaves no sulfur trace on the DNA molecule, cleanly converting it to $-\text{OH}$ group by rapid hydrolysis of the resulting intermediate, $-\text{OCH}_2\text{SH}$, after reductive cleavage. Because of this, such linkers may be better alternatives to the traditional disulfide linkers. In traditional disulfide based linkers ($-\text{SS}-$), the resulting thiol group ($-\text{SH}$) can undergo side reactions when cleaved by reducing reagents such as TCEP as presented in the following Figure 4 (Ref: Desulfurization of Cysteine-Containing Peptides Resulting from Sample Preparation for Protein Characterization by MS, Zhouxi Wang et all, Rapid Commun Mass Spectrom, 2010, 24(3), 267-275 [39]).

In another embodiment, the reporter groups may be attached to the pyrimidine bases (dT, dC) at 5-C position and to purine bases (dA, dG) at 7-N of natural bases, or 7-C of de-aza analogs.

In another embodiment, the structure of the labeled nucleotides may be as shown in Figure 5, where the spacer of the cleavable linker includes the propargyl ether linker. The nucleobases with propargyl ether can be synthesized following prior arts of chemical synthesis.

The L₁ and L₂ represent chemical spacers, and substituents R₁, R₂, R₃ and R₄ are group of atoms that modulate stability and cleavability to the cleavable linker. They can be hydrogen atom, geminal dimethyl, or any alkyl, phenyl, or substituted alkyl group, such as methyl, ethyl, n-butyl, phenyl, etc. They may also contain a hydrocarbon chain with –O, =O,NH, -N=N, acid, amide, poly ethyleneglycol chain (PEG) etc. The label on the nucleotides may be fluorescent dyes, energy transfer dyes, radioactive label, chemi-luminiscence probe, heptane and other form of label that allows detection by chemical or physical methods.

In another embodiment, the structure of the labeled nucleotides may be as shown Figure 6. The spacers of the cleavable linker include the propargylamine linker. Again, the L₁ and L₂ represent spacers, and substituents R₁, R₂, R₃ and R₄ are group of atoms that provide stability and modulate cleavability of the linker as described earlier. They may be hydrogen atoms, alkylgroups such as methyl, ethyl and other substituted groups or their salts. Geminal dialkyl group on the α-carbon of the cleavable disulfide linker (e.g. germinal dimethyl analogue

according to the following structure:  provides better stability to the linker allowing modular synthesis of labeled nucleotides. It presumably prevents disproportional reactions prevalent among disulfide based organic compounds. It also adds greater hydrophobicity to the linker which helps the synthesis and purification of labeled nucleotide analogues [42-44]. The gem dimethyl functionality present in the linker is believed to not only serve to stabilize the disulfide bond electronically, but also prevents disulfide exchange from occurring both inter- and intra-molecularly, likely via steric effects. It has been demonstrated that in the presence of cystamine, the disulfide functionality on the terminator participates in disulfide exchange, while linkers equipped with gem dimethyl groups do not. The linker study in Figure 42 compares linkers with and without the gem dimethyl group. As can be seen from this

study, linkers G and L without the gem dimethyl group quickly exchange with cystamine leading to degradation of the product. As expected, this phenomenon is not observed with our chosen linker A, nor with analogous linker B. In addition, since the labelled nucleotides contain two disulfides, one on the terminator and one on the linker portion of the molecule, it is believed that this stabilizing effect prevents scrambling between the dye and the terminator from occurring. This stability is important to performance of our nucleotides in sequencing.

In another embodiment, the structure of the labeled nucleotides may be as in Figure 7. The spacer of the cleavable linker include the methylene $-(\text{CH}_2)_n-$ directly attached to the nucleobases at 5- position for pyrimidine, and at 7- de-aza-carbon for purines. This linker may be methylene ($n= 1$) or polymethylene ($n> 1$) where after cleavage, the linker generates $-(\text{CH}_2)_n\text{OH}$ group at the point of attachment on the nucleobases, and where the L_1 and L_2 represent spacers, and substituents R_1 , R_2 , R_3 and R_4 are group of atoms that provide stability to the cleavable linker as described earlier.

In another embodiment, the invention relates to synthetic methods for the nucleotides claimed. The capping group and linker may be synthesized modifying prior arts described. For example, the unlabeled dT analog (compound 5) can be synthesized as shown in Figure 8.

In one embodiment the invention involves: (a) nucleoside triphosphates with 3'-O capped by a group comprising methylenedisulfide (e.g. of the formula $-\text{CH}_2\text{SS-R}$) as a cleavable protecting group (see Figure 1); and (b) their labeled analogs (see Figure 2), where labels are attached to the nucleobases via a cleavable oxymethylenedisulfide linker ($-\text{OCH}_2\text{SS}-$). Such nucleotides can be used in nucleic acid sequencing by synthesis (SBS) technologies. General methods for the synthesis of the nucleotides claimed are also described.

In one embodiment, as shown in Figure 1, the general structures of unlabeled nucleotides have the 3'-O group protected by a group comprising methylenedisulfide with a common structure

—CH₂-SS-R, where the R can be regular alkyl or substituted alkyl groups such as —Me, -Et, -nBu, -tBu, -CH₂CH₂NH₂, —CH₂CH₂NMe etc., and B, can be natural or non-natural nucleobases. Some specific examples of non-natural nucleobases are 7-deaza dG and dA, 2-amino-dA etc.

In Figure 2, the general structures of labeled analogs are shown with 3'-O protected by a group comprising methylenedisulfide as in Figure 1, in addition to that a detectable reporter (label) such as fluorophore is attached to the nucleobases *via* a cleavable linker having a general structure —L₁-OCH₂-SS-L₂—. L₁ represents molecular spacer that separates nucleobase from the cleavable linker, while L₂ between cleavable linker and the label, respectively. Both L₁ and L₂ can have appropriate functional groups for connecting to the respective chemical entities such as —CO-, -CONH-, -NHCONH-, -O-, -S-, -C=N, -N=N-, etc. The label may be fluorophore dyes, energy transfer dyes, mass-tags, biotin, haptene, etc. The label may be different on different nucleotides for detection of multiple bases simultaneously, or the same for step-wise detection of spatially separated oligonucleotides or their amplified clones on solid surface.

In one embodiment, the invention relates to a new class of nucleotide that has 3'-O capped with —CH₂-SS-R group and a label attached to the nucleobase through a cleavable linker having a general structure —O-CH₂-SS—. Such capping group and linker can be cleanly cleaved simultaneously by single treatment with TCEP or related chemicals leaving no sulfur traces on the DNA molecules.

This class of nucleotides may be stable enough to endure the relatively high temperature (~65 °C) necessary for nucleotide incorporation onto the DNA templates catalyzed by thermo active polymerases, yet labile enough to be cleaved under DNA compatible conditions such as reduction with TCEP etc. In some embodiments, cleavage may be accomplished by exposure to dithiothreitol.

The nucleotide when exposed to reducing agents such as TCEP de-cap the 3'-O protection

group via step-wise mechanism shown in Figure 3, thus restoring the natural state of the 3'-OH group. TCEP and its analogs are known to be benign to bio-molecules which is a pre-requisite for application in SBS.

In one embodiment, the invention relates to a generic universal building blocks structures comprising new cleavable linkers, shown in Figure 28. PG = Protective Group, L1, L2 – linkers (aliphatic, aromatic, mixed polarity straight chain or branched). RG = Reactive Group. In one embodiment of present invention such building blocks carry an Fmoc protective group on one end of the linker and reactive NHS carbonate or carbamate on the other end. This preferred combination is particularly useful in modified nucleotides synthesis comprising new cleavable linkers. A protective group should be removable under conditions compatible with nucleic acid/nucleotides chemistry and the reactive group should be selective. After reaction of the active NHS group on the linker with amine terminating nucleotide, an Fmoc group can be easily removed using base such as piperidine or ammonia, therefore exposing amine group at the terminal end of the linker for the attachment of cleavable marker. A library of compounds comprising variety of markers can be constructed this way very quickly.

In one embodiment, the invention relates to a generic structure of nucleotides carrying cleavable marker attached via novel linker, shown in Figure 29. S = sugar (i.e., ribose, deoxyribose), B = nucleobase, R = H or reversibly terminating group (protective group). Preferred reversibly terminating groups include but are not limited to: Azidomethyl (-CH₂N₃), Dithio –alkyl (-CH₂-SS-R), aminoxy (-ONH₂).

EXAMPLES

The following examples are provided in order to demonstrate and further illustrate certain

preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1

Synthesis of 3'-O-(methylthiomethyl)-5'-O-(tert-butyldimethylsilyl)-2'-deoxythymidine (2)

5'-O-(tert-butyldimethylsilyl)-2'-deoxythymidine (1) (2.0g, 5.6 mmol) was dissolved in a mixture consisting of DMSO (10.5 mL), acetic acid (4.8 mL), and acetic anhydride (15.4 mL) in a 250 mL round bottom flask, and stirred for 48 hours at room temperature. The mixture was then quenched by adding saturated K_2CO_3 solution until evolution of gaseous CO_2 was stopped. The mixture was then extracted with EtOAc (3X100 mL) using a separating funnel. The combined organic extract was then washed with a saturated solution of $NaHCO_3$ (2X150 mL) in a partitioning funnel, and the organic layer was dried over Na_2SO_4 . The organic part was concentrated by rotary evaporation. The reaction mixture was finally purified by silica gel column chromatography (Hex: EtOAc/ 7:3 to 1:1), see Figure 8. The 3'-O-(methylthiomethyl)-5'-O-(tert-butyldimethylsilyl)-2'-deoxythymidine (2) was obtained as white powder in 75% yield (1.75g, R_f = 0.6, hex: EtOAc/1:1). 1H -NMR ($CDCl_3$): δ_H 8.16 (s, 1H), 7.48 (s, 1H), 6.28 (m, 1H), 4.62 (m, 2H), 4.46 (m, 1H), 4.10 (m, 1H), 3.78-3.90 (m, 2H), 2.39 (m, 1H), 2.14, 2.14 (s, 3H), 1.97 (m, 1H), 1.92 (s, 3H), 0.93 (s, 9H), and 0.13 (s, 3H) ppm.

EXAMPLE 2

Synthesis of 3'-O-(ethyldithiomethyl)-2'-deoxythymidine (4)

Compound 2 (1.75g, 4.08 mmol), dried overnight under high vacuum, dissolved in 20 mL dry CH_2Cl_2 was added with Et_3N (0.54 mL, 3.87 mmol) and 5.0g molecular sieve-3A, and stirred for 30 min under Ar atmosphere. The reaction flask was then placed on an ice-bath to bring the

temperature to sub-zero, and slowly added with 1.8 eq 1M SO_2Cl_2 in CH_2Cl_2 (1.8 mL) and stirred at the same temperature for 1.0 hour. Then the ice-bath was removed to bring the flask to room temperature, and added with a solution of potassium thiotosylate (1.5 g) in 4 mL dry DMF and stirred for 0.5 hour at room temperature.

Then 2 eq EtSH (0.6 mL) was added and stirred additional 40 min. The mixture was then diluted with 50 mL CH_2Cl_2 and filtered through celite-S in a funnel. The sample was washed with adequate amount of CH_2Cl_2 to make sure that the product was filtered out. The CH_2Cl_2 extract was then concentrated and purified by chromatography on a silica gel column (Hex:EtOAc/1:1 to 1:3, $R_f = 0.3$ in Hex:EtOAc/1:1). The resulting crude product was then treated with 2.2g of NH_4F in 20 mL MeOH. After 36 hours, the reaction was quenched with 20 mL saturated NaHCO_3 and extracted with CH_2Cl_2 by partitioning. The CH_2Cl_2 part was dried over Na_2SO_4 and purified by chromatography (Hex:EtOAc/1:1 to 1:2) , see Figure 8.. The purified product (**4**) was obtained as white powder in 18% yield, 0.268g, $R_f = 0.3$, Hex:EtOAc/1:2).

^1H NMR in CDCl_3 : δ_{H} 11.25 (1H, S), 7.65 (1H, S), 6.1 (1H, m), 5.17 (1H, m), 4.80 (2H, S), 4.48 (1H, m), 3.96 (1H, m), 3.60 (2H, m), 3.26 (3H, s), 2.80 (2H, m), 2.20 (2H, m) and 1.14 (3H, m) ppm.

EXAMPLE 3

Synthesis of the triphosphate of 3'-O-(ethyldithiomethyl)-2'-deoxythymidine (**5**)

In a 25 mL flask, compound **4** (0.268g, 0.769 mmol) was added with proton sponge (210 mg), equipped with rubber septum. The sample was dried under high vacuum for overnight. The material was then dissolved in 2.6 mL $(\text{MeO})_3\text{PO}$ under argon atmosphere. The flask, equipped with Ar-gas supply, was then placed on an ice-bath, stirred to bring the temperature to sub-zero.

Then 1.5 equivalents of POCl_3 was added at once by a syringe and stirred at the same temperature for 2 hour under Argon atmosphere. Then the ice-bath was removed and a mixture consisting of tributylammonium-pyrophosphate (1.6g) and Bu_3N (1.45 mL) in dry DMF (6 mL) was prepared. The entire mixture was added at once and stirred for 10 min. The reaction mixture was then diluted with TEAB buffer (30 mL, 100 mM) and stirred for additional 3 hours at room temperature. The crude product was concentrated by rotary evaporation, and purified by C18 Prep HPLC (method: 0 to 5min 100%A followed by gradient up to 50%B over 72min, A = 50 mM TEAB and B = acetonitrile). After freeze drying of the target fractions, the semi-pure product was further purified by ion exchange HPLC using PL-SAX Prep column (Method: 0 to 5min 100%A, then gradient up to 70%B over 70min, where A = 15% acetonitrile in water, B = 0.85M TEAB buffer in 15% acetonitrile). Final purification was carried out by C18 Prep HPLC as described above resulting in ~ 25% yield of compound **5**, see Figure 8.

Example 4

Synthesis of $\text{N}^4\text{-Benzoyl-5'-O-(tert-butyldimethylsilyl)-3'-O-(methylthiomethyl)-2'$ deoxycytidine (7)

The synthesis of 3'-O-(ethyldithiomethyl)-dCTP (10) was achieved according to Figure 9. $\text{N}^4\text{-benzoyl-5'-O-(tert-butyldimethylsilyl)-2'-deoxycytidine}$ (**6**) (50.0g, 112.2 mmol) was dissolved in DMSO (210mL) in a 2L round bottom flask. It was added sequentially with acetic acid (210 mL) and acetic anhydride (96 mL), and stirred for 48 h at room temperature. During this period of time, a complete conversion to product was observed by TLC (R_f = 0.6, EtOAc:hex/10:1 for the product).

The mixture was separated into two equal fractions, and each was transferred to a 2000mL beaker and neutralized by slowly adding saturated K_2CO_3 solution until CO_2 gas

evolution was stopped (pH 8). The mixture was then extracted with EtOAc in a separating funnel. The organic part was then washed with saturated solution of NaHCO₃ (2X1L) followed by with distilled water (2X1L), then the organic part was dried over Na₂SO₄.

The organic part was then concentrated by rotary evaporation. The product was then purified by silica gel flash-column chromatography using puriflash column (Hex:EtOAc/1:4 to 1:9, 3 column runs, on 15um, HC 300g puriflash column) to obtain *N*⁴-benzoyl-5'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*-(methylthiomethyl)-2'-deoxycytidine (7) as grey powder in 60% yield (34.0g, *R*_f = 0.6, EtOAc:hex/9:1), see Figure 9.

¹H-NMR of compound 7 (CDCl₃): δ_H 8.40 (d, *J* = 7.1 Hz, 1H), 7.93 (m, 2H), 7.64 (m, 1H), 7.54 (m, 3H), 6.30 (m, 1H), 4.62 & 4.70 (2Xd, *J* = 11.59 Hz, 2H), 4.50 (m, 1H), 4.19 (m, 1H), 3.84 & 3.99 (2Xdd, *J* = 11.59 & 2.79 Hz, 2H), 2.72 (m, 1H), 2.21 (m, 1H), 2.18 (s, 3H), 0.99 (s, 9H), and 0.16 (s, 6H) ppm.

EXAMPLE 5

***N*⁴-Benzoyl-3'-*O*-(ethyldithiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxycytidine (8)**

*N*⁴-Benzoyl-5'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*-(methylthiomethyl)-2'-deoxycytidine (7) (2.526g, 5.0 mmol) dissolved in dry CH₂Cl₂ (35 mL) was added with molecular sieve-3A (10g). The mixture was stirred for 30 minutes. It was then added with Et₃N (5.5 mmol), and stirred for 20 minutes on an ice-salt-water bath. It was then added slowly with 1M SO₂Cl₂ in CH₂Cl₂ (7.5 mL, 7.5 mmol) using a syringe and stirred at the same temperature for 2 hours under N₂-atmosphere. Then benzenethiosulfonic acid sodium salt (1.6g, 8.0 mmol) in 8 mL dry DMF was added and stirred for 30 minutes at room temperature. Finally, EtSH was added (0.74 mL) and stirred additional 50 minutes at room temperature. The reaction mixture was filtered through celite-S, and washed the product out with CH₂Cl₂. After concentrating the resulting CH₂CH₂ part,

it was purified by flash chromatography using a silica gel column (1:1 to 3:7/Hex:EtOAc) to obtain compound **8** in 54.4% yield (1.5g) , see Figure 9. $^1\text{H-NMR}$ of compound **8** (CDCl_3): δ_{H} 8.40 (m, 1H), 7.95 (m, 2H), 7.64 (m, 1H), 7.54 (m, 3H), 6.25 (m, 1H), 4.69 & 4.85 (2Xd, J = 11.60 Hz, 2H), 4.50 (m, 1H), 4.21 (m, 1H), 3.84 & 3.99 (2Xdd, J = 11.59 & 2.79 Hz, 2H), 2.75 (m, 3H), 2.28 (m, 1H), 1.26 (m, 3H), 0.95 (s, 9H), and 0.16 (s, 6H) ppm.

EXAMPLE 6

$\text{N}^4\text{-Benzoyl-3'-O-(ethyldithiomethyl)-2'-deoxycytidine (9)}$

$\text{N}^4\text{-Benzoyl-3'-O-(ethyldithiomethyl)-5'-O-(tert-butyldimethylsilyl)-2'-deoxycytidine (8, 1.50g, 2.72 \text{ mmol})$ was dissolved in 50 mL THF. Then 1M TBAF in THF (3.3 mL) was added at ice-cold temperature under nitrogen atmosphere. The mixture was stirred for 1 hour at room temperature. Then the reaction was quenched by adding 1 mL MeOH, and solvent was removed after 10 minutes by rotary evaporation. The product was purified by silica gel flash chromatography using gradient 1:1 to 1:9/Hex:EtOAc to result in compound **9** (0.78g, 65% yield, $R_f = 0.6$ in 1:9/Hex:EtOAc) , see Figure 9. $^1\text{H-NMR}$ of compound **9** (CDCl_3): δ_{H} 8.41 (m, 1H), 8.0 (m, 2H), 7.64 (m, 2H), 7.50 (m, 2H), 6.15 (m, 1H), 4.80 & 4.90 (2Xd, J = 11.60 Hz, 2H), 4.50 (m, 1H), 4.21 (m, 1H), 4.00 & 3.85 (2Xdd, J = 11.59 & 2.79 Hz, 2H), 2.80 (m, 2H), 2.65 (m, 1H), 2.40 (m, 1H), and 1.3 (s, 3H) ppm.

Finally, the synthesis of compound **10** was achieved from compound **9** following the standard synthetic protocol described in the synthesis of compound **5** (see Figure 8).

EXAMPLE 7

The synthesis of the labeled nucleotides can be achieved following the synthetic routes

shown in Figure 10 and Figure 11. Figure 10 is specific for the synthesis of labeled dT intermediate, and other analogs could be synthesized similarly.

Synthesis of 5'-O-(tert-butyldimethylsilyl)-5-(N-trifluoroacetyl-aminopropargyl)-2'-deoxyuridine (12)

5'-O-(tert-butyldimethylsilyl)-5-iodo-2'-deoxyuridine (**11**, 25.0g, 53.4 mmol) was dissolved in dry DMF (200 mL) in a 2-neck-round bottom flask. The reaction flask is flushed with Ar-gas filled balloon. It was then added with, freshly opened, vacuum dried tetrakis(triphenylphosphine)palladium (0) (6.16g, 5.27 mmol) and CuI (2.316g, 12.16 mmol) and stirred at room temperature for 10 minutes under argon atmosphere. Next, *N*-trifluoroacetyl-propargylamine (23.99g, 157.8 mmol, 2.9 eq) and Et₃N (14.7 mL, 105.5 mmol) were added sequentially. The mixture was stirred for 3.0 hours at room temperature and reaction completion was confirmed by TLC (*R_f* = 0.5 in EtOAc:Hex/3:2 for the product).

Solvent was then removed by rotary evaporation. The resulting crude product was dissolved in 500 mL EtOAc and transferred into a separating funnel. The organic part was then washed with saturated NaHCO₃ (2X400 mL) and saturated NaCl (2X400 mL) solutions, respectively. The EtOAc part was then dried over anhydrous Na₂SO₄. After filtering off the Na₂SO₄ salt, the filtrate was concentrated using a rotary evaporator. It was then purified by a silica gel flash chromatography (1:1 Hex:EtOAc to 2:3 Hex:EtOAc, 200gm, 15um HP puriflash column, 3 column runs) after binding to 3X40gm silica gel resulting in 21.994g of **12** (83.88% yield), see Figure 10.

¹H-NMR in compound **12** (DMF-d₇): δH 11.65 (brs, 1H), 10.15 (brs, 1H), 8.15 (brs, 1H, H6), 6.37 (t, *J* = 5.99 Hz, 1H, H1'), 5.42 (m, 1H), 4.41 (m, 1H), 4.37 (brs, 2H, for NH-CH₂ of propargylamine group), 4.00 (m, 1H), 3.84-3.97 (m, 2H), 2.30 (m, 1H, H2'), 2.20 (m, 1H, H2'), 0.97 (s, 9H, 3X-CH₃, TBDMS) and 0.19 (s, 6H, 2X CH₃, TBDMS) ppm.

EXAMPLE 8

Synthesis of 5'-O-(tert-butyldimethylsilyl)-3'-O-(methylthiomethyl)-5-(N-trifluoroacetyl-aminopropargyl)-2'-deoxyuridine (13)

Compound **12** (21.99g, 44.77 mmol) was dissolved in DMSO (90 mL) in a 1000mL round bottom flask. It was then added sequentially with AcOH (40 mL) and acetic anhydride (132 mL) and stirred for 48 hours at room temperature. The reaction completion was confirmed by TLC (R_f = 0.5; Hex:EtOAc/1:1 for the product).

The reaction mixture was then transferred to 2,000 mL beaker, and neutralized by saturated K_2CO_3 until the evolution of CO_2 gas was ceased (~pH 8.0). The mixture was then transferred into a separating funnel and extracted (2X500mL CH_2Cl_2). The combined organic part was then washed with saturated $NaHCO_3$ (1X500mL) and dried over Na_2SO_4 . After filtering off the Na_2SO_4 , the organic part was concentrated by rotary evaporation and purified by silica gel flash chromatography (Hex:EtOAc/7:3 to 1:1) producing 12.38g of compound **13** (~50% yield), see Figure 10. TLC: R_f = 0.5; Hex:EtOAc/1:1, 1H -NMR of compound **13** (DMSO-d₆): δ H 11.69 (s, 1H), 10.01 (s, 1H), 7.93 (s, 1H, H6), 6.07 (m, 1H, H1'), 4.69 (m, 2H), 4.38 (m, 1H), 4.19 (m, 2H), 4.03 (m, 1H), 3.75 (m, 2H), 2.34 (m, 1H), 2.14 (m, 1H), 2.07 (s, 3H), 0.86 (s, 9H) and 0.08 (s, 6H) ppm.

The synthesis of the compounds **14**, **15** and **16** can be achieved following the synthetic protocols of the related steps described for compounds **5** and **10**. Synthesis of other N-trifluoroacetyl-aminopropargyl nucleobases by described as in U.S. Patent 8,017,338 [38], incorporated herein by reference. Removal of the N-trifluoroacetyl group to produce the aminopropargyl nucleobases may be produced by solvolysis under mild conditions [45].

On the other hand, the cleavable linker synthesis can be achieved as shown in Figure 11, starting from an 1,4-dutanediol and is described in Example 9.

EXAMPLE 9

Synthesis of 4-O-(*tert*-butyldiphenylsilyl)-butane-1-O-(methylthiomethyl), **18**

18.3g 1,4-butanediol, **17** (18.3g, 203.13 mmol) dissolved in 100 mL dry pyridine in a 1L flask was brought to sub-zero temperature on an ice-bath under nitrogen atmosphere. It was added with *tert*-butyldiphenylsilylchloride (TBDPSCl, 19.34g, 70.4 mmol) slowly with a syringe. The reaction flask was allowed to warm up to room temperature with the removal of the ice-bath and stirring continued for overnight at room temperature. The solvent was then removed by rotary evaporation and purified by flash chromatography using silica gel column (7:3 to 1:1/Hex:EtOAc) resulting in 4-O-(*tert*-butyldiphenylsilyl)-butane-1-ol (13.7g, 59.5% yield, R_f = 0.7 with 1:1/Hex:EtOAc, ^1H NMR (CDCl_3): δ 7.70 (4H, m), 7.40 (4H, m), 3.75 (2H, m), 3.65 (m, 2H), 3.70 (4H, m) and 1.09 (9H, m) ppm. Of the resulting product, 6.07g (18.5 mmol) was dissolved in 90 mL dry DMSO, see Figure 11. It was then added with acetic acid (15 mL) and acetic anhydride (50 mL). The mixture was stirred for 20 hours at room temperature. It was then transferred to a separating funnel and washed with 300 mL distilled water by partitioning with the same volume of EtOAc. The EtOAc part was then transferred to a 1,000 mL beaker and neutralized with saturated K_2CO_3 solution. The aqueous part was removed by partitioning and the EtOAc part was then further washed with distilled water (3X300 mL) and dried over MgSO_4 . The EtOAc part was then concentrated and purified by flash chromatography on a silica gel column (Hex:EtOAc/97:3 to 90:10) to obtain 4-O-(*tert*-butyldiphenylsilyl)-1-O-(methylthiomethyl)-butane, **18** (5.15g, 71.7% yield, R_f = 0.8 in 9:1/Hex:EtOAc). ^1H NMR

(CDCl₃): δH 7.70 (4H, m), 7.40 (6H, m), 4.62 (2H, s), 3.70 (2H, m), 3.50 (2H, m), 2.15 (2H, s), 1.70 (4H, m) and 1.08 (9H, m) ppm.

EXAMPLE 10

Synthesis of compound 19

Compound **18** (2.0g, 5.15 mmol) was dissolved in 40 mL dry CH₂Cl₂, and added with 10g molecular sieve-3A and 0.78 mL Et₃N (5.66 mmol). The mixture was stirred under N₂ gas at room temperature for 30 min. Then the flask was placed on an ice-bath to bring the temperature to sub-zero. It was then added slowly with 7.7 mL of 1M SO₂Cl₂/CH₂Cl₂ solution (7.7 mmol) and stirred under N₂ for 1 hour. Then the ice-bath was removed and benzenethiosulfonic acid-Na salt (1.6g, 8.24 mmol) in 8 mL DMF was added and stirred for 30 minutes at room temperature. Then 4-mercaptophenylacetic acid (1.73g, 10.3 mmol, 2.0 eq) in 7 mL dry DMF was added and stirred for 2 hours. The entire crude sample was then filtered through celite-S and the product was washed out by EtOAc. EtOAc extract was then concentrated by rotary evaporation and purified on a silica gel column (1:1 to 3:7/Hex:EtOAc) to obtain 1.19g of compound **19** in 43% yield, see Figure 11, R_f = 0.5 Hex:EtOAc/3:7. ¹H NMR (CDCl₃): 7.65 (4H, m), 7.55 (2H, m), 7.45 (6H, m), 7.20 (2H, s), 4.80 (2H, m), 3.65 (4H, m), 3.50 (2H, m), 1.60 (4H, m), and 1.09 (9H, s) ppm.

EXAMPLE 11

Synthesis of compound 20

Compound **19** (0.6g, 1.11 mmol) dissolved in 20 mL dry DMF was treated with DSC (0.426 g, 1.5 eq) and Et₃N (0.23 mL) at room temperature and stirred for 1.5 hours under nitrogen atmosphere. Then a mixture consisting of 11-azido-3,6,9-trioxadecan-1-amine (2.0 eq)

and Et₃N (2.0 eq) was prepared in 5 mL DMF. The entire solution was added to the reaction mixture at once and stirred for 1 hour. The solvent was then removed under vacuum and purified by silica gel flash chromatography using gradient 0 to 10% CH₂Cl₂:MeOH to obtain compound **20** in 36% yield (0.297g, R_f = 0.8, 10% MeOH:CH₂Cl₂), see Figure 11. ¹H NMR (MeOH-d₄): δ_H 7.70 (4H, m), 7.55 (2H, m), 7.40 (6H, m), 7.45 (2H, m), 4.85 (2H, s), 3.65-3.30 (22H, m), 1.65 (4H, m), and 1.09 (9H, m) ppm.

Then, the product **20** (0.297g) was dissolved in 7 mL dry THF in a flask and placed on an ice-bath to bring to sub-zero temperature under nitrogen atmosphere. Then 0.6 mL 1M TBAF in THF was added drop-wise and stirred for 3 hours at ice-cold temperature. The mixture was quenched with 1 mL MeOH and volatiles were removed by rotary evaporation and purified by flash chromatography to obtain 165mg of the product **21**, see Figure 11, ¹H NMR (MeOH-d₄): δ_H 7.55 (2H, m), 7.25 (2H, m), 4.85 (2H, s), 3.75-3.30 (22H, m) and 1.50 (4H, m) ppm. This product can be coupled to alkyne substituted dye using click chemistry and to nucleotide using CDI as activating agent to result in compound **22**.

Another variant of cleavable linker, where the stabilizing gem-dimethyl group attached to α-carbon of the cleavable linker, can be achieved following Figure 12.

EXAMPLE 12

In another aspect, the cleavable linker can be compound **30**, where the disulfide is flanked by gem-dimethyl groups and attached to a flexible ethylene glycol linker (PEG). The linker is attached to the PA-nucleotide (e.g. compound **33**) *via* carbamate group (-NH-C(=O)O-). The resulting nucleotide analogue in such case can be as in compound **35** (dUTP analogue), which can be synthesized according to the Figure 13. Other nucleotide analogues (e.g. analogues of dATP, dGTP, dCTP) can be synthesized similarly by replacing PA-nucleotide **33**

with appropriate PA-nucleotide analogues at the last step of the reaction sequence.

EXAMPLE 13

Synthesis of compound 28

Compound **18** (15.53g, 40 mmol) (see Example 9) for synthesis of compound **18**) was dissolved in 450 mL of dry dichloromethane in a round bottom flask. Molecular sieves (3 Å, 80g) and triethylamine (5.6 mL) were added, and the reaction mixture was stirred at 0 °C for 0.5 hour under nitrogen atmosphere. Next, SO₂Cl₂ (1 M in DCM, 64 mL) was added slowly by a syringe and stirred for 1.0 hour at 0 °C temperature. Then, ice-water bath was removed, and a solution of potassium-thiotosylate (10.9g, 48.1 mmol) in 20 mL anhydrous DMF was added at once and stirred for 20 minutes at room temperature. The reaction mixture was then poured into 3-mercaptopropanoic acid (4.4 mL, 36 mmol) dissolved in 20 mL DMF in a 2 L round-bottom flask. The resulting mixture was stirred for 0.5 hours at room temperature, and filtered through celite. The product was extracted with ethyl acetate. The combined organic extracts were washed with distilled water in a separatory funnel, followed by concentrating the crude product by rotary evaporation. The product (**28**) was obtained in 26% yield (5.6g) after purification by flash chromatography on silica gel using EtOAc:Hexane as mobile phase, see Figure 13. ¹H NMR (CDCl₃): δ_H 7.67-7.70 (m, 4H), 7.37-7.47 (m, 6H), 4.81 (s, 2H), 3.81 (t, *J* = 6.73 Hz, 2H), 3.70 (t, *J* = 6.21 Hz, 2H), 3.59 (t, *J* = 6.55, 2H), 1.90 (t, *J* = 6.95 Hz, 2H), 1.58-1.77 (m, 4H), 1.34 (s, 6H), and 1.07 (s, 9H) ppm.

EXAMPLE 14**Synthesis of compound 29**

Compound **28** (5.1g, 10.36 mmol) was dissolved in 100 mL anhydrous pyridine in a 500 mL round bottom flask. To this solution, 1,1'-carbonyldiimidazole (CDI) (3.36g, 20.7 mmol) was added in one portion and the reaction was stirred for 1.0 hour at room temperature under a nitrogen atmosphere. Then, the reaction mixture was poured into a solution consisting of 2,2'-(ethylenedioxy)bis(ethylamine) (7.6 mL, 51.8 mmol) and anhydrous pyridine (50 mL). The mixture was stirred for 1.0 hour at room temperature, and the volatiles were removed by rotary evaporation. The resulting crude product was purified by flash chromatography on silica using MeOH:CH₂Cl₂/9.5:0.5 to furnish pure compound **29** (4.4g, 65% yield), see Figure 13. ¹H NMR (CDCl₃): δ _H 7.63-7.68 (m, 4H), 7.34-7.44 (m, 6H), 4.76 (s, 2H), 4.17 (t, *J* = 7.07 Hz, 2H), 3.65 (t, *J* = 6.16 Hz, 2H), 3.60 (s, 4H), 3.49-3.51 (m, 6H), 3.31-3.39 (m, 2H), 2.88 (m, 2H), 1.9 (t, *J* = 7.06 Hz, 2H), 1.57-1.73 (m, 4H), 1.31 (s, 6H) and 1.03 (s, 9H) ppm.

EXAMPLE 15**Synthesis of compound 31**

Compound **29** (0.94g, 1.42 mmol) was dissolved in 40 mL dry THF and treated with 1M TBAF in THF (1.6 mL, 1.6 mmol) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred for 2.0 hours at 0 °C, during which time LC-MS confirmed complete removal of the TBDPS protecting group. After removing solvent by rotary evaporation, the product was purified by flash chromatography on C18 Flash Column (gradient: 0-100% B over 50 minutes, where A = 50mM TEAB and B = acetonitrile). The target fractions were combined and lyophilized resulting in pure compound **30** (0.284g, 47% yield), MS (ES+) calculated for (M+H) 429.21,

observed m/z 429.18. Next, compound **30** (0.217g, 0.51 mmol) was dissolved in 13 mL of dry acetonitrile under a nitrogen atmosphere. To this solution, DIPEA (97.7uL, 0.56 mmol) and Fmoc-NHS ester (273.6mg, 0.81 mmol) were added at 0 °C temperature and stirred for 2.0 hours at the same temperature. The product was then purified by flash chromatography on silica gel, 1:1 to 1:9/hex:EtOAc gradient, leading to a semi-pure product, which was further purified using 2-5%/MeOH-CH₂Cl₂ gradient to obtain compound **31** (0.245g, 74% yield), see Figure 13. ¹H NMR (CDCl₃): δ_H 7.70 (2H, d, *J* = 7.3 Hz), 7.59 (2H, d, *J* = 7.6 Hz), 7.32 (2H, m), 7.24 (2H, m), 4.69 (2H, s), 4.35 (2H, m), 4.16 (1H, m), 4.09 (2H, m), 3.60-3.45 (12H, m), 3.36-3.26 (4H, m), 1.82 (2H, m), 1.60 (4H, m) and 1.22 (6H, s) ppm.

EXAMPLE 16

Synthesis of compound **32**

Compound **31** (93 mg, 0.143 mmol) was dissolved in dry acetonitrile (12.0 mL) in a round bottom flask equipped with magnetic bar and a nitrogen gas source. To this solution, DSC (56 mg, 0.21 mmol) and DIPEA (37.4μL, 0.21 mmol) were added sequentially, and the resulting mixture was stirred at room temperature for 5.0 hours. Additional DSC (48 mg, 0.18 mmol) and DIPEA (37.4μL, 0.21 mmol) were added and stirring continued for 15.0 hours at room temperature, during which time TLC showed full conversion to the activated NHS ester. The product **32** was obtained (59mg, 53% yield) as a thick oil following silica gel flash chromatography purifications using hexane-ethyl acetate (3:7 to 1:9) gradient and was used in the next step, see Figure 13. ¹H NMR (CDCl₃): δ_H 7.70 (2H, d, *J* = 7.53 Hz), 7.53 (2H, d, *J* = 7.3 Hz), 7.33 (2H, m), 7.24 (2H, m), 4.69 (2H, s), 4.34 (2H, m), 4.28 (2H, m), 4.16 (1H, m), 4.09 (2H, m), 3.57-3.46 (10H, m), 3.35-3.26 (4H, m), 2.75 (4H, s), 1.74 (4H, m), 1.62 (2H, m) and

1.23 (6H, s) ppm.

EXAMPLE 17

Synthesis of compound 34

An aliquot of compound **33** (10 μ mol) (synthesized according to Ref. US 2013/0137091 A1) was lyophilized to dryness in a 15 mL centrifuge tube. It was then re-suspended in 1.0 mL of dry DMF with 60 μ mol DIPEA. In a separate tube, compound **32** (30 μ mol, 3 eq) was dissolved in 3.33 mL dry DMF, and added all at once. The reaction was mixed well by rigorous shaking by hand and placed on the shaker for 12h at room temperature. Next, piperidine (0.33 mL) was added and shaking continued for 30 minutes at room temperature. The product was then purified by HPLC using C18 column (gradient: 0-70% B over 40 minutes, where A = 50 mM TEAB and B = acetonitrile). The product **34** was obtained in 73.3% yield (7.33 μ mol) after lyophilization of the target fractions, see Figure 13.

EXAMPLE 18

Synthesis of compound 35

An aliquot of compound **34** (4.9 μ mol) was dissolved in 1.0 mL distilled water and 0.5M Na₂HPO₄ (0.49 mL) in a 15 mL centrifuge tube. In a separate tube, 10mg of 5-CR₆G-NHS ester (17.9 μ mol) was dissolved in 0.9 mL of dry DMF. This solution was then added to the reaction mixture all at once and stirred at room temperature for 6.0 hours. The reaction mixture was then diluted with 50mM TEAB (25 mL). The product was purified by HPLC C18 (gradient: 0-60% B over 70 minutes). Compound **35** was obtained after lyophilization of the target fractions (2.15 μ mol, 44% yield in ~ 98% purity by HPLC, and the structure was confirmed by MS (ES+): calculated for (M-H) C₅₈H₇₆N₁₀O₂₅P₃S₂⁻, 1469.36, found m/z 1469.67, see Figure 13.

Similarly, analogs of dATP, dCTP and dGTP were synthesized following similar procedure described for compound **35**, and characterized by HPLC and LC-MS resulting a full set of A-series (**98, 100, 101, and 102**, Figure 45). For dATP analog calculated for (M-H) $C_{66}H_{83}N_{12}O_{23}P_3S_2$, 1,568.4348, found m/z 1,568.4400; For dCTP analog calculated for (M-H) $C_{52}H_{65}N_{11}O_{30}P_3S_4$, 1,545.2070, found m/z 1,545.2080 and for dGTP analog calculated for (M-H) $C_{66}H_{93}N_{12}O_{27}P_3S_4$, 1,706.4369, found m/z 1,706.4400. In another aspect, the invention involves nucleotides with cleavable linker as in compound **43** for dATP analogue where the cleavable disulfide is flanked by gem-dimethyl group and the linker is attached to PA-nucleotide via urea group (-NH(C=O)NH-). The compound can be synthesized according to Figure 14 (for dATP analogue). For other nucleotide analogues (e.g. for analogues of dCTP, dGTP, dUTP) can be synthesized similarly replacing **42** by appropriate PA-analogues at the last step of the reaction sequence.

EXAMPLE 19

Synthesis of compound **37**

In a 1L round bottom flask with equipped with stir bar, 5-(fmoc-amino)-1-pentanol (**36**, 20g, 62 mmol) was dissolved in DMSO (256 mL) at room temperature. To the solution, AcOH (43 mL) and Ac₂O (145 mL) were added sequentially. The flask was closed with a rubber septum, placed under N₂, and stirred at room temperature for 20h. Reaction completion was confirmed by TLC. The reaction mixture was then transferred to a 3 L beaker and the flask was washed with water. The beaker was cooled in an ice bath and the reaction mixture was neutralized with 50% saturated K₂CO₃ (400 mL) for 30 minutes. The mixture was transferred to a separatory funnel and extracted with EtOAc (2x700mL). The organic phase was then washed with 50% saturated K₂CO₃ (2x400mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*.

The crude oil was purified by silica gel chromatography (0 to 20% B over 20min, A = Hex, B = EtOAc). Collection and concentration of fractions yields compound **37** (17.77g, 75%) as a white solid, see Figure 14. ^1H NMR (CDCl_3): δ_{H} 7.79 (d, J = 7.33, 2H), 7.63 (d, J = 7.83, 2H), 7.441 (t, J = 7.33, 2H), 7.357 (t, J = 7.58, 2H), 4.803 (bs, 1H), 4.643 (s, 2H), 4.43 (d, J = 6.82, 2H), 4.24 (t, J = 6.82, 1H), 3.54 (t, J = 6.32, 2H), 3.251 (m, 1H), 2.167 (s, 3H), 1.657-1.550 (m, 4H), and 1.446-1.441 (m, 2H) ppm.

EXAMPLE 20

Synthesis of compound **38**

Compound **37** (2.77g, 7.2 mmol) was dissolved in DCM (60 mL) in a 250 mL round bottom flask equipped with stir bar and septum under N_2 . To the flask, triethylamine (3.0 mL, 21.6 mL, 3eq) and 4 \AA Molecular Sieves (28g) were added. The suspension was stirred for 10min at room temperature, followed by 30min in an ice bath. To the flask was added SO_2Cl_2 (1M solution in DCM, 14.4 mL, 14.4 mmol, 2eq) and the reaction mixture was stirred in the ice bath for 1h. Reaction progress was monitored by the disappearance of starting material via TLC (1:1 Hex:EtOAc). Once SO_2Cl_2 activation was complete, a solution of potassium thiotosylate (2.45g, 10.8 mmol, 1.5eq) in DMF (60 mL) was rapidly added. The reaction mixture was allowed to slowly warm to room temperature for 1h. The flask was then charged with 3-mercaptop-3-methylbutanol (1.8 mL, 14.4 mmol, 2eq) and stirred at room temperature for 1h. The reaction mixture was filtered and concentrated *in vacuo* at 40°C. Purification by FCC (0 to 50% B over 30min, A = Hex, B = EtOAc) afforded **38** (482mg, 14%) as a yellow oil, see Figure 14. ^1H NMR (CDCl_3): δ_{H} 7.76 (d, J = 7.81, 2H), 7.59 (d, J = 7.32, 2H), 7.40 (t, J = 7.32, 2H), 7.31 (t, J = 7.32, 2H), 4.87 (bs, 1H), 4.79 (s, 2H), 4.40 (d, J = 6.84, 2H), 4.21 (t, J = 6.84 1H), 3.78 (t, J = 6.84, 2H), 3.57 (t, J = 6.35, 2H), 3.20 (m, 2H), 1.88 (t, J = 6.84, 2H), 1.64-1.50 (m,

4H), 1.42-1.39 (m, 2H) and 1.32 (s, 6H) ppm.

EXAMPLE 21

Synthesis of compound 39

Compound **38** (135mg, 0.275 mmol) was desiccated under vacuum for 2h in a 50 mL round bottom flask. The vacuum was removed and the flask placed under N₂. Compound **38** was dissolved in DMF (3.1 mL) and the flask was charged with DIPEA (96 μ L, 0.55 mmol, 2eq). The solution was stirred for 10min and then DSC (120mg, 0.468 mmol, 1.7eq) was added in one dose as a solid. The reaction mixture was allowed to stir for 2h and completion was verified via TLC (1:1 Hex:EtOAc). The reaction was then concentrated *in vacuo* at 35 °C and further dried under high vacuum for 1h. The crude oil was loaded on to silica gel and purified by FCC (0 to 50% B over 14min, A = hex, B = EtOAc). The fractions were checked by TLC and concentrated to afford compound **39** (133mg, 76%) as an oil that crystallized over time, see Figure 14. ¹H NMR (CDCl₃): δ _H 7.78 (d, *J* = 7.58, 2H), 7.61 (d, *J* = 7.58, 2H), 7.42 (t, *J* = 7.58, 2H), 7.33 (t, *J* = 7.58, 2H), 4.87 (bs, 1H), 4.80 (s, 2H), 4.48 (t, *J* = 7.07, 2H), 4.44 (d, *J* = 6.82, 2H), 4.24 (t, *J* = 7.07, 1H), 3.58 (t, *J* = 6.32, 2H), 3.22 (m, 2H), 2.83 (s, 4H), 2.08 (m, 2H), 1.649-1.562 (m, 4H), 1.443-1.390 (m, 2H) and 1.366 (s, 6H) ppm.

EXAMPLE 22

Synthesis of compound 40

2,2'-(Ethylenedioxy)bis(ethylamine) (92 μ L, 635 μ mol, 10eq) and triethylamine (176 μ L, 1270 μ mol, 20eq) were dissolved in DMF (10 mL). A separate solution of 6-ROX, NHS ester (40mg, 64umol, 1eq) in DMF (2.7 mL) was also prepared. The 6-ROX, NHS ester solution was

added drop-wise to a rapidly stirring solution containing the diamine. The reaction stirred for 2h and progress was monitored by C18 HPLC-MS (0 to 100% B over 10min, A = 50mM TEAB, B = MeCN). Once complete, the reaction was purified via preparative C18-HPLC (10 to 100% B over 50min, A = 50 mM TEAB, B = MeCN). The fractions were combined and lyophilized to yield compound **40** (20mg, 48%) as a purple-red solid, see Figure 14. MS (ES-) calculated for (M-H) $C_{39}H_{45}N_4O_6$ 664.33, found m/z 664.56.

EXAMPLE 23

Synthesis of compound **41**

Compound **40** (10mg, 15 μ mol) was dissolved in DMF (1 mL) and charged with DIPEA (8 μ L, 45 μ mol, 3eq). Separately, compound **39** (28mg, 45 μ mol, 3eq) was dissolved in DMF (0.21 mL). The solution of compound **39** was rapidly added to the solution with compound **40**. The reaction was placed on a shaker plate for 1.5h at which time analytical C18-HPLC (0-100% B over 10min, A = 50 mM Acetate Buffer pH 5.2, B = MeCN) revealed remaining compound **40**. Additional compound **39** (13mg, 21 μ mol, 1.4eq) was added and the reaction was placed on a shaker plate for an additional hour. Without additional analytics, piperidine (300 μ L) was added and allowed to react for 10min. The reaction mixture was then directly injected on to a preparative C18-HPLC (10-100% B over 50min, A = 50 mM TEAB, B = MeCN). The fractions were collected and lyophilized to yield compound **41** (4.7mg, 34%) as a purple-red solid, see Figure 14. MS (ES+) calculated for (M+H) $C_{51}H_{68}N_5O_9S_2^+$ 959.45, found m/z 959.76

EXAMPLE 24

Synthesis of compound 43

A 5 mL sample vial was charged with amine **41** (2 mg, 2 μ mol), DSC (0.8 mg, 3 μ mol, 1.5eq), DIPEA (0.7 μ L, 4 μ mol, 2eq), and *N,N*-dimethylformamide (1.7 mL). The reaction mixture was placed on a shaker for 1h. Reaction progress was monitored by C18-HPLC (0 to 100% B over 10min, A = 50mM Acetate Buffer pH 5.2, B = MeCN). Next, nucleotide **42** (6 μ mol, 3eq, Ref. US 2013/0137091 A1) in 0.1 Na_2HPO_4 (3.3 mL) was added and the reaction mixture was placed on a shaker overnight. The reaction was next diluted with water and purified by preparative C18-HPLC (0 to 60% B over 70min, A = 50 mM TEAB, B = MeCN) to give the title compound **43** (0.5 μ mol, 25%), see Figure 14. MS (ES-) calculated for (M-H) $\text{C}_{67}\text{H}_{87}\text{N}_{13}\text{O}_{22}\text{P}_3\text{S}_2$ 1581.47, found m/z 1581.65.

EXAMPLE 25

In another aspect, the cleavable linker can be compound **45**, where the linker is tethered to PA-nucleotides *via* urea functionality and the disulfide is connected to the dye by a two carbon linker. The resulting nucleotide analogue in such case can be as in compound **49** (dGTP analogue), which can be synthesized according to the Figure 15. Other nucleotide analogues (e.g. analogues of dATP, dUTP, dCTP) can be synthesized similarly by replacing nucleotide **46** with appropriate PA-nucleotide analogues in the third step of the reaction sequence.

EXAMPLE 26

Synthesis of compound 44

A 100 mL round bottomed flask equipped with a magnetic stir bar was charged with **37**

(1.00g, 2.59 mmol) in CH_2Cl_2 , molecular sieves and triethylamine (0.72 mL, 5.18 mmol). The reaction mixture was stirred for 10 minutes at room temperature and cooled to 0 °C. Sulfuryl chloride (4.40 mL, 4.40 mmol) was added slowly and the resultant mixture was stirred for 1 hour at 0 °C. TLC analysis using 20% ethyl acetate in hexanes indicated the disappearance of starting material, and a solution of benzenethionosulfonic acid sodium salt (648 mg, 3.89 mmol) in *N,N*'-dimethylformamide (5 mL) was added in one portion at 0 °C and the reaction mixture was stirred for 20 min at room temperature. Next, N-(trifluoroacetamido)ethanethiol (896 mg, 5.18 mmol) was added in one portion and the resulting mixture was stirred for 30 minutes at room temperature. The molecular sieves were filtered off and the solvents were removed under reduced pressure and the residue was purified via column chromatography on silica gel using 0-20% ethyl acetate-hexanes gradient, to give the title compound **44** (529 mg, 39%) as a yellowish oil. ^1H NMR (CDCl_3), see Figure 15: δ_{H} 7.76 (d, J = 7.52 Hz, 2H), 7.57 (d, J = 7.50 Hz, 2H), 7.40-7.38 (m, 2H), 7.30-7.25 (m, 2H), 4.82 (s, 2H), 4.42 (d, 2H), 4.21-4.20 (m, 1H), 3.70-3.67 (m, 2H), 3.59-3.55 (m, 2H), 3.17-3.16 (m, 2H) and 1.64-1.40 (m, 6H) ppm.

EXAMPLE 27

Synthesis of compound **45**

A 25 mL round bottomed flask equipped with a magnetic stir bar was charged with carbamate **44** (100mg, 0.184 mmol), and 1 mL of 20% piperidine solution in *N,N*-dimethylformamide at room temperature. The reaction mixture was stirred at room temperature for 10 minutes, then diluted with acetonitrile (5 mL) and purified *via* reverse phase preparative HPLC using a 0-30% acetonitrile-TEAB buffer gradient to give the title compound **45** (11mg, 20%) as a clear oil, see Figure 15. ^1H NMR (400 MHz, CD_3OD) δ_{H} 4.90 (s, 2H),

3.64-3.60 (m, 2H), 3.32 (s, 2H), 2.98-2.93 (m, 2H), 2.86-2.82 (m, 2H), 1.66-1.60 (m, 2H), 1.50-1.48 (m, 2H) and 1.33-1.30 (m, 2H) ppm.

EXAMPLE 28

Synthesis of compound 47

A 5 mL sample vial was charged with amine **45** (0.960mg, 3.0 μ mol), DSC (1.15mg, 4.5 μ mol) and triethylamine (60 μ L, 6.0 μ mol) and shaken for 2 hours at room temperature. Then a solution consisting of 3 eq of nucleotide **46** in 200 μ L (ref. US 2013/0137091 A1) in *N,N*-dimethylformamide was added. The reaction mixture was placed on a shaker for 12 hours. The reaction was next diluted with TEAB buffer and purified by preparative reverse phase HPLC using a 0-30% acetonitrile: 50 mM TEAB buffer gradient to give the title compound **47** (in 14% yield), see Figure 15. MS (ES-): calculated for (M-H) $C_{26}H_{37}F_3N_{10}O_{16}P_3S_2^-$, 959.10, found m/z 959.24.

EXAMPLE 29

Synthesis of compound 48

Nucleotide **47** (1 μ mol) was dissolved in TEAB buffer (200 μ L of 50 mM aqueous soln.) and treated with 200 μ L of ammonium hydroxide (30% aqueous soln.) for 50 minutes at room temperature. The reaction was then diluted with TEAB buffer (1 mL of 1M solution) and distilled water (5 mL). The resulting mixture was purified via C18-HPLC, 0-30% Acetonitrile: 50 mM TEAB buffer gradient to afford the title compound **48** (0.40 μ mol, 90%), see Figure 15. MS (ES-): calculated for (M-H) $C_{24}H_{38}N_{10}O_{15}P_3S_2^-$, 863.12, found m/z 863.45.

EXAMPLE 30

Synthesis of compound 49

An aliquot of compound **48** (0.04 µmols) was dissolved in 0.1 mL distilled water and 0.5M Na₂HPO₄ (20 µL) in a 3 mL eppendorf tube. In a separate tube, 1 mg of ROX-NHS ester (0.168 µmol) was dissolved in 48 µL of dry DMF. This solution was then added to the reaction mixture all at once and stirred at room temperature for 6.0 hours. The reaction mixture was then diluted with 50 mM TEAB (5 mL). The product was purified by C18-HPLC using (O-60% B gradient, A = 50mM TEAB, B = acetonitrile). Compound **49** was obtained after lyophilization of the target fractions (0.03 µmol, 30% yield), see Figure 15. MS (ES-) calculated for (M-H), C₅₇H₆₇N₁₂O₁₉P₃S₂⁻ 1380.33, found 1380.25.

Cleavage comparison with regular disulfide linked nucleotides

This new class of nucleotides containing cleavable oxymethylenedisulfide (-OCH₂-SS-) linker, disclosed herein, was compared with regular disulfide (-SS-) linked nucleotide (e.g. nucleotide **50**, described in US Pat. Appln. 2013/0137091 [46]) under reducing phosphine based cleavage conditions. A stark difference in these two classes of nucleotides was observed. When labeled nucleotide **50** was exposed to 10 eq of TCEP at 65 °C, it generated a number of side products including compound **52** along with the expected product **51** identified by LC-MS (Figure 16, and Figure 17, 5 minutes exposure). The proportion of the unwanted side products increased over time (Figure 18, 15 minutes exposure). Under identical cleavage conditions, the oxymethylenedisulfide linked nucleotide **35** cleanly produced the desired cleavage products, compounds **53** and **54**. The methylene thiol segment (-CH₂SH) of the linker was fully eliminated from the nucleotide upon cleavage of the disulfide group (Figure 20 and

Figure 21, 5 minutes exposure). In addition, a prolonged exposure to TCEP did not generate further side products as revealed by LC-MS (Figure 22, 15 minutes exposure). Therefore, this new class of nucleotides could offer significant advantages in the use of DNA sequencing by synthesis (SBS) by eliminating side reactions inherent to the presence of a thiol group as shown in Figure 4.

EXAMPLE 31

Synthesis of compound 57

In another embodiment, the 3'-OH group of the nucleotides can be capped with **-CH₂-SS-Et** or **-CH₂-SS-Me**, and the fluorophore dyes are attached to the nucleobases *via* one of the cleavable **-OCH₂-SS-** linkers described earlier (e.g. as in compound **35**, **43**, and **49**).

The synthesis of PA nucleotides with 3'-**OCH₂-SS-Et** and **-OCH₂-SS-Me**, can be achieved according to Figure 10 and Figure 22, respectively. The difference in the synthesis of 3'-**OCH₂-SS-Me** analogues from that of 3'-**OCH₂-SS-Et** (Figure 10) is the replacement of mercaptoethanol (EtSH) by methanethiol or sodium thiomethoxide at the appropriate step as shown in Figure 22. The **-OCH₂-SS-Me** group is the smallest structure among all possible 3'-**O-CH₂-SS-R** analogues. Therefore, nucleotide analogues with 3'-**OCH₂-SS-Me** capping group should perform better than those of other analogues in terms of enzymatic incorporation rates and cleavability by reducing agents such as TCEP.

Next, the resultant PA-nucleotide (e.g. **57**) can be coupled to the appropriate cleavable **-OCH₂-SS-** linkers, and finally to fluorophore dye as shown in the Figure 23 using the activated linker **32**. And other nucleotides with differing dyes can be synthesized similarly using the appropriate PA-nucleotides (e.g. PA analogues of dATP, dGTP, dCTP) and NHS activated dyes

(Alexa488-NHS, ROX-NHS, Cy5-NHS ester etc.) achieving nucleotide analogues labeled with different fluorophore reporting groups.

EXAMPLE 32

Nucleotide analogues with different linker can be achieved following the protocols described, as shown in in the synthesis of compounds **60** and **61** (Figure 24).

Diverse sets of 3'-**OCH₂-SS-Et** and 3'-**OCH₂-SS-Me** nucleotides with cleavable linkers **-OCH₂-SS-**, but differing in the chain lengths and substitution at the α -carbons can be synthesized similarly. The resulting classes of nucleotides are shown in the Figure 25, Figure 26, and Figure 27. Among nucleotides shown in the Figure 25, the cleavable linker is flanked by stabilizing gem-dimethyl group attached to flexible ethylene-glycol linker and attached to PA-nucleobase via carbamate functional group (**-NH-C(C=O)-O-**), while in Figure 26, the carbamate group is replaced by urea group (**-NH-C(C=O)-NH-**). On the other hand, among nucleotides shown in Figure 27, the disulfide group is attached to primary carbon chain, and tethered to the PA-nucleobase by urea functional group.

EXAMPLE 33

Synthesis of compound **64**:

A 250 mL round bottom flask was charged with compound **62** (3.0 g, 4.58 mmol), 25 mL dry CH₂Cl₂, 3-Å molecular sieves (5.0 g) and cyclohexene (0.55 mL, 5.4 mmol). The resulting mixture was stirred for 10 minutes at room temperature under a nitrogen atmosphere. The reaction flask was then placed on an ice-bath and SO₂Cl₂ (6.8 mL, 1M in CH₂Cl₂, 1.5 eq) was added slowly via a syringe, and stirred for 1 hour at 0 °C. Next, an extra 0.5 eq of SO₂Cl₂ were added to ensure complete conversion to compound **63**. The volatiles were removed under

vacuum while keeping the temperature close to 10 °C. The resulting solid was re-suspended in 20 mL of dry DMF and kept under a nitrogen atmosphere.

In a separate flask, (2,4,6-trimethoxyphenyl)methanethiol (2.45 g, 11.44 mmol) was dissolved in dry DMF (30 mL) under nitrogen atmosphere, and treated with NaH (274.5 mg, 60% in oil) producing a grey slurry. To this, compound **63** was added at once and stirred at room temperature for 3 hrs under nitrogen atmosphere. The reaction mixture was then filtered through celite®-S (20 g) in a funnel eluting the product with EtOAc (100mL). The EtOAc solution was then washed with distilled water (2X100 mL). The EtOAc extract was dried over Na₂SO₄, concentrated by rotary evaporation, and purified by flash chromatography (column: 120 g RediSepRfGold, gradient: 80% Hex to 50 Hex:EtOAc). See **Figure 43**. The target compound (**64**) was obtained as white solid (1.2 g, 32% yield, R_f: 0.4, Hex:EtOAc/3:2). ¹H NMR (CDCl₃): δH 8.13 (m, 3H), 7.43 (m, 1H), 7.32 (m, 2H), 6.12 (m, 1H), 6.00 (s, 2H), 4.62 (m, 2H), 4.31 (m, 3H), 4.00 (m, 1H), 3.82-3.60 (m, 13H), 2.39 (m, 1H), 1.84 (m, 1H), 0.78 (m, 9H), and 0.01 (m, 6H) ppm.

EXAMPLE 34

Synthesis of compound **65**:

Compound **64** (1.2 g 1.46 mmol) was dried under high vacuum with P₂O₅ in a desiccator overnight and dissolved in 30 mL of anhydrous CH₂Cl₂ in a 100 mL flask equipped with a magnetic stirrer. To this was added dimethyldisulfide (0.657 mL, 7.3 mmol), and the reaction flask was placed on an ice-bath. Dimethyl(methylthio)sulfonium tetrafluoroborate (DMTSF, 316 mg, 1.1 eq) was then added and stirred for 1.5 hr at 0 °C. The reaction mixture was transferred to a 250 mL separatory funnel and neutralized with 50 mL of 0.1M aq. solution of NaHCO₃, and extracted with CH₂Cl₂ (2X 50mL). See **Figure 43**. The organic portion was dried over

Na_2SO_4 and concentrated by rotary evaporation. The crude product was purified on a silica gel column (80 g RediSepRf gold) using gradient 80-50% Hex-EtOAc to result in 0.82 g of compound **65** (82% yield, $R_F = 0.5$, Hex:EtOAc/3:2). ^1H NMR (CDCl_3): δ 8.15 (m, 3H), 7.42 (m, 1H), 7.35 (m, 2H), 6.11 (m, 1H), 4.80-4.65 (m, 2H), 4.34 (m, 1H), 4.28 (m, 2H), 4.10 (m, 1H), 3.83-3.67 (m, 2H), 2.49 (m, 1H), 2.34 (s, 3H), 1.90 (m, 1H), 0.78 (m, 9H), and 0.10 (m, 6H) ppm.

EXAMPLE 35

Synthesis of compound **66**:

A round bottomed flask equipped with a magnetic stirrer was charged with compound **65** (0.309 g, 0.45 mmol) and 10.0 mL dry CH_2Cl_2 (10.0 mL) and placed on an ice-bath under a nitrogen atmosphere. TBAF (0.72 mL, 0.72 mmol, in 1M solution) was added slowly via syringe. The reaction mixture was stirred for 3 hours at 0 °C. The reaction mixture was then transferred to a separatory funnel and quenched with 0.5 M NaHCO_3 solution (50mL). The resulting mixture was extracted with EtOAc (2 X100 mL) and dried over Na_2SO_4 . The product **66** was obtained as a white powder after silica gel column chromatography in 76% yield (196 mg, $R_F = 0.3$, Hex:EtOAc/1:1) on a 40 g RediSepRf column using gradient 7:3 to 2:3 Hex:EtOAc. See **Figure 43**. ^1H NMR (CDCl_3): δ 8.40 (s, 1H), 8.25 (m, 2H), 7.60 (m, 1H), 7.52 (m, 2H), 6.21 (m, 1H), 4.90-80 (m, 2H), 4.65 (m, 1H), 4.40 (m, 2H), 4.25 (m, 1H), 4.05-3.85 (m, 2H), 2.62 (m, 1H), 2.50 (s, 3H) and 2.31 (m, 1H) ppm.

The product **67** was obtained after phosphorylation of compound **66** (confirmed by LC-MS m/z (M-H) 611.19 for $\text{C}_{14}\text{H}_{23}\text{N}_4\text{O}_{13}\text{P}_3\text{S}_2$ for **67**) via standard triphosphate synthesis method (see the synthesis of compound **5** for detail and see Figure 8). It was further converted to dye labeled products according to procedure described for compounds presented in Figure 13,

Figure 14, and Figure 15.

EXAMPLE 36

Synthesis of compound 70:

Compound **68** (7.3 g, 13.8 mmol) was dried in a desiccator overnight and dissolved in anhydrous DCM (70 mL) in a dry 500 mL round bottom flask equipped with a stirbar and rubber septum under an atmosphere of N₂. Cyclohexene (1.54 mL, 15.2 mmol, 1.1 equiv) and dry 3-Å molecular sieves (16.6 g) were added to the reaction mixture and the resulting suspension was stirred for 20 min at 0 °C in an ice-water bath. Next, SO₂Cl₂ (1 M solution in DCM, 32.7 mL, 2.36 equiv) was added and the resulting mixture was stirred at 0 °C for 1 h. Reaction progress was monitored by the disappearance of the starting material *via* TLC (100% EtOAc). Once the SO₂Cl₂ activation was complete, a mixture of (MeO)₃BnSH (7.4 g, 34.5 mmol, 2.5 equiv) and NaH (1.32 g, 33.12 mmol, 60% in mineral oil) in DMF (120mL) was prepared and rapidly added in one portion. The reaction was allowed to slowly warm to room temperature and stirred for 1h. The reaction mixture was filtered and concentrated *in vacuo* at 40 °C. Purification by column chromatography on silica gel (eluted with 0 to 60% ethyl acetate : hexanes gradient 15 mins, followed by 60% ethyl acetate : hexanes for 45 mins) afforded the desired compound **70** (4.2 g, 43.7% yield) as a clear oil. See Figure 44. ¹H NMR (CDCl₃): δ_H 8.72 (s, 1H), 8.31 (s, 1H), 7.94 (m, 2H), 7.52 (m, 1H), 7.44 (m, 2H), 6.41 (m, 1H), 6.03 (s, 2H), 4.67 (s, 2H), 4.50 (m, 1H), 4.10 (m, 1H), 3.73 (m, 13H), 2.52 (m, 2H), 0.81 (s, 9H) and 0.002 (d, 6H) ppm.

EXAMPLE 37

Synthesis of compound 71:

Compound **70** (2 g, 2.87 mmol) was dissolved in anhydrous DCM (38 mL) in a 200 mL round bottom flask equipped with stirbar and a rubber septum under an atmosphere of N₂ and

cooled on an ice-water bath. To this mixture was added dimethyldisulfide (1.3 mL, 14.36 mmol, 5 equiv), followed by addition of DMTSF (620 mg, 3.15 mmol, 1.1 equiv) as a solution in DCM (20 mL), in one portion. The resulting mixture was allowed to slowly warm to room temperature and then stirred for an additional 4 h. The reaction was quenched by addition of a saturated aqueous solution of NaHCO₃ (100 mL), extracted with DCM (150 mL x 2) and EtOAc (200mL) dried over Na₂SO₄ and concentrated *in vacuo*. Purification by column chromatography on silica gel (eluted with 0 to 60% ethyl acetate : hexanes gradient 15 mins, followed by 60% ethyl acetate : hexanes for 45 mins) afforded the desired compound **71** (1 g, 62% yield) as a white powder. See Figure 44. ¹H NMR (CDCl₃): δ_H 8.69 (s, 1H), 8.24 (s, 1H), 7.94 (m, 1H), 7.51 (m, 1H), 7.42 (m, 2H), 6.41 (m, 1H), 4.82 (m, 2H), 4.57 (m, 1H), 4.15 (m, 1H), 3.77 (m, 2H), 2.61 (m, 2H), 2.40 (s, 3H), 0.81 (s, 9H) and 0.00 (d, 6H) ppm.

EXAMPLE 38

Synthesis of compound **72**:

Compound **71** (562 mg, 1.25 mmol) was dissolved in anhydrous THF (30 mL) in a 100 mL round bottom flask equipped with a stirbar and rubber septum under an atmosphere of N₂ and cooled on an ice-water bath. TBAF (1.5mL of 1 M soln. in THF, 1.5 equiv) was then added dropwise and stirred at 0 °C for 2 h. The reaction progress was monitored by TLC (100% ethyl acetate R_f for compound **72** = 0.205, R_f for compound **71** = 0.627). Upon reaction completion methanol (5 mL) was added, the reaction was concentrated on the rotary and the residue was purified *via* column chromatography on silica gel (eluted with 0 to 60% ethyl acetate : hexanes gradient 15 mins, followed by 60% ethyl acetate : hexanes for 45 mins) to afford the desired compound **72** (280 mg, 62% yield) as white powder. See Figure 44. ¹H NMR (CDCl₃): δ_H 8.69 (s, 1H), 8.02 (s, 1H), 7.95 (m, 2H), 7.53 (m, 1H), 7.44 (m, 2H), 6.25 (m, 1H), 4.83 (m, 2H), 4.70 (m,

1H), 4.29 (m, 1H), 3.93 (m, 1H), 3.74 (m, 1H), 2.99 (m, 1H), 2.43 (s, 3H) and 2.41 (m, 1H) ppm.

Compound **72** was then converted to triphosphate **73** following standard triphosphate synthesis described earlier (see the synthesis of compound **5** in Figure 8).

EXAMPLE 39

Synthesis of compound **108**:

A 1 L round bottom flask equipped with a stirbar was charged with 1,4-butanediol (18.3 g, 203.13 mmol) in 100 mL of anhydrous pyridine and cooled to 0 °C under a nitrogen atmosphere. *tert*-Butyldiphenylsilylchloride (13.8 mL, 70 mmol) was then added dropwise *via* syringe, the reaction was allowed to gradually warm to room temperature and stirring continued at rt for 12 h. The volatiles were removed by rotary evaporation and the residue absorbed onto 80 grams of silica gel. Purification *via* flash column chromatography on silica gel using 30 to 50 % ethyl acetate in hexanes gradient resulted in 4-O-(*tert*-butyldiphenylsilyl)-butane-1-ol, **108** (13.7g, 59.5% yield, R_f = 0.7 with 1:1/hexanes:ethyl acetate, ^1H NMR (CDCl_3): δ_{H} 7.70 (m, 4H), 7.40 (m, 6H), 3.75 (m, 2H), 3.65 (2H, m), 1.70 (m, 4H), 1.09 (m, 9H,) ppm. The synthesis is illustrated in Figure 53.

EXAMPLE 40

Synthesis of compound **109**:

A 250 mL round bottom flask equipped with a magnetic stir bar and was charged with compound **108** (6.07 g, 18.5 mmol) and 90 mL anhydrous DMSO. Acetic acid (15 mL) and acetic anhydride (50 mL) were sequentially added and the reaction was stirred for 20 h at room temperature, transferred to a separatory funnel and partitioned between 300 mL distilled water and 300 mL of ethyl acetate. The organic layer was then transferred to a 1 L beaker and

neutralized using a saturated aqueous K_2CO_3 solution (500 mL). The organic layer was washed with distilled water (3 x 300 mL) and dried over $MgSO_4$. The volatiles were removed under reduced pressure and the residue was purified *via* flash column chromatography on a silica gel (hexanes:ethyl acetate /97:3 to 90:10) to obtain 4-O-(*tert*-butyldiphenylsilyl)-1-O-(methylthiomethyl)-butane, **109** (5.15g, 71.7% yield, R_f = 0.8 in 9:1/hexanes:ethyl acetate). 1H NMR ($CDCl_3$): δ_H 7.70 (m, 4H), 7.40 (m, 6H), 4.62 (s, 2H), 3.70 (m, 2H), 3.50 (m, 2H), 2.15 (s, 2H), 1.70 (m, 4H), 1.08 (m, 9H) ppm. The synthesis is illustrated in Figure 53.

EXAMPLE 41

Synthesis of compound **110**:

A 1 L round bottom flask equipped with a magnetic stirbar was charged with compound **109** (15.5 g, 40 mmol), anhydrous dichloromethane (450 mL), 3 \AA molecular sieves (80 g) and triethylamine (5.6 mL) and the reaction was stirred at 0 °C for 30 min under a nitrogen atmosphere. Next, SO_2Cl_2 (64 mL of 1 M soln. in dichloromethane) was added slowly *via* syringe and stirred for 1 h at 0 °C. Ice bath was then removed and a solution of potassium-thiotosylate (10.9 g, 48.1 mmol) in 20 mL anhydrous DMF was added at once. The resulting mixture was stirred for 20 min at room temperature, added at once to a 2 L round bottom flask containing a solution of 3-mercaptop-3-methylbutan-1-ol (4.4 mL, 36 mmol) in DMF (20 mL). The reaction was stirred for 30 min at room temperature, and then filtered through celite-S. The product was partitioned between equal amounts of ethyl acetate and water. The organic extracts were washed with distilled water in a separatory funnel, followed by concentrating the crude product by rotary evaporation. Purification by flash column chromatography on silica gel using ethyl acetate : hexanes gradient gave the title compound **110** (5.6 g, 26%). 1H NMR ($CDCl_3$): δ_H 7.67-7.70 (m, 4H), 7.37-7.47 (m, 6H), 4.81 (s, 2H), 3.81 (t, J

= 6.73 Hz, 2H), 3.70 (t, J = 6.21 Hz, 2H), 3.59 (t, J = 6.55, 2H), 1.90 (t, J = 6.95 Hz, 2H), 1.58-1.77 (m, 4H), 1.34 (s, 6H), and 1.07 (s, 9H) ppm. The synthesis is illustrated in Figure 53.

EXAMPLE 42

Synthesis of compound 111:

A 500 mL round bottom flask equipped with a magnetic stir bar was charged with compound **110** (5.1 g, 10.36 mmol), anhydrous pyridine (100 mL) and 1,1'-carbonyldiimidazole (CDI) (3.36 g, 20.7 mmol) under a nitrogen atmosphere. The reaction mixture was stirred for 1 h at room temperature and poured into a solution of 2,2'-(ethylenedioxy)bis(ethylamine) (7.6 mL, 51.8 mmol) in anhydrous pyridine (50 mL). Stirring continued for 1 h and the volatiles were removed by rotary evaporation. The resulting crude was purified *via* flash column chromatography on silica gel using (0-15% methanol in CH_2Cl_2) to furnish compound **111** (4.4 g, 65% yield). ^1H NMR (CDCl_3): δ 7.63-7.68 (m, 4H), 7.34-7.44 (m, 6H), 4.76 (s, 2H), 4.17 (t, J = 7.07 Hz, 2H), 3.65 (t, J = 6.16 Hz, 2H), 3.60 (s, 4H), 3.49-3.51 (m, 6H), 3.31-3.39 (m, 2H), 2.88 (m, 2H), 1.9 (t, J = 7.06 Hz, 2H), 1.57-1.73 (m, 4H), 1.31 (s, 6H) and 1.03 (s, 9H) ppm. The synthesis is illustrated in Figure 53.

EXAMPLE 43

Synthesis of compound 113:

A 50 mL round bottom flask equipped with a magnetic stir bar was charged with compound **111** (0.94 g, 1.42 mmol), anhydrous THF (40 mL) and of TBAF (1.6 mL of 1 M soln. in THF, 1.6 mmol) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred for 2.0 h at 0 °C, during which time LC-MS showed complete removal of the TBDPS protecting group. After removing the volatiles on the rotary, the product was purified *via* flash chromatography on

silica gel (0-5% methanol in dichloromethane gradient, to give pure compound **112** (0.284 g, 47% yield), MS (ES+) calculated for (M+H) 429.21, observed m/z 429.18.

Next, compound **112** (0.217g, 0.51 mmol) was dissolved in anhydrous acetonitrile (13 mL) under a nitrogen atmosphere and cooled to 0 °C. DIPEA (97.7 μ L, 0.56 mmol) and Fmoc-NHS ester (273.6 mg, 0.81 mmol) were added and the reaction stirred at 0 °C for 2 h. Purification by flash column chromatography on silica gel, using 50 to 90% ethyl acetate in hexanes gradient, produced a semi-pure product, which was further purified *via* column chromatography on silica gel using 2-5% methanol in CH₂Cl₂ gradient to furnish compound **113** (0.245 g, 74% yield). ¹H NMR (CDCl₃): δ _H 7.70 (2H, d, *J* = 7.3 Hz), 7.59 (2H, d, *J* = 7.6 Hz), 7.32 (2H, m), 7.24 (2H, m), 4.69 (2H, s), 4.35 (2H, m), 4.16 (1H, m), 4.09 (2H, m), 3.60-3.45 (12H, m), 3.36-3.26 (4H, m), 1.82 (2H, m), 1.60 (4H, m) and 1.22 (6H, s) ppm. The synthesis is illustrated in Figure 53.

EXAMPLE 44

Synthesis of compound **114**:

A 50 mL round bottom flask equipped with a magnetic stir bar was charged with compound **7** (170 mg, 0.26 mmol), anhydrous acetonitrile (15 mL), DSC (100 mg, 0.39 mmol) and DIPEA (68 μ L, 0.39 mmol). The reaction mixture was stirred at room temperature for 3 h and additional DSC (100 mg, 0.39 mmol) and DIPEA (68 μ L, 0.39 mmol) were added. The resulting mixture was stirred at room temperature for 12 h. Reaction progress was followed by TLC (R_f = 0.4 for starting material, product R_f = 0.8 in 9:1/ethyl acetate: hexanes). The volatiles were removed by rotary evaporation, and the residue remaining was purified *via* 3- successive silica gel columns using hexanes-ethyl acetate gradient to give compound **114** (121 mg, 59% yield). ¹H NMR (CDCl₃): δ _H 7.81 (m, 2H), 7.63 (m, 2H), 7.42 (m, 2H), 7.33 (m, 2H), 4.78 (s, 2H),

4.43 (m, 2H), 4.37 (t, J = 7.65 Hz, 2H), 4.25 (m, 2H), 4.18 (m, 2H), 3.67-3.55 (m, 10H), 3.39 (m, 4H), 2.84 (s, 4H), 1.88 (m, 4H), 1.73 (m, 4H), and 1.32 (s, 6H) ppm. The synthesis is illustrated in Figure 53.

EXAMPLE 45

Synthesis of compound 117:

A 500 mL round bottom flask equipped with a magnetic stir bar was charged with compound **68** (7.3 g, 13.8 mmol, pre-dried in a desiccator overnight), anhydrous dichloromethane (70 mL), cyclohexene (1.54 mL, 15.2 mmol) and 3-Å molecular sieves (16.6 g) and the resulting suspension was stirred for 20 min at 0 °C under a nitrogen atmosphere. Next, SO_2Cl_2 (1 M solution in dichloromethane, 32.7 mL, 2.36 equiv) was added and the resulting mixture was stirred at 0 °C for 1 h. Reaction progress was monitored *via* TLC for disappearance of the starting material (100% ethyl acetate). Once the SO_2Cl_2 activation was complete, a mixture of $(\text{MeO})_3\text{BnSH}$ (7.4 g, 34.5 mmol, 2.5 equiv) and NaH (1.32 g, 33.12 mmol, 60% in mineral oil) in DMF (120 mL) was prepared and rapidly added in one portion. The reaction was allowed to slowly warm to room temperature and stirred for 1h. The reaction mixture was filtered and concentrated *in vacuo* at 40 °C. Purification by column chromatography on silica gel using 0 to 60% ethyl acetate in hexanes gradient afforded the desired compound **70** (4.2 g, 43.7% yield) as a clear oil. ^1H NMR (CDCl_3): δ_{H} 8.72 (s, 1H), 8.31 (s, 1H), 7.94 (m, 2H), 7.52 (m, 1H), 7.44 (m, 2H), 6.41 (m, 1H), 6.03 (s, 2H), 4.67 (s, 2H), 4.50 (m, 1H), 4.10 (m, 1H), 3.73 (m, 13H), 2.52 (m, 2H), 0.81 (s, 9H) and 0.002 (d, 6H) ppm. The synthesis is illustrated in Figure 54.

EXAMPLE 46

Synthesis of compound 71:

A 200 mL round bottom flask equipped with a magnetic stir bar was charged with compound **117** (2.0 g, 2.87 mmol) and dichloromethane (38 mL) under an atmosphere of N₂ and cooled on an ice-water bath. To this mixture was added dimethyldisulfide (1.3 mL, 14.36 mmol, 5 equiv), followed by addition of DMTSF (620 mg, 3.15 mmol, 1.1 equiv) as a solution in dichloromethane (20 mL). The resulting mixture was allowed to slowly warm to room temperature and stirred for an additional 4 h. The reaction was then quenched by addition of a saturated aqueous solution of NaHCO₃ (100 mL), extracted with dichloromethane (150 mL x 2) and ethyl acetate (200 mL) dried over Na₂SO₄ and concentrated *in vacuo*. Purification by column chromatography on silica gel (eluted with 0 to 60% ethyl acetate in hexanes gradient) gave the desired compound **71** (1.0 g, 62%) as a white powder. ¹H NMR (CDCl₃): δ_H 8.69 (s, 1H), 8.24 (s, 1H), 7.94 (m, 1H), 7.51 (m, 1H), 7.42 (m, 2H), 6.41 (m, 1H), 4.82 (m, 2H), 4.57 (m, 1H), 4.15 (m, 1H), 3.77 (m, 2H), 2.61 (m, 2H), 2.40 (s, 3H), 0.81 (s, 9H) and 0.00 (d, 6H) ppm. The synthesis is illustrated in **Figure 54**.

EXAMPLE 47

Synthesis of compound 119:

Compound **71** (562 mg, 1.25 mmol) was dissolved in anhydrous THF (30 mL) in a round bottom flask equipped with a stir bar and rubber septum under an atmosphere of N₂ and cooled on an ice-water bath. TBAF (1.5mL of 1 M soln. in THF, 1.5 equiv) was then added dropwise and stirred at 0 °C for 2 h. The reaction progress was monitored by TLC (100% ethyl acetate R_f for compound **119** = 0.2, R_f for compound **71** = 0.6). Upon reaction completion methanol (5 mL) was added, the reaction was concentrated on the rotary and the residue was purified *via*

column chromatography on silica gel (eluted with 0 to 60% ethyl acetate : hexanes gradient 15 mins, followed by 60% ethyl acetate in hexanes for 45 mins) to afford the desired compound **119** (280 mg, 62% yield) as white powder. ^1H NMR (CDCl_3): δ_{H} 8.69 (s, 1H), 8.02 (s, 1H), 7.95 (m, 2H), 7.53 (m, 1H), 7.44 (m, 2H), 6.25 (m, 1H), 4.83 (m, 2H), 4.70 (m, 1H), 4.29 (m, 1H), 3.93 (m, 1H), 3.74 (m, 1H), 2.99 (m, 1H), 2.43 (s, 3H) and 2.41 (m, 1H) ppm.

Compound **119** was then converted to triphosphate **120** using the standard triphosphate synthesis method *vide infra*, except the de-protection was carried out by treating with 10% NH_4OH for 5 h at room temperature to minimize $-\text{SSMe}$ cleavage. Yield 25%; HRMS-ES $^+$: calculated for $\text{C}_{12}\text{H}_{20}\text{N}_5\text{O}_{12}\text{P}_3\text{S}_2$, 582.976, observed m/z 582.975 The synthesis is illustrated in **Figure 54**.

EXAMPLE 48

Synthesis of compound **123**:

Compound **121** (2.5 g, 4.94 mmol) was dried in a desiccator overnight and dissolved in anhydrous dichloromethane (25 mL) in a dry round bottom flask equipped with a stirbar and rubber septum under an atmosphere of N_2 . Cyclohexene (0.55 mL, 1.1 equiv) and dry 3- \AA molecular sieves (6.0 g) were added to the reaction mixture and the resulting suspension was stirred for 20 min at room temperature. The reaction flask was then placed on an ice-salt-water bath to bring the temperature to sub-zero and SO_2Cl_2 (7.4 mL, 1 M solution in dichloromethane) was added slowly with a syringe. The resulting mixture was stirred at 0 °C for 1 h followed by addition of 0.5 equivalents of SO_2Cl_2 to bring the reaction to completion. Reaction progress was monitored *via* TLC by the disappearance of the starting material. Next, a suspension of $(\text{MeO})_3\text{BnSH}$ (2.65 g, 12.35 mmol, 2.5 equiv) and NaH (0.472 g, 11.85 mmol, 60% in mineral oil) in DMF (40 mL) was prepared in a separate flask. The reaction mixture was combined and slowly warmed to room temperature and stirred for 1 h. The reaction mixture was then filtered

through a glass sintered funnel to remove MS, the filtrate was quenched by addition of 50 mM aqueous NaH_2PO_4 solution (50 mL) and extracted with dichloromethane. The combined organics were dried over Na_2SO_4 and concentrated *in vacuo*. Purification by column chromatography on silica gel using hexanes:ethyl acetate gradient gave the desired compound **123** (1.4 g, 42.2% yield). ^1H NMR (CDCl_3): δ_{H} 8.29 (m, 1H), 7.77 (m, 2H), 7.48 (m, 1H), 7.38 (m, 2H), 6.15 (m, 1H), 5.99 (m, 2H), 4.55 (m, 2H), 4.32 (m, 1H), 4.00 (m, 1H), 3.80 (m, 1H), 3.75 (m, 1H), 3.69 (m, 9H), 2.52 (m, 1H), 1.97 (m, 1H), 0.80 (m, 9H) and 0.01 (m, 6H) ppm. The synthesis is illustrated in **Figure 55**.

EXAMPLE 49

Synthesis of compound **124**:

Compound **123** (1.4 g, 2.08 mmol) was dissolved in anhydrous dichloromethane (42 mL) in a 200 mL round bottom flask equipped with stirbar and a rubber septum under an atmosphere of N_2 and cooled to at 0 °C. To this mixture was added dimethyldisulfide (0.93 mL, 10.4 mmol, 5 equiv), followed by addition of DMTSF (450 mg, 2.28 mmol, 1.1 equiv). The resulting mixture was stirred at 0 °C for 2 h. The reaction was quenched by addition 50 mM NaHCO_3 (100 mL), extracted with dichloromethane (100 mL x 2) and dried over Na_2SO_4 and concentrated *in vacuo*. The product was purified by column chromatography on silica gel (eluted with 0 to 30% ethyl acetate in dichloromethane gradient to afford the desired compound **124** (0.93 g, 83.1%) as a white powder. ^1H NMR (CDCl_3): δ_{H} 8.48 (m, 1H), 7.93 (m, 2H), 7.56 (m, 1H), 7.47 (m, 1H), 7.37 (m, 2H), 6.00 (m, 1H), 4.73 (m, 2H), 4.34 (m, 1H), 4.07 (m, 1H), 3.84 (m, 1H), 3.73 (m, 1H), 2.44 (m, 1H), 2.33 (m, 3H), 2.25 (m, 1H), 0.76 (m, 9H) and 0.01 (m, 6H) ppm. The synthesis is illustrated in **Figure 55**.

EXAMPLE 50

Synthesis of compound 125:

Compound **124** (930 mg, 1.73 mmol) was dissolved in anhydrous THF (52 mL) in a 100 mL round bottom flask equipped with a stirbar and rubber septum under an atmosphere of N₂ and cooled to 0 °C on an ice-water bath. TBAF (3.5mL of 1 M soln. in THF, 1.5 equiv) was then added drop-wise and stirred at 0 °C for 4 h. Upon reaction completion methanol (5 mL) was added to quench the reaction, the volatiles were removed under reduced pressure, and the residue was purified *via* column chromatography on silica gel (0 to 75% ethyl acetate in hexanes gradient) to afford the desired compound **125** (425 mg, 58% yield) as white powder. ¹H-NMR (CDCl₃): δ_H 8.24 (m, 1H), 7.81 (m, 1H), 7.51-7.42 (m, 2H), 7.41 (m, 2H), 6.09 (m, 1H), 4.80 (m, 2H), 4.50 (m, 1H), 4.17 (m, 1H), 3.94 (m, 1H), 3.80 (m, 1H), 2.58 (m, 1H), 2.40 (m, 3H) and 2.41 (m, 1H) ppm. The synthesis is illustrated in **Figure 55**.

EXAMPLE 51

Synthesis of compound 126:

Compound **125** was then converted to triphosphate **126** using the standard triphosphate synthesis procedure *vide infra*; the final de-protection step was carried out by treating with 10% NH₄OH for 2 h at room temperature to minimize –SSMe cleavage. 30% yield, HR MS-ES⁺: calculated for C₁₁H₂₀N₃O₁₃P₃S₂, 558.965; observed m/z 558.964. The synthesis is illustrated in **Figure 55**.

EXAMPLE 52

Synthesis of compound 130:

A 100 mL round bottom flask equipped with a magnetic stir bar was charged with **127**

(2.0 g, 2.8 mmol) and dried in a desiccator over P_2O_5 under high vacuum for 12 h. Dichloromethane (40 mL) was added under N_2 and the resulting solution cooled on a salt-ice bath for 15 minutes. Cyclohexene (0.34 mL, 3.4 mmol) was added, followed by dropwise addition of SO_2Cl_2 (3.4 mL, 1 M soln. in dichloromethane, 3.4 mmol). The resulting mixture was stirred for 30 minutes, and the reaction progress was monitored by TLC (ethyl acetate : hexanes / 1:1, **127** R_f = 0.5, **128** R_f = 0.15 for $-CH_2Cl$ decomposed product). Additional SO_2Cl_2 (3.1 mL, 1 M soln. in dichloromethane, 3.1 mmol) was added drop-wise and the reaction mixture was stirred for another 40 minutes to ensure complete conversion to compound **128**. This mixture was then concentrated under high vacuum at 0 °C.

Anhydrous dichloromethane (40 mL) was then added to the residue under N_2 and the mixture was stirred at 0 °C until all solids dissolved. A solution of potassium *p*-toluenethiosulfonate (0.96 g, 425 mmol) in DMF (8 mL) was added slowly and the resulting reaction mixture was stirred at 0 °C for 1 h. The mixture was first concentrated under reduced pressure at 0 °C, and then at room temperature. The residue was purified by flash column chromatography on silica gel column using 0 to 100% ethyl acetate in hexanes gradient to give compound **130** as a cream solid (1.1 g, 51%; TLC R_f 0.35, ethyl acetate : hexanes 2:1). MS (ES) m/z: 733 [M+1⁺]. ¹H NMR ($CDCl_3$, 400 MHz): δ_H 8.02 (br.s, 1H), 7.94 (s, 1H), 7.88 (d, J =8.3 Hz, 2H), 7.45 (m, 4H), 7.38 (m, 6H), 7.27 (m, 2H), 6.01 (t, J =6.6 Hz 1H), 5.46 & 5.38 (AB, J_{AB} =12.1 Hz, 2H), 4.97 (m, 1H), 3.86 (m, 1H), 3.74 (dd, J =12.5, 2.8 Hz, 1H), 3.55 (dd, J =12.5, 2.9 Hz, 1H), 2.87 (m, 1H), 2.65 (m, 1H), 2.43 (s, 3H), 2.17 (m, 1H), 1.26 (d, J =6.8 Hz, 3H), 1.25 (d, J =6.9 Hz, 3H) ppm. The synthesis is illustrated in **Figure 56**.

EXAMPLE 53

Synthesis of compound 131:

To a solution of **130** (1.1 g 1.5 mmol) in dichloromethane (anhydrous, 40 mL) cooled in on an ice-water bath was added dimethyldisulfide (0.66 mL, 7.5 mmol) under N_2 . The resulting mixture was stirred for 15 min and NaSMe (0.23 g, 3.3 mmol) was added in one portion. The resulting reaction mixture was stirred at 0°C for 4 h (the reaction progress was monitored by TLC (ethyl acetate:hexanes /2:1, **130** R_f = 0.35, **131** R_f = 0.45). The mixture was filtered through Celite-S and concentrated under reduced pressure. The residue was purified on silica gel column, eluted with ethyl acetate in hexanes (0 ~ 100%) to afford compound **131** as a white solid (0.68 g, 75%; TLC R_f : 0.45, Ethyl acetate /hexanes /2:1). MS (ES) m/z: 625 [M+1 $^+$]. 1H NMR ($CDCl_3$): δ_H 8.02 (s, 1H), 8.00 (br. s, 1H), 7.45 (m, 4H), 7.39 (m, 4H), 7.28 (m, 2H), 6.24 (t, J =6.2 Hz, 1H), 5.05 (m, 1H), 4.99 & 4.94 (AB, J_{AB} =11.4 Hz, 2H), 4.27 (m, 1H), 3.99 (dd, J =12.5, 2.3 Hz, 1H), 3.86 (dd, J =12.5, 2.3 Hz, 1H), 3.12 (m, 1H), 2.74 (m, 1H), 2.52 (s, 3H), 2.50 (m, 1H), 1.30 (d, J =6.6 Hz, 3H) and 1.29 (m, 3H) ppm. The synthesis is illustrated in **Figure 56**.

EXAMPLE 54

Synthesis of compound 132:

Compound **131** was then converted to triphosphate **132** via standard triphosphate synthesis method described in standard method section. 25% yield; HRMS-ES $^+$: calculated for $C_{12}H_{20}N_5O_{13}P_3S_2$, 598.971, observed m/z 598.970. The synthesis is illustrated in **Figure 56**.

EXAMPLE 55

Synthesis of compound 134:

Compound **133** (4.47 g, 10.7 mmol) and (2,4,6-trimethoxyphenyl)methanethiol

(TMPPM-SH) were dried under high vacuum for 2 h and then placed in a desiccator with P₂O₅ for 12 h. Compound **133** was dissolved in anhydrous CH₂Cl₂ (50.0 mL) and cyclohexene (10 mL, 96.6 mmol) was added. The resulting mixture was stirred for 15 minutes at -10 °C under a nitrogen atmosphere. Next a freshly prepared solution of 1 M SO₂Cl₂ in CH₂Cl₂ (25 mL, 26.75 mmol) was added drop-wise *via* addition funnel, and the resulting mixture stirred for 1 hour at -10 °C. The volatiles were removed *in vacuo* while keeping the bath temperature at 10 °C. The residue was then dissolved in anhydrous DMF (52 mL) and kept under a nitrogen atmosphere.

In a separate flask, (2,4,6-trimethoxyphenyl)methanethiol (4 g, 18.7 mmol) was dissolved in anhydrous DMF (48 mL) under a nitrogen atmosphere and cooled to 0 °C. NaH (1.1 g, 26.8 mmol, 60% in mineral oil) was then added and the resulting grey slurry was stirred for 15 minutes at 0 °C. It was added to the former solution in one portion and the reaction was stirred at room temperature for 1 h. The reaction mixture was then partitioned in a reparatory funnel (150 : 300 mL/ brine : ethyl acetate). The organic layer was then washed with brine (2x150 mL). The aqueous layer was back-extracted (4 x 50 mL ethyl acetate). The combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed and product was purified by flash chromatography on silica gel column (column: 120 g RediSepRfGold- ISCO, gradient 0-100% ethyl acetate in hexanes). The target compound **134** was obtained as white solid in 22% yield (1.35 g). ¹H NMR (CDCl₃): δ_H 8.17 (s, 1H), 7.39 (d, 1H), 6.30 (m, 1H), 6.12 (s, 2H), 4.71 (dd, 2H), 4.43 (m, 1H), 4.04 (m, 1H), 3.87 (m, 1H), 3.83 (m, 9H), 3.74(dd, 1H), 2.74 (ddd, 1H), 2.34 (ddd, 1H), 1.93 (m, 2H) 1.53 (s, 3H), 0.93 (m, 9H), 0.11(m, 6H) ppm. LCMS (ESI) [M-H⁺] observed 581, R_f = 0.59 (4:6/hexanes- ethyl acetate). And compound **135** was also isolated as a side product in 22.5% yield (1.13 g). ¹H NMR (CDCl₃): δ_H 8.55 (s, 1H), 7.41 (m, 1H), 6.12 (M, 3H), 4.76 (dd, 2H), 4.47 (m, 1H), 4.01 (m, 1H), 3.90 (m, 1H), 3.82 (m, 9H), 3.75(m, 1H), 2.29 (m, 2H), 2.04 (s, 3H) and 1.91 (m, 2H) ppm. LCMS (ESI) [M-H⁺] observed

467. The synthesis is illustrated in **Figure 57**.

EXAMPLE 56

Synthesis of compound 136:

Compound **134** (3.6 g, 6.2 mmol) in a 100 mL round bottom flask was dried under high vacuum for 2 h and then placed in a vacuum desiccator with P_2O_5 for 12 h. Anhydrous CH_2Cl_2 (96 mL) and dimethyldisulfide (2.8 mL, 30.9 mmol) were added, and the reaction cooled to 0 °C. Dimethyl(methylthio)sulfonium tetrafluoroborate (DMTSF, 1.34 g, 6.82 mmol) was then added and the reaction stirred for 1 h at 0 °C. The reaction mixture was next transferred to a 250 mL separatory funnel and neutralized with 90 mL of 0.1 M aqueous solution of $NaHCO_3$, and extracted with ethyl acetate (2 x 200 mL). Combined organic layer was dried over anhydrous sodium sulfate and concentrated on the rotary. The residue was purified by flash chromatography on a silica gel column using 30-50% ethyl acetate in hexanes gradient. The target compound **136** was obtained as white solid (2.1 g, 77% yield). 1H NMR ($CDCl_3$): δ_H 7.99 (s, 1H), 7.47 (d, 1H), 6.29 (dd, 1H), 4.87 (dd, 2H), 4.49 (m, 1H), 4.13 (m, 1H), 3.88 (m, 2H), 3.5 (m, 1H), 2.47 (s, 3H), 2.45 (dd, 1H), 2.04 (dd, 1H) and 1.54 (s, 2H), 0.93 (m, 9H) and 0.13 (m, 6H) ppm. LCMS (ESI) $[M-H^+]$ observed 447.0. The synthesis is illustrated in **Figure 57**.

EXAMPLE 57

Synthesis of compound 137:

Compound **136** (2.16 g, 4.8 mmol) in a 100 mL round bottom flask dried under high vacuum for 2 h, was dissolved in anhydrous THF (40 mL) followed by addition of acetic acid (1.2 mL) and TBAF in THF (6.7 mL of 1 M solution, 6.72 mmol). The reaction mixture was stirred for 1 hour at 0 °C and then for 2 additional hours at room temperature. The volatiles were

removed *in vacuo* and the residue purified *via* flash chromatography on 40 g RediSepRf gold column using 0-8% Methanol in dichloromethane gradient. The target compound **137** was obtained as white solid (1.45 g, 90% yield). ^1H NMR (CDCl_3): δ_{H} 8.12 (s, 1H), 7.36 (d, 1H), 6.11 (t, 1H), 4.87 (dd, 2H), 4.57 (m, 1H), 4.14 (q, 1H), 3.94 (dd, 1H), 3.83 (m, 1H), 2.50 (s, 3H), 2.4 (m, 2H), 1.93 (s, 3H) ppm; LCMS (ESI) [$\text{M}-\text{H}^+$] observed 333. The synthesis is illustrated in **Figure 57**.

EXAMPLE 58

Synthesis of compound **138**:

The product **138** was obtained after phosphorylation of compound **137** using the standard triphosphate synthesis method *vide infra*. 40% yield, HR LC-MS: calculated for $\text{C}_{12}\text{H}_{21}\text{N}_2\text{O}_{14}\text{P}_3\text{S}_2$, 573.965; observed m/z 573.964. The synthesis is illustrated in **Figure 57**.

EXAMPLE 59

Synthesis of compound **141**:

A 100 mL round bottom flask equipped with a magnetic stir bar was charged with compound **139** (2.23 g, 3.55 mmol), CH_2Cl_2 (20 mL), 3-Å molecular sieves (3.5 g) and cyclohexene (0.60 mL). The resulting mixture was stirred for 20 minutes at room temperature under a nitrogen atmosphere. The reaction was cooled to 0 °C and SO_2Cl_2 (5.4 mL, 1 M in CH_2Cl_2 , 1.5 equiv) were added slowly via a syringe. The reaction was stirred for 1.5 h at 0 °C and an additional 1.8 mL of SO_2Cl_2 (1 M soln. in dichloromethane) was added and stirring continued for 40 minutes at 0 °C to ensure complete conversion to compound **140**. The volatiles were removed under reduced pressure while keeping the bath temperature close to 10 °C. The resulting solid was re-suspended in 20 mL of anhydrous DMF and kept under a nitrogen atmosphere.

In a separate flask, (2,4,6-trimethoxyphenyl)methanethiol (1.98 g, 9.25 mmol) was dissolved in anhydrous DMF (15 mL) and treated with NaH (247 mg, 60% in mineral oil, 6.17 mM) producing a dark grey slurry. Next, compound **140** solution was added in one portion and the reaction was stirred at room temperature for 1 h. The reaction mixture was then partitioned between distilled water (150 mL) and ethyl acetate (150 mL). The organic layer was further washed with distilled water (2 x 150 mL) and dried over Na₂SO₄. The volatiles were removed under reduced pressure and the residue was purified by flash column chromatography on silica gel column using 80 to 100% ethyl acetate in hexanes gradient. The target compound **141** was obtained as white solid (798 mg, 28%). ¹H NMR (CDCl₃): δ_H 8.33 (s, 1H), 7.57 (m, 1H), 6.53 (m, 2H), 6.00 (s, 2H), 4.62 (m, 2H), 4.44 (m, 1H), 4.32 (m, 2H), 3.97 (m, 1H), 3.80-3.60 (m, 11H), 3.10 (m, 6H), 2.36 (m, 1H), 2.24 (m, 1H), 0.80 (m, 9H) and 0.01 (m, 6H) ppm. Further confirmed by LC-MS: observed *m/z* 795.25 for (M-H). The synthesis is illustrated in **Figure 58**.

EXAMPLE 60

Synthesis of compound **142**:

A 100 mL round bottomed flask equipped with a magnetic stir bar was charged with compound **141** (0.779 gm, 0.98 mmol, vacuum dried over P₂O₅ for 12 h) and dry THF (20.0 mL), and cooled to 0 °C under a nitrogen atmosphere. TBAF (1.17 mL, 1M solution in THF, 1.17 mmol) was added slowly *via* a syringe and the reaction mixture was stirred for 1.5 h at 0 °C. Next, an additional TBAF (1 mL, 1M solution in THF, 1 mmol) was added and reacted for 3 h at 0 °C. The reaction mixture was then transferred to a separatory funnel and quenched by addition of methanol (5 mL), distilled water (100 mL) was added and the reaction extracted with ethyl acetate (2 x 100 mL). The organics were dried over Na₂SO₄ and concentrated *in vacuo*. Column chromatography of the residue on silica gel using 80-100% ethyl acetate in hexanes

gradient afforded compound **142** as a white powder (525 mg, 79%). ¹H NMR (Methanol-d4): δ _H 8.33 (s, 1H), 7.19 (m, 1H), 6.06 (m, 2H), 6.03 (m, 1H), 4.72 (m, 2H), 4.64 (m, 1H), 4.57 (m, 1H), 4.35 (m, 2H), 4.17 (m, 1H), 3.75 (m, 9H), 3.16 (m, 6H), 2.80 (m, 1H) and 2.28 (m, 1H) ppm; LC-MS: M-H observed m/z 680.0. The synthesis is illustrated in **Figure 58**.

EXAMPLE 61

Synthesis of compound **143**:

Compound **143** was synthesized from compound **142** via standard triphosphate synthesis procedure described in the standard methods section. Yield 65%, LRMS-ES⁻: calculated for C₂₅H₃₃N₅O₁₅P₃S⁻, 768.09; observed m/z 768.54 (M-H). The synthesis is illustrated in **Figure 58**.

EXAMPLE 62

Synthesis of compound **144**:

A 50 mL conical tube was charged with compound **143** (3.80 mL of 5.25 mM soln. in HPLC grade water, 20 μ mol) and pH 4.65 acetate buffer (4.75 mL), and quickly combined with 9.0 mL of freshly prepared DMTSF (80 mM) solution in pH 4.65 acetate buffer. The resulting mixture was shaken at room temperature for 2 h and quenched by addition of saturated aqueous solution of NaHCO₃ (2 mL). The product was immediately purified on preparative HPLC (column: 30x250mm C₁₈ Sunfire, method: 0 to 2.0 min 100% A, followed by 50% B over 70 min, flow: 25mL/min, A = 50mM TEAB, B = acetonitrile). The appropriate fractions were lyophilized and combined after dissolving in HPLC grade water to furnish 23.4 umols of compound **144** (73% yield). LRMS-ES⁻: calculated for C₁₆H₂₃N₅O₁₂P₃S₂⁻, 634.00, m/z observed 634.42 for (M-H). The synthesis is illustrated in **Figure 58**.

Compound **144** was converted to dye labeled product (**76**) according to procedure

described in standard methods section (**Figure 59**). Compound **146** was obtained in 75% yield in two steps, LRMS-ES⁺: calculated for C₃₄H₅₉N₇O₁₉P₃S₄, 1090.20, m/z observed 1090.24 for (M+H). Compound **76** was obtained in 50-70% yield from **146**, HRMS-ES⁻: calculated for C₆₇H₈₆N₉O₂₃P₃S₄, 1605.393; observed m/z 1605.380 for (M-H).

EXAMPLE 63

Synthesis of compound **150**:

A 250 mL round bottom flask was charged with compound **148** (3.0 g, 4.58 mmol), 25 mL dry CH₂Cl₂, 3-Å molecular sieves (5.0 g) and cyclohexene (0.55 mL, 5.4 mmol). The resulting mixture was stirred for 10 minutes at room temperature under a nitrogen atmosphere. The reaction flask was then placed on an ice-bath, SO₂Cl₂ (6.8 mL, 1M in CH₂Cl₂, 1.5 eq) was added slowly via a syringe, and the reaction stirred for 1 h at 0 °C. Next, an extra 0.5 eq of SO₂Cl₂ was added to ensure complete conversion to compound **149**. The volatiles were removed under vacuum while keeping the temperature close to 10 °C. The resulting solid was re-suspended in 20 mL of dry DMF and kept under a nitrogen atmosphere.

In a separate flask, (2,4,6-trimethoxyphenyl)methanethiol (2.45 g, 11.44 mmol) was dissolved in dry DMF (30 mL) under nitrogen atmosphere, and treated with NaH (274.5 mg, 60% in silicon oil) producing a grey slurry. To this, compound **149** was added at once and the reaction stirred at room temperature for 3 h under nitrogen atmosphere. The reaction mixture was then filtered through celite®-S washed with ethyl acetate (100mL). The ethyl acetate solution was washed with distilled water (2 x 100 mL), the organic extract was dried over Na₂SO₄, concentrated *in vacuo* and purified *via* flash column chromatography on silica gel column using 20 to 50% ethyl acetate in hexanes gradient. The target compound **150** was obtained as white solid (1.2 g, 32% yield, R_f: 0.4, hexanes:ethyl acetate /3:2). ¹H NMR (CDCl₃):

δ_H 8.13 (m, 3H), 7.43 (m, 1H), 7.32 (m, 2H), 6.12 (m, 1H), 6.00 (s, 2H), 4.62 (m, 2H), 4.31 (m, 3H), 4.00 (m, 1H), 3.82-3.60 (m, 13H), 2.39 (m, 1H), 1.84 (m, 1H), 0.78 (m, 9H), and 0.01 (m, 6H) ppm. The synthesis is illustrated in **Figure 60**.

EXAMPLE 64

Synthesis of compound 151:

Compound **150** (1.2 g 1.46 mmol) was dried under high vacuum with P_2O_5 in a desiccator overnight and dissolved in 30 mL of anhydrous CH_2Cl_2 in a 100 mL flask equipped with a magnetic stir bar. To this was added dimethyldisulfide (0.657 mL, 7.3 mmol), and the reaction flask was placed on an ice-bath. Dimethyl(methylthio)sulfonium tetrafluoroborate (DMTSF, 316 mg, 1.1 eq) was added and stirred for 1.5 hr at 0 °C. The reaction mixture was transferred to a 250 mL separatory funnel and neutralized with 50 mL of 0.1 M aq. solution of $NaHCO_3$, and extracted with CH_2Cl_2 (2 x 50 mL). The organic layer was dried over Na_2SO_4 and concentrated by rotary evaporation. The crude product was purified on a silica gel column using gradient 80-50% ethyl acetate in hexanes gradient to result in 0.82 g of compound **151** (82% yield, R_F = 0.5, hexanes:ethyl acetate /3:2). 1H NMR ($CDCl_3$): δ_H 8.15 (m, 3H), 7.42 (m, 1H), 7.35 (m, 2H), 6.11 (m, 1H), 4.80-4.65 (m, 2H), 4.34 (m, 1H), 4.28 (m, 2H), 4.10 (m, 1H), 3.83-3.67 (m, 2H), 2.49 (m, 1H), 2.34 (s, 3H), 1.90 (m, 1H), 0.78 (m, 9H), and 0.10 (m, 6H) ppm. The synthesis is illustrated in **Figure 60**.

EXAMPLE 65

Synthesis of compound 152:

A 100 mL round bottomed flask equipped with a magnetic stir bar was charged with compound **151** (0.309 g, 0.45 mmol), and 10.0 mL dry THF (10.0 mL) and placed on an ice-bath

under a nitrogen atmosphere. TBAF (0.72 mL, 1 M soln. in THF, 0.72 mmol) was added slowly via syringe. The reaction mixture was stirred for 3 h at 0 °C. The reaction mixture was then transferred to a separatory funnel and quenched with 0.5 M aqueous soln. of NaHCO₃ (50 mL). The resulting mixture was then extracted with ethyl acetate (2 x 100 mL) and dried over Na₂SO₄. The product **152** was obtained as a white powder after silica gel column chromatography in 76% yield (196 mg, R_f = 0.3, hexanes:ethyl acetate /1:1) on silica gel column using gradient 7:3 to 2:3 hexanes:ethyl acetate. ¹H NMR (CDCl₃): δ_H 8.40 (s, 1H), 8.25 (m, 2H), 7.60 (m, 1H), 7.52 (m, 2H), 6.21 (m, 1H), 4.90-80 (m, 2H), 4.65 (m, 1H), 4.40 (m, 2H), 4.25 (m, 1H), 4.05-3.85 (m, 2H), 2.62 (m, 1H), 2.50 (s, 3H) and 2.31 (m, 1H) ppm. The synthesis is illustrated in **Figure 60**.

EXAMPLE 66

Synthesis of compound **153**:

Compound **153** was obtained after phosphorylation of compound **152** in 30% yield using the standard triphosphate synthesis method *vide infra* (LC-MS: calculated for C₁₄H₂₃N₄O₁₃P₃S₂, 610.98; observed m/z 611.11 (M-H). It was further converted to dye labeled product (**72**) according to procedure described in standard method section (**Figure 61**). Compound **155** was obtained in 49% yield in two steps, and compound **72** in 60-85% yield, HRMS-ES⁻: calculated C₅₃H₆₈N₈O₃₀P₃S₆⁻, 1581.156 (M-H); found m/z 1582.160.

EXAMPLE 67

Synthesis of compounds **159** & **160**:

A 100 mL round bottom flask equipped with a magnetic stir bar was charged with compound **157** (2.04 g, 2.39 mmol) and was dried on high vacuum over 12 h. After flushing the reaction vessel with argon, 13 mL anhydrous CH₂Cl₂ and cyclohexanesene (0.30 mL, 2.86 mmol) were added

sequentially. The reaction flask was then placed on an ice-water-salt bath and stirred for 10 min to bring the mixture below 0 °C. SO₂Cl₂ (4.0 mL, 1M in CH₂Cl₂, 4.0 mmol) was added drop-wise *via* a syringe over 2 min, and the reaction mixture stirred for 1 h at 0 °C. An additional 0.8 equiv. of SO₂Cl₂ (2.0 mL, 2.0 mmol) was added drop-wise over 1 min and the reaction was stirred for an additional ½ h at 0 °C. Next, the volatiles were removed *in vacuo* while keeping the bath temperature at ~ 10 °C. The resulting solid was re-suspended in 15 mL of dry DMF and kept under an argon atmosphere.

In a separate 100 mL flask, (2,4,6-trimethoxyphenyl)methanethiol (TMPPM-SH, 1.27 g, 6.0 mmol, vacuum dried overnight) was dissolved in dry DMF (16 mL) under argon atmosphere and treated with NaH (195 mg, 60% in oil, 4.88 mmol) producing a grey slurry TMPMT-SNa salt. The mixture was stirred until gas formation subsided (Ca. 10 min). To this, TMPMT-SNa salt was added at once and the mixture was stirred at room temperature under argon atmosphere until TLC (micro-workup: dichloromethane /water; solvent: hexanes: ethyl acetate/1:1) confirmed complete conversion (1 h). The reaction mixture was then filtered through celite®-S (10 g) in a filtration funnel eluting the product with dichloromethane (100 mL). The dichloromethane solution was then washed with water (3x100 mL). The aqueous layer was extracted with 3x100 mL dichloromethane. Combined dichloromethane extract was dried over Na₂SO₄ and concentrated by rotary evaporation. It was then purified by flash chromatography (column: 100 g, gradient: 25% - 50% hexanes:ethyl acetate 5 CV, then 50% EE 10 CV). The target compound **160** was obtained as a white foam (1.22 g, 51% yield). ¹H NMR (DMSO-d₆): δ_H 10.63 (s, 1H), 10.15 (s, 1H), 7.95 (s, 1H), 7.3-7.5 (m, 8H), 7.20-7.3 (m, 2H), 6.40 (m, 1H), 6.15 (m, 1H), 4.69 (m, 2H), 4.50 (dd, 1H), 4.30 (m, 2H), 3.95 (m, 1H), 3.81 (m, 1H), 3.3 (m, 4H), 2.7 (m, 1H), 1.05 (m, 8H), 0.8 (m, 9H) and 0.11 (m, 6H) ppm. LCMS: 1019.371 Da. The synthesis is illustrated in **Figure 62**.

Additionally, the TBDMS-deprotected product **159** was obtained as a side product in 25% yield (0.48 g). $R_f = 0.2$ /hexanes: ethyl acetate /1:1. ^1H NMR (DMSO-d₆): δ_{H} 10.63 (s, 1H), 10.15 (s, 1H), 7.95 (s, 1H), 7.3-7.5 (m, 8H), 7.20-7.3 (m, 2H), 6.40 (m, 1H), 6.15 (m, 1H), 4.69 (m, 2H), 4.50 (dd, 1H), 4.30 (m, 2H), 3.95 (m, 1H), 3.81 (m, 11H), 3.5 (m, 1H), 3.3 (m, 4H), 2.7 (m, 1H), and 1.04 (m, 8H) ppm. LCMS: 905.286 Da.

EXAMPLE 68

Synthesis of compound **161**:

A 100 mL round bottom flask equipped with a magnetic stir bar and rubber septum was charged with compound **160** (0.36 g, 0.35 mmol) and dried for 12 h on high vacuum. After flushing with argon, 7 mL dry dichloromethane and dimethyldisulfide (0.16mL, 1.76 mmol) were added. The reaction flask was placed on an ice-bath and stirred for 10 min to bring the mixture to 0 °C. Dimethyl(methylthio)sulfonium tetrafluoroborate (DMTSF, 80 mg, 0.4 mmol) was then added and the reaction was stirred for at 0 °C until TLC (micro-workup: dichloromethane /water; solvent: Hexanes: Ethyl acetate/1:1). The reaction mixture was transferred to a 250 mL separatory funnel, neutralized with 50 mL of 0.1 M aq. solution of NaHCO₃ and extracted with CH₂Cl₂ (3 x 50 mL). The organic layer was dried over Na₂SO₄ and concentrated by rotary evaporation. The crude product was purified on a silica gel column (column: 25 g, gradient: 10% - 50% hexanes:ethyl acetate 3 CV, then 50% ethyl acetate 5 CV). The target compound **161** was obtained as yellow foam (0.23 g, 74% yield). The synthesis is illustrated in **Figure 62**.

EXAMPLE 69

Synthesis of compound **162**:

A 100 mL round bottomed flask equipped with a magnetic stir bar was charged with

compound **161** (0.18 g, 0.20 mmol), dissolved in 7.0 mL dry THF and placed on an ice-bath under an argon atmosphere. The mixture was stirred for 10 min to bring it to 0 °C and 0.28 mL Acetic acid were added. TBAF (1 M in THF, 0.47 mL, 0.47 mmol) was added dropwise via syringe over 1 min. The reaction mixture was stirred for 0.5 h at 0 °C and then 1 h at rt. TLC (hexanes:ethyl acetate/1:1) still showed starting material. Additional TBAF (1 M in THF, 0.47 mL, 0.47 mmol) was added dropwise via syringe over 1 min and the reaction mixture was stirred for 1 h at room temperature. Next, the mixture was quenched with 2 mL methanol and stirred for 10 min at rt. The solvent was removed by rotary evaporation, and the crude product was purified by silica gel column chromatography (column: 10 g, hexanes: ethyl acetate/ 1:1 to 100% over 2 CV, then 100% Ethyl acetate over 20 CV to yield compound **162** as a white foam (96 mg, 62%). The synthesis is illustrated in **Figure 62**.

EXAMPLE 70

Synthesis of compound **163**:

Compound **163** was obtained after phosphorylation of compound **162** using the standard triphosphate synthesis method *vide infra*; except in the de-protection step AMA or methanolic ammonia were used instead of ammonium hydroxide. It was further converted to the dye labeled product **78** according to the standard procedure below. Compound **78** was obtained in 97% yield from compound **165**. HRMS-ES⁻ calculated C₆₇H₉₆N₉O₂₇P₃S₆ (M-H) 1743.395, found 1743.390. The synthesis is illustrated in **Figure 62**.

EXAMPLE 71

Synthesis of compound 169:

A 100 mL round bottom flask was charged with compound **167** (3.120 g, 5.66 mmol), 30.0 mL dry CH_2Cl_2 , 3-Å molecular sieves (5.0 g) and cyclohexanesene (0.70 mL, 6.9 mmol). The resulting mixture was stirred for 10 minutes at room temperature under a nitrogen atmosphere. The reaction flask was then placed on an ice-bath. To this, SO_2Cl_2 (8.5 mL, 1M in CH_2Cl_2 , 1.5 equiv) was added slowly *via* a syringe, and stirred for 1 hour at 0 °C. Next, an additional 4.0 mL of 1 M SO_2Cl_2 was added and stirred for 40 minutes to ensure complete conversion to compound **168**. The volatiles were removed under vacuum while keeping the temperature close to 10 °C. The resulting solid was re-suspended in 20 mL of dry DMF and kept under a nitrogen atmosphere.

In a separate flask, (2,4,6-trimethoxyphenyl)methanethiol (3.028 g, 14.15 mmol) was dissolved in dry DMF (40 mL) under nitrogen atmosphere, and treated with NaH (566mg, 60% in oil, 14.15 mM) producing a grey slurry. To this, compound **168** solution was added at once and stirred at room temperature for 2.5 h under nitrogen atmosphere. The reaction mixture was then filtered through celite®-S (20 g) with ethyl acetate (200 mL). The ethyl acetate solution was then washed with distilled water (3 x 200 mL) and dried over Na_2SO_4 , concentrated by rotary evaporation, and purified by flash chromatography on 120 g RediSepRfGold, gradient: hexanes:ethyl acetate (7:3 to 3:7). The target compound (**169**) was obtained as white solid (1.43 g, 35.5% yield, R_f : 0.5, hexanes:ethyl acetate /1:1). ^1H NMR (CDCl_3): δ_{H} 7.98 (m, 1H), 6.09 (m, 1H), 6.00 (m, 2H), 4.67-4.51 (m, 2H), 4.30 (m, 1H), 4.22 (m, 2H), 4.00 (m, 1H), 3.80-3-60 (m, 11H), 2.31 (m, 1H), 1.83 (m, 1H), 0.80 (m, 9H) and 0.01 (m, 6H) ppm. The synthesis is illustrated in **Figure 64**.

EXAMPLE 72

Synthesis of compound 170:

Compound **169** (1.43 g 1.99 mmol) was dried under high vacuum over P_2O_5 for 12 h and dissolved in of anhydrous CH_2Cl_2 (25 mL) in a flask equipped with a magnetic stir bar and a nitrogen gas source. To this was added dimethyldisulfide (0.89 mL, 9.88 mmol), and the reaction flask was stirred on an ice-bath. Dimethyl(methylthio)sulfonium tetrafluoroborate (**DMTSF**, 430 mg, 2.19 mmol) was then added and stirred for 1.0 h at 0 °C. The reaction mixture was transferred to a 500 mL separatory funnel and quenched with 100 mL of 50 mM aq. solution of $NaHCO_3$, and extracted with CH_2Cl_2 (2X 150 mL). The organic portion was dried over Na_2SO_4 and concentrated by rotary evaporation. The crude product was purified on a silica gel column (80 g RediSepRf gold) using hexanes-ethyl acetate (8:2 to 3:7) gradient to result in 0.622 gm of compound **170** (54% yield, $R_F = 0.6$, hexanes:ethyl acetate/1:1). 1H NMR ($CDCl_3$): δ_H 7.99 (brs, 1H, NH), 7.98 (s, 1H), 6.12 (m, 1H), 4.69 (m, 2H), 4.35 (m, 1H), 4.19 (m, 2H), 4.06 (m, 1H), 3.80 (m, 1H), 3.60 (m, 2H), 2.40 (m, 1H), 2.33 (s, 3H), 1.88 (m, 1H), 0.78 (m, 9H), and 0.10 (m, 6H) ppm. The synthesis is illustrated in **Figure 64**.

EXAMPLE 73

Synthesis of compound 171:

A 100 mL round bottomed flask equipped with a magnetic stir bar was charged with compound **170** (0.623 g, 1.06 mmol, vacuum dried over P_2O_5 for 12 h) and anhydrous THF (20.0 mL) and placed on an ice-bath under a nitrogen atmosphere. TBAF (1.27 mL, 1 M solution in THF, 1.27 mmols) was added slowly via syringe. The reaction mixture was stirred for 1.5 h at 0 °C, and an additional 0.9 mL of 1 M TBAF soln. in THF was added and stirred a total of 4 h at 0 °C. The reaction mixture was then transferred to a separatory funnel and quenched with 0.5 M

NaHCO₃ solution (50 mL). The resulting mixture was extracted with ethyl acetate (2 X100 mL) and dried over Na₂SO₄. The product **171** was obtained as a white powder after silica gel column chromatography in 63% yield (311 mg) on a 80 g RediSepRf column using gradient 7:3 to 3:7 ethyl acetate in hexanes. ¹H NMR (methanol-d₄): δ_H 8.16 (s, 1H), 6.06 (m, 1H), 4.79 (m, 2H), 4.69 (m, 1H), 4.40 (m, 1H), 4.14 (m, 2H), 3.99 (m, 1H), 3.63 (m, 2H), 2.36 (m, 3H), 2.32 (m, 1H), and 2.08 (m, 1H) ppm, LRMS-ES-: M-H observed m/z 468.0 Da. The synthesis is illustrated in **Figure 64**.

EXAMPLE 74

Synthesis of compound **172**:

The product **172** was obtained in 58% yield after phosphorylation of compound **171** using *via* standard triphosphate synthesis method. LRMS calculated C₁₄H₂₁N₃O₁₄P₃S₂⁻ (M-H), 611.97, found 612.15. Compound **171** was further elaborated to the dye labeled product (**74**) according to standard procedure described in standard method section *vide infra* (**Figure 65**). Compound **174** was obtained in 74% yield in two steps (HRMS-ES⁻ calculated C₃₂H₅₅N₅O₂₁P₃S₄⁻(M-H) 1066.15, found 1066.42. Compound **74** was obtained in 62% yield (HRMS-ES⁻ calculated C₅₉H₈₀N₇O₂₅P₃S₄⁻ (M-H), 1507.330, found 1507.325.

EXAMPLE 75

Standard Method for Triphosphate Synthesis:

Nucleoside (160 μmol) and proton sponge (1.5 equiv) pre-dried under high vacuum over P₂O₅, were dissolved in trimethylphosphate (0.8 mL) in a 25 mL pear-shaped flask under N₂-atmosphere and stirred for 20 minutes until all solids were completely dissolved. The flask was then placed on an ice-water bath to bring the reaction to (-5 to 0 °C). Then, POCl₃ (1.5 eq.)

was added in one portion *via* syringe and the reaction stirred for 1 h.

A mixture of n-butylammonium-pyrophosphate (0.36 g), n-Bu₃N (0.36 mL) and anhydrous DMF (1.3 mL) was prepared in a 15 mL conical tube producing a thick slurry. Once completely dissolved, it was rapidly added at once to the vigorously stirring mixture and stirred for 15 mins at room temperature.

The reaction mixture was then poured into 100 mL of 0.1 M TEAB buffer in a 250 mL round bottom flask and stirred for 3 h at room temperature. It was then concentrated down to 25 mL *in vacuo* and treated with 25 mL of ammonium hydroxide (28-30% NH₃ content) for 8 h at room temperature. After removing most of the volatiles under reduced pressure, the reaction crude was resuspended in 0.1M TEAB buffer (30 mL) and purified by C18 preparative - HPLC (30x250mm, C18 Sunfire column, method: 0 to 2 min 100%A, followed by 50%B over 70 mins, flow 25mL/min; A = 50 mM TEAB, B = ACN). The target fractions were lyophilized, and combined after dissolving in HPLC grade water (20 mL). This semi-pure product was further purified by ion exchange HPLC on PL-SAX Prep column (method: 0 to 5 min 100%A, then linear gradient up to 70%B over 70 min, where A = 15% acetonitrile in water, B = 0.85 M TEAB buffer in 15% acetonitrile). Final purification was carried out by C18 Prep HPLC as described above. The nucleoside triphosphates were obtained in 20 – 65% yield following lyophilization.

EXAMPLE 76

Standard Method for Converting of 3'-OCH₂S-(2,4,6-Trimethoxyphenyl)methane-dNTP to 3'-(OCH₂SSMe)-dNTP Using DMTSF:

A 50 mL conical tube was charged with 3'-OCH₂S-(2,4,6-trimethoxyphenyl)methane-dNTP (3.80 mL of 5.25 mMolar soln. in HPLC grade water, 20 µmols) and pH=4.65 acetate buffer (5.20 mL), and quickly combined with 9.0

mL of DMTSF (80 mMolar soln. in pH=4.65 acetate buffer). The resulting mixture was shaken at room temperature for 2 h and the reaction was quenched by 2.0 mL of saturated NaHCO₃ solution, and immediately purified by prep-HPLC on 30X250mm C18 Sunfire column, method: 0 to 2.0 min 100% A, followed by linear gradient up to 50% B over 70 min, flow: 25 mL/min, A = 50 mM TEAB, B = acetonitrile. The target fractions were lyophilized and combined after dissolving in HPLC grade water to result in 50-75% yield of 3'-(OCH₂SSMe)-dNTP depending on nucleotide. Structural examples of 3'-OCH₂S-(2,4,6-trimethoxyphenyl)methane-dNTPs are illustrated in **Figure 66**.

EXAMPLE 77

Standard Method for Conjugation of NHS Activated Linker:

MeSSdNTP-PA (10 ummol) dissolved in HPLC grade water (2 mL) was diluted with freshly prepared 0.5 M aqueous soln. of Na₂HPO₄ (1 mL). In a conical tube, the NHS-activated linker (NHS-A-Fmoc, **114**, 35 mg, 2.5 eq.) was dissolved in anhydrous DMF (2.0 mL). It was then added to the MeSSdNTP-PA/ Na₂HPO₄ solution at once and stirred for 8 h at room temperature.

The reaction was then diluted with 0.1 M TEAB buffer (2.0 mL) and treated with piperidine (0.6 mL). The mixture was stirred at room temperature for 1 h, diluted further with 0.1 M TEAB (10 mL) and quickly purified by prep HPLC on 30X250mm C18 Sunfire column, method: 0 to 2.0 min 100% A, followed by linear gradient up to 50% B over 70 min, flow rate: 25 mL/min, A = 50 mM TEAB, B = acetonitrile. The target fractions were lyophilized and combined after dissolving in HPLC grade water resulting in 45-75% yield of MeSSdNTP-A-NH₂.

EXAMPLE 78

Standard Method for Labeling with NHS Dye:

MeSSdNTP-A-NH₂ (4.55 μmol) in 2.0 mL of HPLC grade water was diluted with Na₂HPO₄ (0.8 mL of 0.5 Molar aqueous soln.) in a 15 mL conical tube, and combined with NHS-activated dye (2.5 eq.) in 1.4 mL of anhydrous DMF. The reaction mixture was stirred for 8 h at room temperature, diluted with 0.1 M TEAB buffer (40 mL) and purified by prep-HPLC on 30X250 mm C18 Sunfire column, method: 0 to 5 min 100%A, followed by linear gradient up to 50%B over 70 mins, flow rate 25 mL/min). The target fractions were lyophilized and combined after dissolving in HPLC grade water to result in 50-80% yield of labeled product.

EXAMPLE 79

Attachment of cleavable linkers and markers to nucleobases

One of the preferred moieties used to attach cleavable linkers is propargyl based or allyl based. Other means of attaching cleavable linkers and dyes are also contemplated. In particular, attachments to the base moiety that result with little or no residual linker after dye cleavage are particularly preferred. Attachments to the base that result with residual linkers after cleavage that do not carry charge are also preferred. These features are important to ensure that the nucleotides are incorporated in the efficient manner by the enzyme into growing strand of nucleic acid after the cleavage of the label/dye. One particular embodiment contemplated by the present invention comprises the use of hydroxymethyl modified base moieties to attach cleavable dyes. Examples of such compounds are shown in **Figure 71**. **Figure 72** shows the structures of hydroxymethyl derivatives after cleavage of the dye and the 3'-O protective group.

EXAMPLE 80

Cleavage of cleavable linkers and 3'-O Protective Groups

A variety of cleaving agents can be used to cleave the linkers and protective groups of the present invention. For example, a variety of thiol carrying compounds can be used as described in ("Thiol-Disulfide Interchange", Singh, R., and Whitesides, G.M., Sulfur-Containing Functional Groups; Supplement S, Patai, S., Eds., J. Wiley and Sons, Ltd., 1993, p633-658,) [15]. In particular compounds with reduced thiol groups pKas can be used to achieve fast and efficient cleavage yields, for example dithiobutylamine, DTBA (Lukesh et. al., J. Am. Chem. Soc., 2012, 134 (9), pp 4057–4059 [16]). Examples of thiol bearing compounds that can be used to perform cleavage of the current invention are shown in **Figure 73**.

Another class of compounds that are suitable for cleaving the dithio terminating groups and linkers of the present invention are phosphines (Harpp et al., J. Am. Chem. Soc. 1968 90 (15) 4181-4182 [12], Burns et al., J. Org. Chem. 1991, 56, 2648-2650 [13], Getz et al., Analytical Biochemistry 273, 73–80 (1999) [14]). Examples of phosphines useful to cleave dithio based protective groups and linkers of the present invention include: triphenylphosphine, tributylphosphine, tris-hydroxymethyl-phosphine (THMP), tris-hydroxypropyl-phosphine (THPP), tris-carboethoxy-phosphine (TCEP). In certain cases it may be desired to be able to selectively cleave either the linker or the 3'- protective group selectively. This can be achieved by designing protective group and linker as well as selection of cleavage reagents. For example, a combination of 3'-azidomethyl ether protecting group and disulfide linker bearing nucleotide can be used for this purpose. In this case, selective cleavage of the disulfide bridge can be accomplished by using thiol based cleaving reagent and removal of 3'-azidomethyl ether protecting groups can be achieved by using phosphine such as TCEP. Example of such procedure is illustrated in **Figure 74**, **Figure 75**, and **Figure 76**. **Figure 74** shows schemes of chemical

reactions taking place and structures of the compounds formed; **Figure 75** shows HPLC chromatograms collected at each stage and **Figure 76** absorption spectra extracted from each peak. Step A) Labeled, 3'-O-protected nucleotide shows one peak (1) and absorption at both nucleotide (280 nm; note the max for the propargyl cpds is shifted towards 280-290 nm) and the dye (575 nm). Step B) Treatment with DTT produces peak 2 with absorption peak of the dye (575 nm) and migrating slower (more hydrophobic) and peak 3 with (278 nm) absorption and faster migration due to more hydrophilic character. Step C) Additional treatment with TCEP produces peak 4 with absorption max at 278 and without the dye at even lower retention time consistent with the loss of the 3'-OH protective group. The cleaved dye splits into additional peak (5, 6) but both peaks have identical absorption.

Another example of cleavage is shown in **Figure 77** and **Figure 78**. **Figure 77** shows scheme for cleavage reaction using nucleotide carrying dithio based protective group on the 3' end and dithio based linker. As this figure shows the cleavage reaction could be performed as one step or 2 step process. **Figure 78** shows results of cleavage experiments performed using variety of cleavage agents: dithiosuccinic acid, L-cysteine, DTT and cysteamine. **Figure 78 (A)** shows RP-HPLC chromatograms generated for starting material and reaction mixtures after incubation with cleavage agents dithiosuccinic acid, L-cysteine, DTT and cysteamine. **Figure 78 (B)** shows identified compositions of reaction mixtures indicating full cleavage of both linker and the 3'-protective groups in case of L-cysteine, DTT and cysteamine, and selective cleavage of 3'-O-protective group in case of dithiosuccinic acid. This indicates that selectivity can be achieved by choosing structures of linker, protecting group and the nature of cleaving agent (i.e., with varying pKa of the SH groups and degree of steric hindrance). In addition to these a variety of suitable cleaving agents can be used such as Bis(2-mercaptoethyl)sulfone (BMS) and N,N'-dimethyl-N,N'-bis(mercaptoacetyl)hydrazine (DMH) (Singh et al., Bioorg. Chem., 22,

109-115 (1994) [17]. Reactions can be further catalyzed by inclusion of selenols (Singh et al. Anal Biochem. 1995 Nov 20;232(1):86-91 [18]). Borohydrides, such as sodium borohydrides can also be used for this purpose (Stahl et al., Anal. Chem., 1957, 29 (1), pp 154–155 [19]) as well as ascorbic acid (Nardai et al., J. Biol. Chem. 276, 8825-8828 (2001) [20]). In addition, enzymatic methods for cleavage of disulfide bonds are also well known such as disulfide and thiooxidase and can be used with compounds of the present invention (Holmgren et. al., Methods in Enzymology, Volume 252, 1995, Pages 199–208 [21]).

EXAMPLE 81

Scavengers

Accordingly to the cleave agent used one skilled in the art needs to choose a scavenger agent which will remove excess of cleave agent after cleavage reaction is completed. For example, for thiol bearing cleave agents, a scavenger capable of reacting with free SH group can be used. For example, alkylating agents such as iodoacetamide or maleimide derivatives can be used (US Patent No: 8,623,598 [47], herein incorporated by reference). For borohydrides, one skilled in the art could use ketone bearing compounds, for example levulinic acid or similar compound. Finally, one could also use oxidizing reagent to oxidize excess cleave agent to non-reactive species, for example periodate (*Molecules* 2007, 12(3), 694-702 [48]).

EXAMPLE 82

Modular Synthesis

Labeled nucleotides of the present invention require several steps of synthesis and involve linking variety of dyes to different bases. It is desirable to be able to perform linker and dye attachment in a modular fashion rather than step by step process. The modular approach involves pre-building of the linker moiety with protecting group on one end and activated group on the other. Such pre-built linker can then be used to couple to propargylamine nucleotide, deprotect the masked amine group and then couple the activated dye. This has the advantage of fewer steps and higher yield as compare to step-by-step synthesis. For example, Compound 32 in **Figure 13** is an example of preactivated linker comprising cleavable functionality, with activated eactive group (disuccinimidyl carbonate) and masked/protected amine (Fmoc). After coupling to free amine on propargylaamine nucleotide the protective group can be conveniently removed for example by treatment with base (aq. Ammonia, piperidine) and can be coupled to activated (NHS) dye molecule.

EXAMPLE 83

Linkers of the present invention were tested to measure their hydrophobicity. The logP value of a compound, which is the logarithm of its partition coefficient between n-octanol and water $\log(c_{\text{octanol}}/c_{\text{water}})$ is a well-established measure of the compound's hydrophilicity (or lack thereof) [49]. Low hydrophilicities and therefore high logP values cause poor absorption or permeation. In this case, the logP value was calculated using predictive software, the table below shows the results, indicating that the linkers (such as those in **Figure 25**) are hydrophobic linkers, while some commercially used linkers are hydrophilic.

Linker	Molecular Formula	LogP			
		Osiris*	ChemDraw	Molinsp. **	MarvinSketch
Legacy	C8H16N2O2S2	0.60	0.49	-0.14	-0.76
New	C22H43N3O8S2	2.57	2.09	1.30	0.71
ILMN PEG11	C43H74N6O18	-1.80	-1.80	-2.37	-1.30
ILMN PEG23	C63H114N6O28	-2.74	-3.60	-4.34	-1.77

Although the invention has been described with reference to these preferred embodiments, other embodiments can achieve the same results. Variations and modifications of the present invention will be obvious to those skilled in the art and it is intended to cover in the appended claims all such modifications and equivalents. The entire disclosures of all applications, patents, and publications cited above, and of the corresponding application are hereby incorporated by reference.

References:

1. Metzker, M. L. (2010) "Sequencing Technologies - the Next Generation," *Nat. Rev. Genet.* 11(1), 31-46.
2. Fuller, C. W. *et al.* (2009) "The Challenges of Sequencing by Synthesis," *Nat. Biotechnol.* 27(11), 1013-1023.
3. Hiatt, A. C. and Rose, F. "Enzyme Catalyzed Template-Independent Creation of Phosphodiester Bonds Using Protected Nucleotides," United States Patent 5,990,300, Application 08/300,484, filed 9/2/1994. (issued 11/23/1999).
4. Buzby, P. R. "Nucleotide Analogs," United States Patent Application Publication Number US 2007-0117104 A1, Application 11/295,406, filed 12/5/2005. (published 5/24/2007).
5. Chen, F. *et al.* (2013) "The History and Advances of Reversible Terminators Used in New Generations of Sequencing Technology," *Genomics Proteomics Bioinformatics* 11(1), 34-40.
6. Tabor, S. and Richardson, C. C. (1995) "A Single Residue in DNA Polymerases of the Escherichia coli DNA Polymerase I Family Is Critical for Distinguishing between Deoxy- and Dideoxyribonucleotides," *Proc. Natl. Acad. Sci. U. S. A.* 92(14), 6339-6343.
7. Perler, F. B. and Southworth, M. W. "Thermostable Dna Polymerase from 9on-7 and and Method for Producing the Same," United States Patent 5,756,334, Application 08/271,364, filed 7/6/1994. (issued 5/26/1998).
8. Southworth, M. W. *et al.* (1996) "Cloning of Thermostable DNA Polymerases from Hyperthermophilic Marine Archaea with Emphasis on Thermococcus Sp. 9 Degrees N-7 and Mutations Affecting 3'-5' Exonuclease Activity," *Proc. Natl. Acad. Sci. U. S. A.* 93(11), 5281-5285.

9. Evans, S. J. *et al.* (2000) "Improving Dideoxynucleotide-Triphosphate Utilisation by the Hyper-Thermophilic DNA Polymerase from the Archaeon *Pyrococcus Furiosus*," *Nucleic Acids Res.* 28(5), 1059-1066.
10. Arezi, B. *et al.* (2002) "Efficient and High Fidelity Incorporation of Dye-Terminators by a Novel Archaeal DNA Polymerase Mutant," *J. Mol. Biol.* 322(4), 719-729.
11. Smith, G. P. *et al.* "Modified Polymerases for Improved Incorporation of Nucleotide Analogues," WIPO PCT Patent Publication Number WO/2005/024010, Application PCT/GB2004/003891, filed 9/10/2004. (published 3/17/2005).
12. Harpp, D. N. *et al.* (1968) "Organic Sulfur Chemistry. I. The Disulfide-Phosphine Reaction. Desulfurization with Tris(Diethylamino)Phosphine," *J. Am. Chem. Soc.* 90(15), 4181-4182.
13. Burns, J. A. *et al.* (1991) "Selective Reduction of Disulfides by Tris(2-Carboxyethyl)Phosphine," *J. Org. Chem.* 56(8), 2648-2650.
14. Getz, E. B. *et al.* (1999) "A Comparison between the Sulfhydryl Reductants Tris(2-Carboxyethyl)Phosphine and Dithiothreitol for Use in Protein Biochemistry," *Anal. Biochem.* 273(1), 73-80.
15. Singh, R. and Whitesides, G. M. (1993) "Thiol-Disulfide Interchange," in *Sulfur-Containing Functional Groups* (Supplement, S. and Patai, S., Eds.), pp 633-658, J. Wiley and Sons, Ltd.
16. Lukesh, J. C. *et al.* (2012) "A Potent, Versatile Disulfide-Reducing Agent from Aspartic Acid," *J. Am. Chem. Soc.* 134(9), 4057-4059.
17. Singh, R. and Whitesides, G. M. (1994) "Reagents for Rapid Reduction of Native Disulfide Bonds in Proteins," *Bioorg. Chem.* 22, 109-115.
18. Singh, R. and Kats, L. (1995) "Catalysis of Reduction of Disulfide by Selenol," *Anal.*

Biochem. 232(1), 86-91.

19. Stahl, C. R. and Siggia, S. (1957) "Determination of Organic Disulfides by Reduction with Sodium Borohydride," *Anal. Chem.* 29(1), 154-155.
20. Nardai, G. *et al.* (2001) "Protein-Disulfide Isomerase- and Protein Thiol-Dependent Dehydroascorbate Reduction and Ascorbate Accumulation in the Lumen of the Endoplasmic Reticulum," *J. Biol. Chem.* 276(12), 8825-8828.
21. Holmgren, A. and Bjornstedt, M. (1995) "[21] Thioredoxin and Thioredoxin Reductase," in *Methods in Enzymology*, pp 199-208, Academic Press.
22. Chen, C.-Y. (2014) "DNA Polymerases Drive DNA Sequencing-by-Synthesis Technologies: Both Past and Present," *Frontiers in Microbiology* 5.
23. Ju, J. *et al.* "Four-Color Dna Sequencing by Synthesis Using Cleavable Fluorescent Nucleotide Reversible Terminators," United States Patent 7,883,869, Application 12/312,903, filed 7/9/2009. (issued 2/8/2011).
24. Ju, J. *et al.* "Massive Parallel Method for Decoding DNA and RNA," United States Patent 8,088,575, Application 12/804,284, filed 7/19/2010. (issued 1/3/2012).
25. Ju, J. *et al.* "Chemically Cleavable 3'-O-Allyl-Dntp-Allyl-Fluorophore Fluorescent Nucleotide Analogues and Related Methods," United States Patent 8,796,432, Application 12/084,457, filed 4/30/2008. (issued 8/5/2014).
26. Balasubramanian, S. "Polynucleotide Sequencing," United States Patent 6,833,246, Application 10/113,221, filed 3/29/2002. (issued 12/21/2004).
27. Balasubramanian, S. *et al.* "Labelled Nucleotides," United States Patent 7,785,796, Application 12/460,741, filed 7/23/2009. (issued 8/31/2010).
28. Milton, J. *et al.* "Labelled Nucleotides," United States Patent 7,414,116, Application 10/525,399, filed 2/23/2005. (issued 8/19/2008).

29. Metzker, M. L. *et al.* (1994) "Termination of DNA Synthesis by Novel 3'-Modified-Deoxyribonucleoside 5'-Triphosphates," *Nucleic Acids Res.* 22(20), 4259-4267.
30. Ju, J. *et al.* (2006) "Four-Color DNA Sequencing by Synthesis Using Cleavable Fluorescent Nucleotide Reversible Terminators," *Proc. Natl. Acad. Sci. U. S. A.* 103(52), 19635-19640.
31. Ruparel, H. *et al.* (2005) "Design and Synthesis of a 3' -O-Allyl Photocleavable Fluorescent Nucleotide as a Reversible Terminator for DNA Sequencing by Synthesis," *Proc. Natl. Acad. Sci. U. S. A.* 102(17), 5932-5937.
32. Bergmann, F. *et al.* "Compound for Sequencing by Synthesis," United States Patent Application Publication Number US 2015-0140561 A1, Application 14/542,980, filed 11/17/2014. (published 5/21/2015).
33. Kwiatkowski, M. "Compounds for Protecting Hydroxyls and Methods for Their Use," United States Patent Application Publication Number US 2002-0015961 A1, Application 09/952,719, filed 9/12/2001. (published 2/7/2002).
34. Gardner, A. F. *et al.* (2012) "Rapid Incorporation Kinetics and Improved Fidelity of a Novel Class of 3' -Oh Unblocked Reversible Terminators," *Nucleic Acids Res.* 40(15), 7404-7415.
35. Litosh, V. A. *et al.* (2011) "Improved Nucleotide Selectivity and Termination of 3' -Oh Unblocked Reversible Terminators by Molecular Tuning of 2-Nitrobenzyl Alkylated Homedu Triphosphates," *Nucleic Acids Res.* 39(6), e39-e39.
36. Bowers, J. *et al.* (2009) "Virtual Terminator Nucleotides for Next Generation DNA Sequencing," *Nat. Meth.* 6(8), 593-595.
37. Zhao, C. *et al.* "Compositions and Methods for Nucleotide Sequencing," United States

Patent 8,399,188, Application 12/442,925, filed 12/23/2009. (issued 3/19/2013).

38. Zon, G. "Reversible Di-Nucleotide Terminator Sequencing," United States Patent 8,017,338, Application 12/275,161, filed 11/20/2008. (issued 9/13/2011).

39. Wang, Z. *et al.* (2010) "Desulfurization of Cysteine-Containing Peptides Resulting from Sample Preparation for Protein Characterization by MS," *Rapid Commun. Mass Spectrom.* 24(3), 267-275.

40. Jung, A. *et al.* (2002) "7-Deaza-2'-Deoxyguanosine Allows PCR and Sequencing Reactions from CpG Islands," *Mol. Pathol.* 55(1), 55-57.

41. Kutyavin, I. V. (2008) "Use of Base-Modified Duplex-Stabilizing Deoxynucleoside 5'-Triphosphates to Enhance the Hybridization Properties of Primers and Probes in Detection Polymerase Chain Reaction," *Biochemistry* 47(51), 13666-13673.

42. Semenyuk, A. *et al.* (2006) "Synthesis of RNA Using 2'-O-Dtm Protection," *J. Am. Chem. Soc.* 128(38), 12356-12357.

43. Semenyuk, A. and Kwiatkowski, M. (2007) "A Base-Stable Dithiomethyl Linker for Solid-Phase Synthesis of Oligonucleotides," *Tetrahedron Lett.* 48(3), 469-472.

44. Semenyuk, A. (2006) "Novel Methods for Synthesis of High Quality Oligonucleotides," Uppsala University.

45. Bellamy, A. J. *et al.* (2007) "The Use of Trifluoroacetyl as an N- and O-Protecting Group During the Synthesis of Energetic Compounds Containing Nitramine and/or Nitrate Ester Groups," *Propellants Explos. Pyrotech.* 32(1), 20-31.

46. Gordon, S. and Olejnik, J. "Methods and Compositions for Incorporating Nucleotides," United States Patent Application Publication Number US 2013-0137091 A1, Application 13/305,415, filed 11/28/2011. (published 5/30/2013).

47. Olejnik, J. *et al.* "Methods and Compositions for Inhibiting Undesired Cleaving of

Labels," United States Patent 8,623,598, Application 12/405,866, filed 3/17/2009. (issued 1/7/2014).

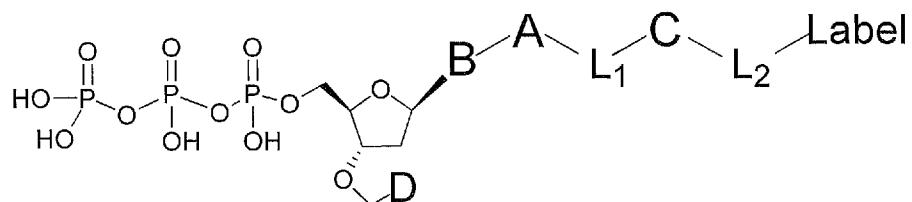
48. Montazerozohori, M. *et al.* (2007) "Fast and Highly Efficient Solid State Oxidation of Thiols," *Molecules* 12(3), 694.

49. Clark, D. E. (1999) "Rapid Calculation of Polar Molecular Surface Area and Its Application to the Prediction of Transport Phenomena. 2. Prediction of Blood-Brain Barrier Penetration," *J. Pharm. Sci.* 88(8), 815-821.

CLAIMS:

We claim:

1. A labeled deoxynucleoside triphosphate according to the following structure:

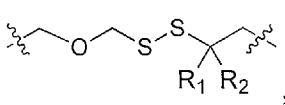
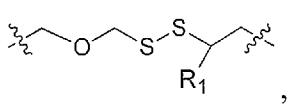
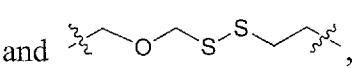


wherein D is selected from the group consisting of an azide, disulfide alkyl, disulfide substituted alkyl groups; B is a nucleobase; A is an attachment group; C is a cleavable site core; L₁ and L₂ are connecting groups; and Label is a label.

2. The labeled deoxynucleoside triphosphate according to claim 1, wherein said nucleobase is a non-natural nucleobase analog selected from the group consisting of 7-deaza guanine, 7-deaza adenine, 2-amino,7-deaza adenine, and 2-amino adenine.

3. The labeled deoxynucleoside triphosphate according to claim 1, wherein said attachment group A is chemical group selected from the group consisting of propargyl, hydroxymethyl, exocyclic amine, propargyl amine, and propargyl hydroxyl.

4. The labeled deoxynucleoside triphosphate according to claim 1, wherein said cleavable site

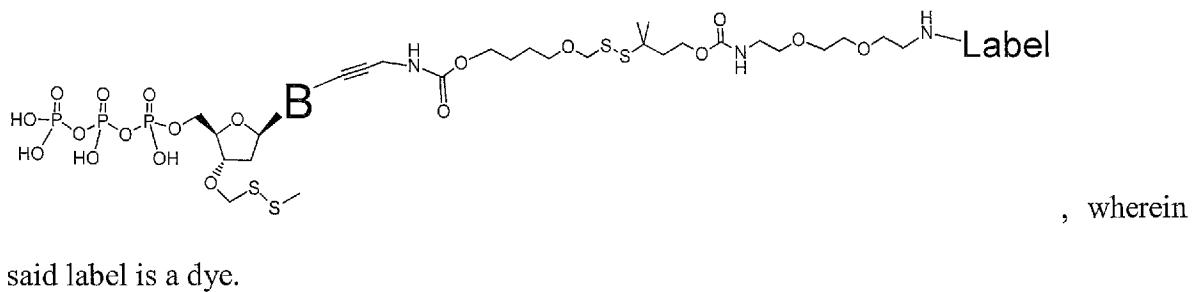
core is selected from the group consisting of:  ,  , and  , wherein R₁ and R₂ are independently selected alkyl groups.

5. The labeled deoxynucleoside triphosphate according to claim 1, wherein L₁ is selected from the group consisting of $-\text{CONH}(\text{CH}_2)_x-$, $-\text{CO-O}(\text{CH}_2)_x-$, $-\text{CONH-(OCH}_2\text{CH}_2\text{O})_x-$, $-\text{CO-O}(\text{CH}_2\text{CH}_2\text{O})_x-$, and $-\text{CO}(\text{CH}_2)_x-$, wherein x is 0-10.

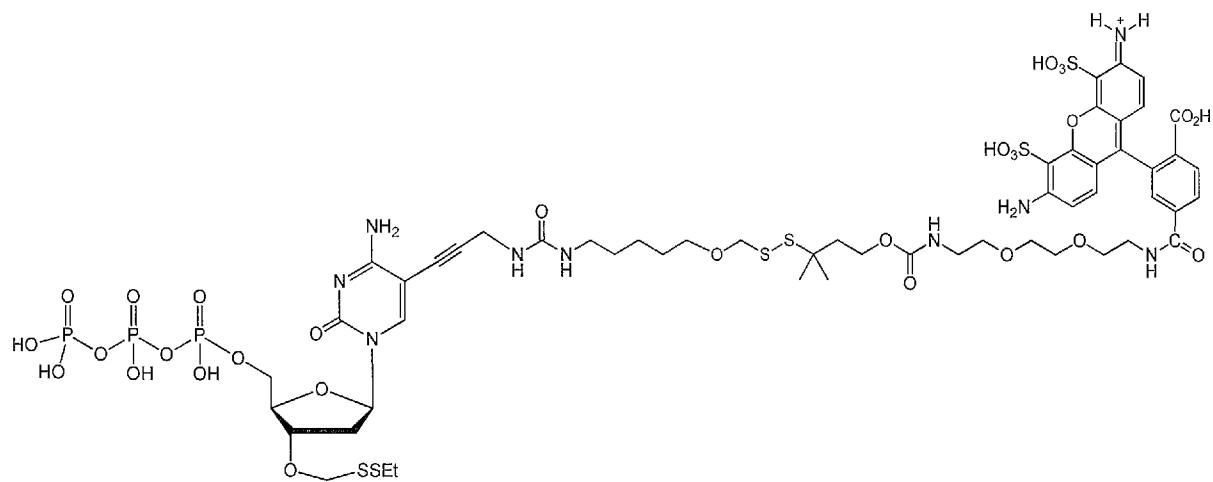
6. The labeled deoxynucleoside triphosphate according to claim 1, wherein L₂ is selected from the group consisting of $-\text{NH}-$, $-(\text{CH}_2)_x\text{NH}-$, $-\text{C}(\text{Me})_2(\text{CH}_2)_x\text{NH}-$, $-\text{CH}(\text{Me})(\text{CH}_2)_x\text{NH}-$, $-\text{C}(\text{Me})_2(\text{CH}_2)_x\text{CO}-$, $-\text{CH}(\text{Me})(\text{CH}_2)_x\text{CO}-$, $-(\text{CH}_2)_x\text{OCONH}(\text{CH}_2)_y\text{O}(\text{CH}_2)_z\text{NH}-$, $-(\text{CH}_2)_x\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_y(\text{CH}_2)_z\text{NH}-$, and $-\text{CONH}(\text{CH}_2)_x-$, $-\text{CO}(\text{CH}_2)_x-$, wherein x, y, and z are each independently selected from is 0-10.

7. The labeled deoxynucleoside triphosphate according to claim 1, wherein said label is selected from the group consisting of fluorophore dyes, energy transfer dyes, mass-tags, biotin, and haptene.

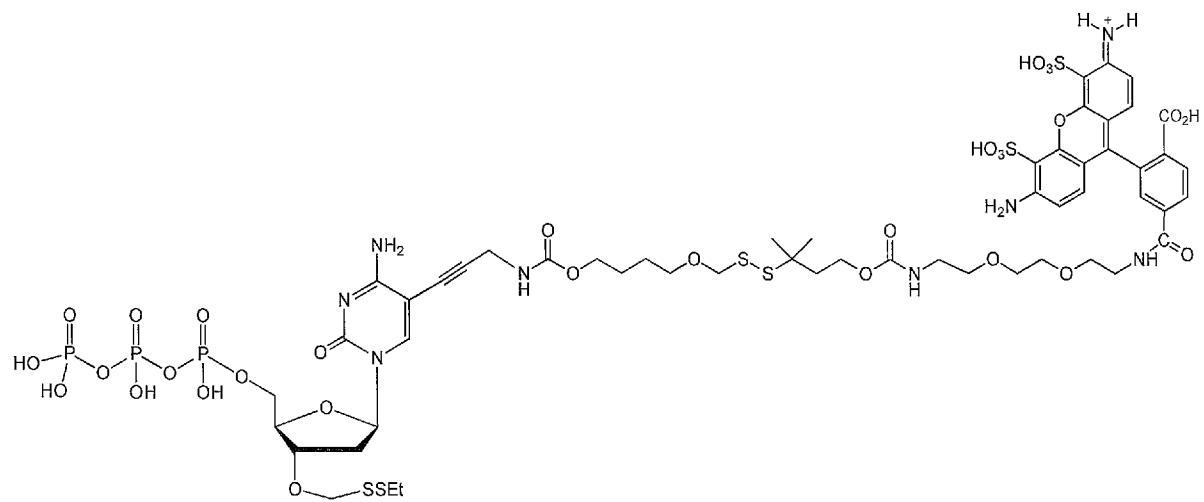
8. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



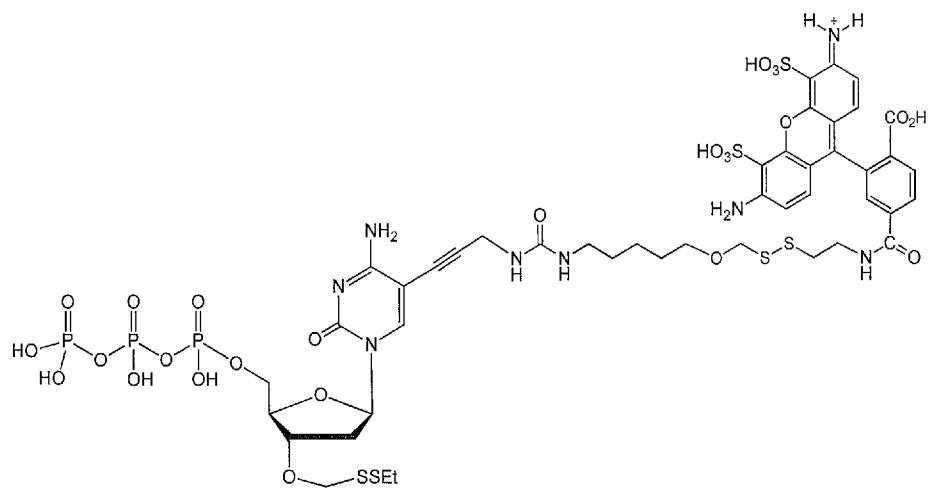
9. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



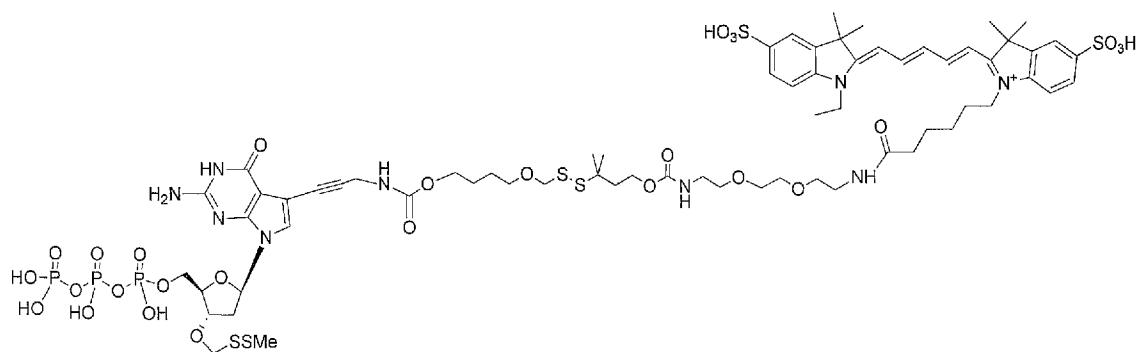
10. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



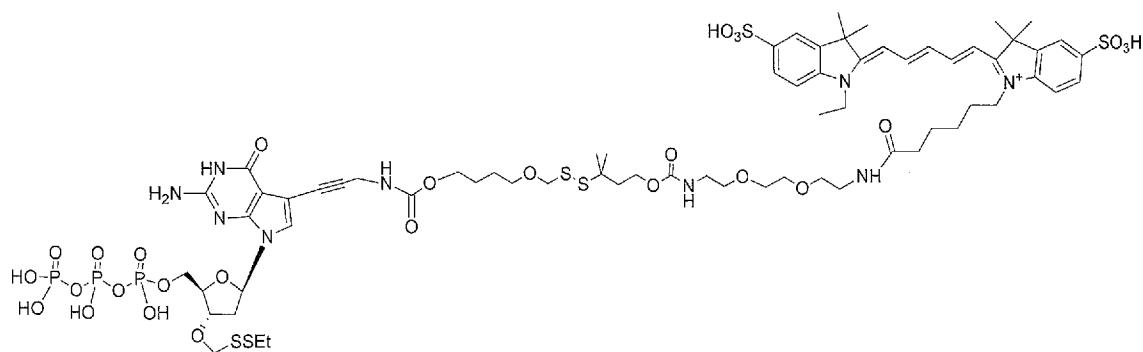
11. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



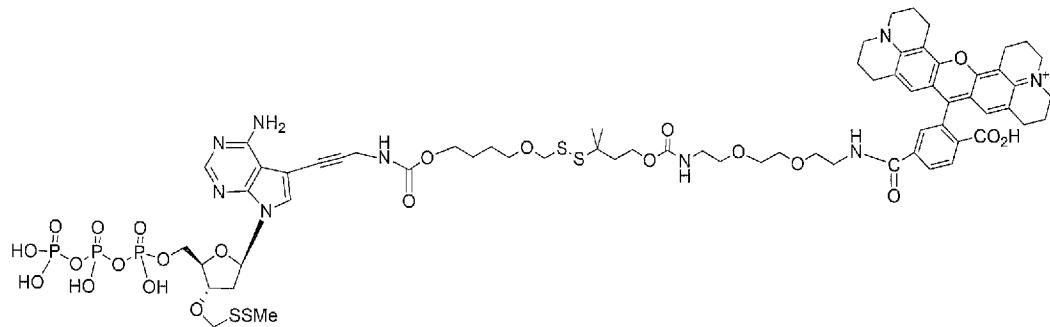
12. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



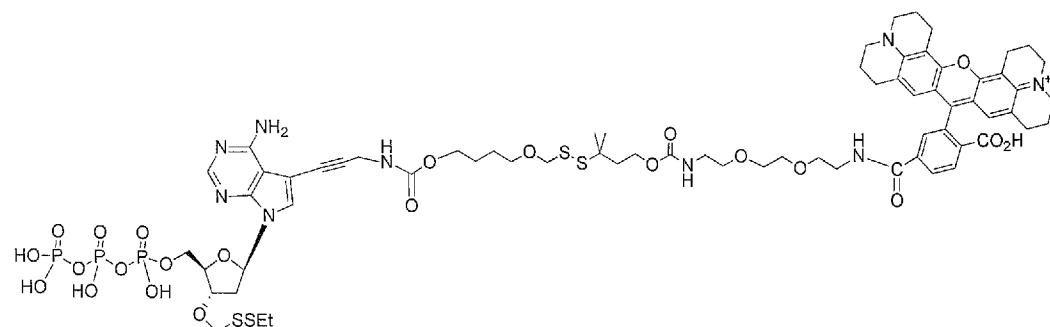
13. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



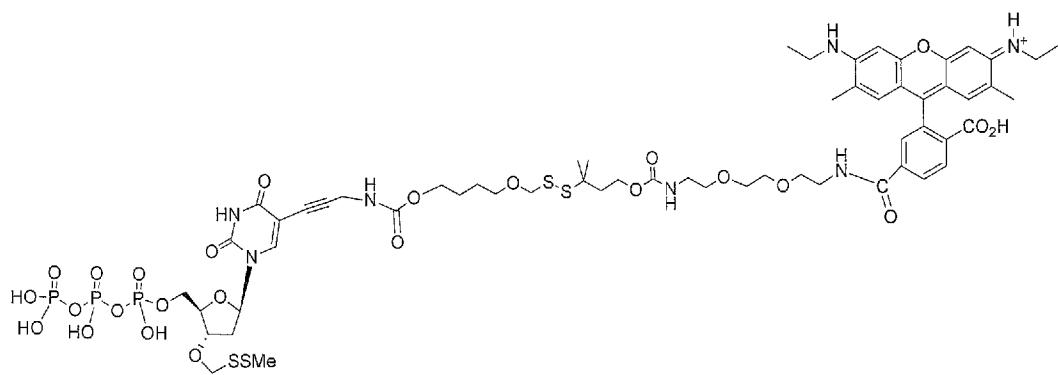
14. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



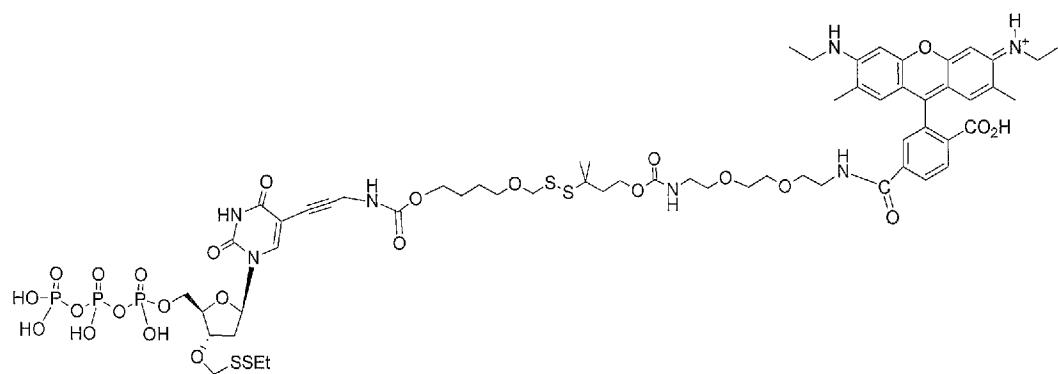
15. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



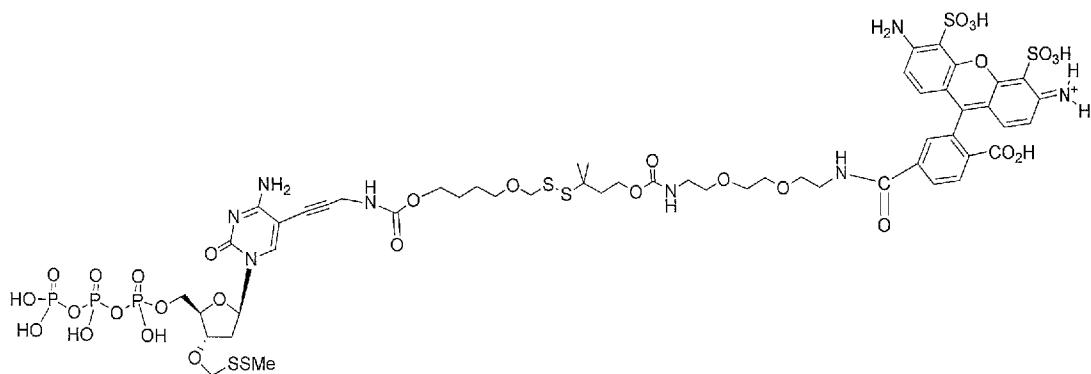
16. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



17. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:

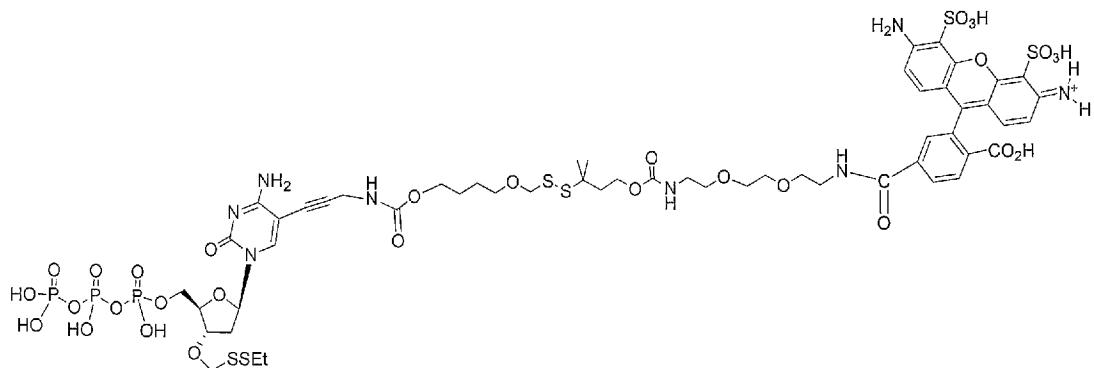


18. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:



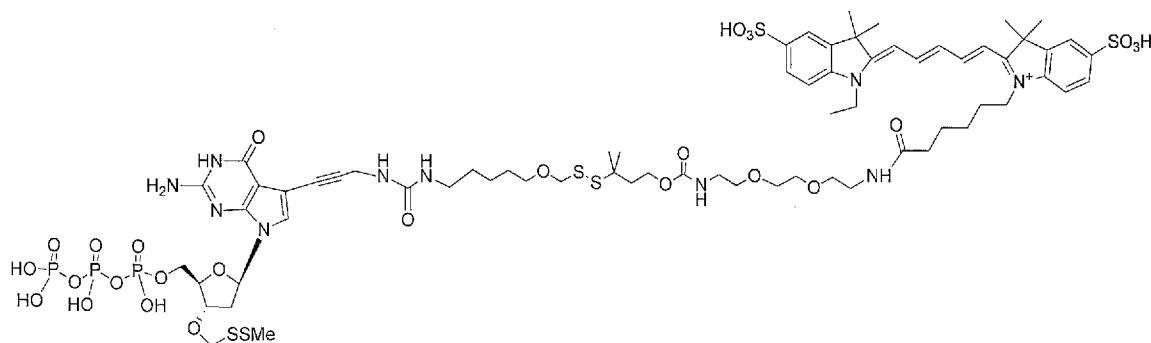
19. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has

the structure:



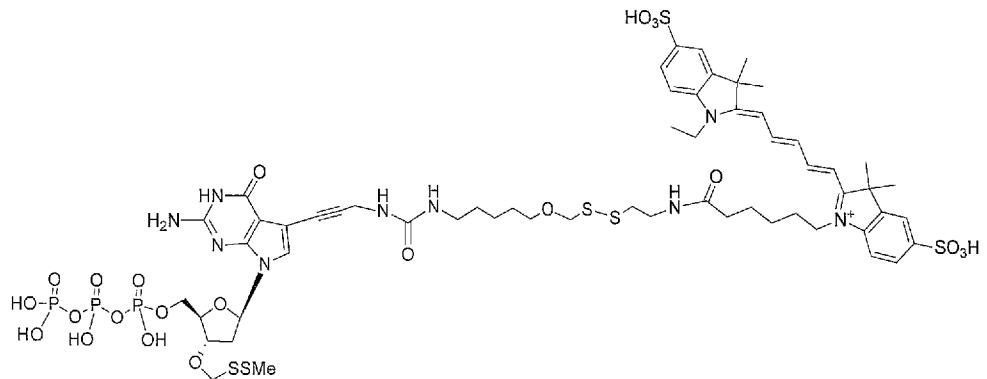
20. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has

the structure:

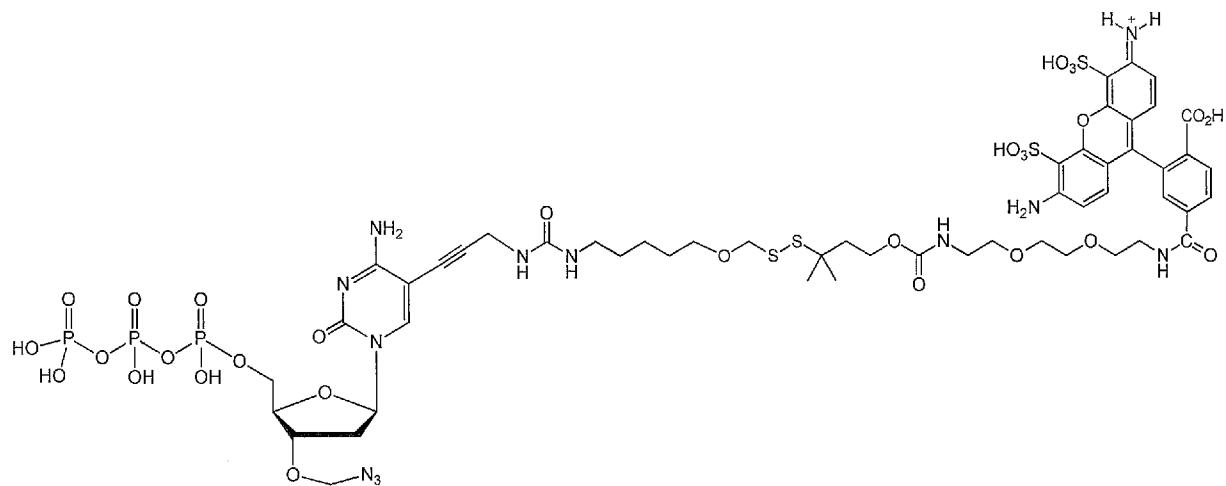


21. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has

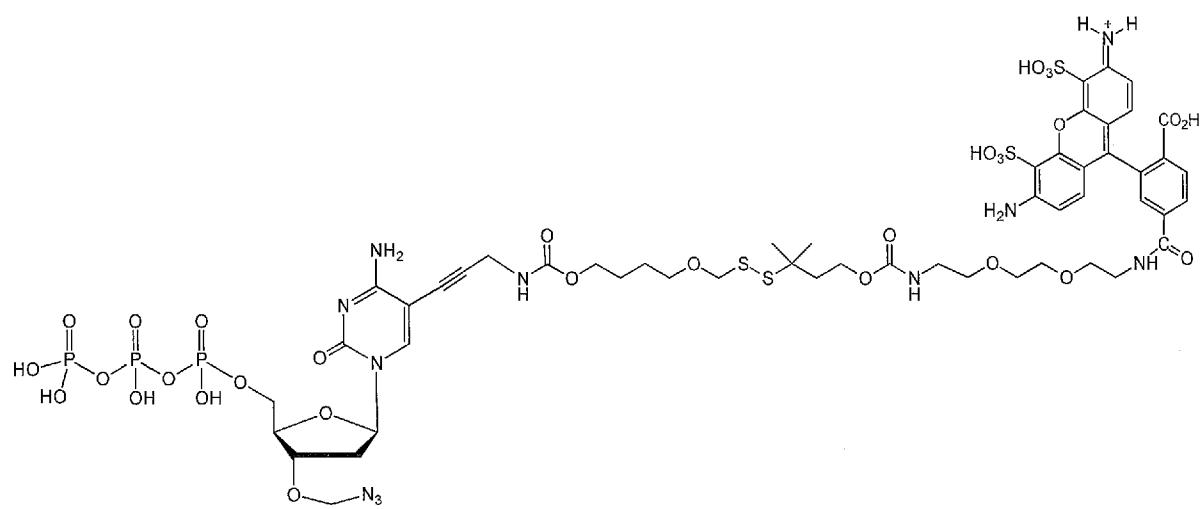
the structure:



22. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:

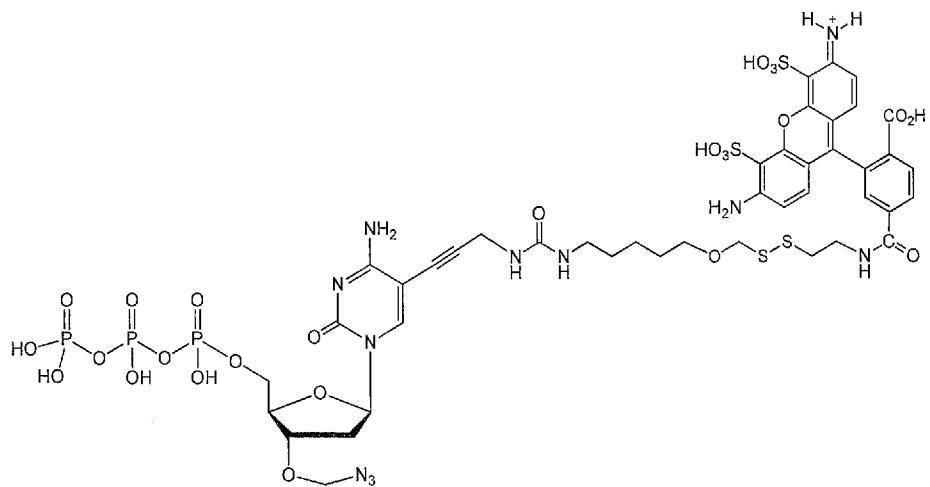


23. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has the structure:

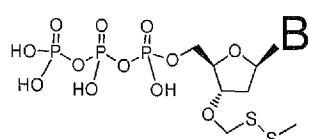


24. The labeled deoxynucleoside triphosphate according to claim 1, wherein the compound has

the structure:

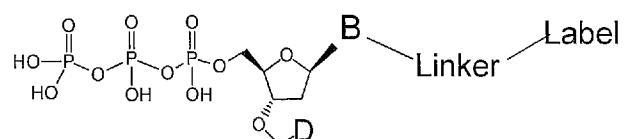


25. A deoxynucleoside triphosphate according to the following structure:

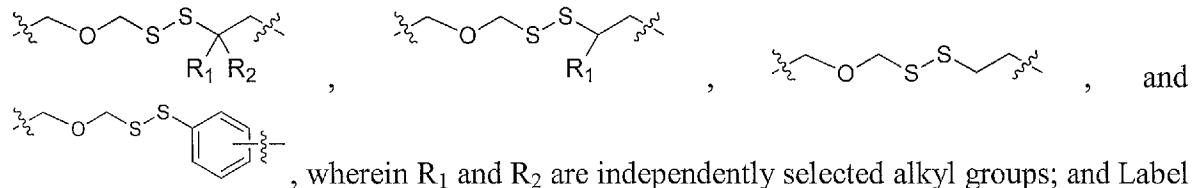


wherein B is a nucleobase.

26. A labeled deoxynucleoside triphosphate according to the following structure:



wherein D is selected from the group consisting of an azide, disulfide alkyl, disulfide substituted alkyl groups, disulfide allyl, and disulfide substituted allyl groups; B is a nucleobase; Linker comprises a cleavable oxymethylenedisulfide-containing site core, wherein said cleavable site core is selected from the group consisting of:



, wherein R₁ and R₂ are independently selected alkyl groups; and Label

is a label.

27. The labeled deoxynucleoside triphosphate according to claim 26, wherein said Linker has a logP value of greater than 0.

28. The labeled deoxynucleoside triphosphate according to claim 26, wherein said Linker has a logP value of greater than 0.1.

29. The labeled deoxynucleoside triphosphate according to claim 26, wherein said Linker has a logP value of greater than 1.0.

30. A deoxynucleoside triphosphate comprising a nucleobase and a sugar, said nucleobase comprising a detectable label attached via a cleavable oxymethylenedisulfide linker, said sugar comprising a 3'-O capped by a cleavable protecting group comprising methylenedisulfide.

31. The deoxynucleoside triphosphate according to claim 30, wherein said nucleoside is in a mixture with a polymerase.

32. The deoxynucleoside triphosphate according to claim 30, wherein the nucleobase of said nucleoside is non-natural.

33. The deoxynucleoside triphosphate according to claim 32, wherein the non-natural nucleobase of said nucleoside is selected from the group comprising 7-deaza guanine, 7-deaza adenine,

2-amino,7-deaza adenine, and 2-amino adenine.

34. The deoxynucleoside triphosphate according to claim 30, wherein said cleavable protecting group is of the formula $-\text{CH}_2\text{-SS-}\text{R}$, wherein R is selected from the group consisting of alkyl and substituted alkyl groups.

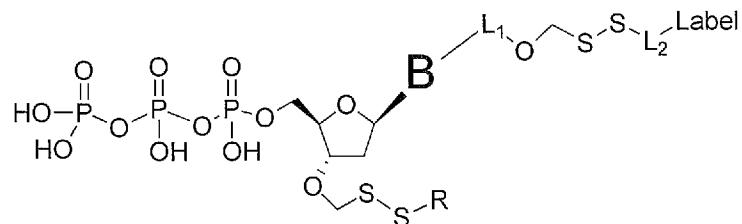
35. The deoxynucleoside triphosphate according to claim 31, wherein said mixture further comprises a primer.

36. The deoxynucleoside triphosphate according to claim 35, wherein said primer is hybridized to nucleic acid template.

37. The deoxynucleoside triphosphate according to claim 30, wherein said detectable label is a fluorescent label.

38. The deoxynucleoside triphosphate according to claim 36, wherein said nucleic acid template is immobilized.

39. A labeled deoxynucleoside triphosphate according to the following structure:



wherein B is a nucleobase, R is selected from the group consisting of alkyl and substituted

alkyl groups, and L₁ and L₂ are connecting groups.

40. The labeled deoxynucleoside triphosphate according to claim 39, wherein L₁ and L₂ are independently selected from the group consisting of -CO-, -CONH-, -NHCONH-, -O-, -S-, -C=N, and -N=N-, alkyl, aryl, branched alkyl, branched aryl and combinations thereof.

41. The labeled deoxynucleoside triphosphate according to claim 39, wherein said label is a detectable label.

42. The labeled deoxynucleoside triphosphate according to claim 40, wherein L₂ is not -S-.

43. The labeled deoxynucleoside triphosphate according to claim 39, wherein said label is selected from the group consisting of fluorophore dyes, energy transfer dyes, mass-tags, biotin, and haptene.

44. A kit comprising a DNA polymerase and at least one deoxynucleoside triphosphate comprising a nucleobase and a sugar, said sugar comprising a cleavable protecting group on the 3'-O, wherein said cleavable protecting group comprises methylenedisulfide, and wherein said nucleoside further comprises a detectable label attached via a cleavable oxymethylenedisulfide linker to the nucleobase of said nucleoside.

45. A reaction mixture comprising a nucleic acid template with a primer hybridized to said template, a DNA polymerase and at least one deoxynucleoside triphosphate comprising a nucleobase and a sugar, said sugar comprising a cleavable protecting group on the 3'-O,

wherein said cleavable protecting group comprises methylenedisulfide, wherein said nucleoside further comprises a detectable label attached via a cleavable oxymethylenedisulfide linker to the nucleobase of said nucleoside.

46. A method of performing a DNA synthesis reaction comprising the steps of a) providing a nucleic acid template with a primer hybridized to said template, a DNA polymerase, at least one deoxynucleoside triphosphate comprising a nucleobase and a sugar, said sugar comprising a cleavable protecting group on the 3'-O, wherein said cleavable protecting group comprises methylenedisulfide, wherein said nucleoside further comprises a detectable label attached via a cleavable oxymethylenedisulfide linker to the nucleobase of said nucleoside, and b) subjecting said reaction mixture to conditions which enable a DNA polymerase catalyzed primer extension reaction.
47. A method according to claim 46, wherein said DNA polymerase catalyzed primer extension reaction is part of a sequencing reaction.
48. A method for analyzing a DNA sequence comprising the steps of
 - a) providing a nucleic acid template with a primer hybridized to said template forming a primer/template hybridization complex,
 - b) adding DNA polymerase, and a first deoxynucleoside triphosphate comprising a nucleobase and a sugar, said sugar comprising a cleavable protecting group on the 3'-O, wherein said cleavable protecting group comprises methylenedisulfide, wherein said nucleoside further comprises a first detectable label attached via a cleavable oxymethylenedisulfide-containing linker to the nucleobase of said nucleoside,

- c) subjecting said reaction mixture to conditions which enable a DNA polymerase catalyzed primer extension reaction so as to create a modified primer/template hybridization complex, and
- d) detecting said first detectable label of said deoxynucleoside triphosphate in said modified primer/template hybridization complex.

49. A method according to claim 48, further comprising the steps of e) removing said cleavable protecting group and optionally said detectable label from said modified primer/template hybridization complex, and f) repeating steps b) to e) at least once.

50. A method according to claim 49, wherein the method further comprises adding a second deoxynucleoside triphosphate is added during repeat of step b), wherein said second deoxynucleoside triphosphate comprises a second detectable label attached via a cleavable oxymethylenedisulfide linker, wherein said second detectable label is different from said first detectable label.

51. A method according to claim 50, wherein the cleavable oxymethylenedisulfide-containing linker has a logP value of greater than 0.

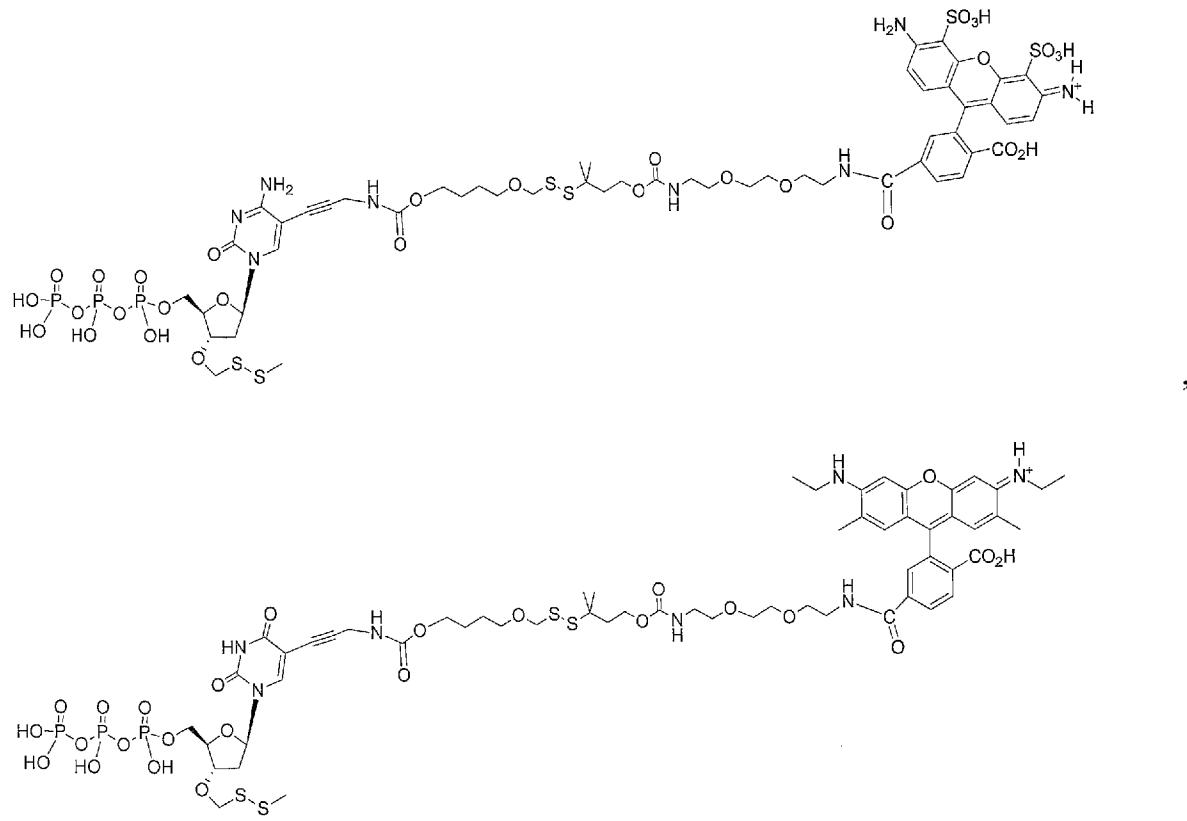
52. A method according to claim 50, wherein the cleavable oxymethylenedisulfide-containing linker has a logP value of greater than 0.1.

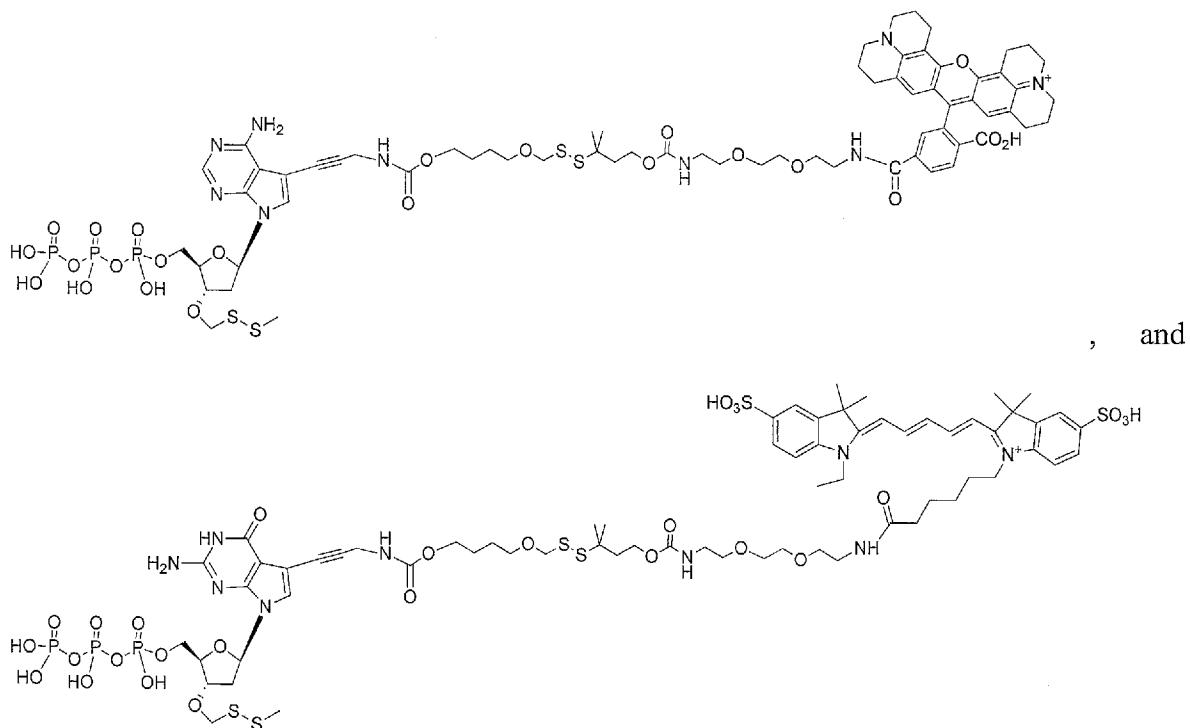
53. A method according to claim 50, wherein the cleavable oxymethylenedisulfide-containing linker has a logP value of greater than 1.0.

54. A method according to claim 50, wherein the nucleobase of said second deoxynucleoside triphosphate is different from the nucleobase of said first deoxynucleoside triphosphate.

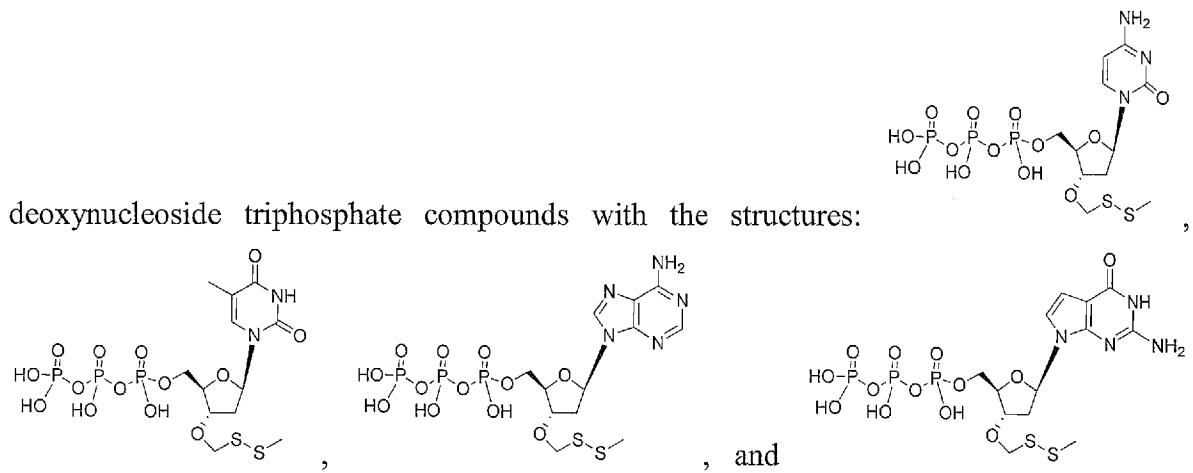
55. A method according to claim 49, wherein a mixture of at least 4 differently labeled, 3'-O methylenedisulfide capped deoxynucleoside triphosphate compounds representing analogs of A, G, C and T or U are used in step b).

56. A method according to claim 49, wherein a mixture of at least 4 differently labeled, 3'-O methylenedisulfide capped deoxynucleoside triphosphate compounds are used in step b), said compounds having the structures:

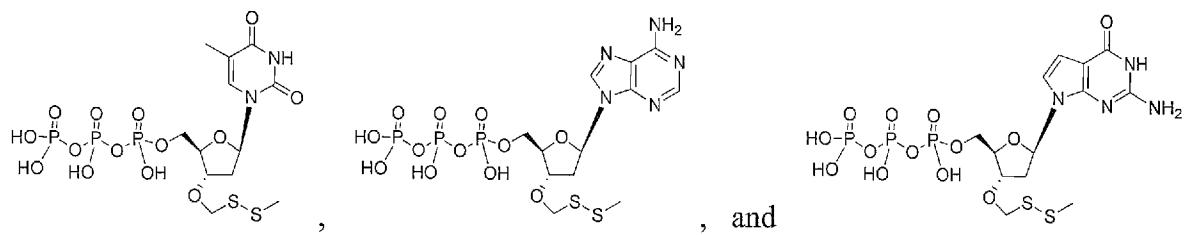




57. A method according to claim 56, wherein unlabeled 3'-O methylenedisulfide capped



deoxynucleoside triphosphate compounds with the structures:



are also used in step b).

58. A method according to claim 49, wherein step e) is performed by exposing said modified primer/template hybridization complex to a reducing agent.

59. A method according to claim 58, wherein said reducing agent is TCEP.

60. A method of preparing a 3'-*O*-(methylthiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside, comprising: a) providing a 5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside, wherein said 5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside comprises a nucleobase and a sugar, and ii) a methylthiomethyl donor ; and b) treating said 5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside under conditions so as to create a 3'-*O*-(methylthiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside.

61. The method according to claim 60, wherein said methylthiomethyl donor is DMSO.

62. The method according to claim 60, wherein said conditions comprise acidic conditions.

63. The method according to claim 60, wherein said 5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside comprises a protecting group on the nucleobase of said nucleoside.

64. The method according to claim 60, wherein said 3'-*O*-(methylthiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside is purified with column chromatography.

65. A method of preparing a 3'-*O*-(R-substituted-dithiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside, comprising: a) providing i) a 3'-*O*-(methylthiomethyl)

-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside, and ii) R-SH, wherein R comprises alkyl or substituted alkyl; and b) treating said 3'-*O*-(methylthiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside under conditions so as to create a 3'-*O*-(R-substituted-dithiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside.

66. The method according to claim 65, wherein said R-SH is ethanethiol.

67. The method according to claim 65, wherein said conditions comprise basic conditions.

68. A method of preparing a 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside, comprising:
a) providing a 3'-*O*-(R-substituted-dithiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside; and b) treating said 3'-*O*-(R-substituted-dithiomethyl)-5'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxynucleoside under conditions so as to create a 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside.

69. The method according to claim 68, wherein said conditions comprise exposing said 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside to NH₄F.

70. A method of preparing a triphosphate of 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside, comprising: a) providing a 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside; and b) treating said 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside under conditions so as to create a triphosphate of 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside.

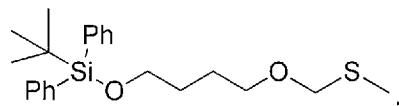
71. The method according to claim 70, wherein said conditions comprises exposing said 3'-*O*-(R-substituted-dithiomethyl)-2'-deoxynucleoside to (MeO)₃PO with POCl₃ and Bu₃N.

72. The method according to claim 70, wherein said method further comprises step c) removal of said nucleobase protecting group.

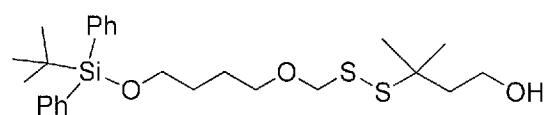
73. The method according to claim 72, wherein said protecting group comprises a N-trifluoroacetyl-aminopropargyl protecting group.

74. The method according to claim 73, wherein said N-trifluoroacetyl-aminopropargyl protecting group is removed by solvolysis to produce a 5'-*O*-(triphosphate)-3'-*O*-(R-substituted-dithiomethyl)-5-(aminopropargyl)-2'-deoxynucleoside.

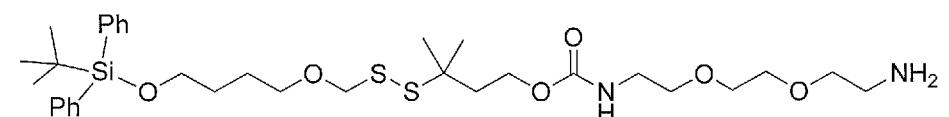
75. A compound wherein the structure is:



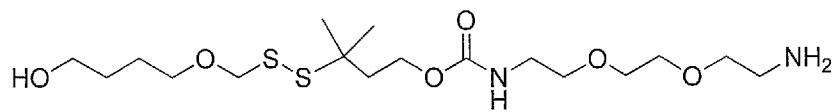
76. A compound wherein the structure is:



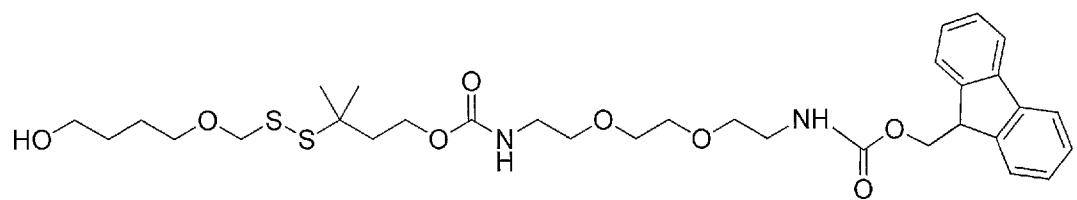
77. A compound wherein the structure is:



78. A compound wherein the structure is:



79. A compound wherein the structure is:



80. A compound wherein the structure is:

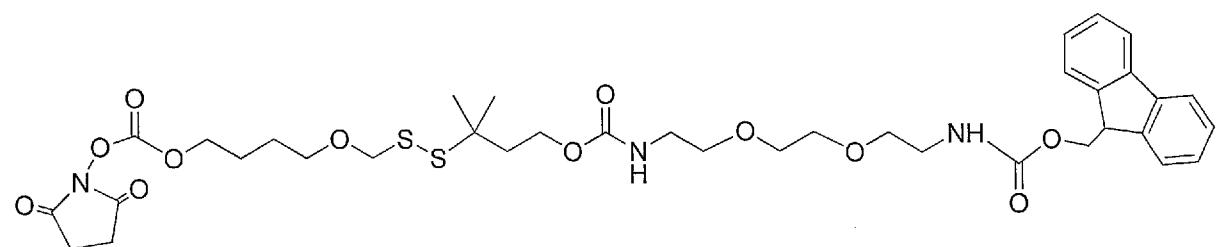


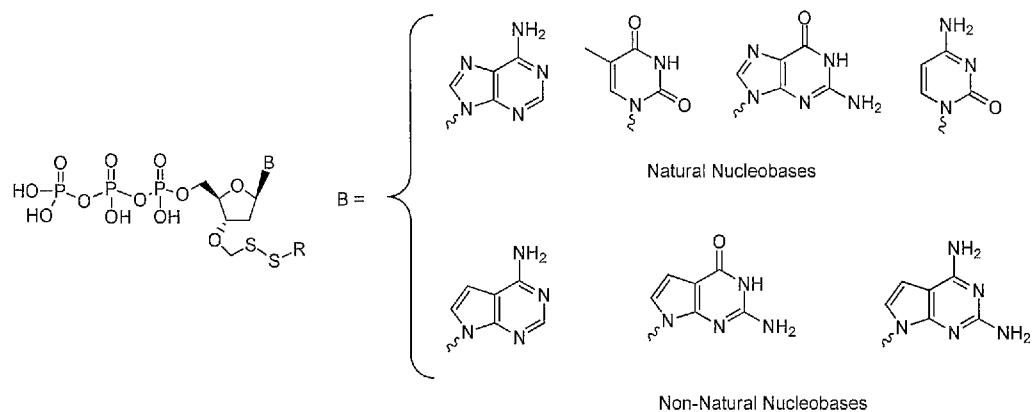
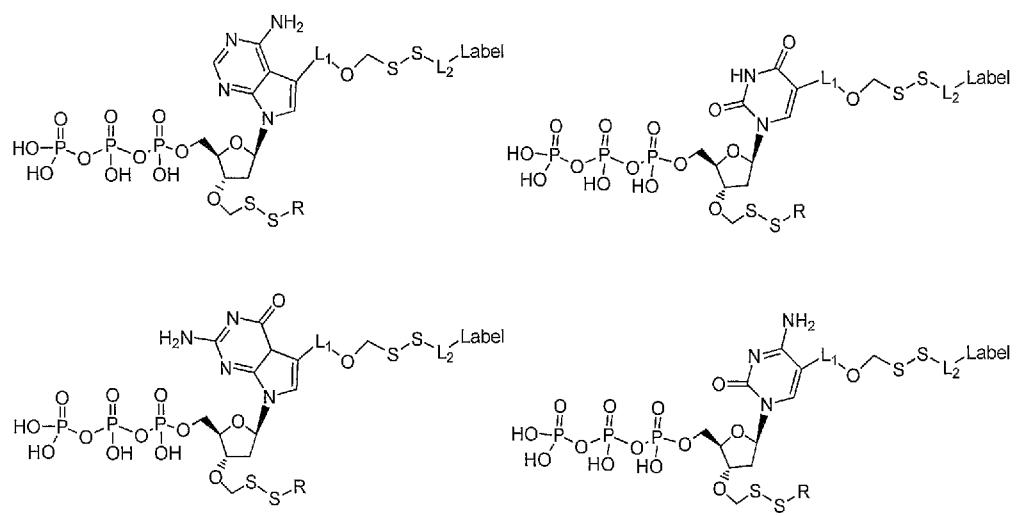
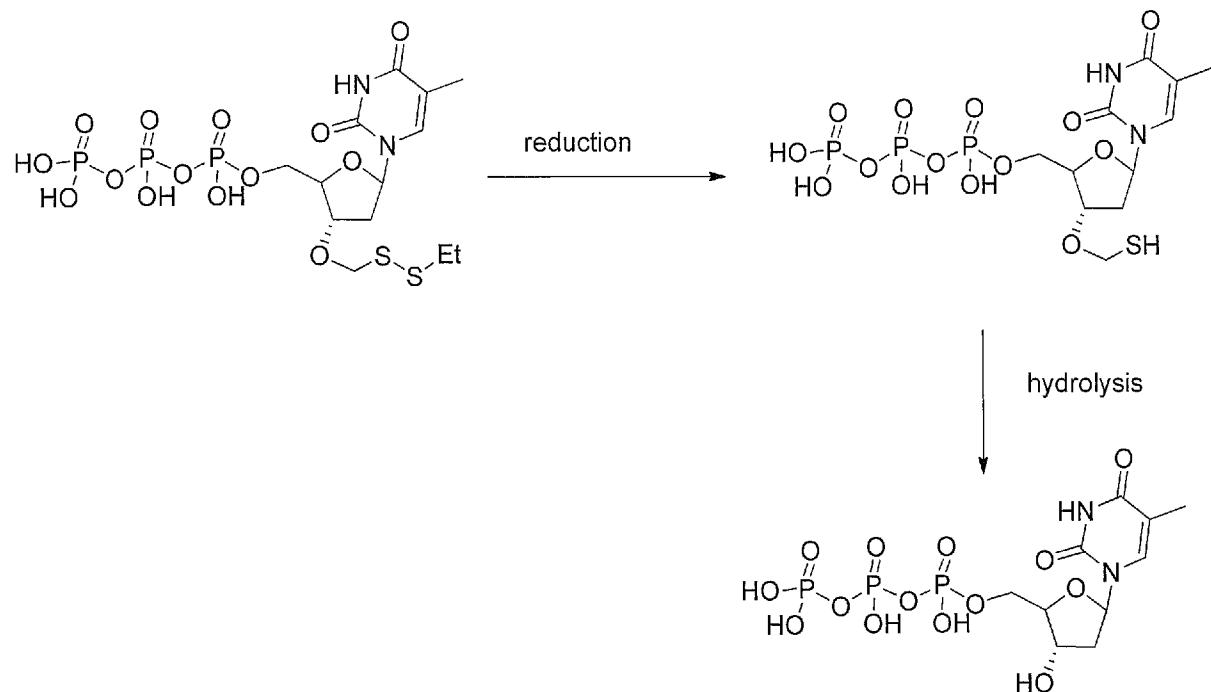
FIGURE 1**FIGURE 2**

FIGURE 3

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FIGURE 4

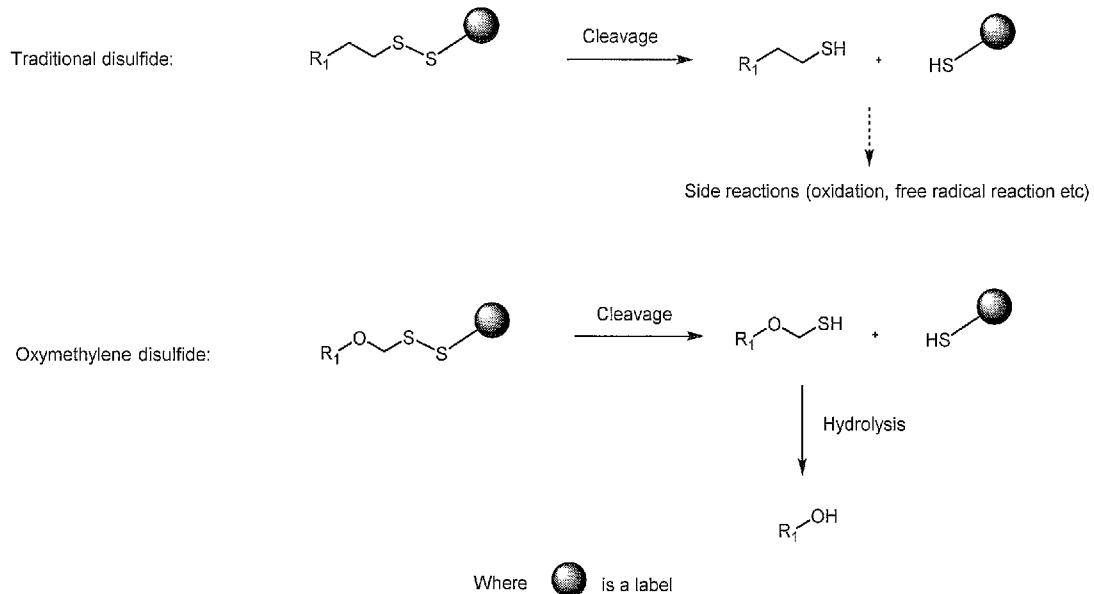


FIGURE 5

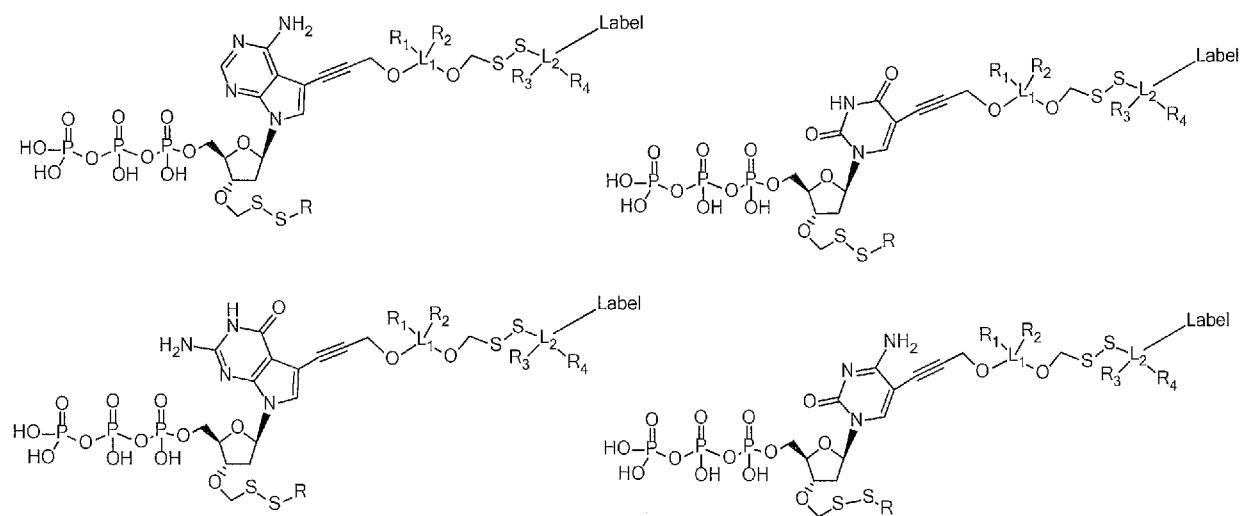


FIGURE 6

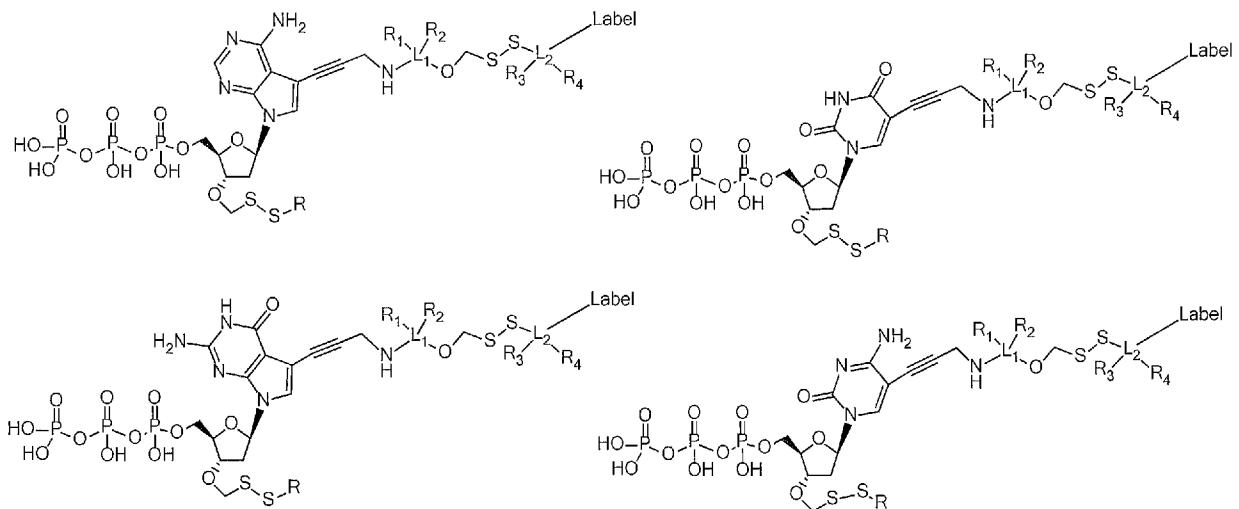


FIGURE 7

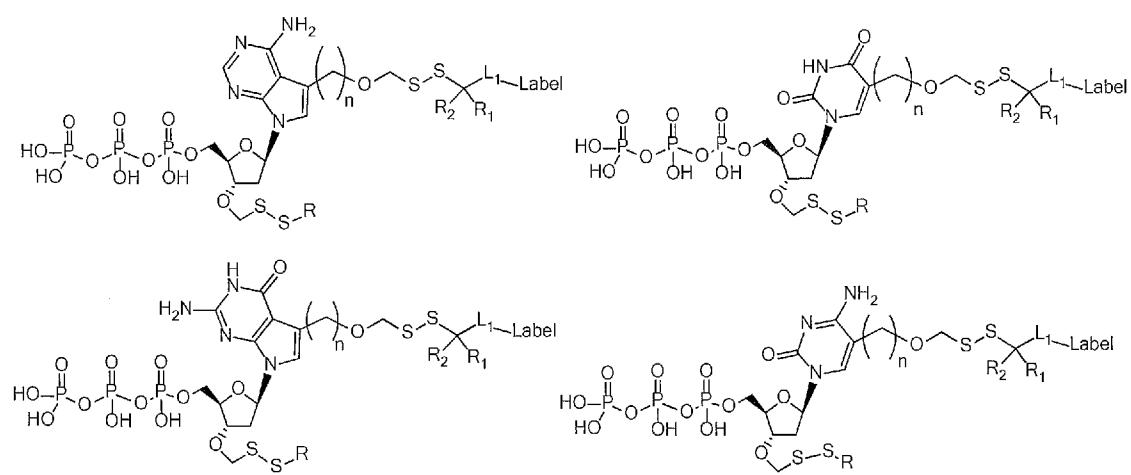


FIGURE 8

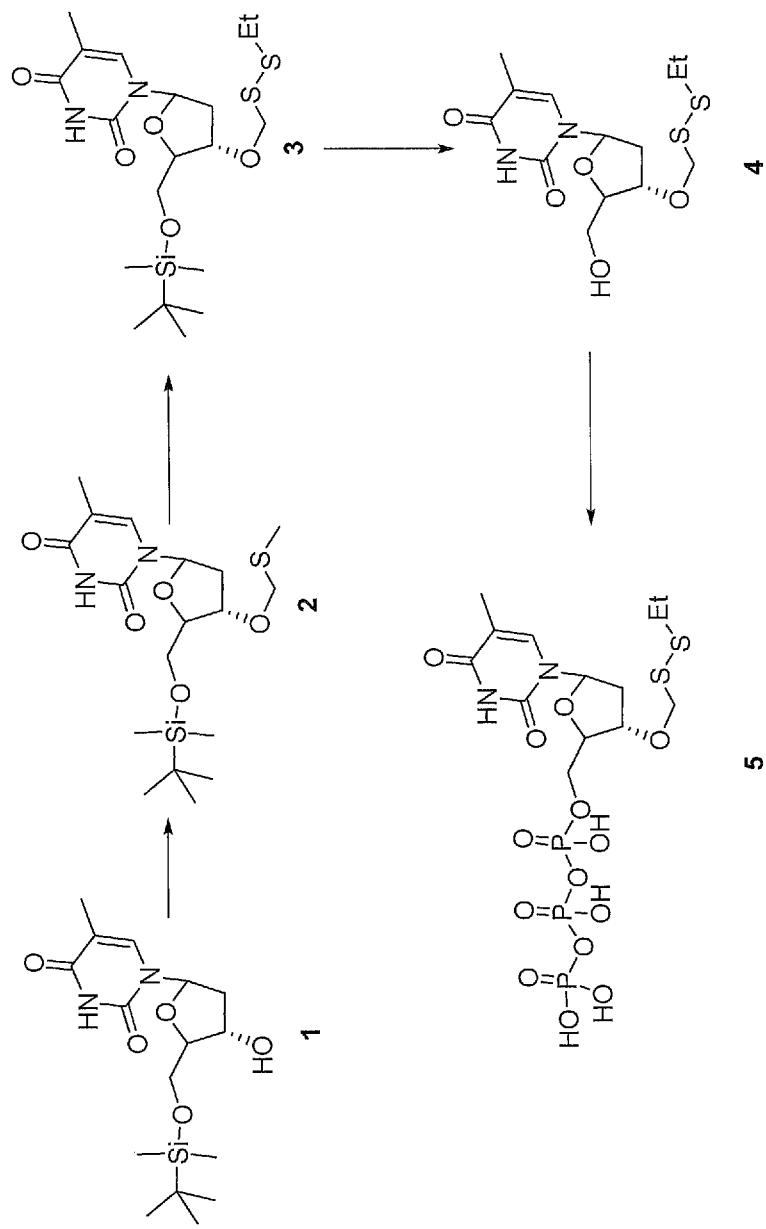


FIGURE 9

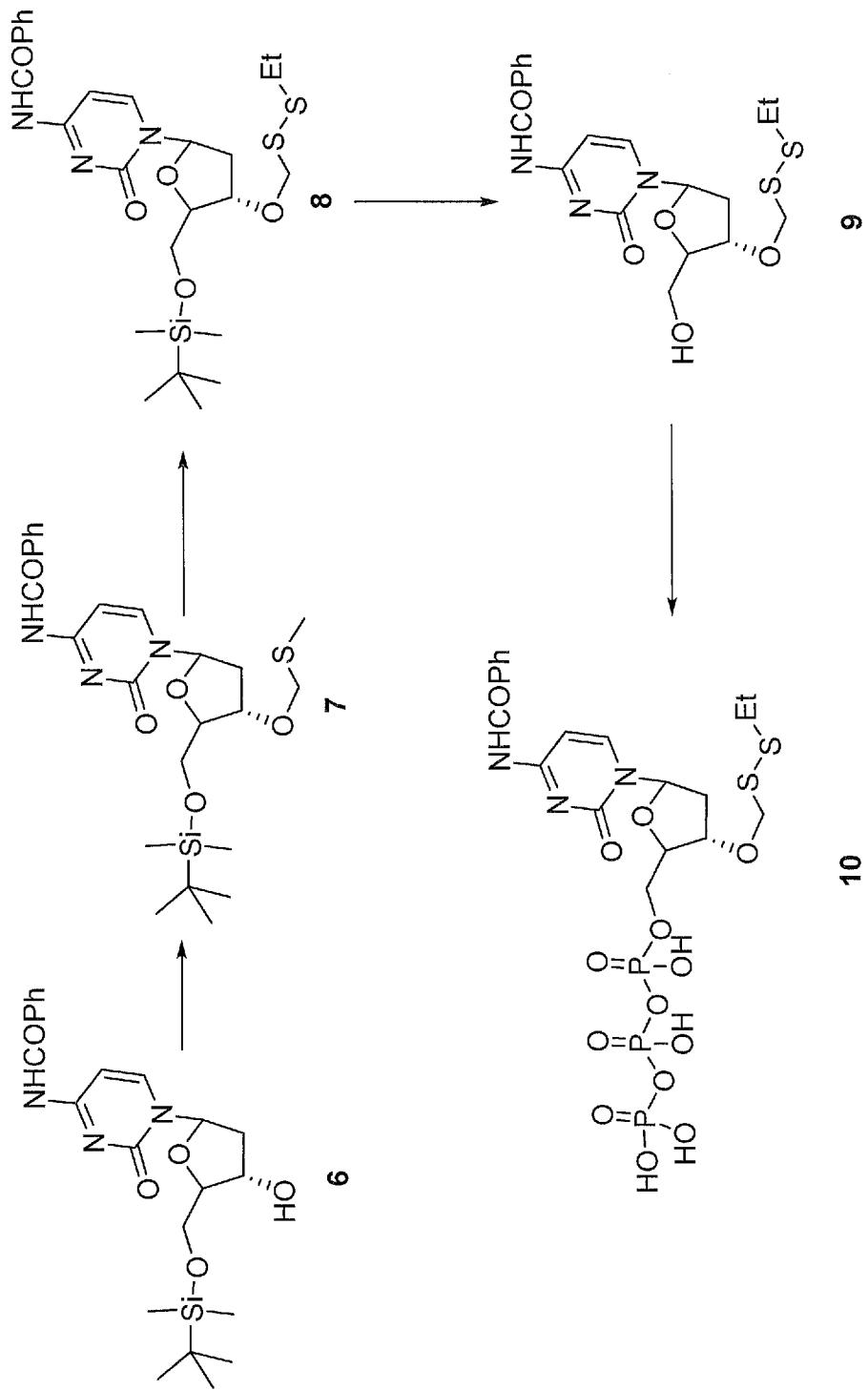


FIGURE 10

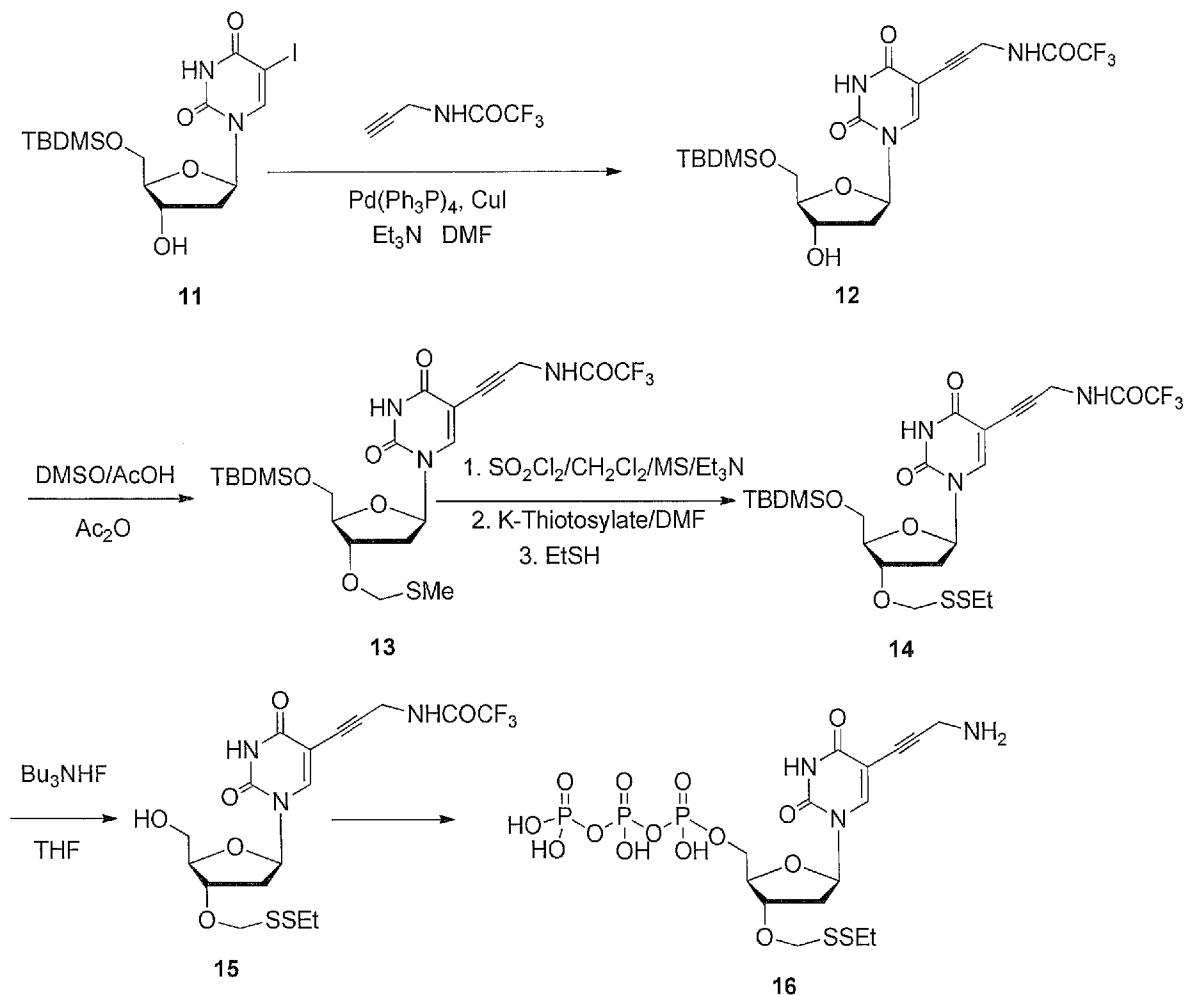


FIGURE 11

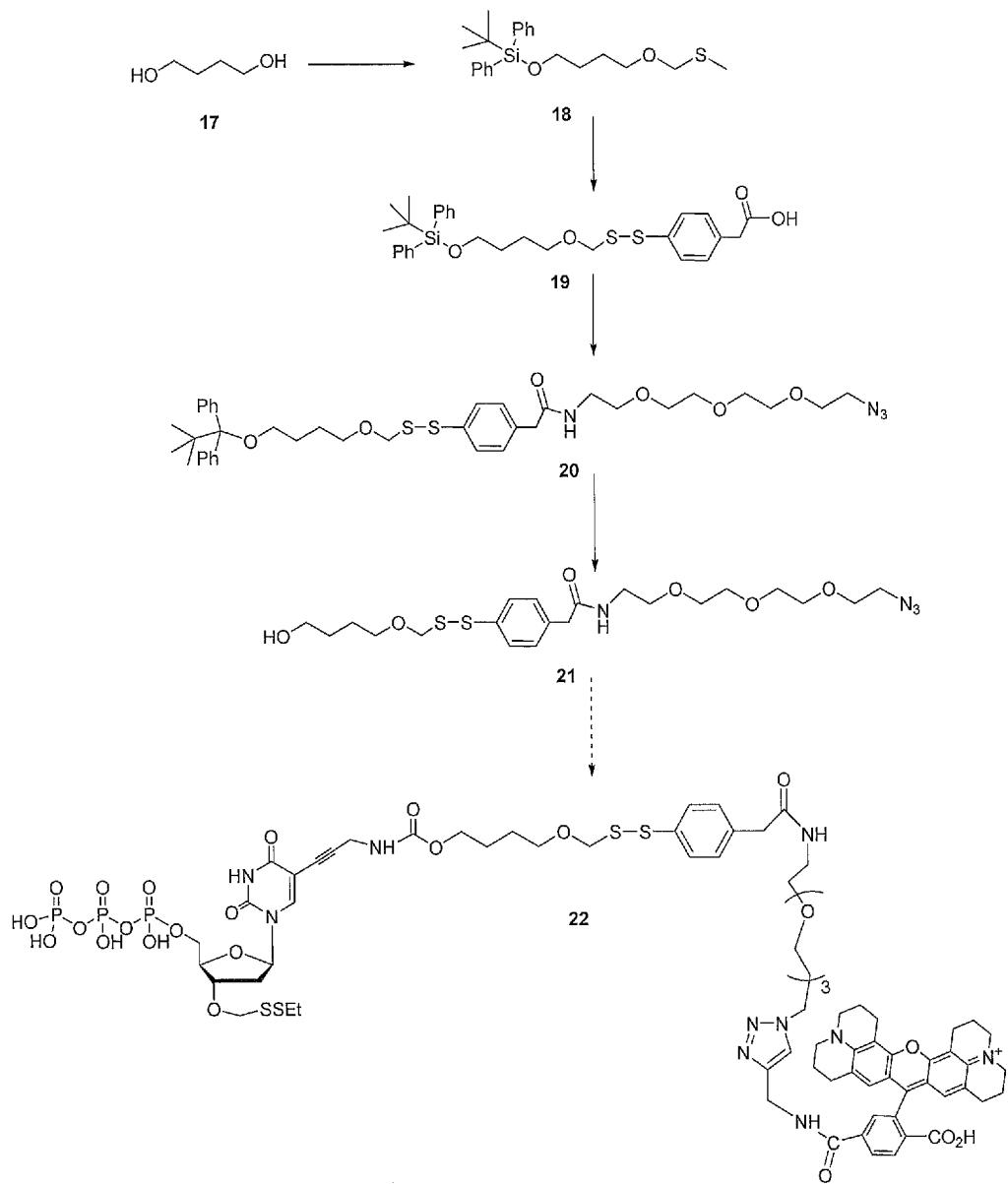


FIGURE 12

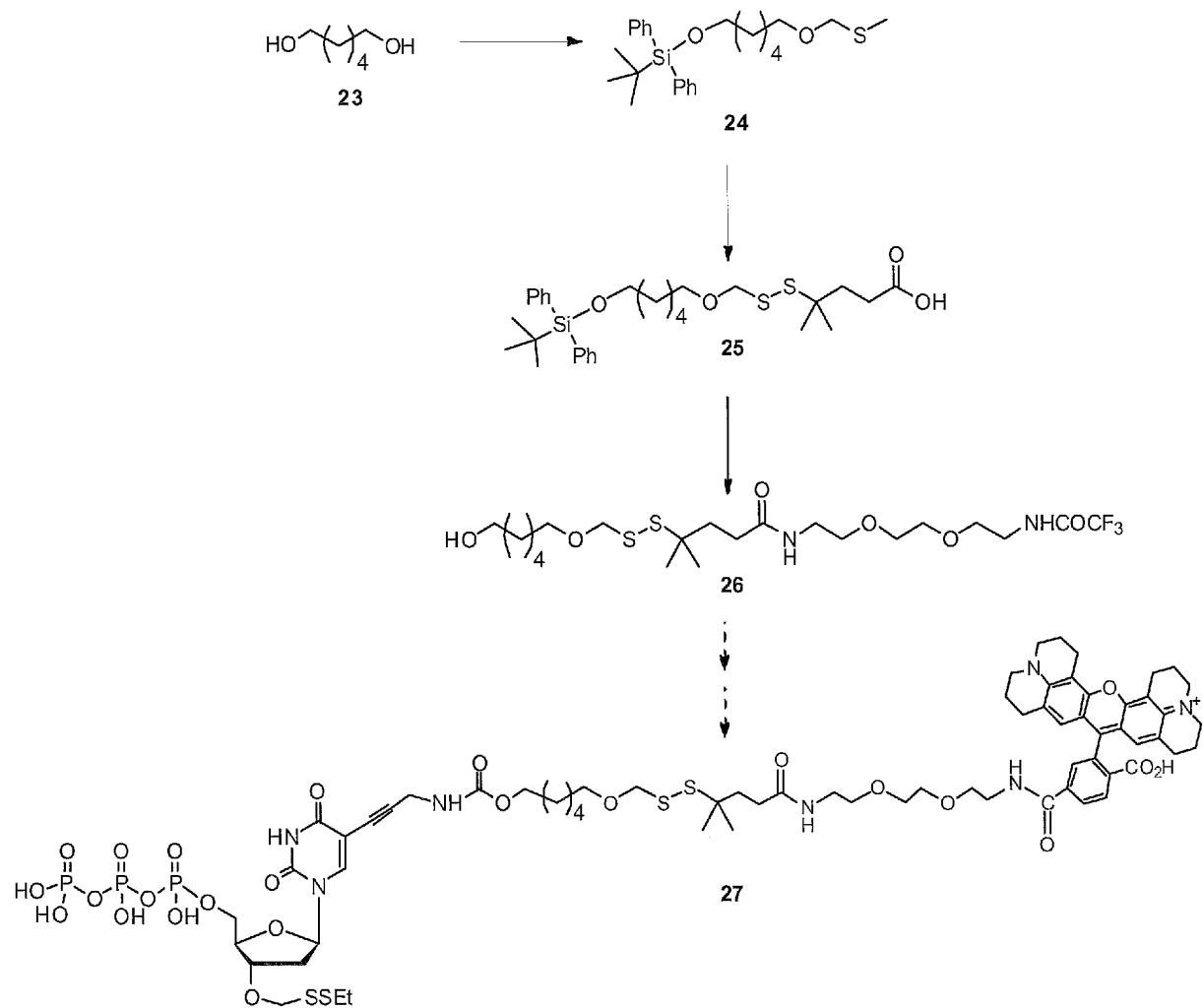


FIGURE 13

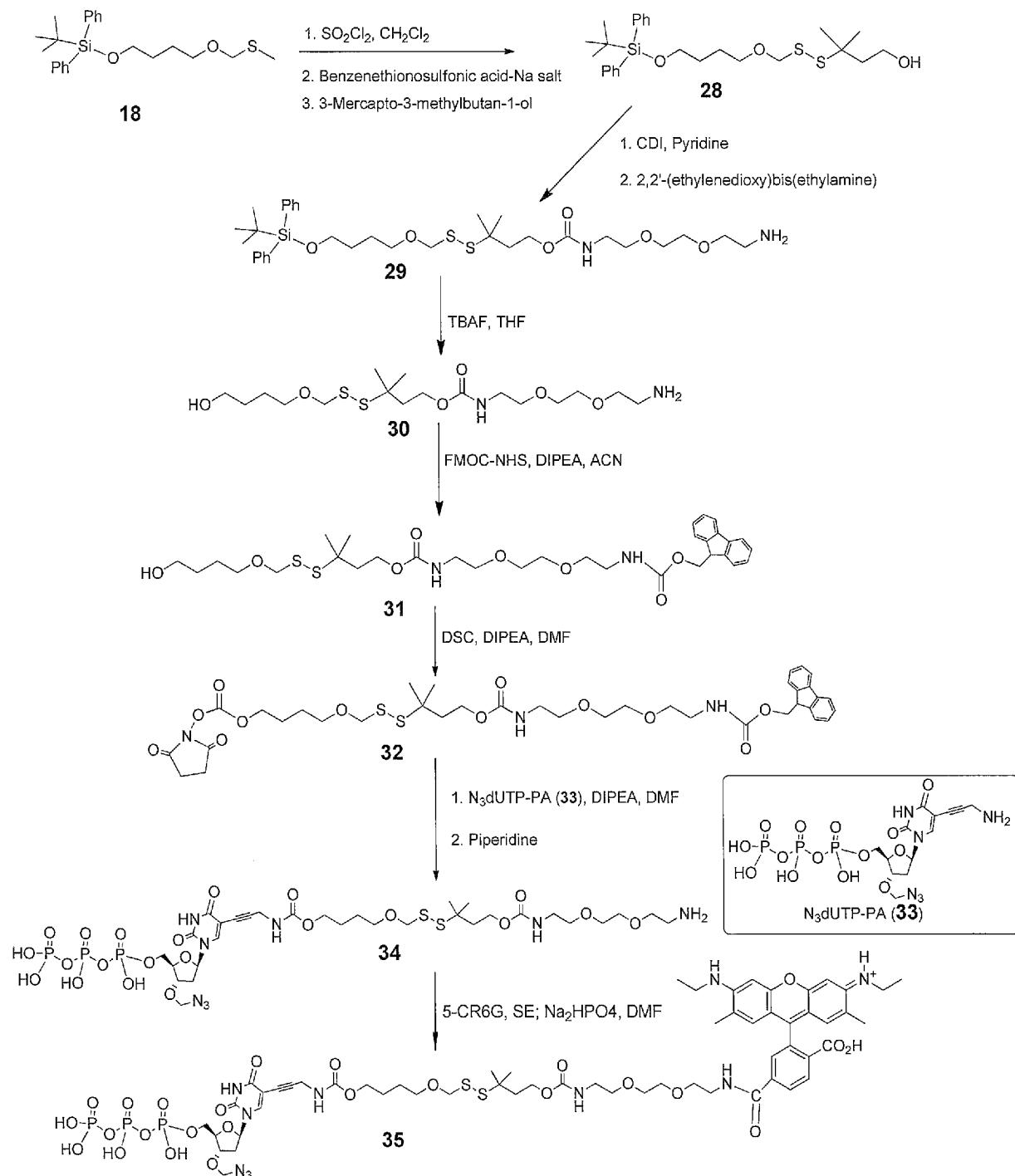


FIGURE 14

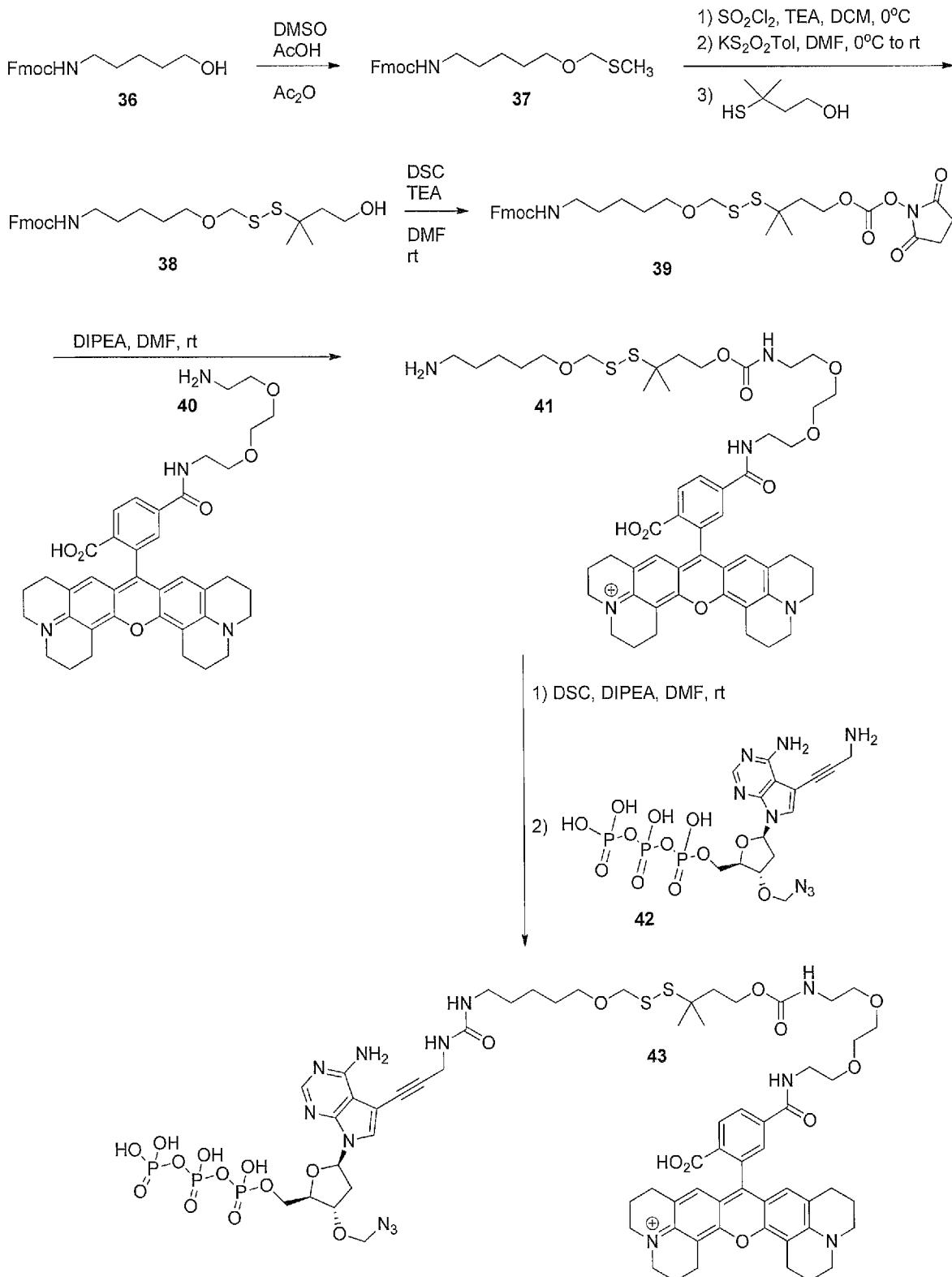


FIGURE 15

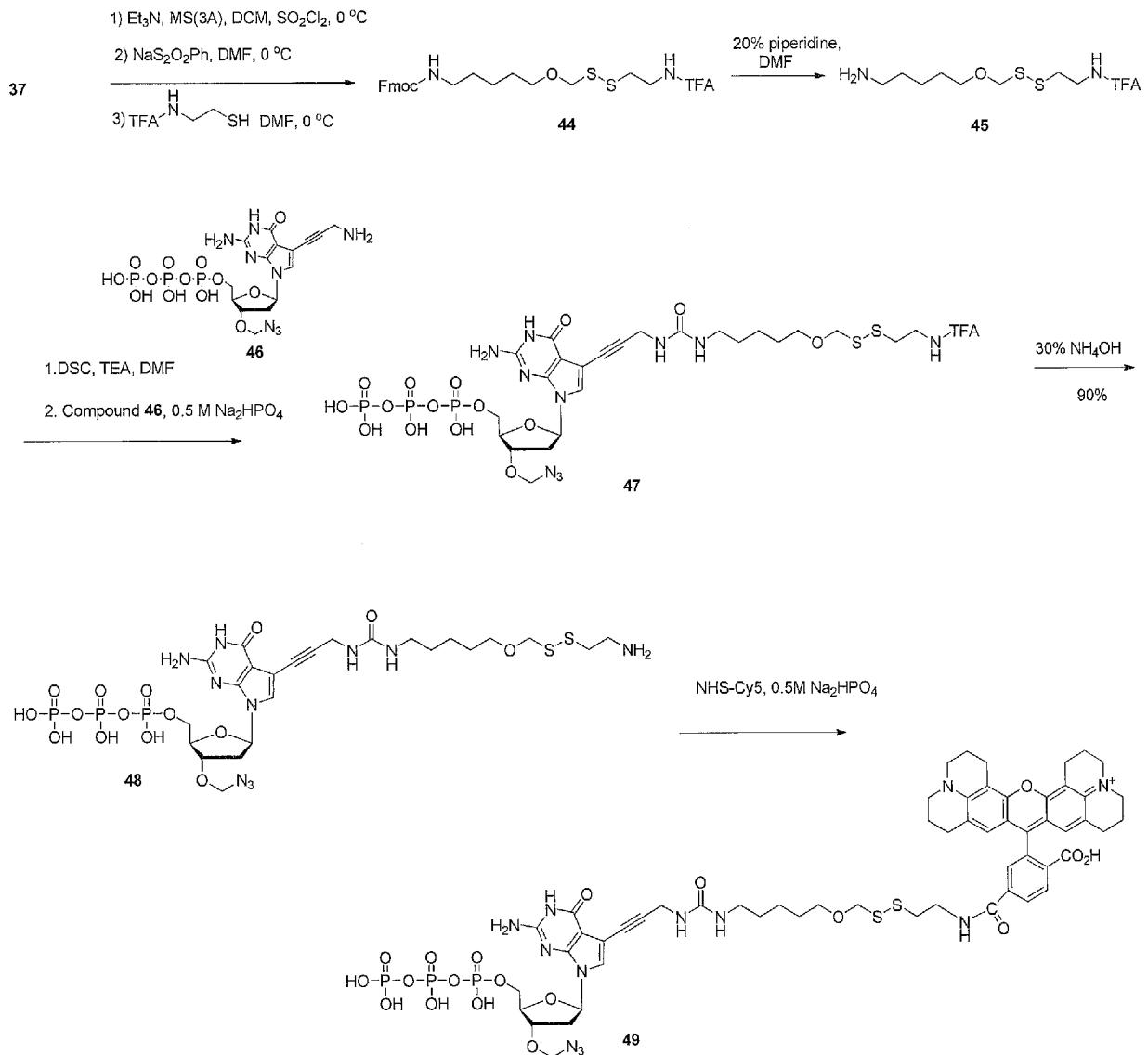
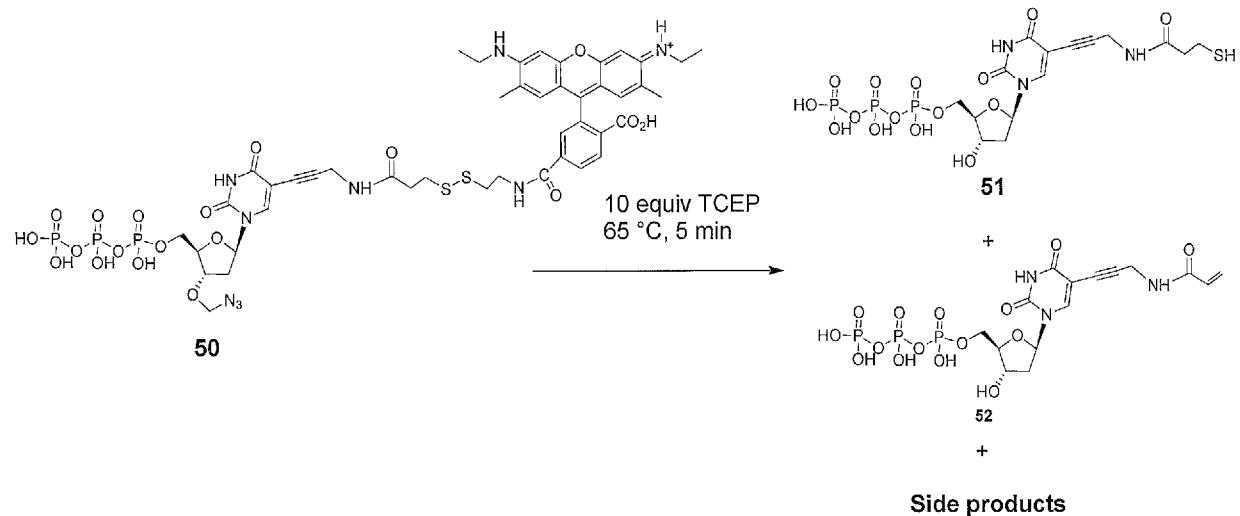


FIGURE 16



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FIGURE 17

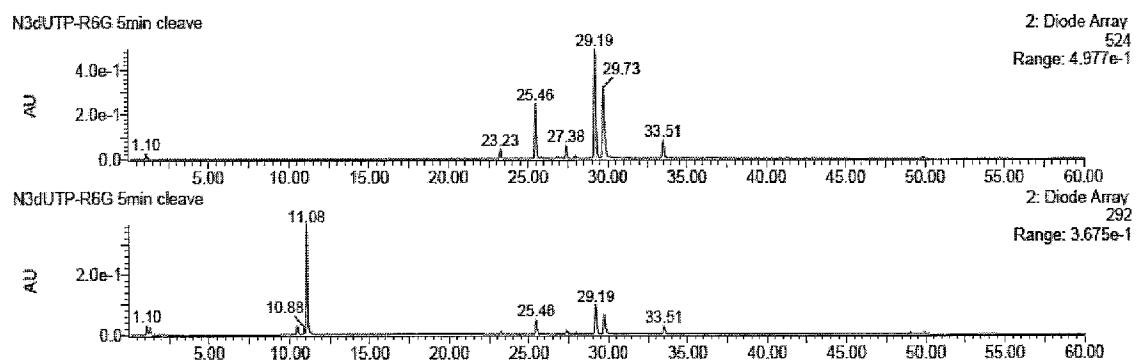


FIGURE 18

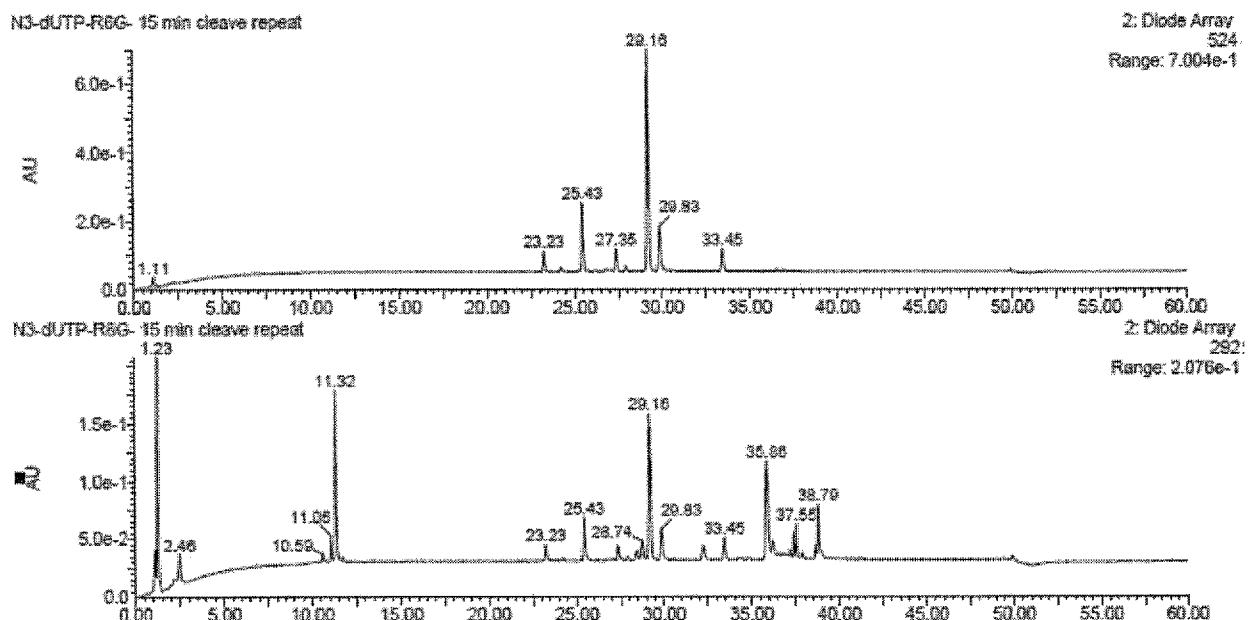
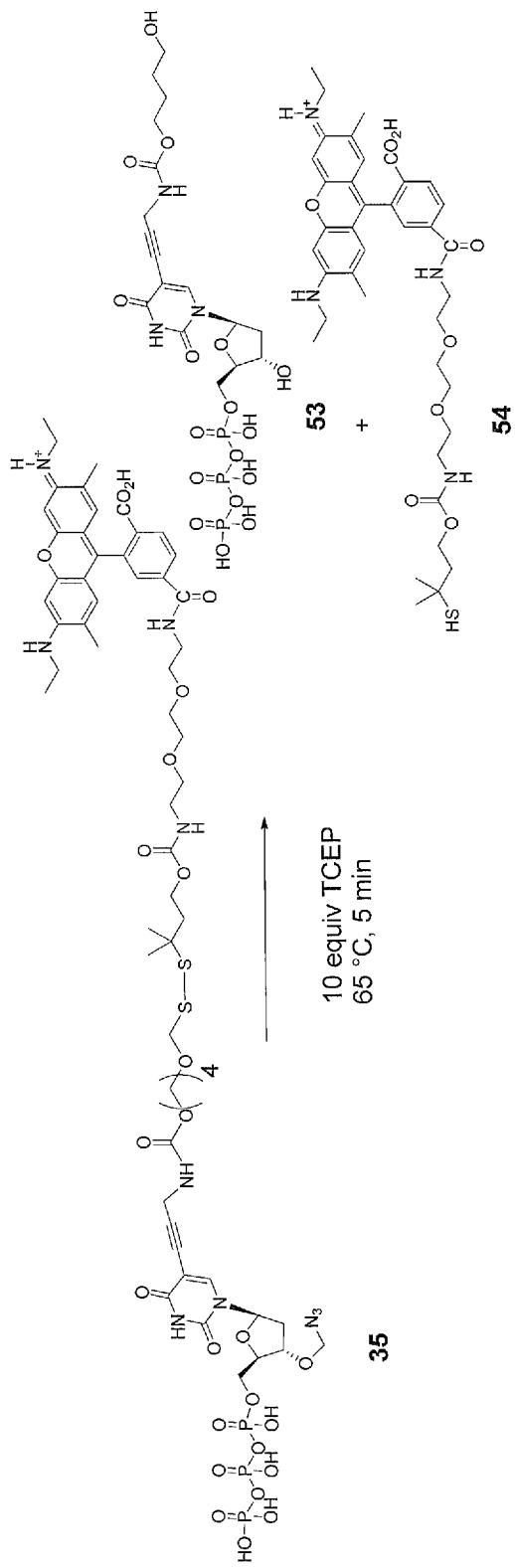
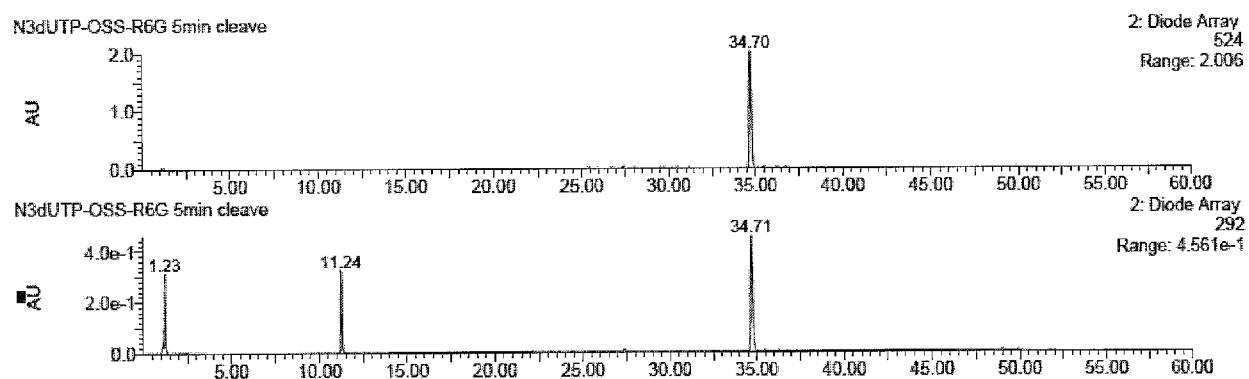


FIGURE 19



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FIGURE 20



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FIGURE 21

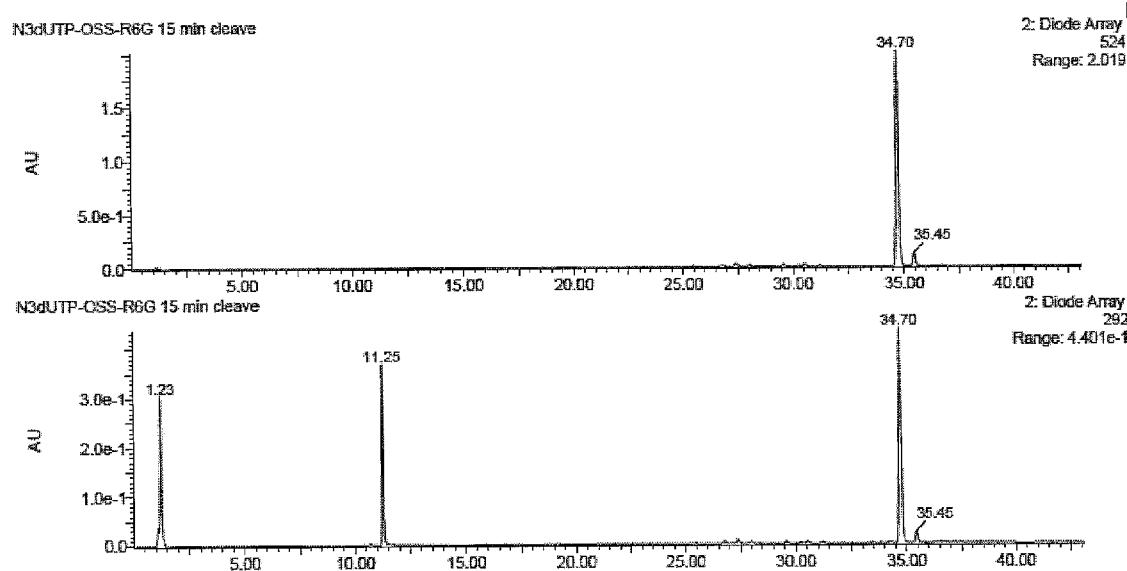


FIGURE 22

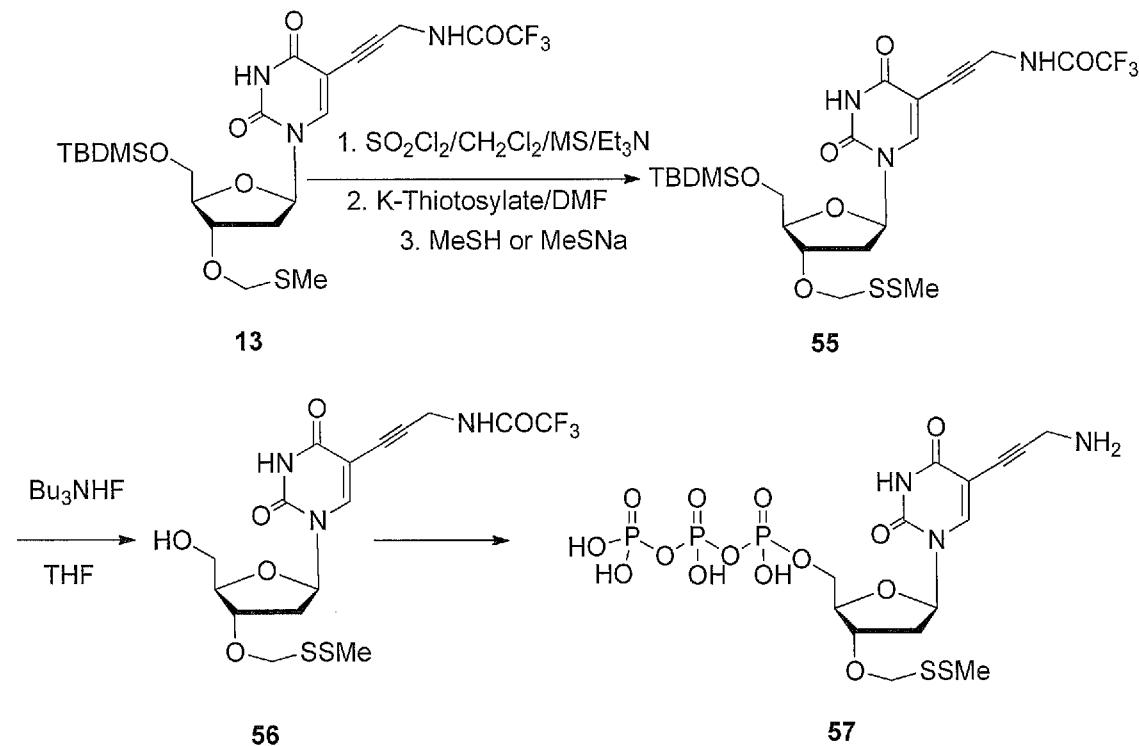


FIGURE 23

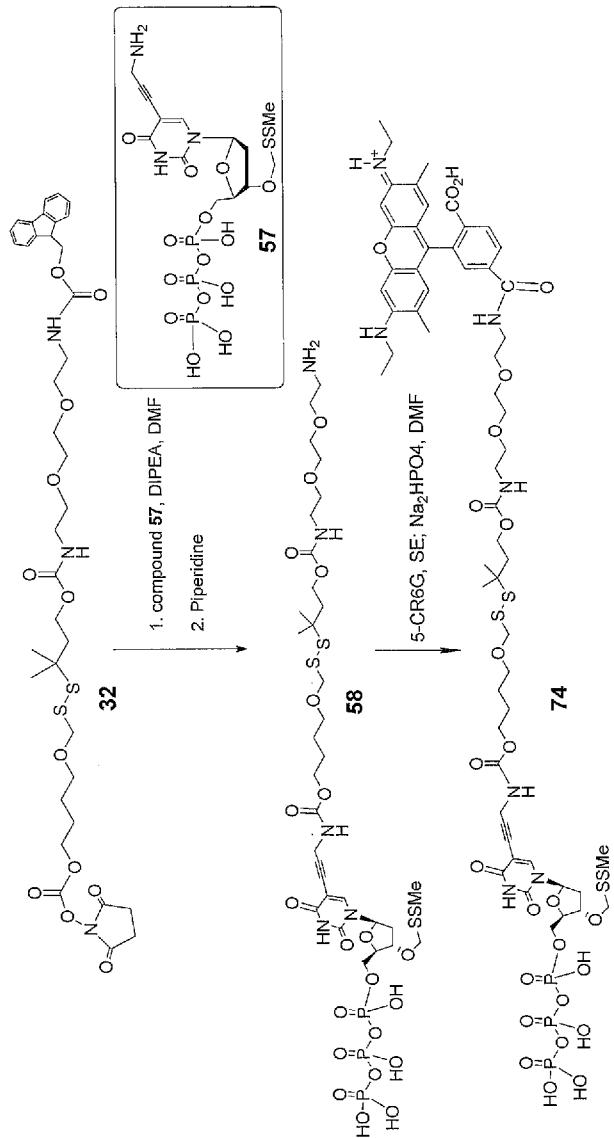


FIGURE 24

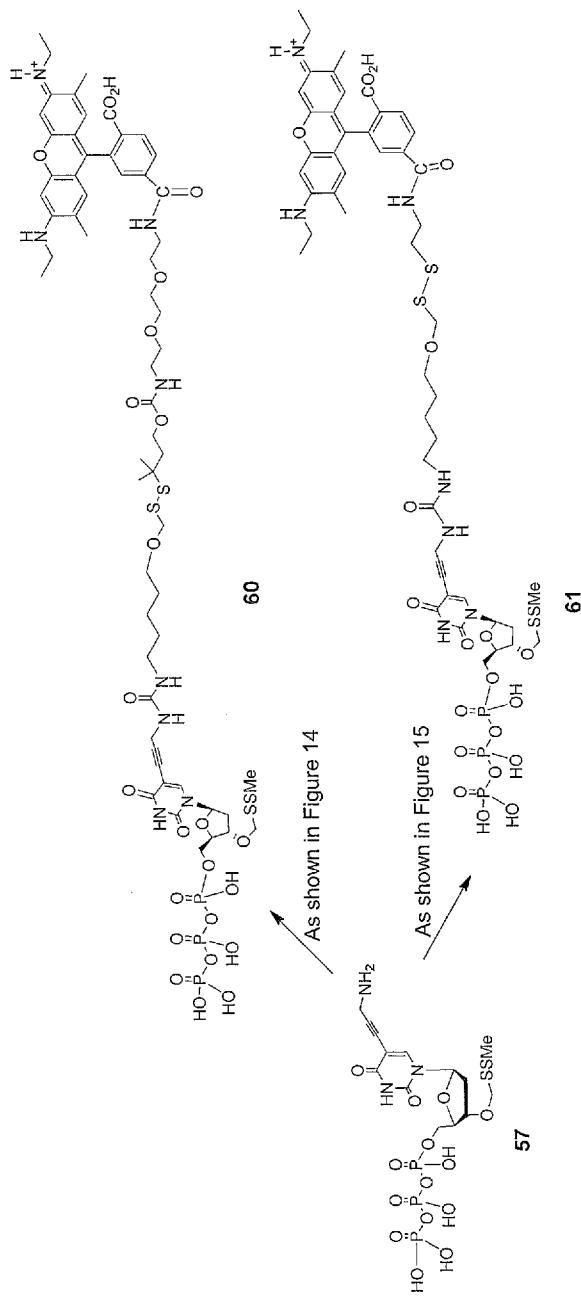


FIGURE 25

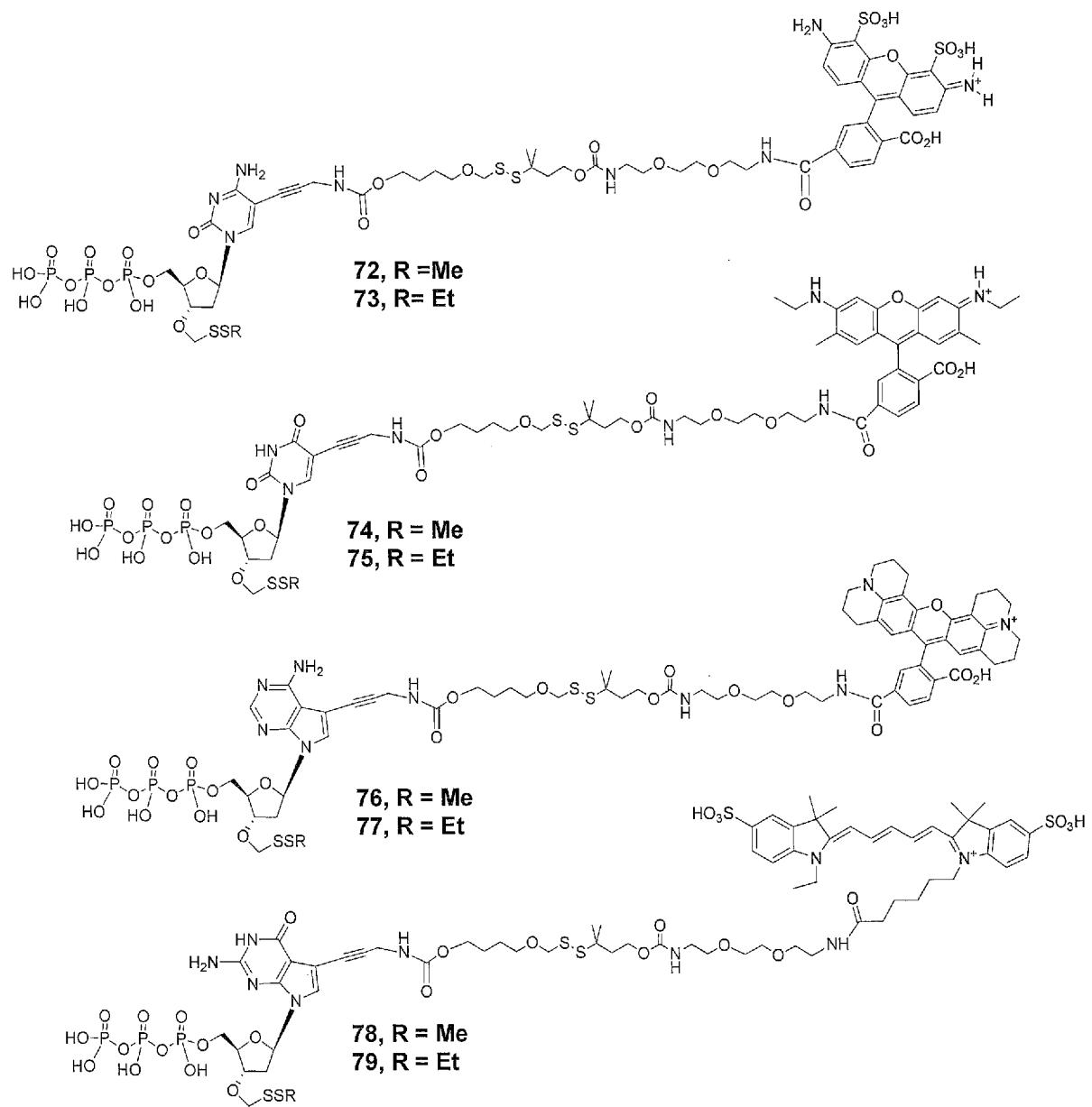


FIGURE 26

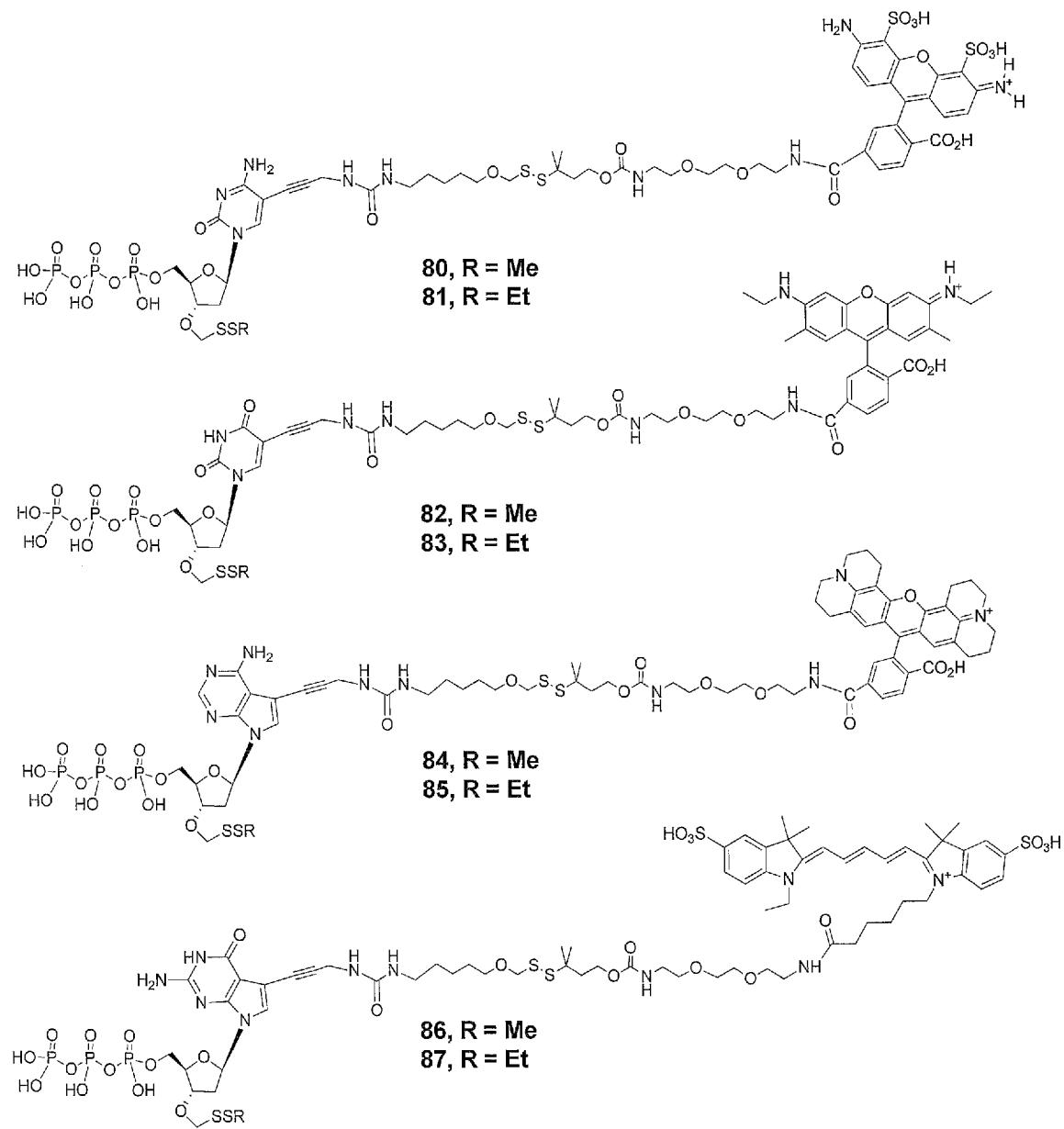
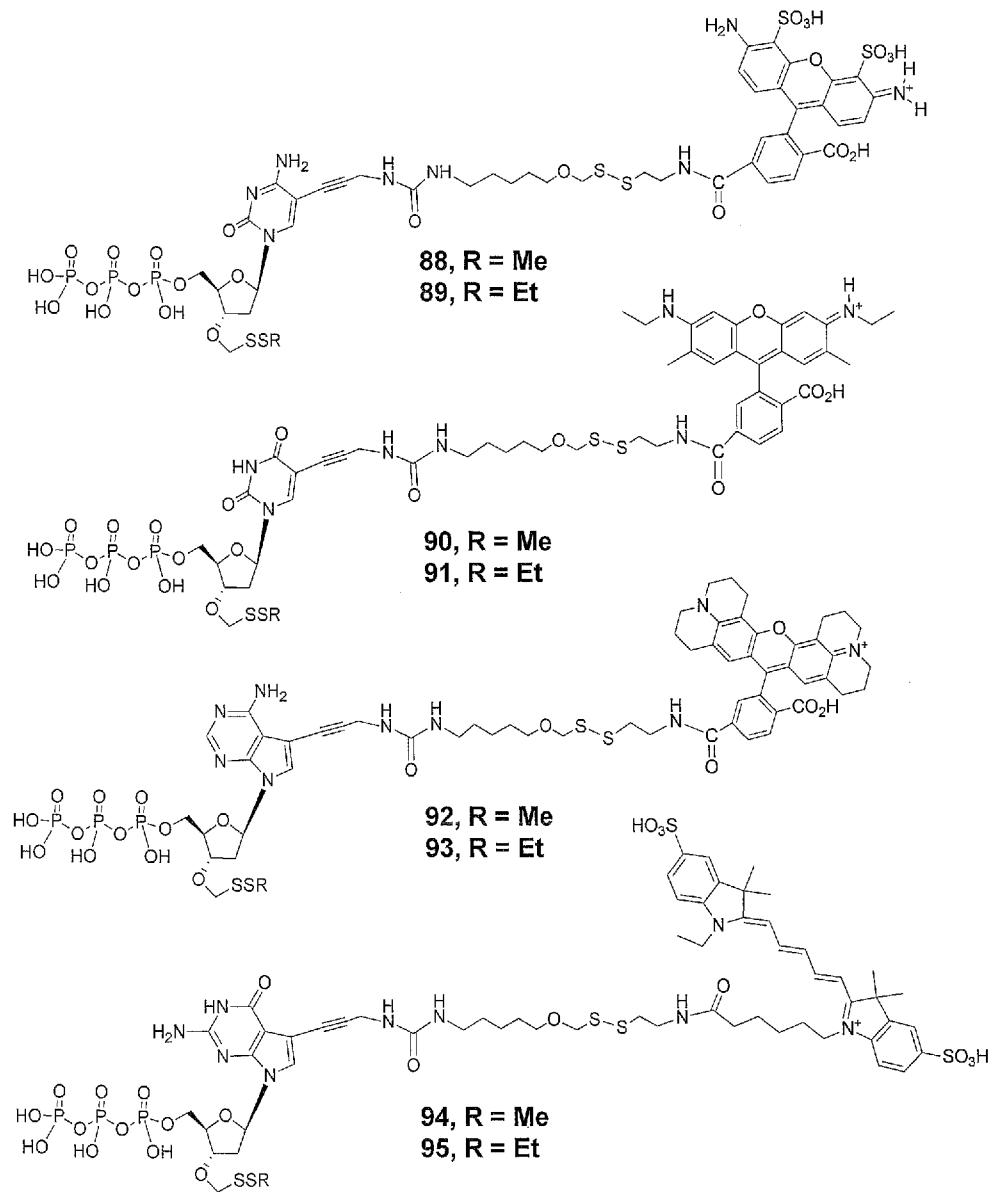


FIGURE 27



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FIGURE 28

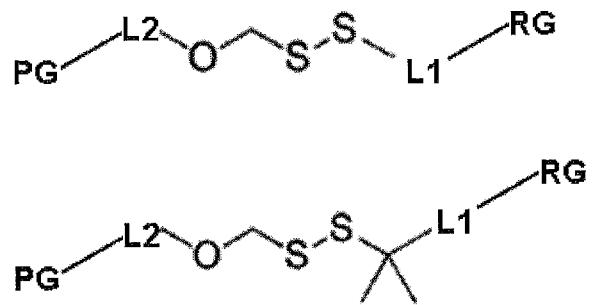


FIGURE 29

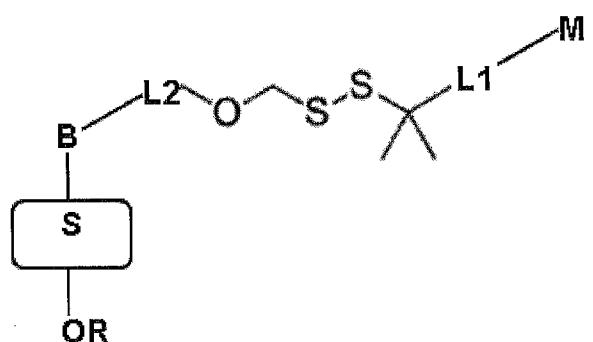


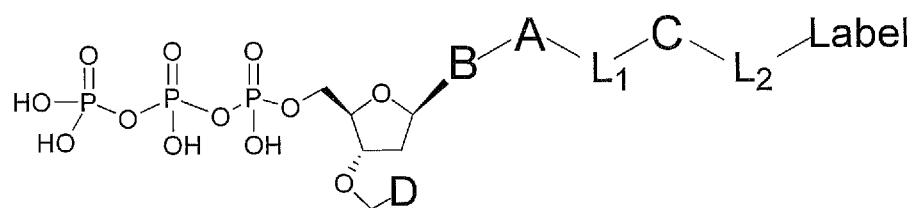
FIGURE 30

FIGURE 31

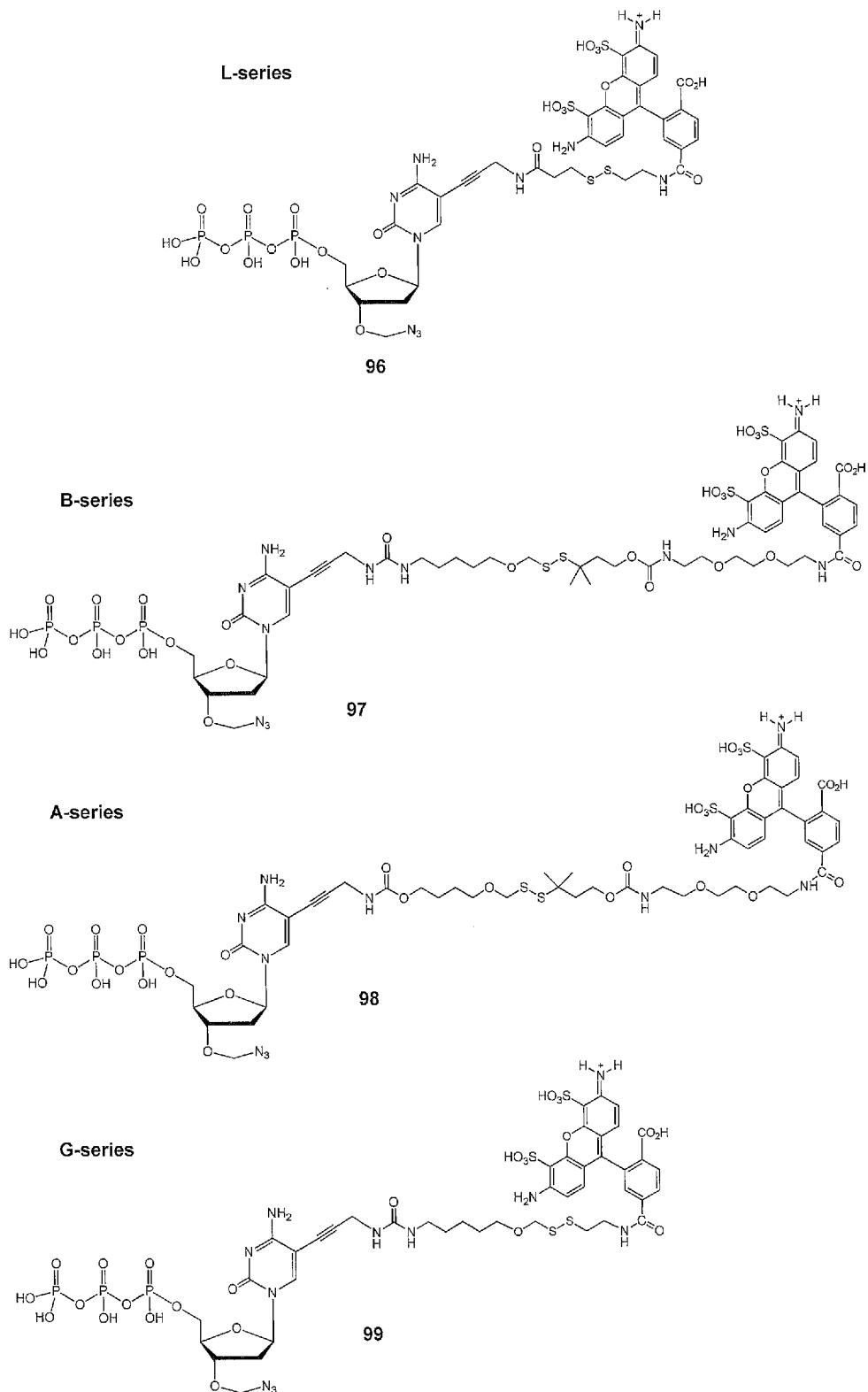
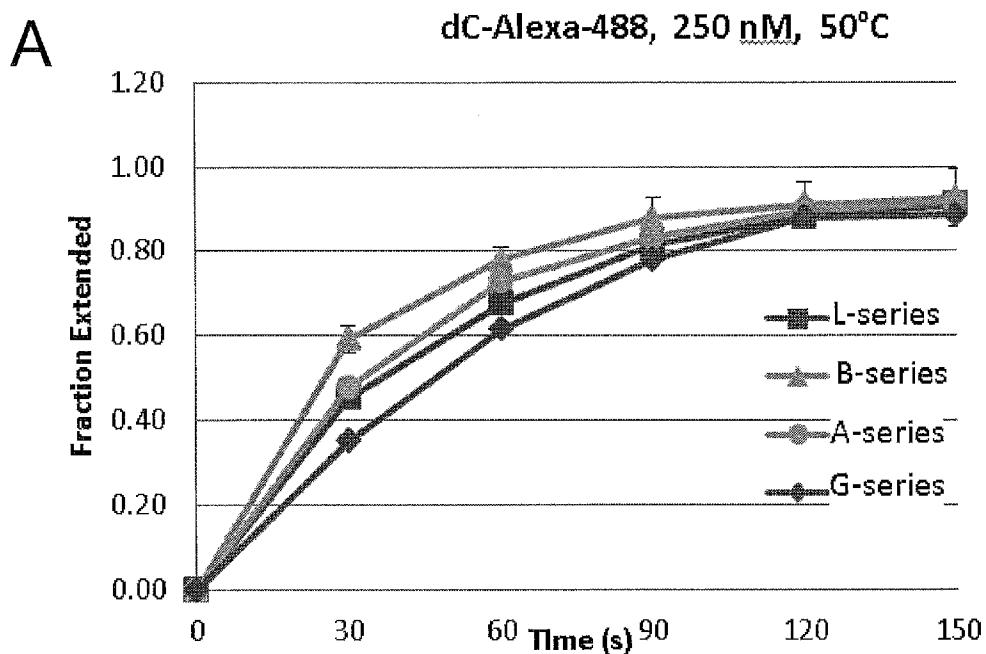


FIGURE 32



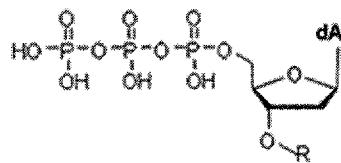
B

	250 nM	AVG rate s ⁻¹
dC-Alexa-488 L-Series		0.021
dC-Alexa-488 B-Series		0.032
dC-Alexa-488 A-Series		0.024
dC-Alexa-488 G-Series		0.015

C

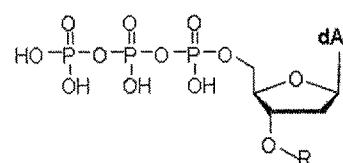
	Rate s ⁻¹		
	125nM	250nM	500nM
dC-Alexa-488 B-Series	0.0021	0.003	0.0051
dC-Alexa-488 A-Series	0.0015	0.0021	0.0024
dC-Alexa-488 L-Series	0.0009	0.0015	0.0035
dC-Alexa-488 G-Series	0.0013	0.0022	0.0033

FIGURE 33



Wherein R =

- CH₂-N₃
- CH₂-SS-Et (Et-O-SS-dA)
- CH₂-SS-Me (Me-O-SS-dA)



Wherein R =

- CH₂-N₃
- CH₂-SS-Et
- CH₂-SS-Me

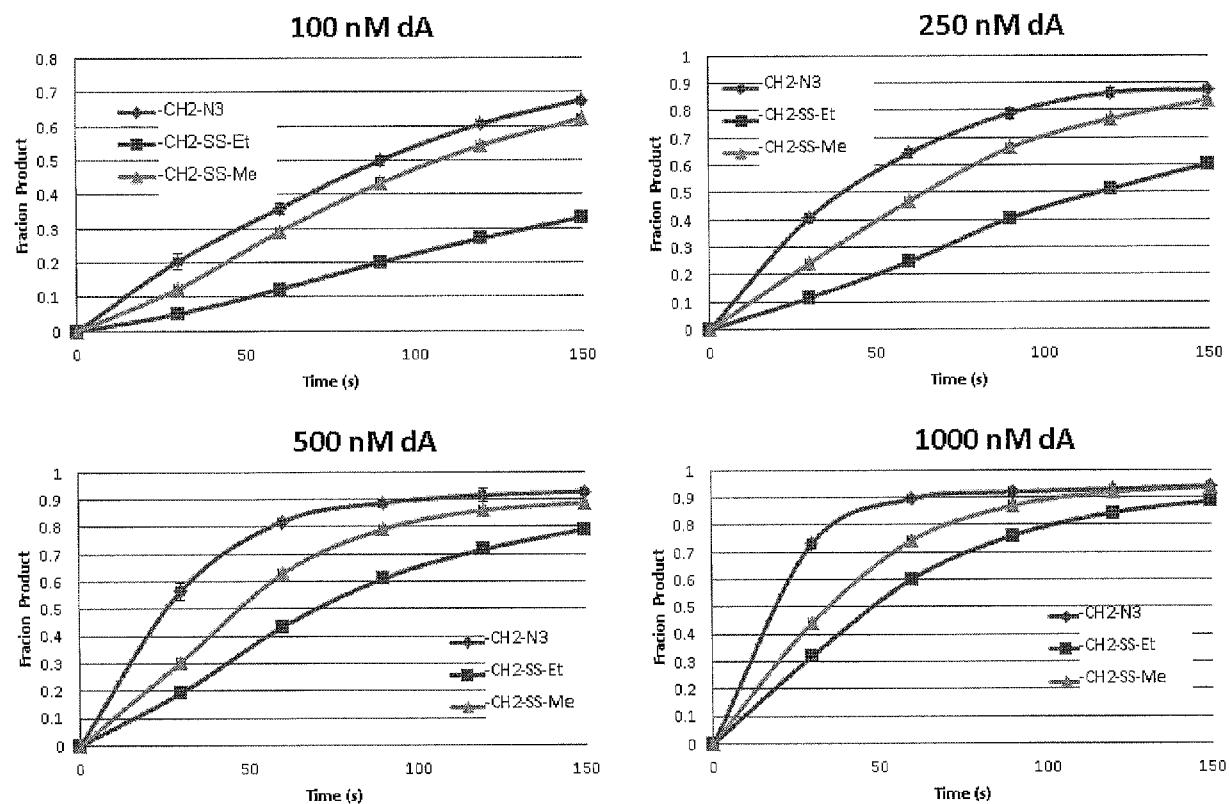


FIGURE 34

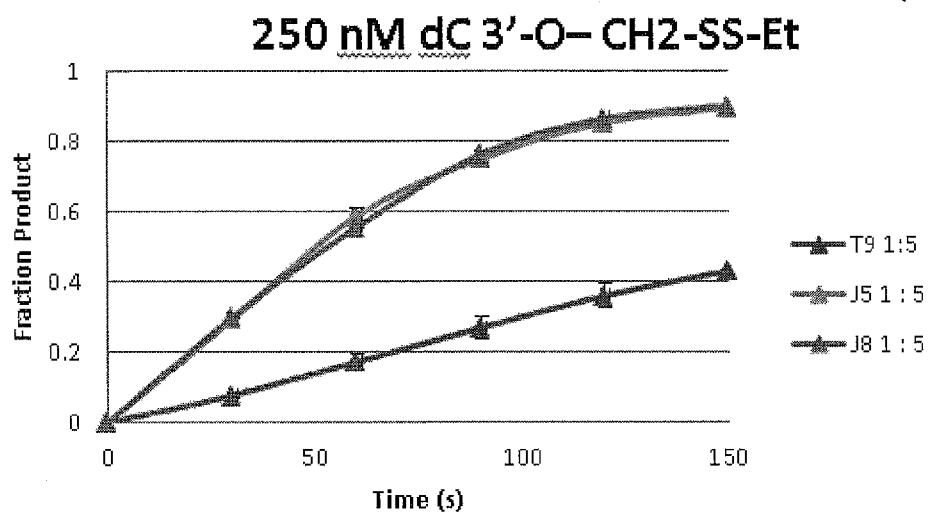


FIGURE 35

	L-series (96)	G-series (99)	A-series (98)
labeled C	400	250	180
labeled T	30	22.5	90
labeled A	100	150	120
labeled G	100	80	120
unlabeled C	2000	2000	2000
unlabeled T	2000	2000	2000
unlabeled A	1000	1000	1000
unlabeled G	500	500	500

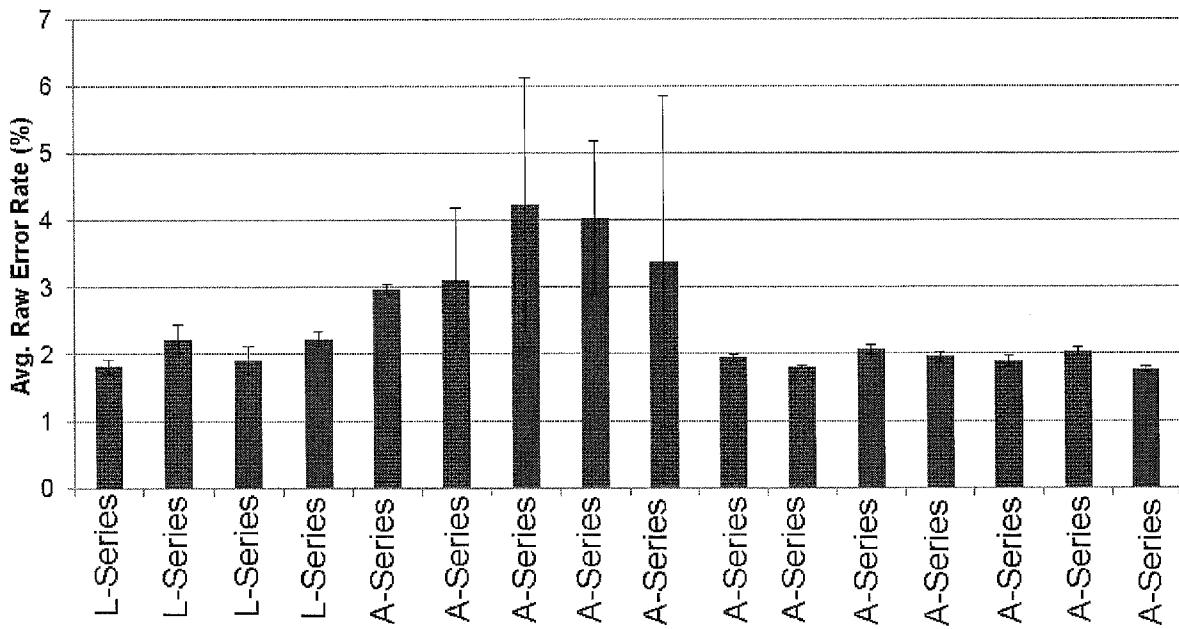
FIGURE 36

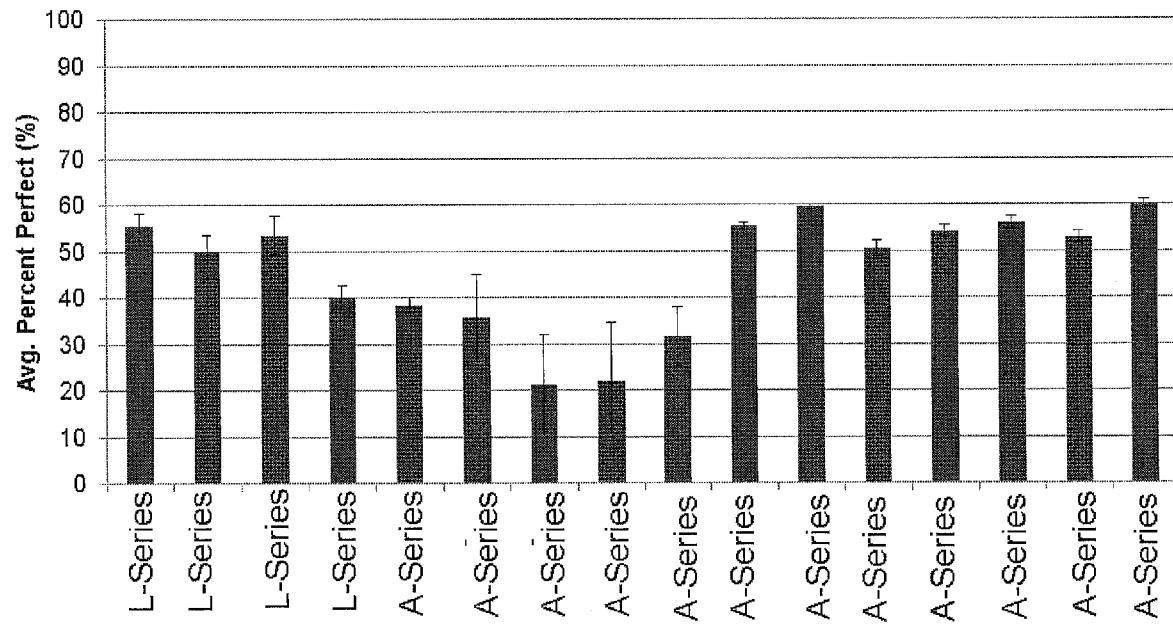
FIGURE 37

FIGURE 38

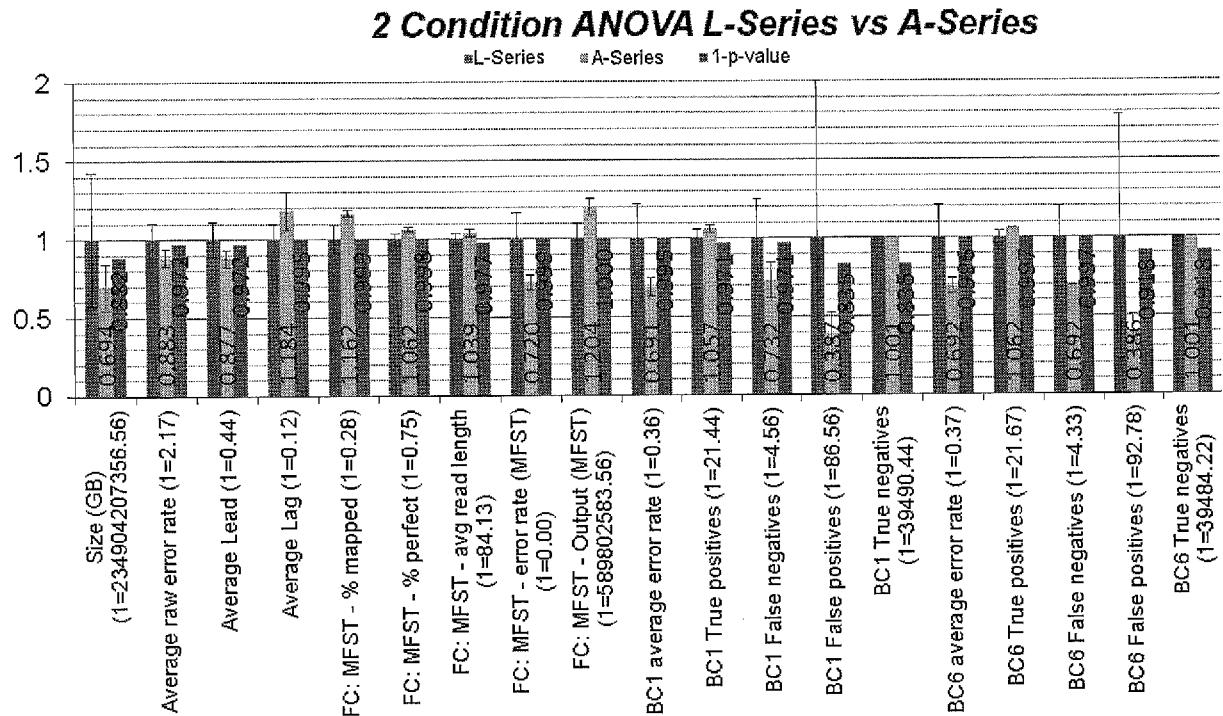


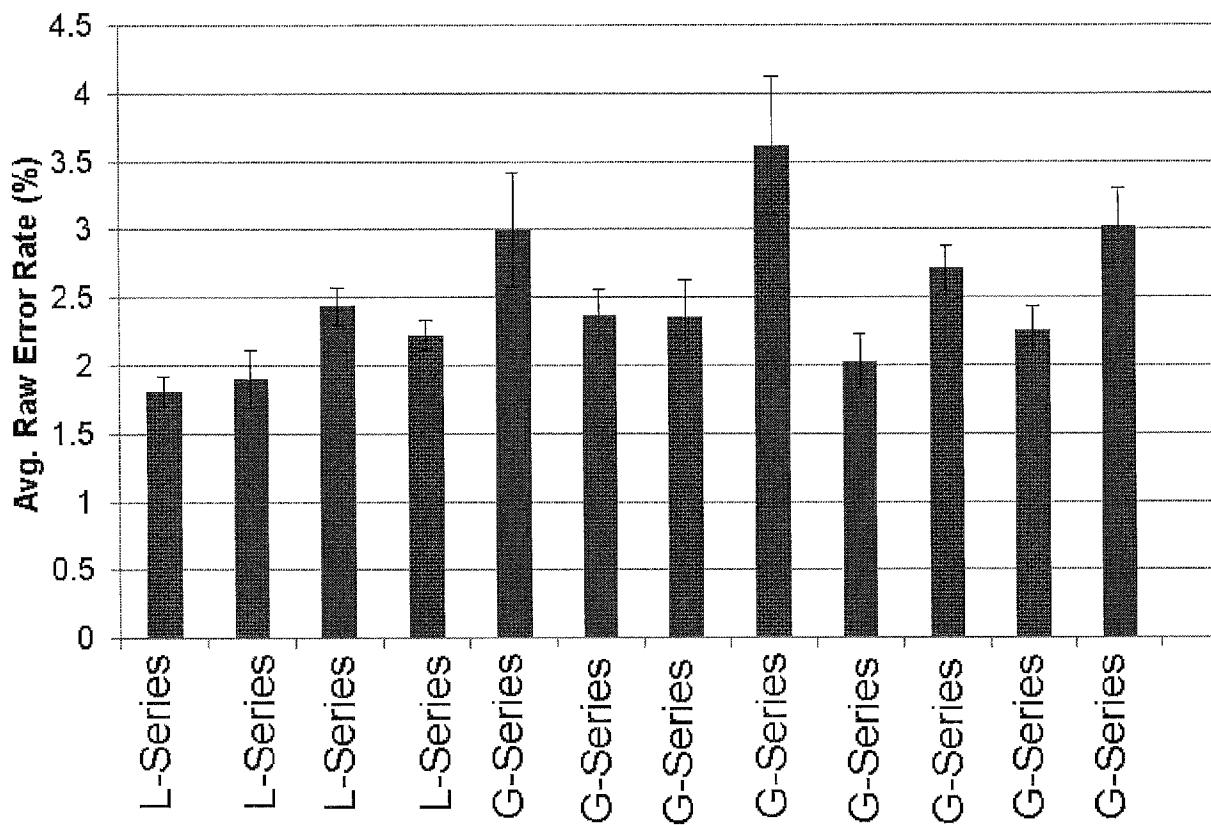
FIGURE 39

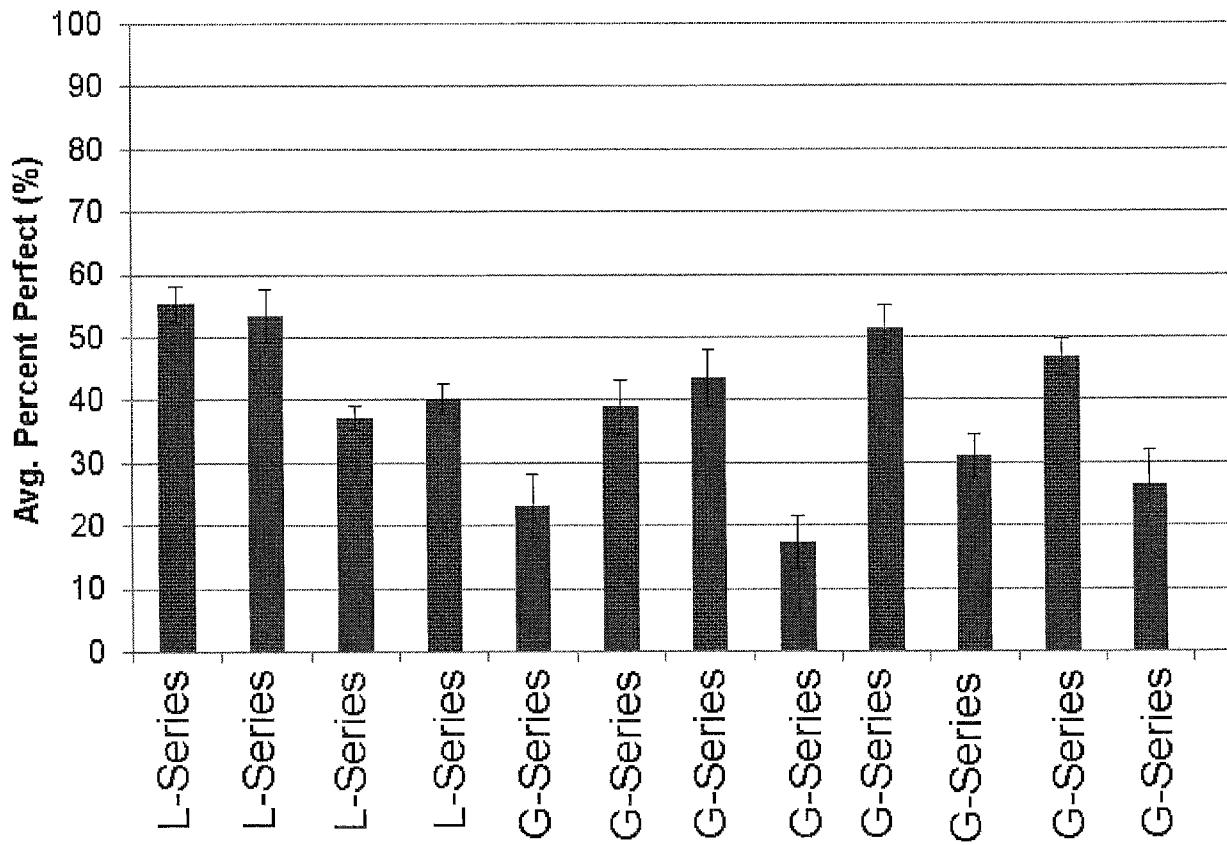
FIGURE 40

FIGURE 41

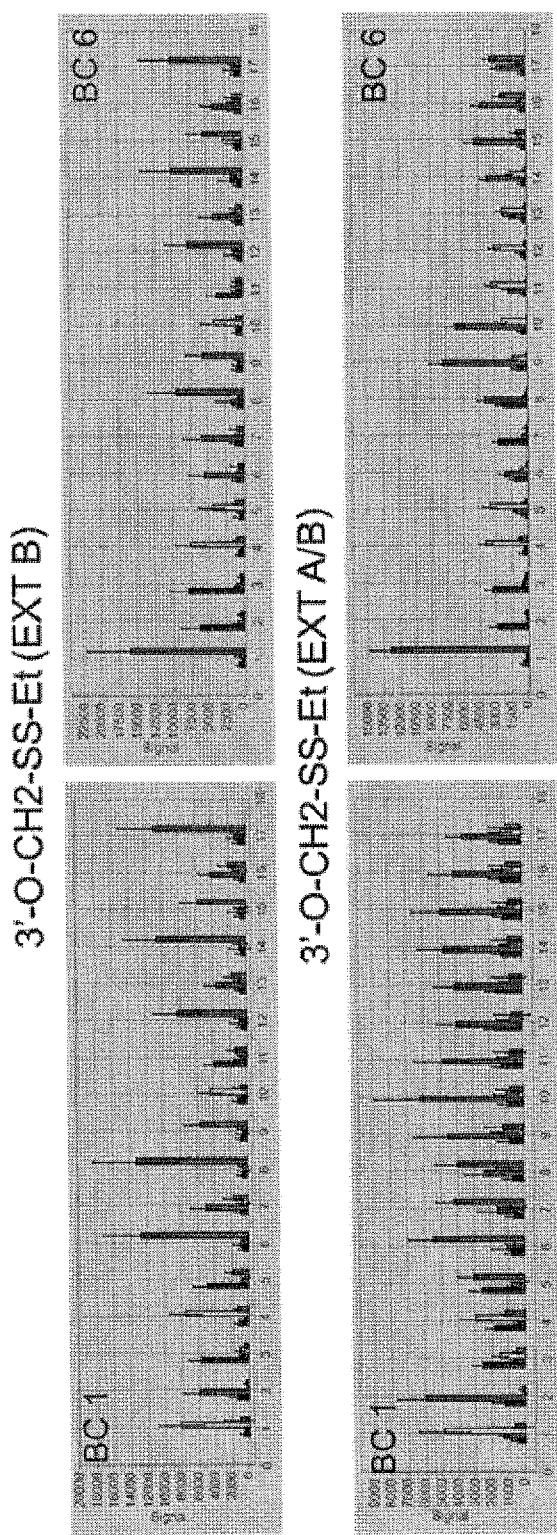


FIGURE 42

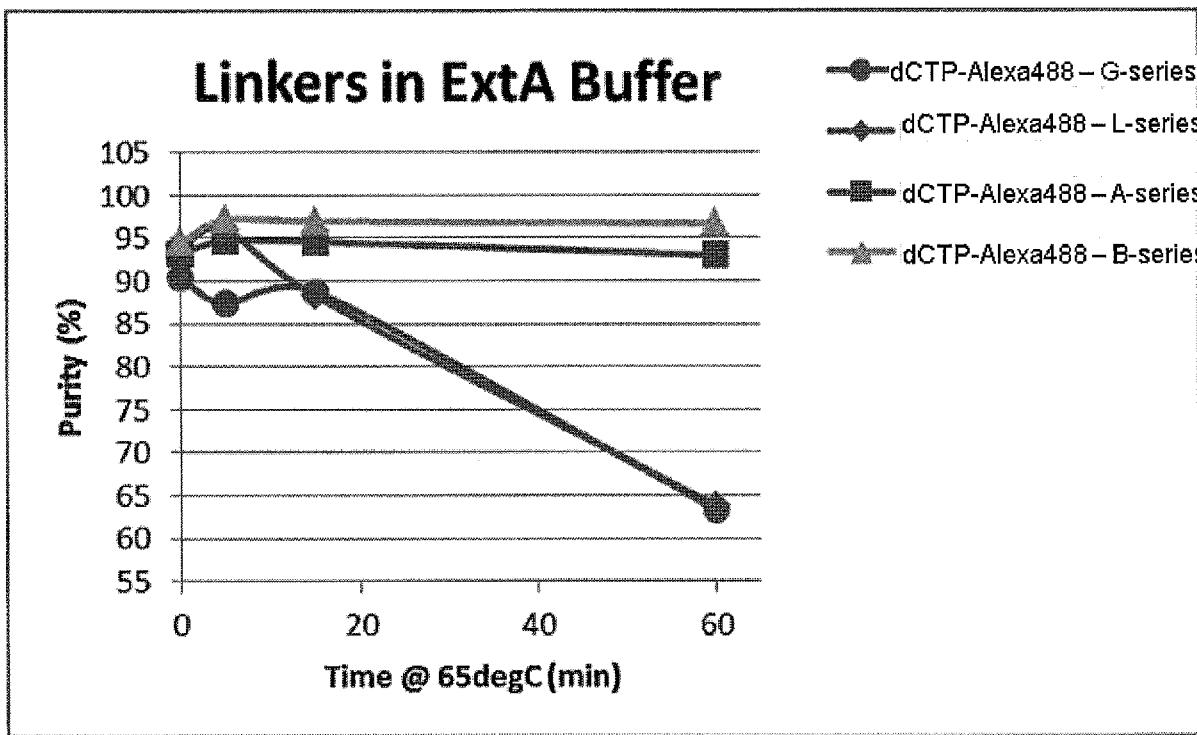


FIGURE 43

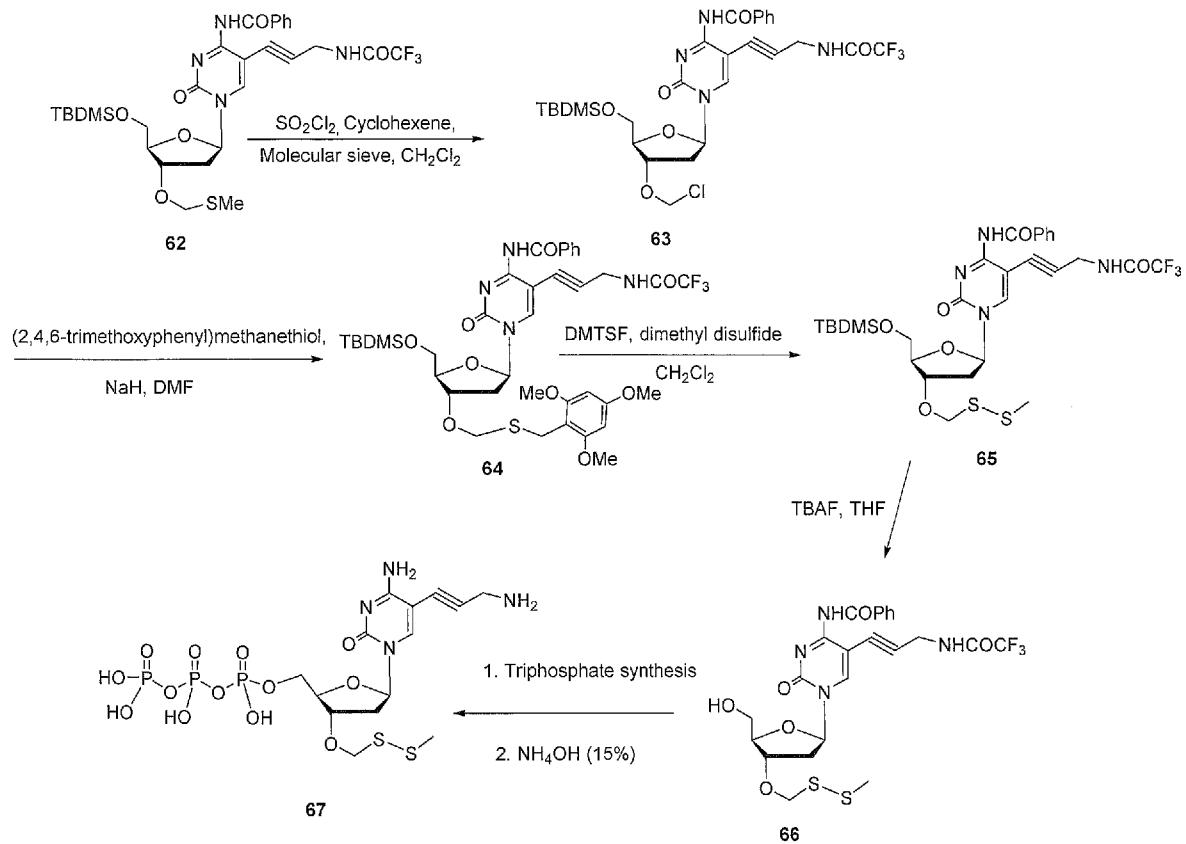


FIGURE 44

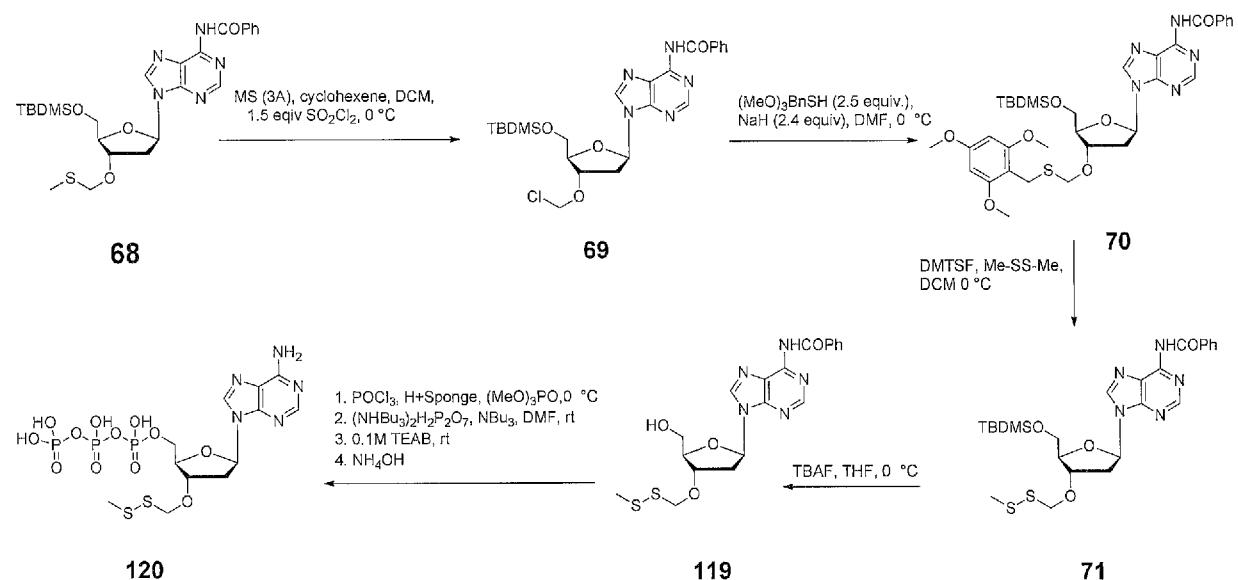


FIGURE 45

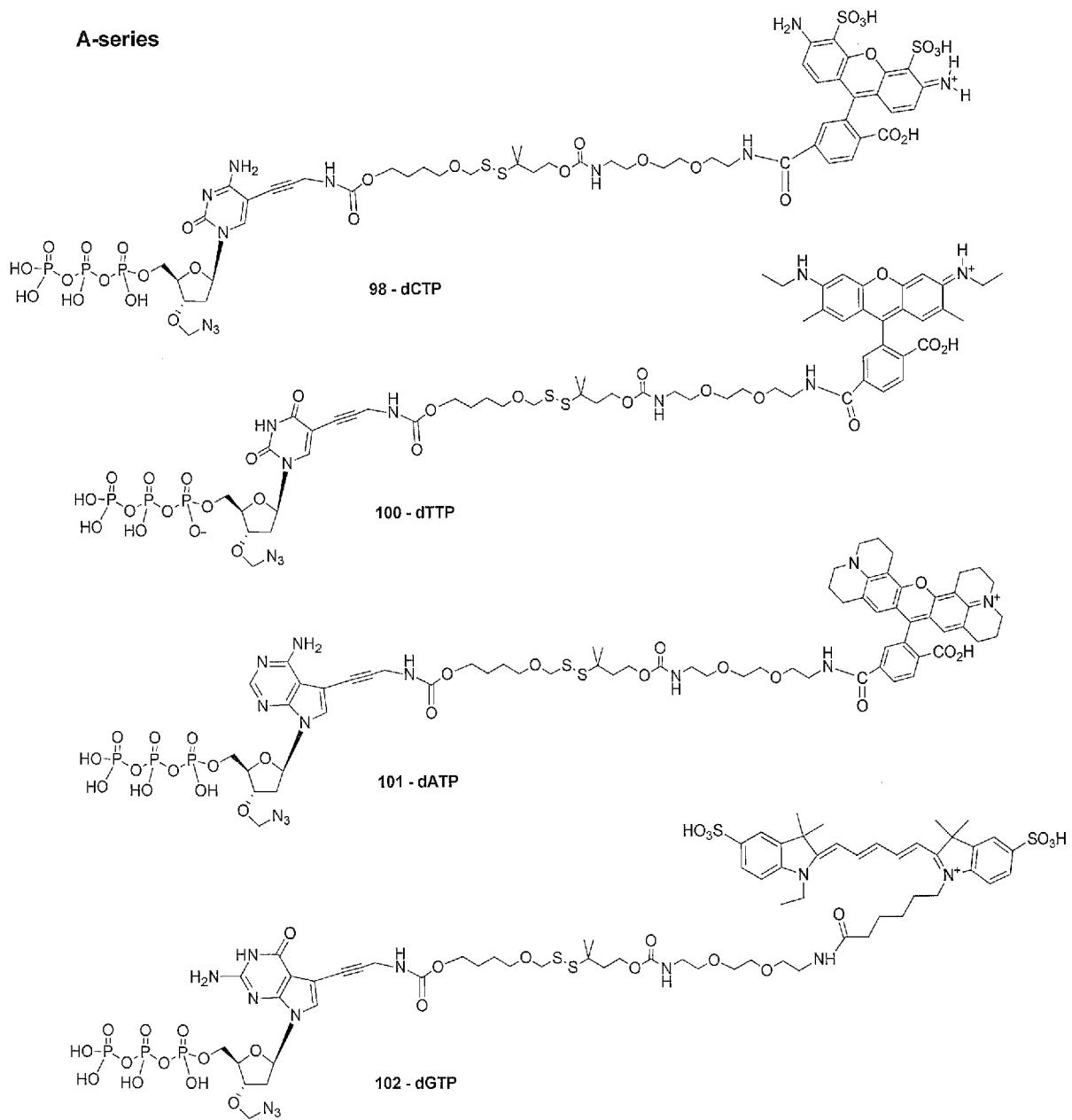


FIGURE 46

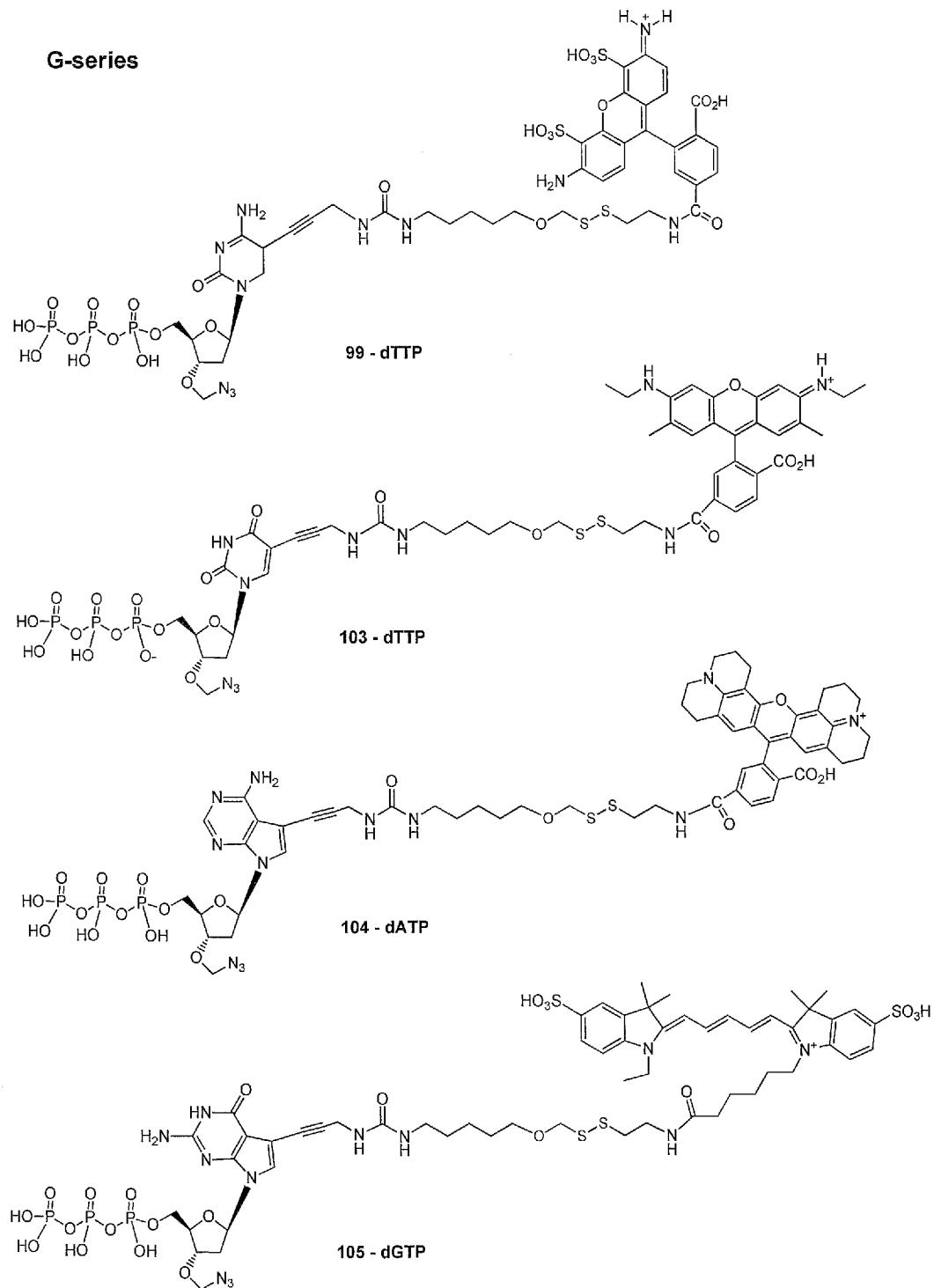


FIGURE 47

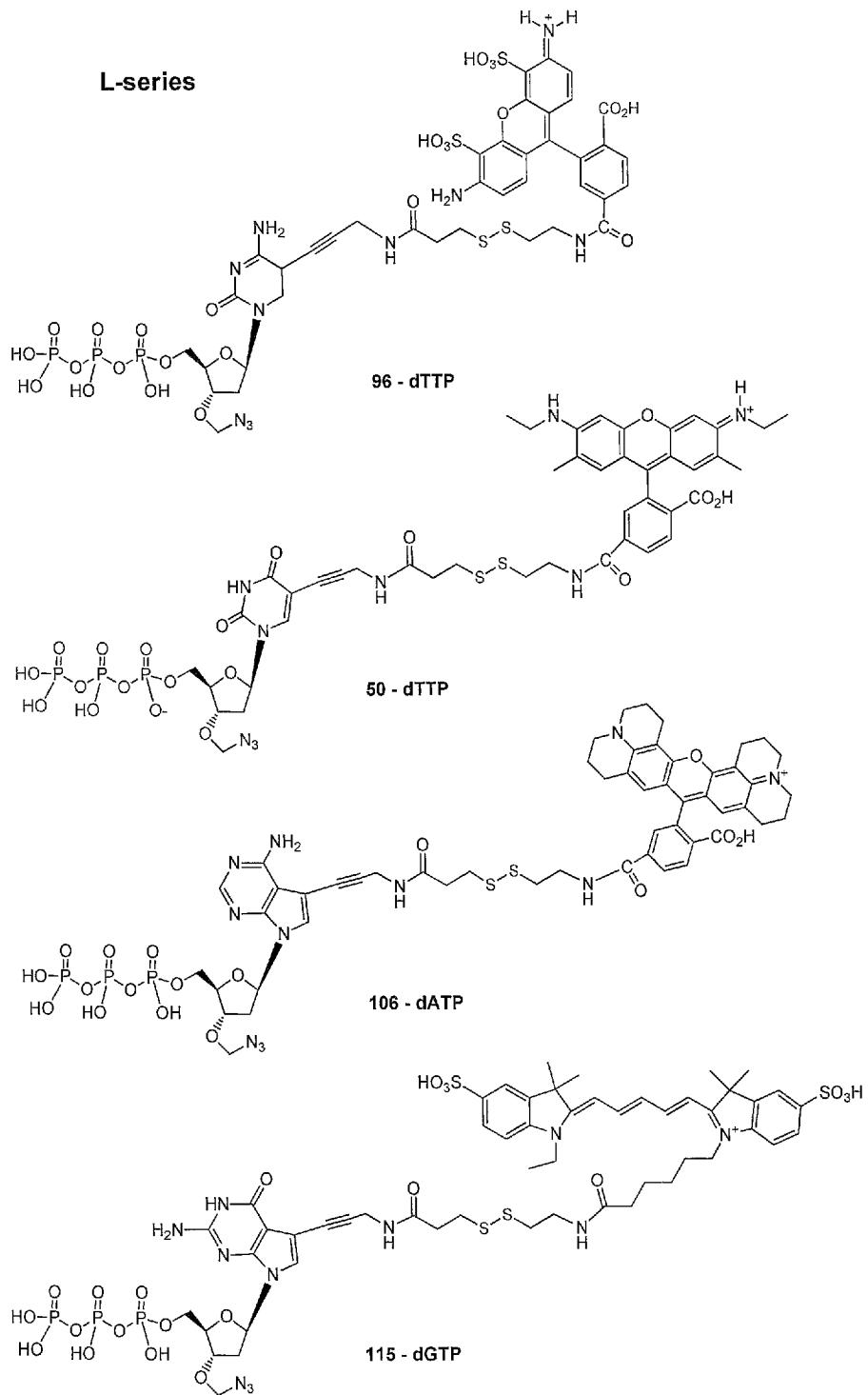


FIGURE 48

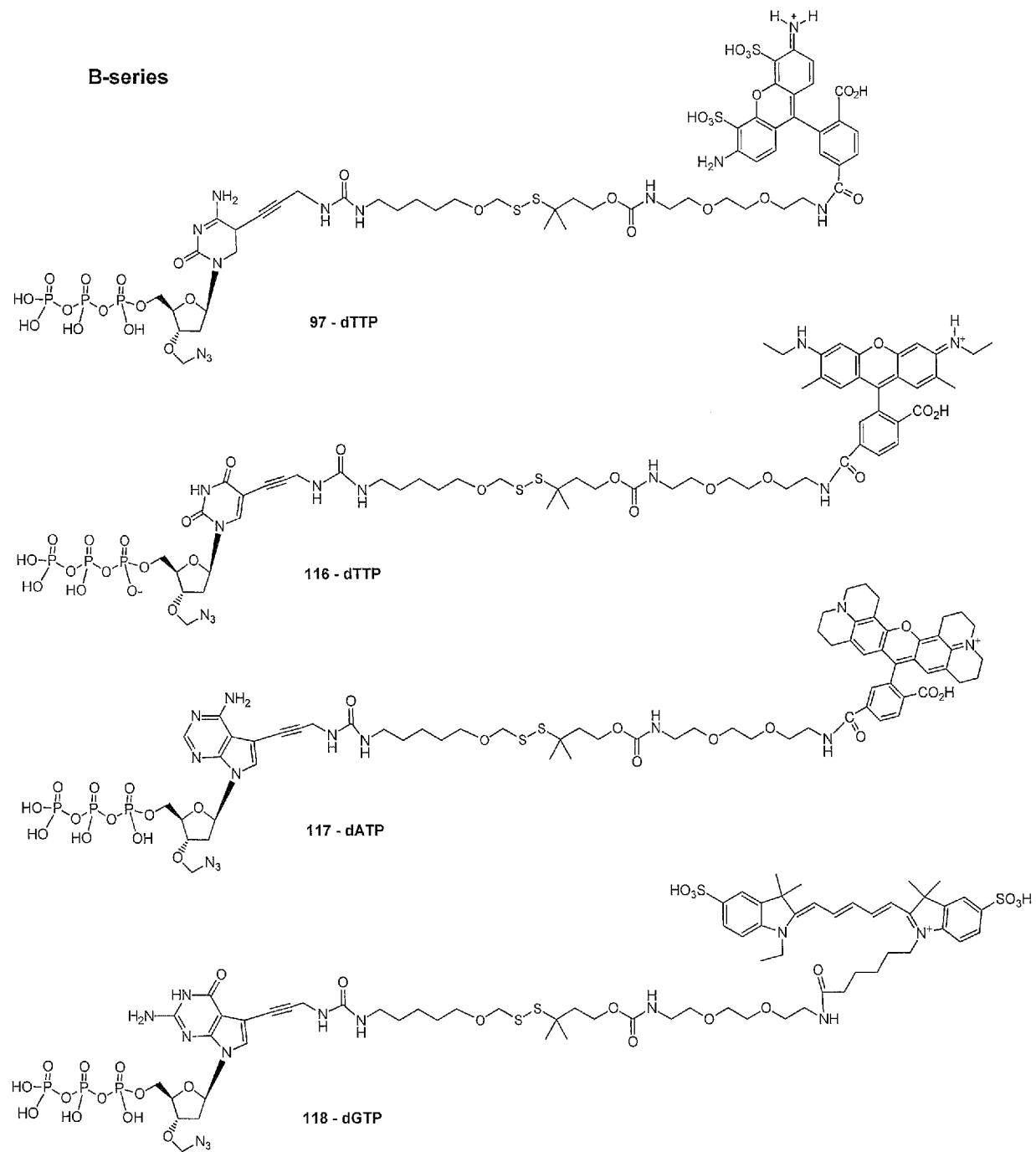


FIGURE 49

	Concentration [nM]	Compound Number
labeled C	180	72
labeled T	270	74
labeled A	360	76
labeled G	120	78
unlabeled C	2000	126
unlabeled T	2000	138
unlabeled A	1000	73
unlabeled G	500	132

FIGURE 50

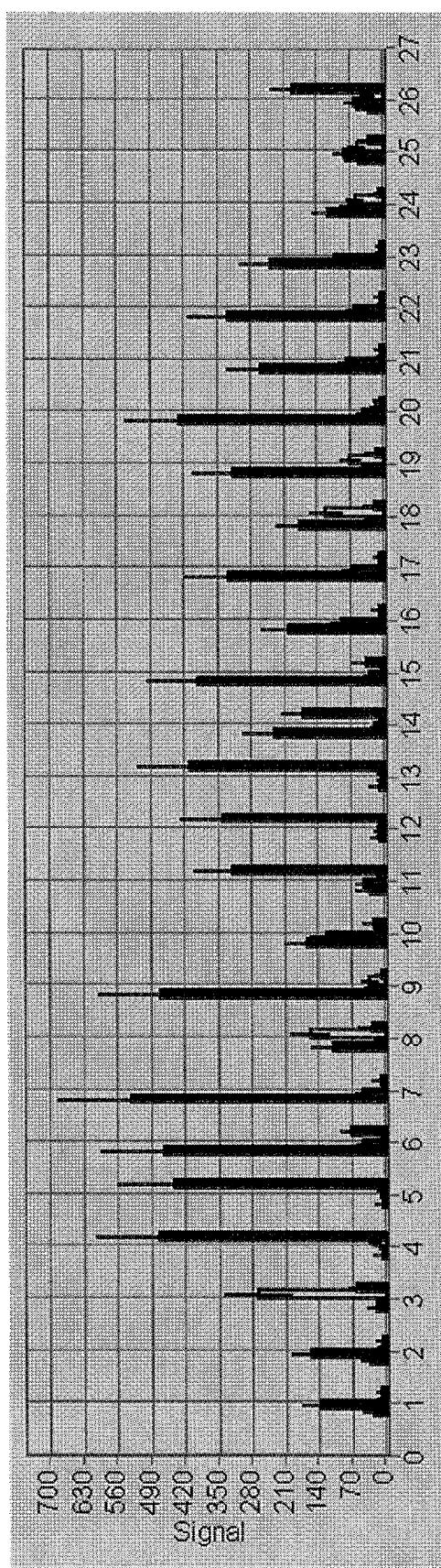


FIGURE 51

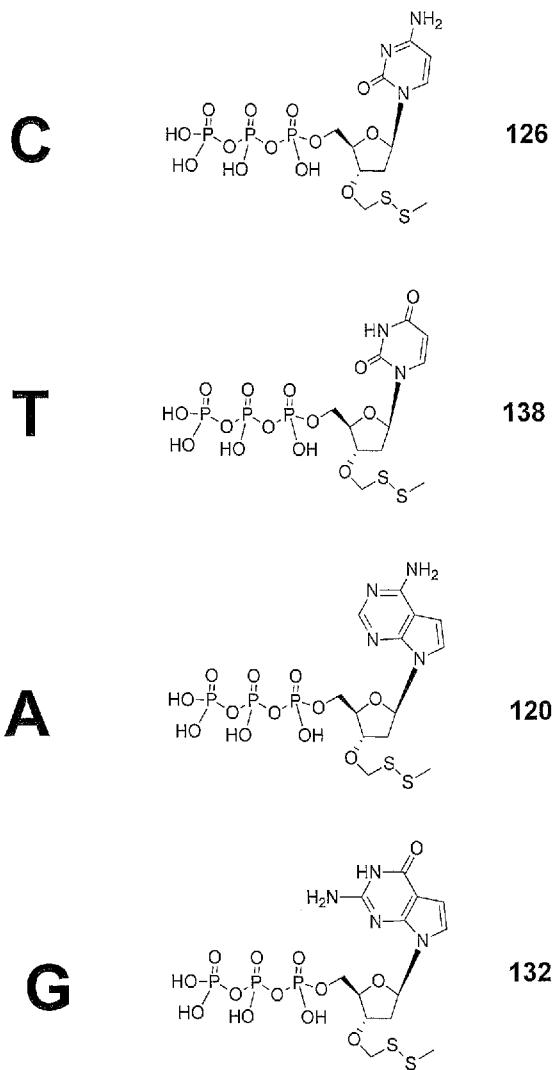


FIGURE 52

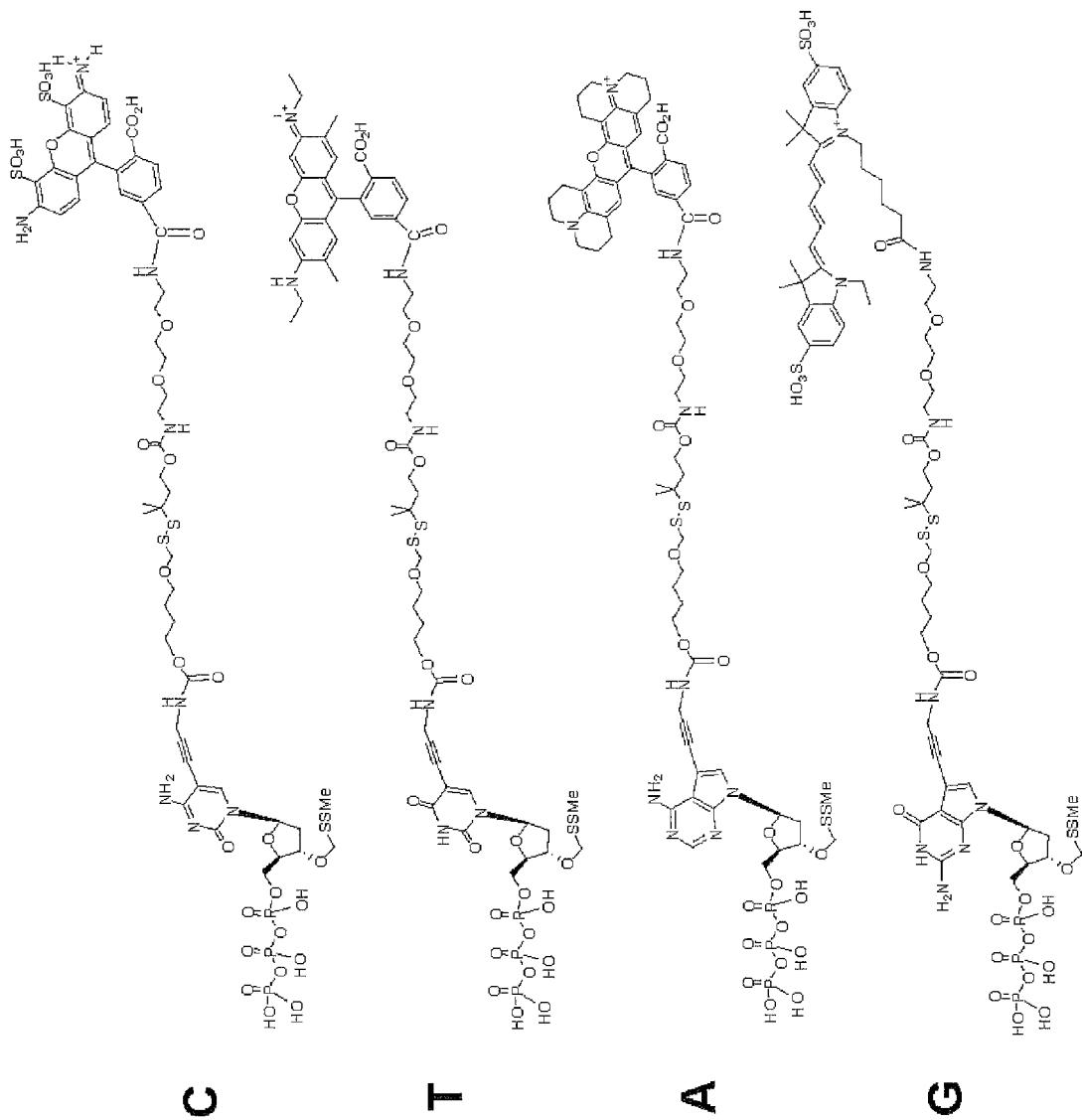


FIGURE 53

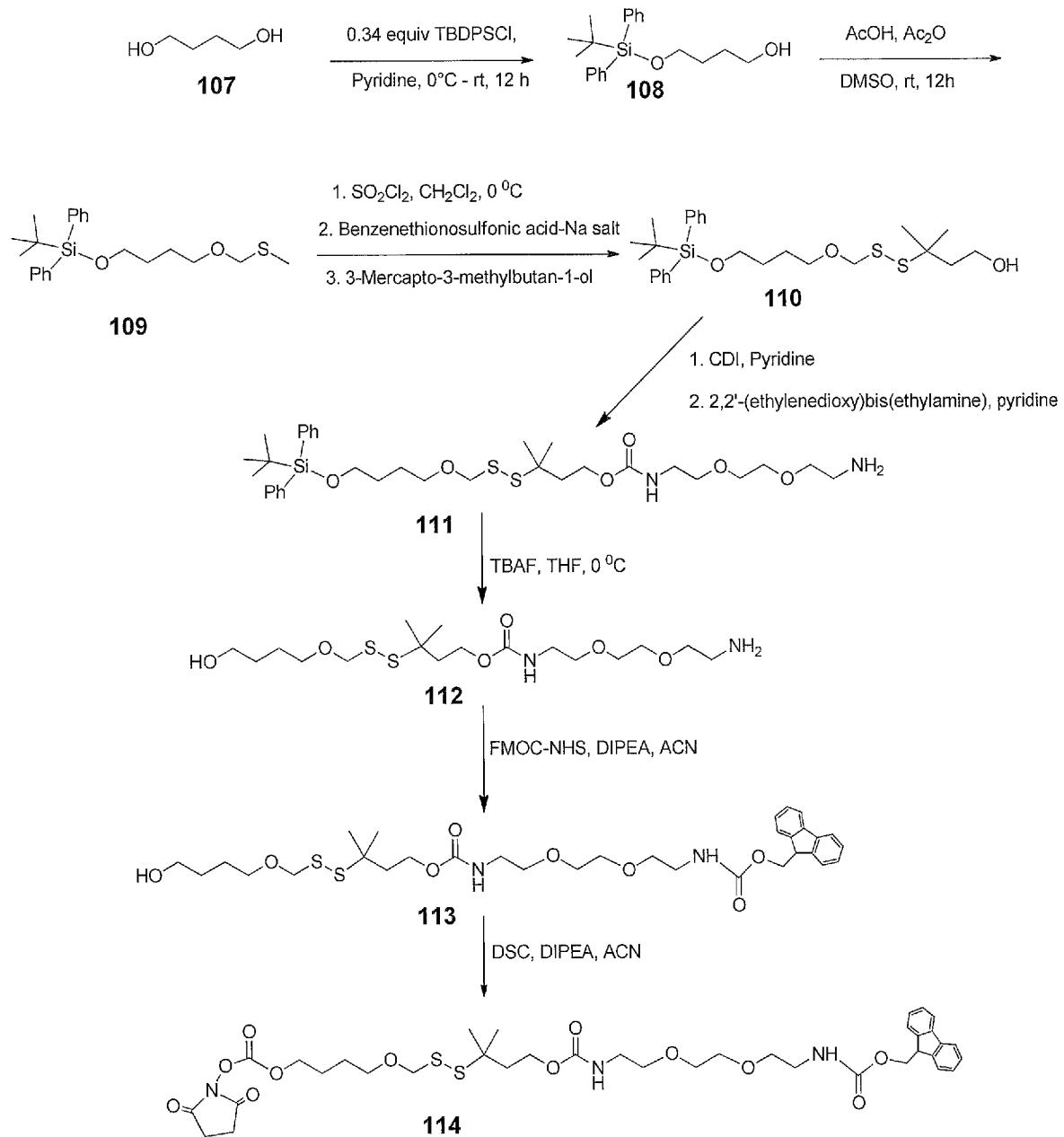


FIGURE 54

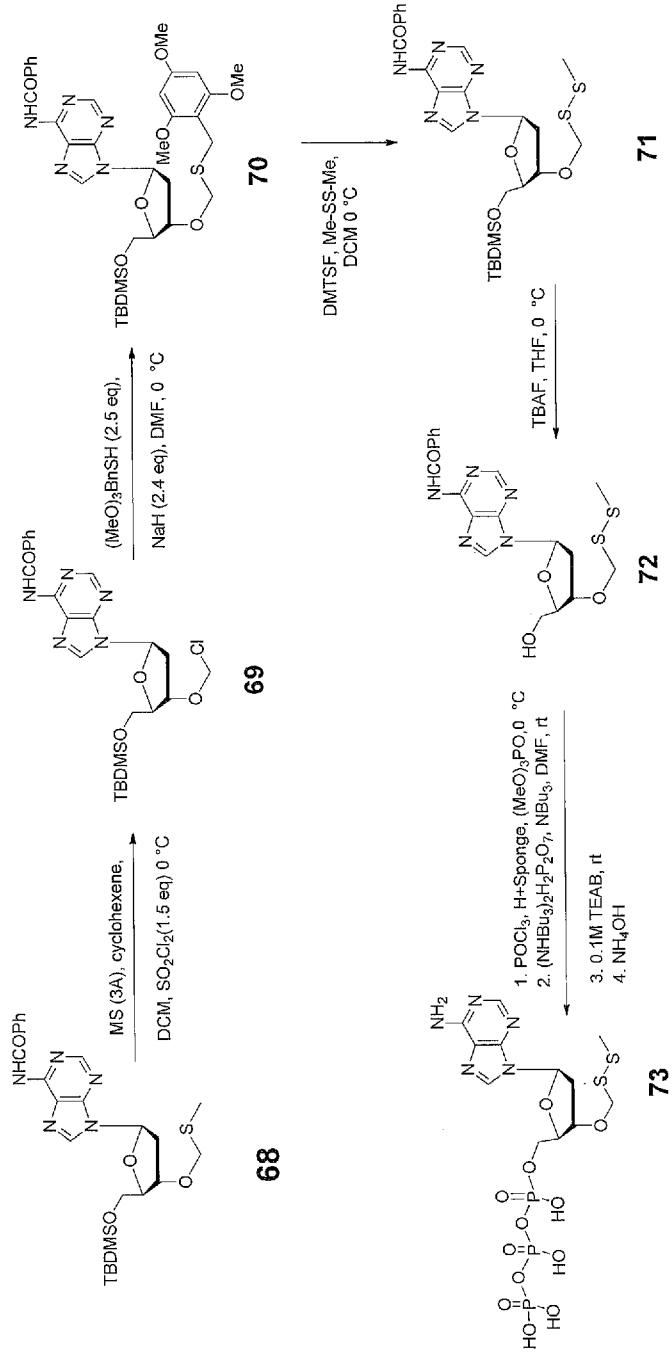


FIGURE 55

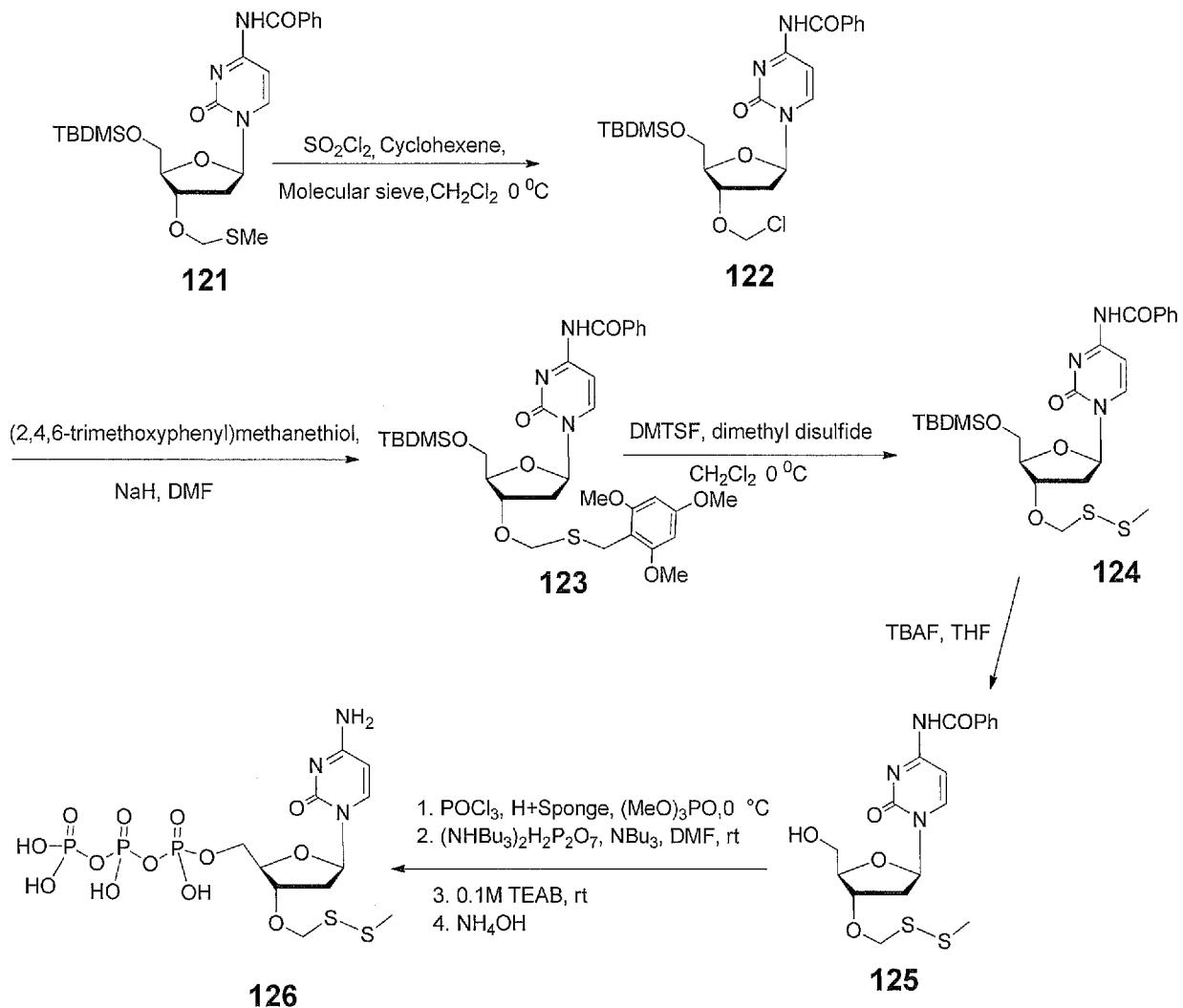


FIGURE 56

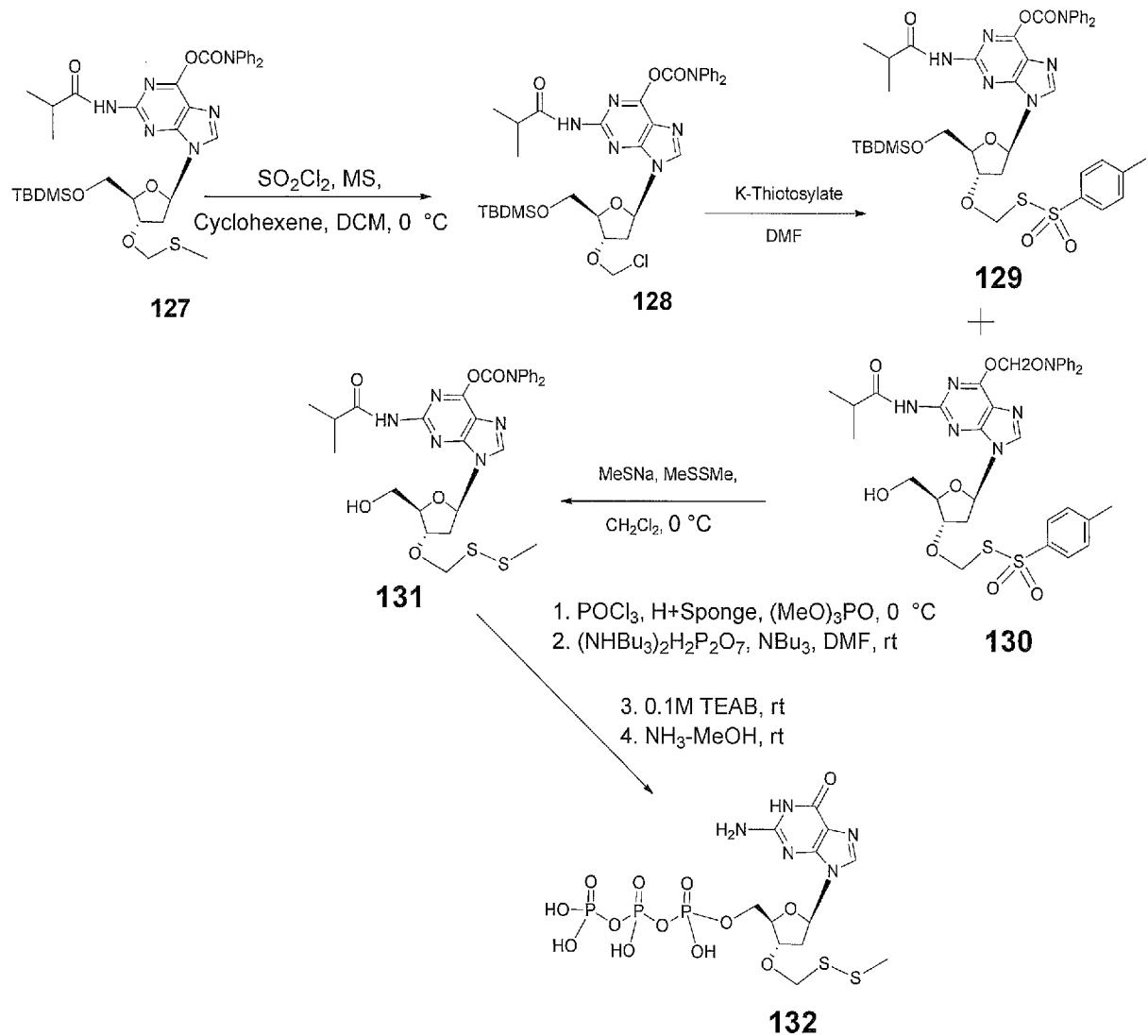


FIGURE 57

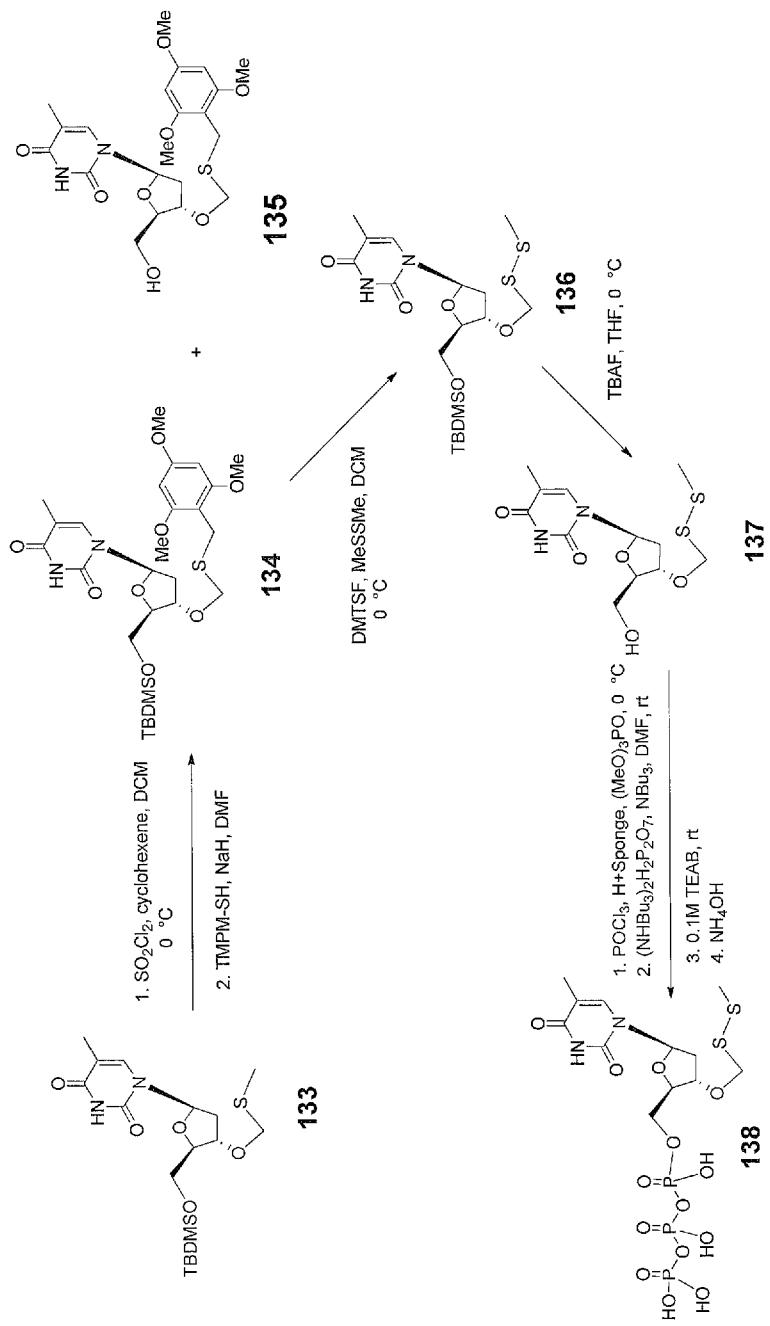


FIGURE 58

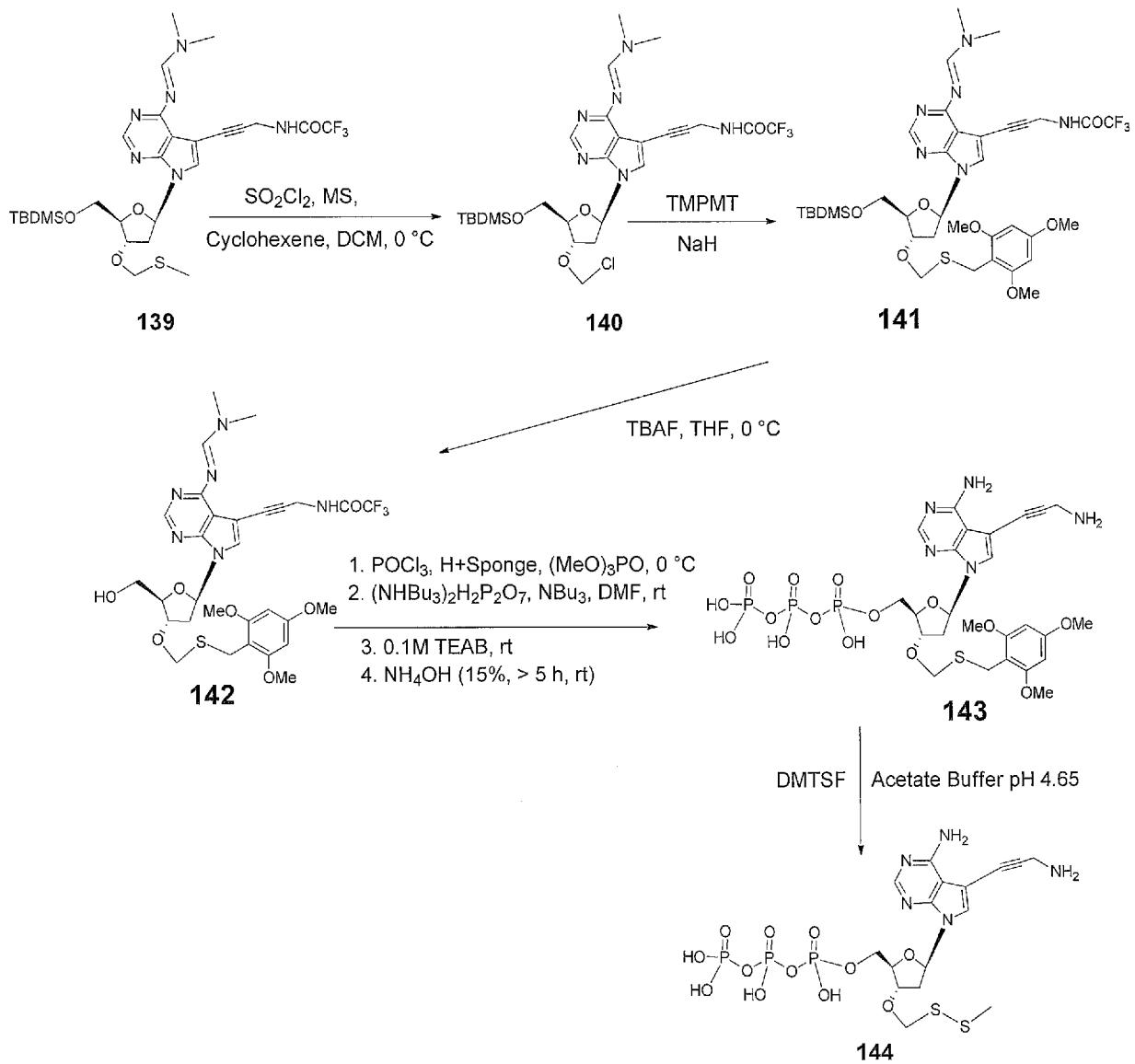


FIGURE 59

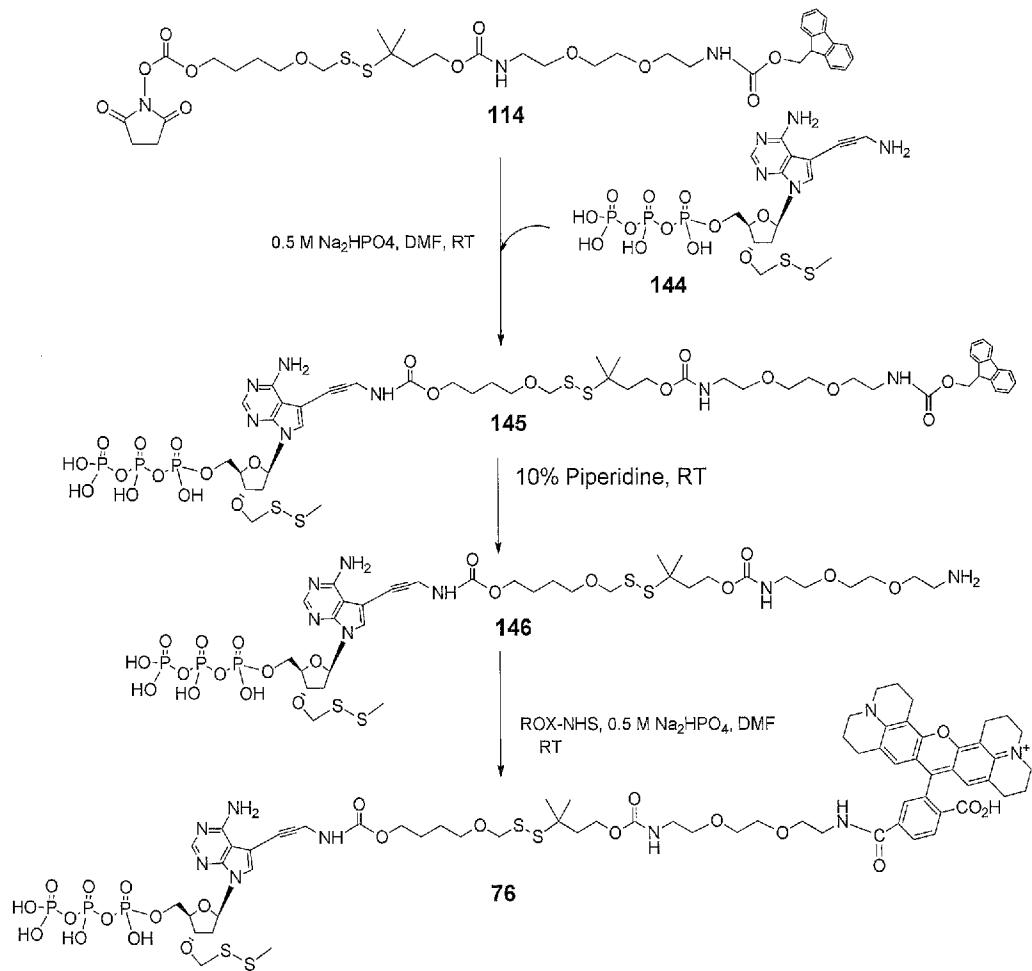


FIGURE 60

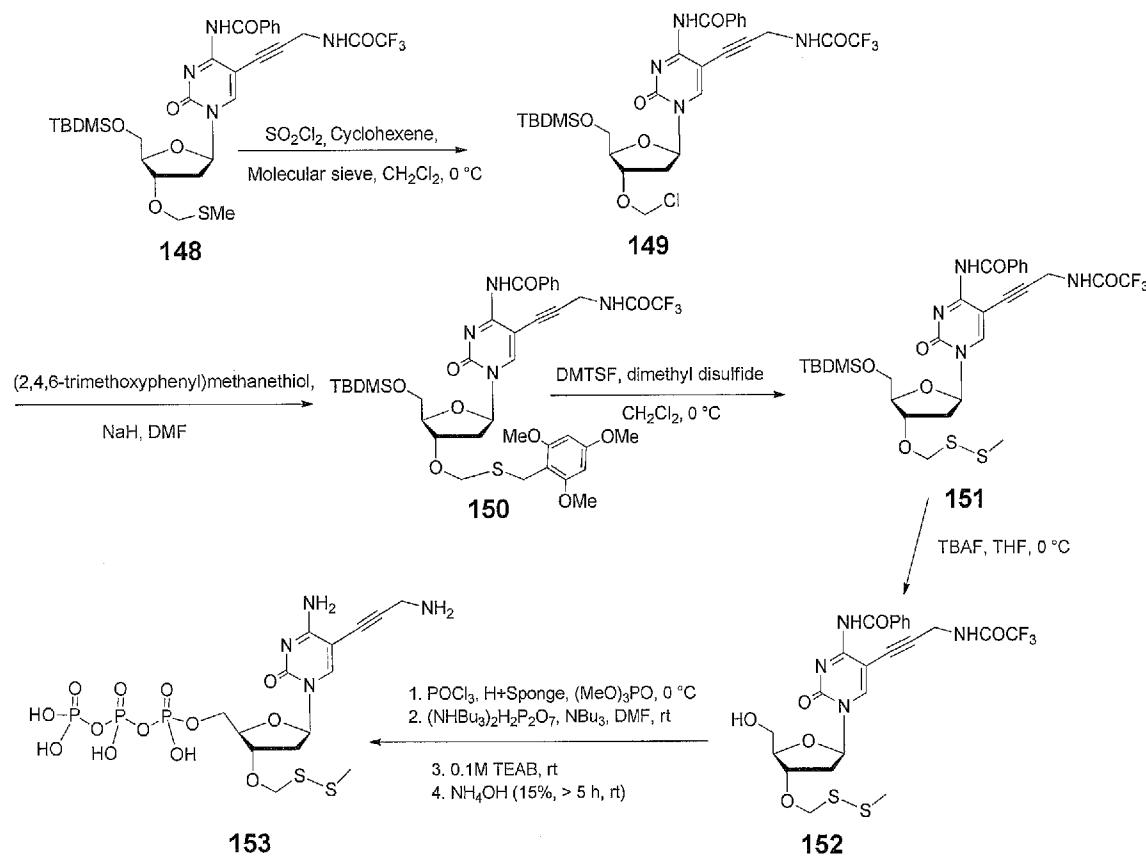


FIGURE 61

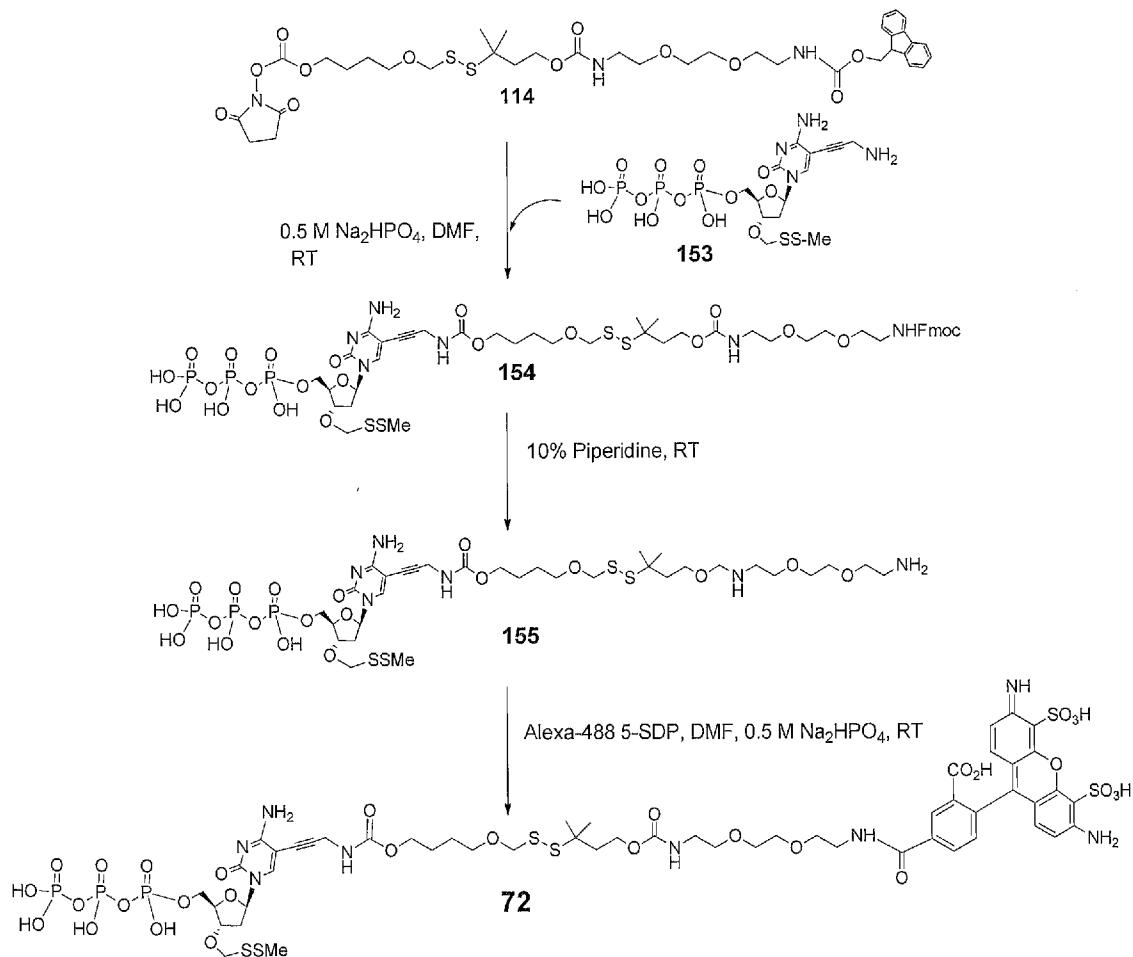


FIGURE 62

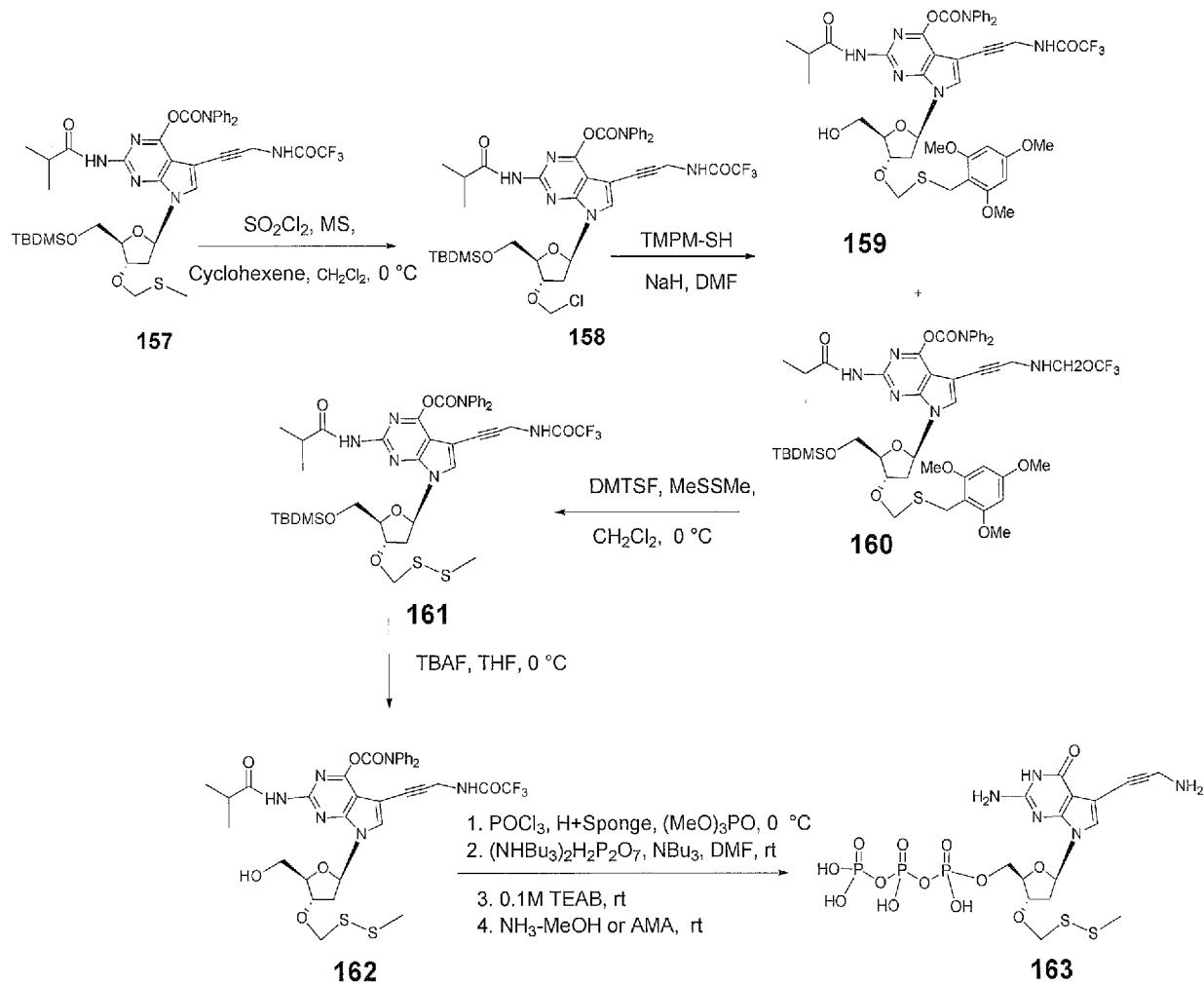


FIGURE 63

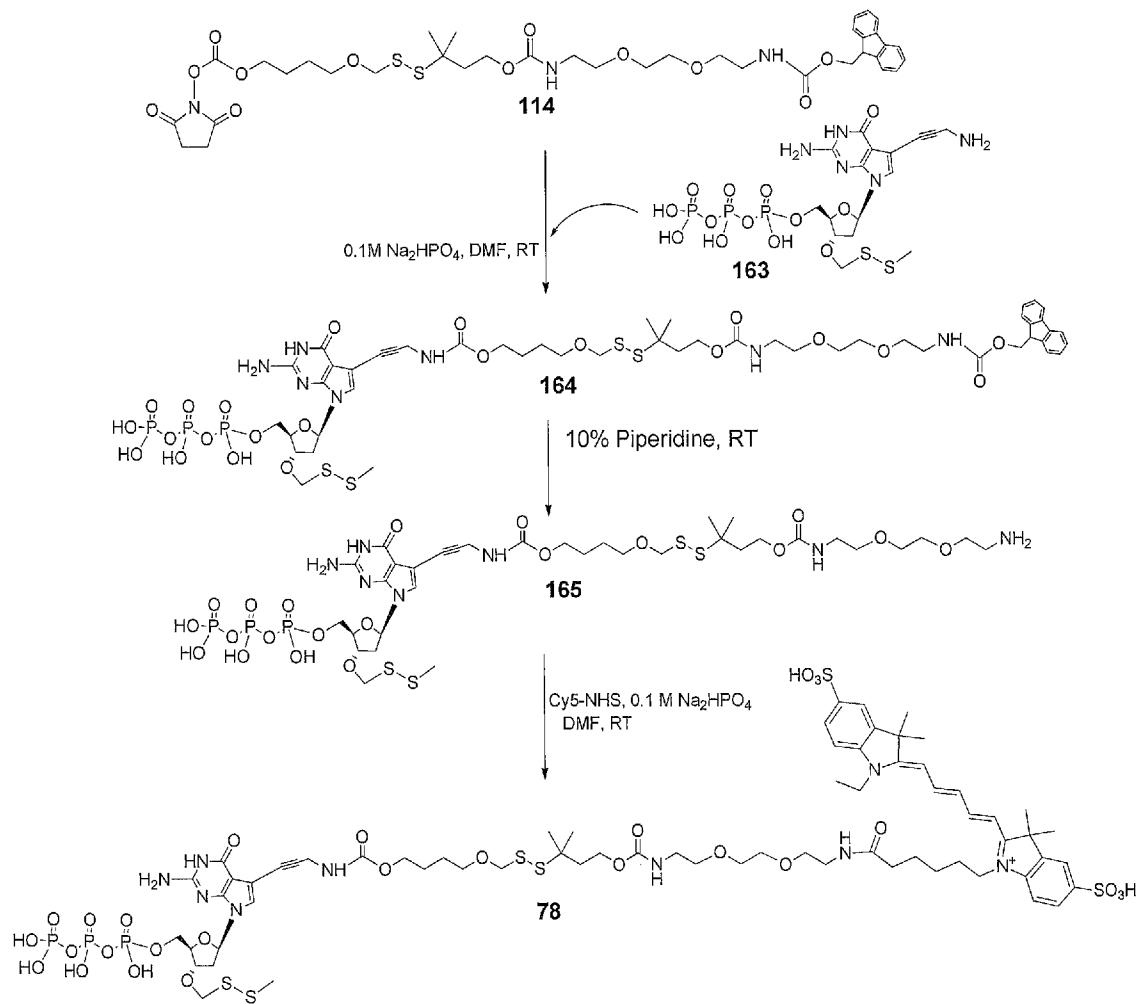


FIGURE 64

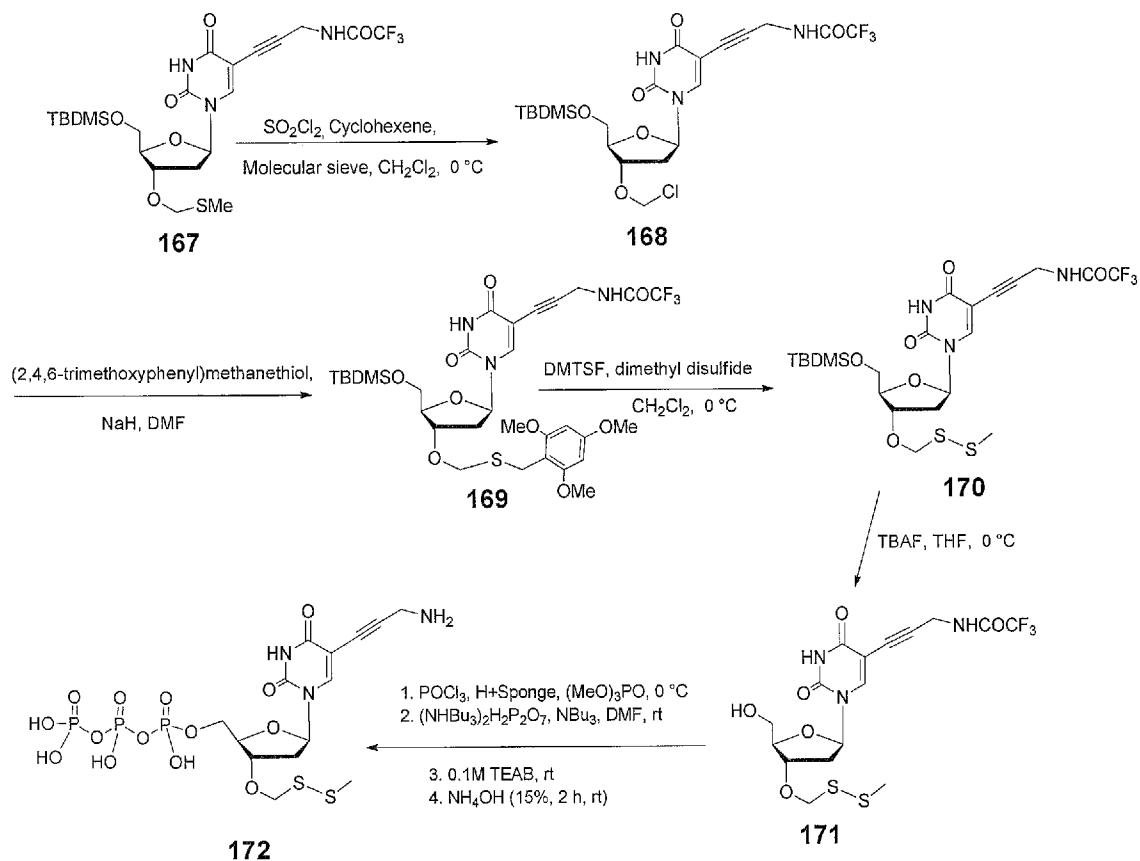


FIGURE 65

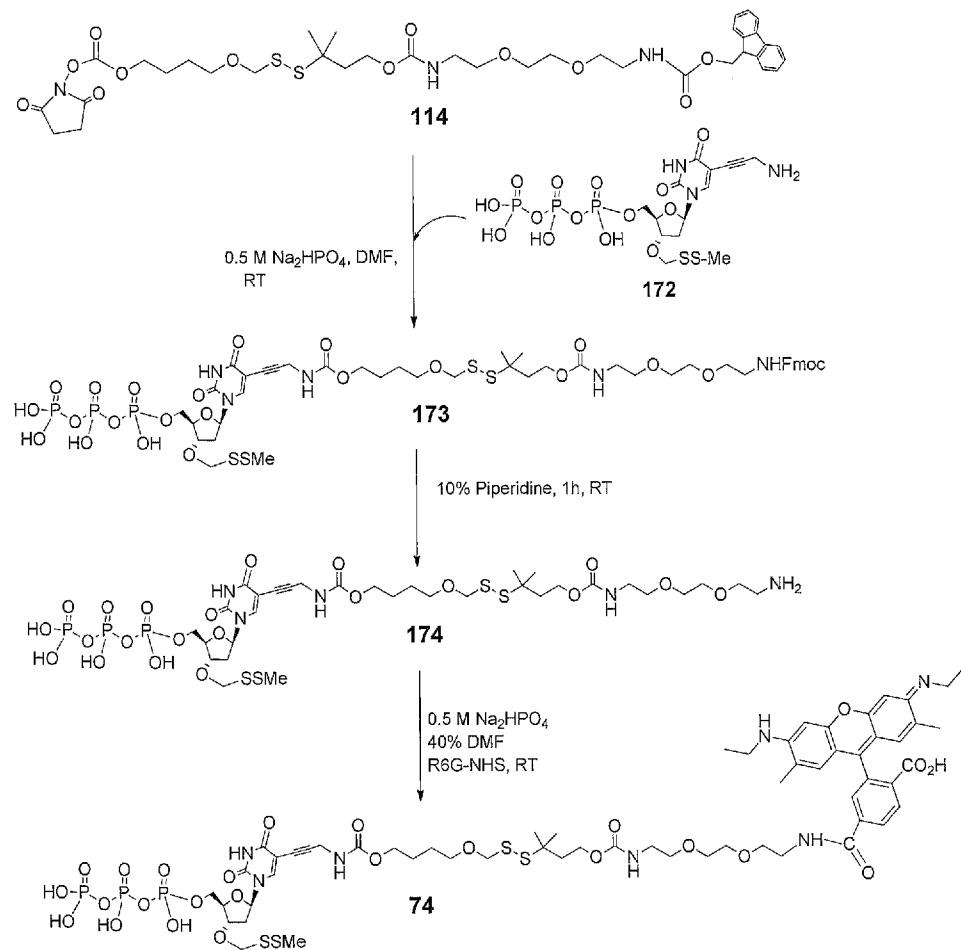


FIGURE 66

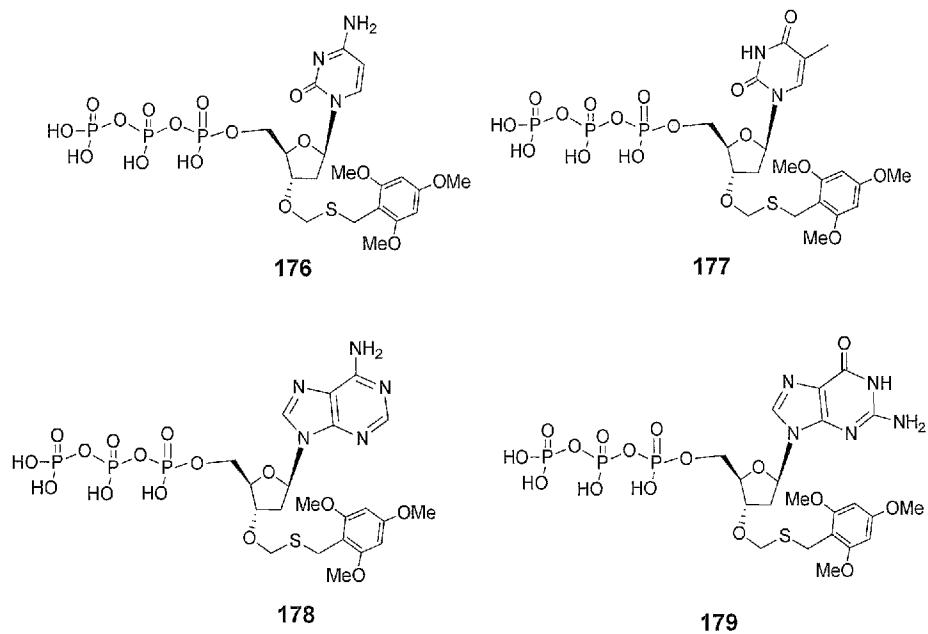


FIGURE 67

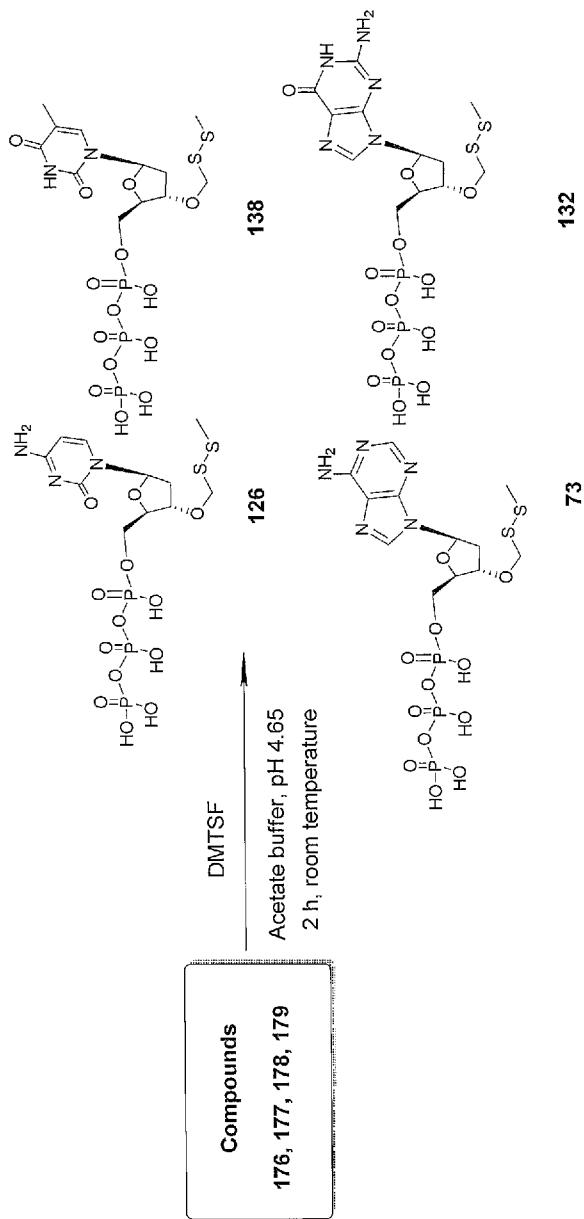


FIGURE 68

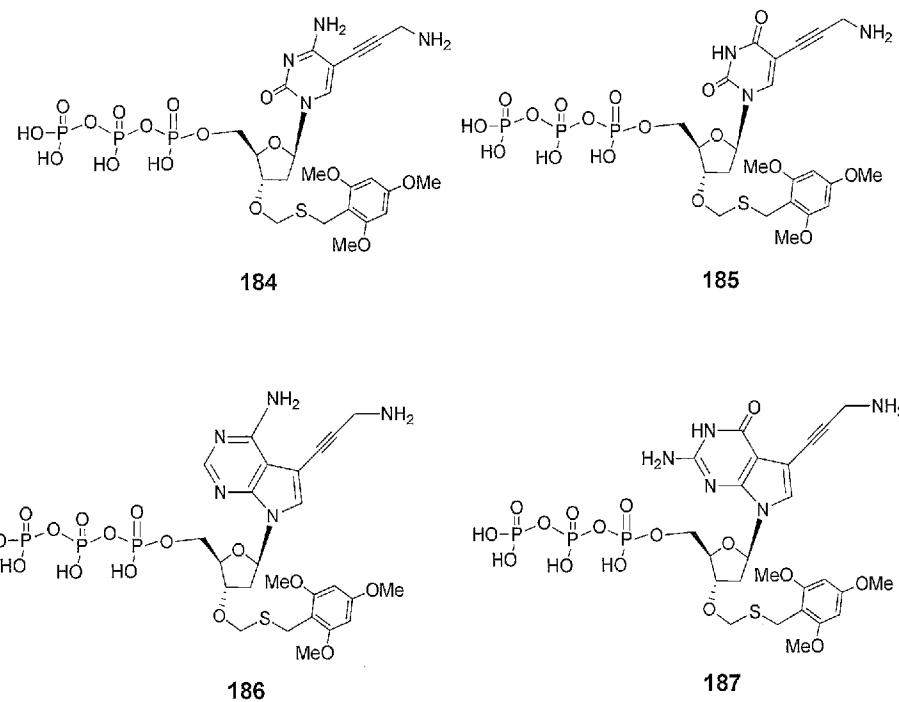


FIGURE 69

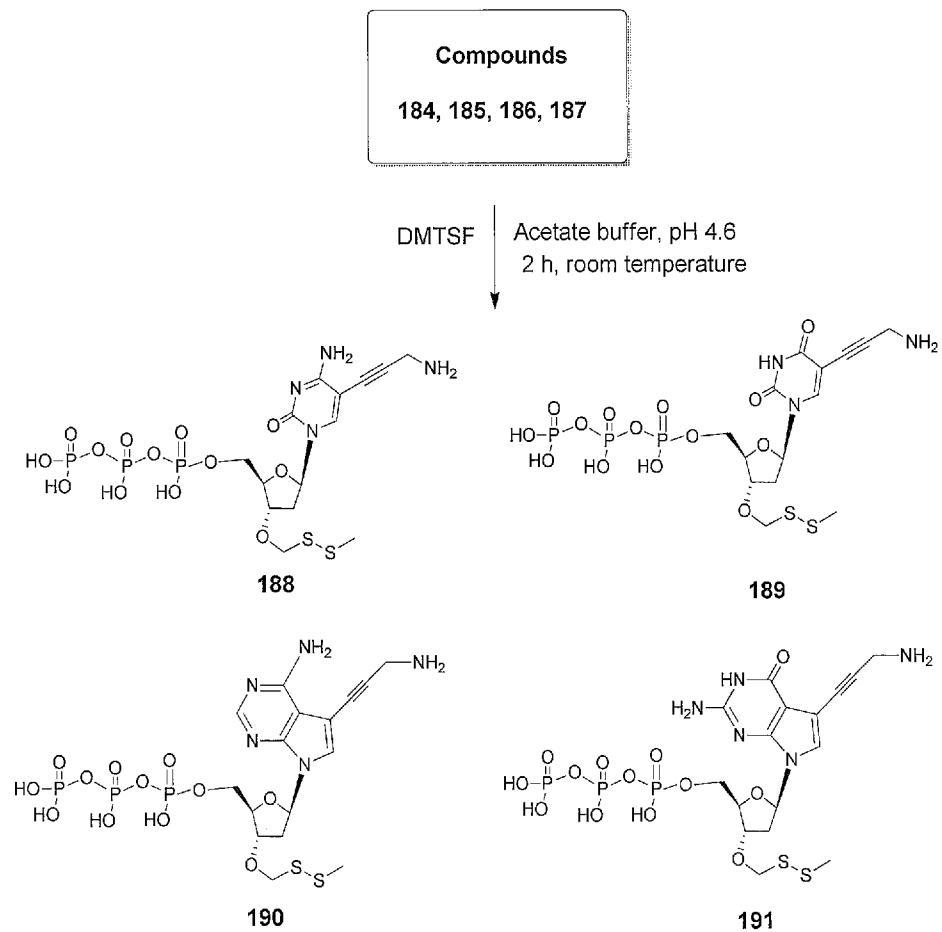


FIGURE 70

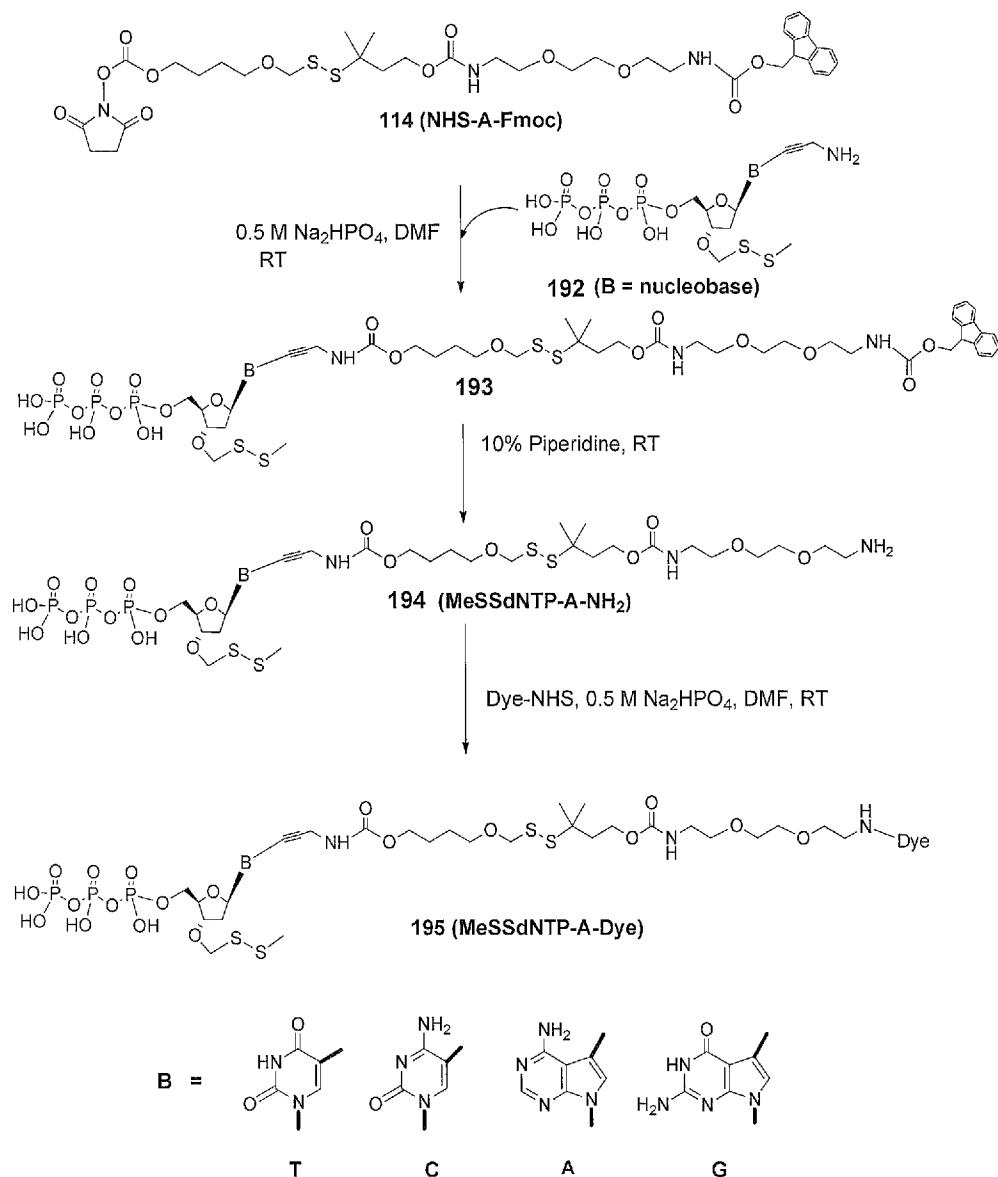


FIGURE 71

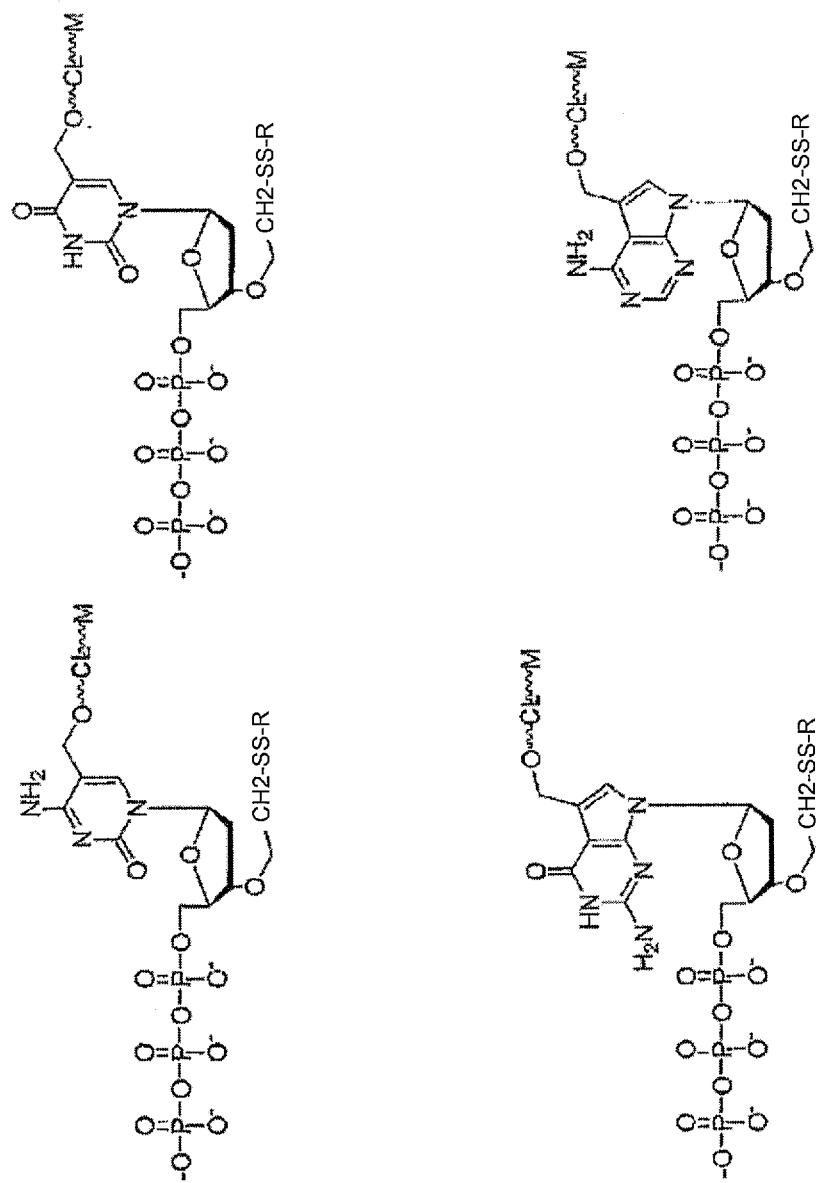


FIGURE 72

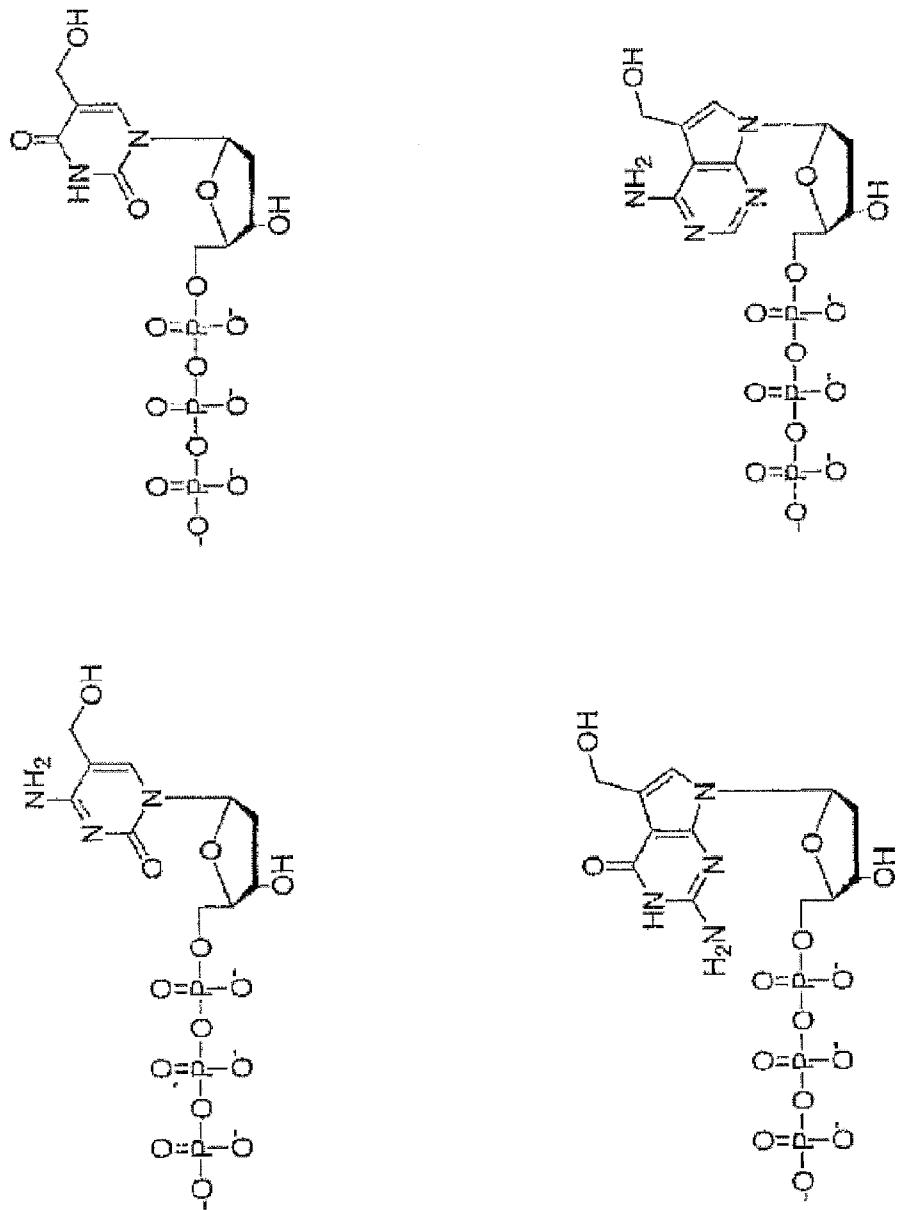


FIGURE 73

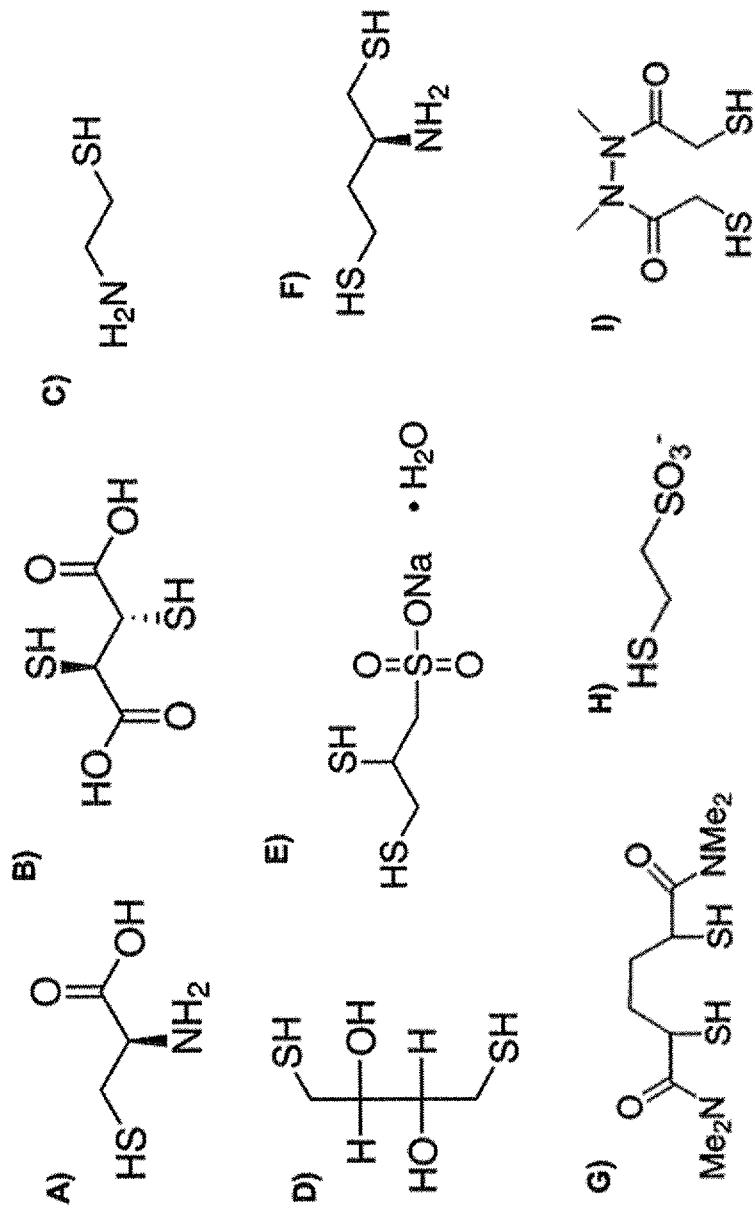


FIGURE 74

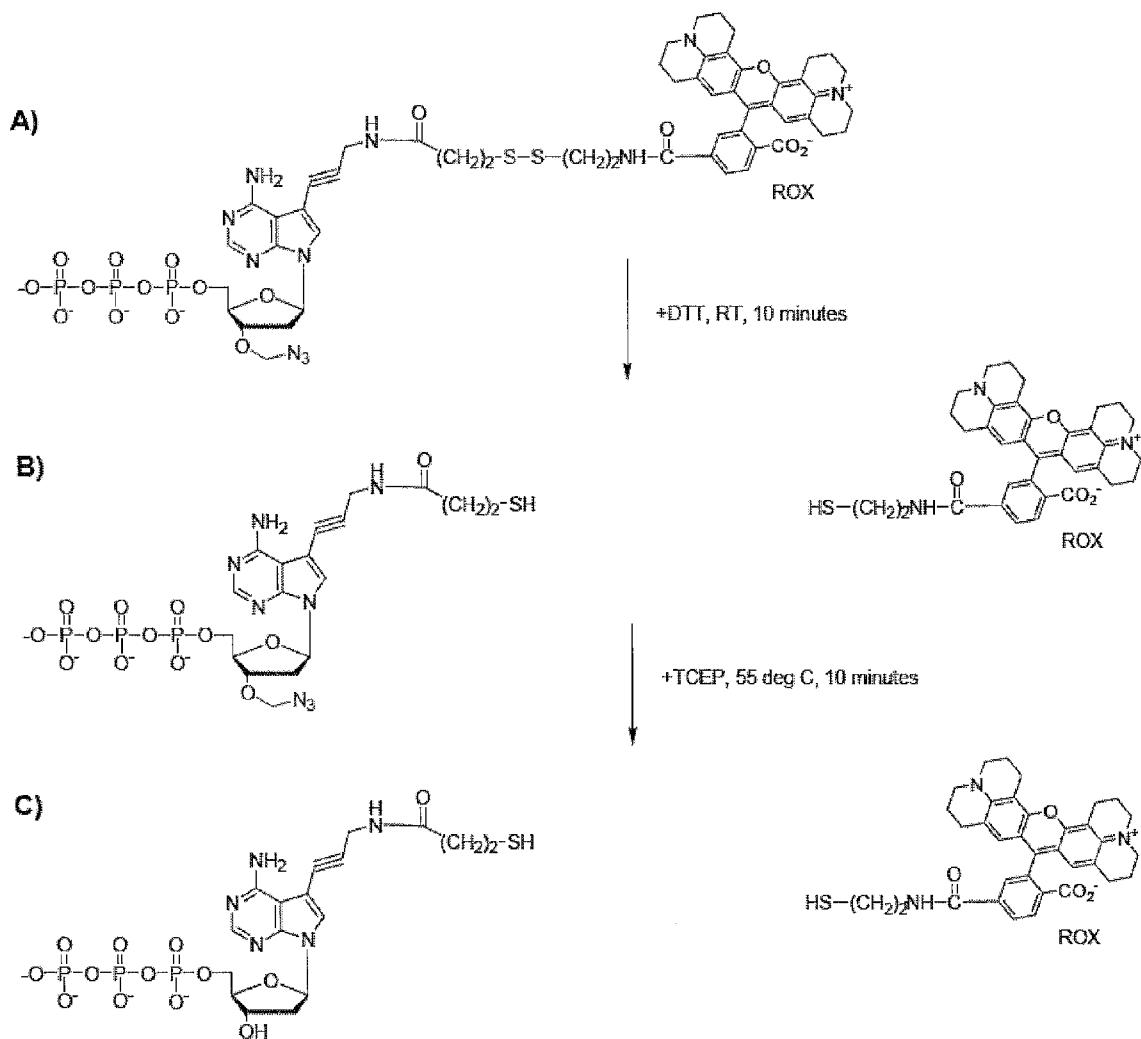
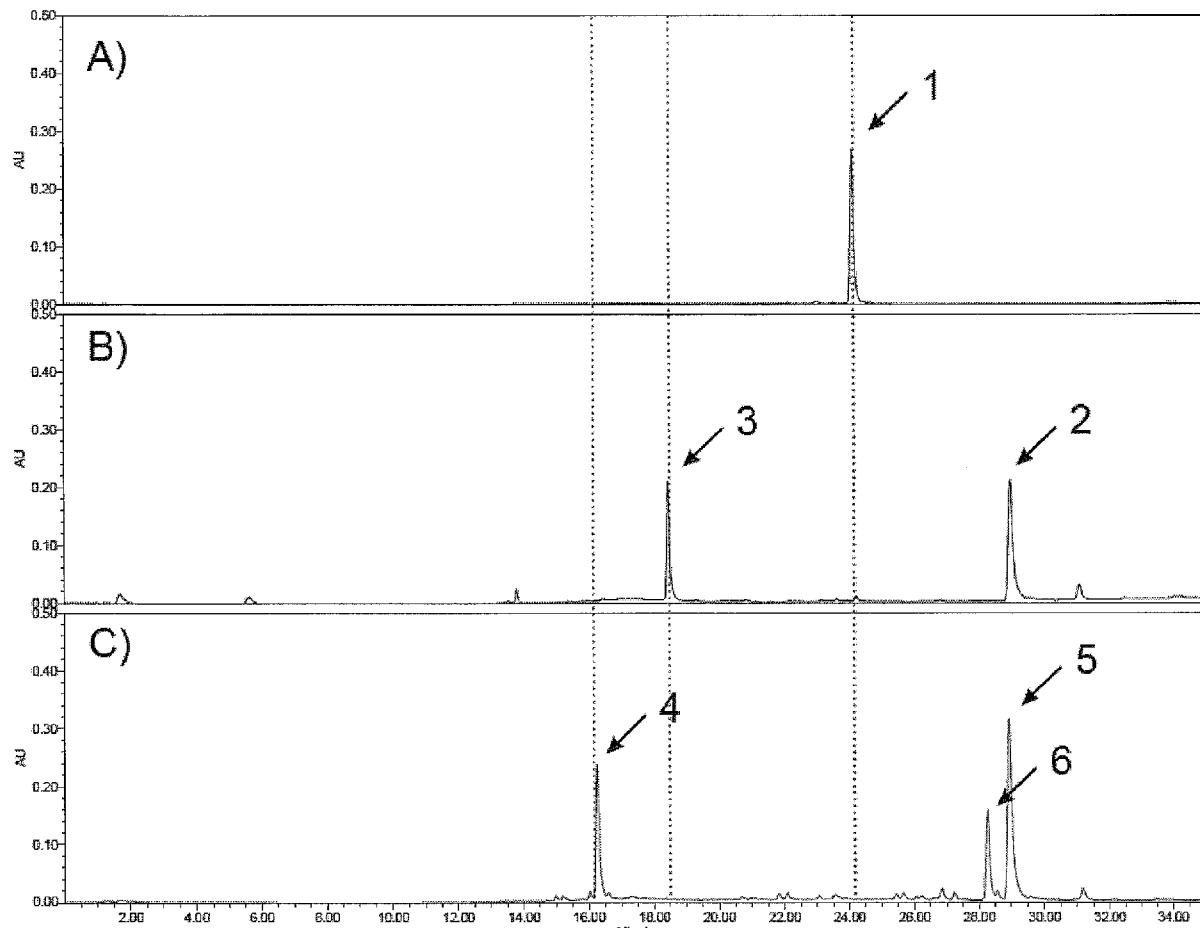


FIGURE 75



70/72

FIGURE 76

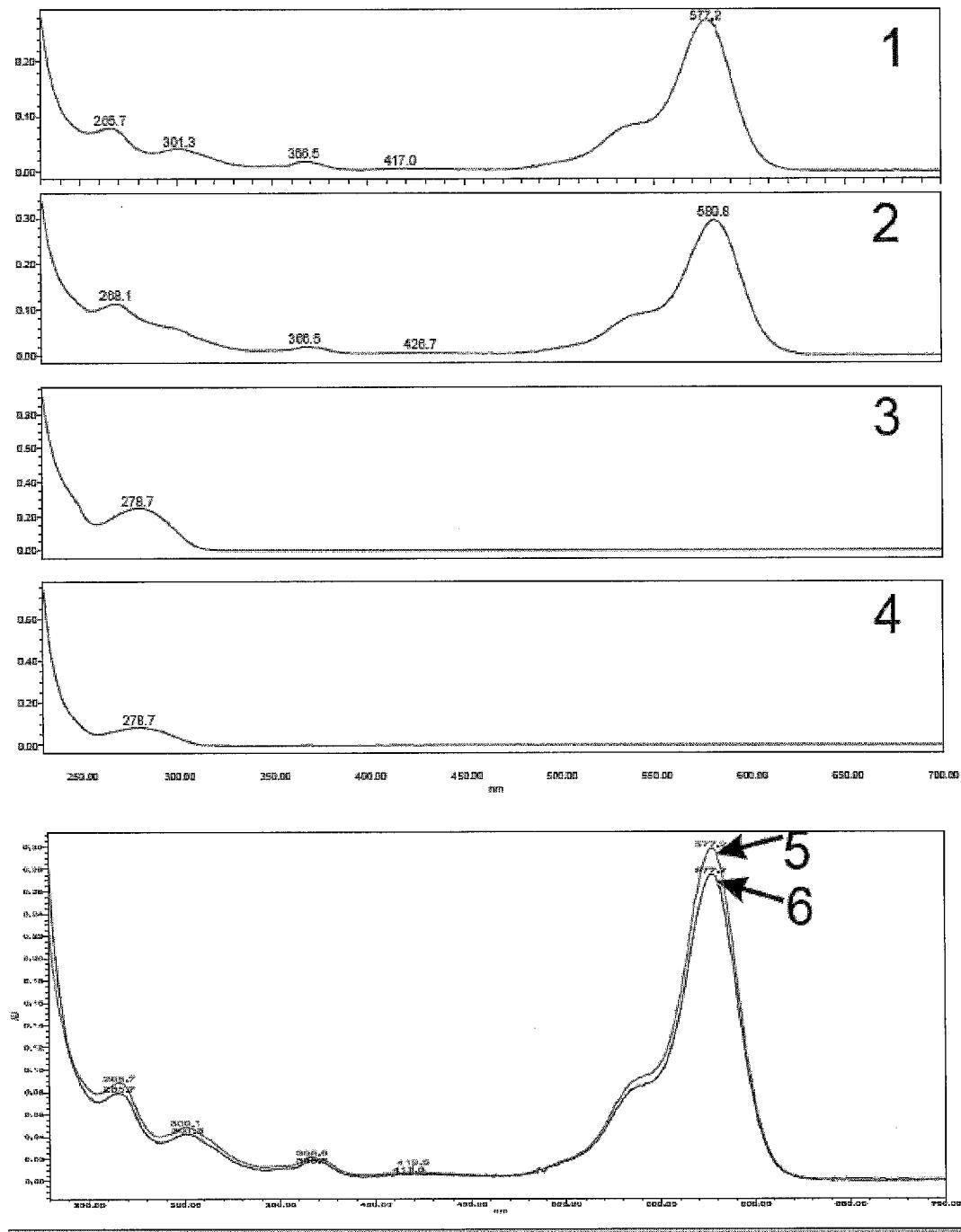


FIGURE 77

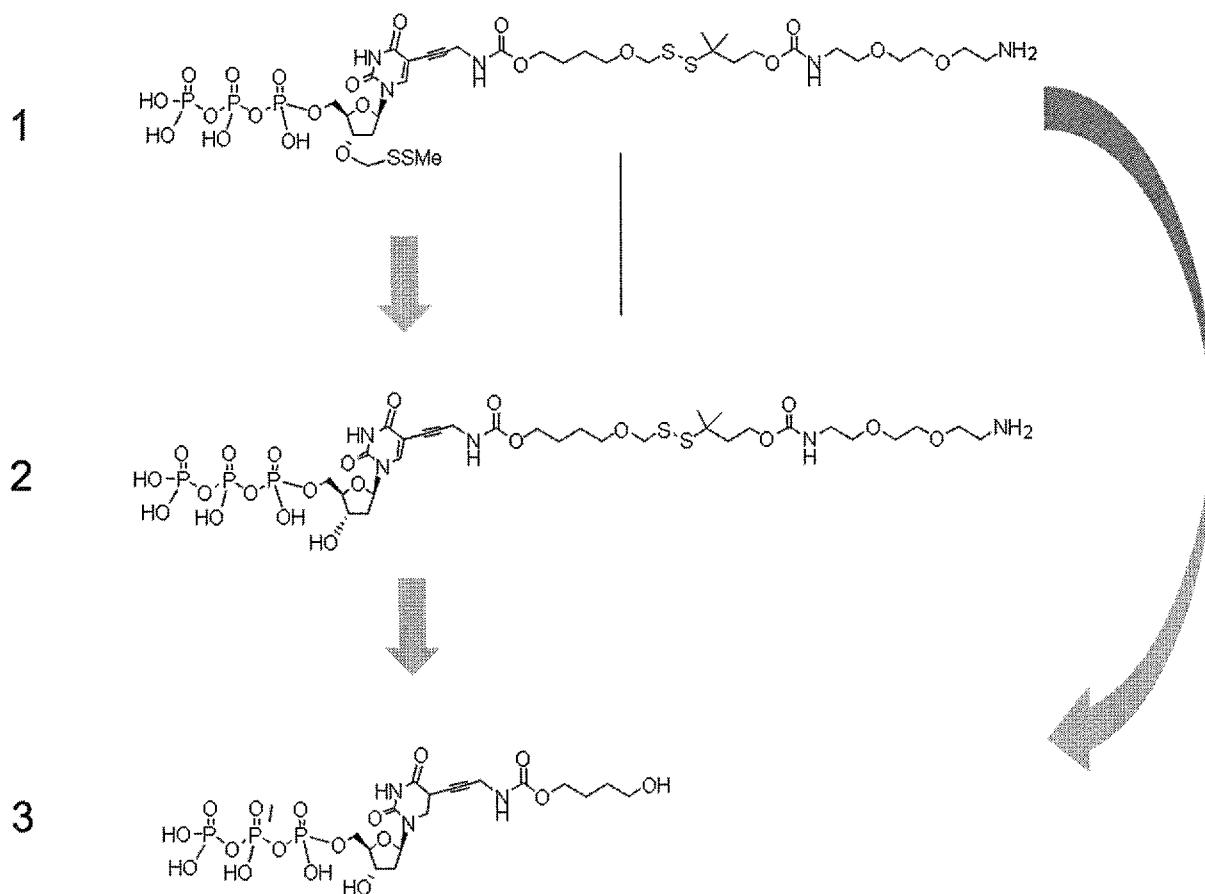
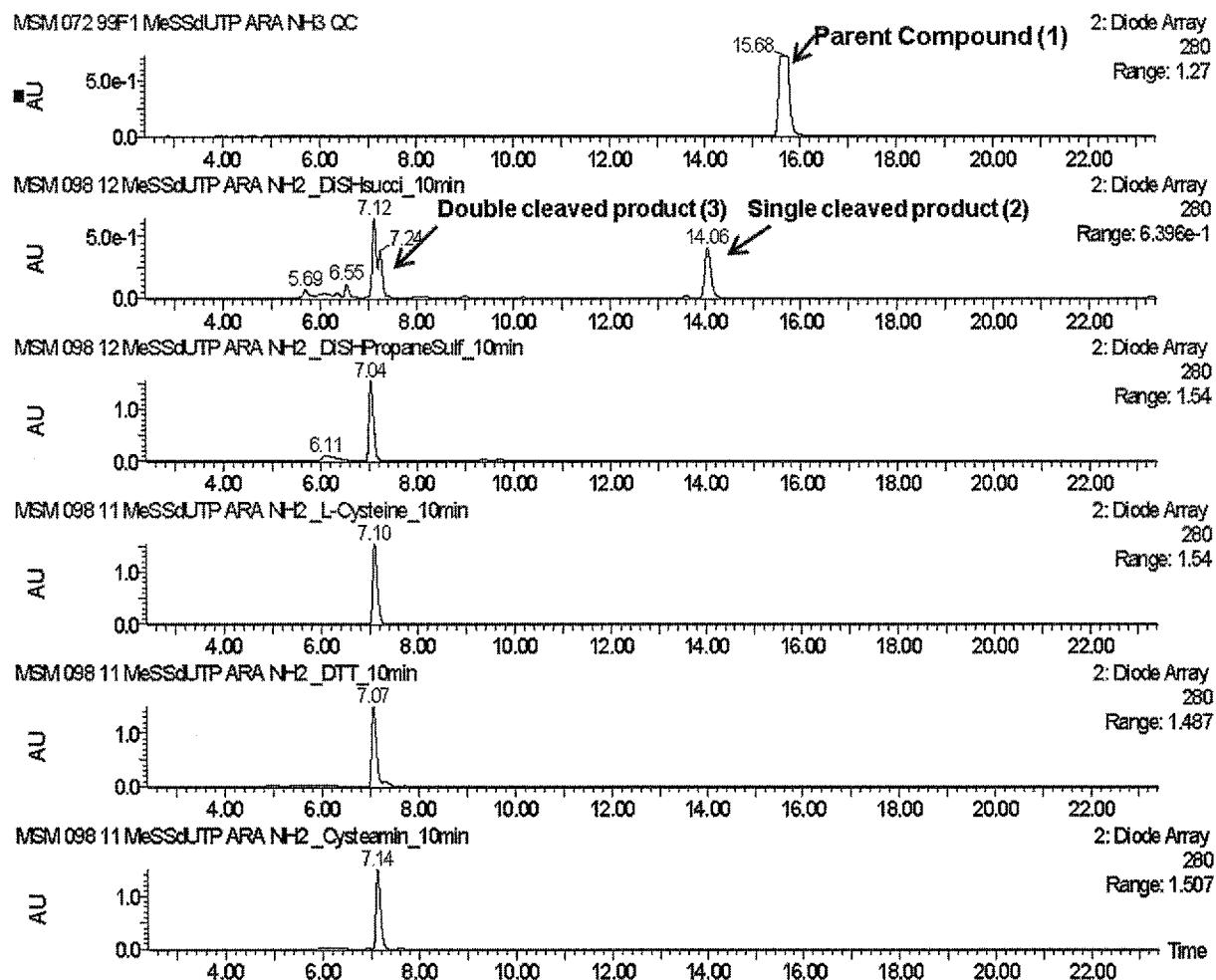


FIGURE 78

A)



B)

Cleaving Agent	Reaction Conditions	Time & Temperature	Compound 1 [%]	Compound 2 [%]	Compound 3 [%]
Dithio-succinic acid	Nucleotide – 0.5 mM, cleave agent - 20 mM, buffer: 200 mM TE, pH 8.5	10 min @ 65 degree C	0	~50	~50
L-Cysteine			0	0	100
DTT			0	0	100
Cysteamine			0	0	100