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(54) **Trifoszforilezett oligonukleotidok tisztítása affinitás-markerek alkalmazásával**

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(54) **PURIFICATION OF TRIPHOSPHORYLATED OLIGONUCLEOTIDES USING CAPTURE TAGS**

AUFREINIGUNG VON TRIPHOSPHORYLIERTEN OLIGONUKLEOTIDEN MIT EINFANG-TAGS

PURIFICATION D'OLIGONUCLÉOTIDES TRIPHOSPHORYLÉS EN UTILISANT DES MARQUAGES DE CAPTURE

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EP 2 691 410 B1

Description

[0001] The present invention relates to a method of preparing triphosphate-modified oligonucleotides using a capture tag, as defined in the claims. The method allows the synthesis and purification of triphosphate-modified oligonucleotides in high yield and purity suitable for pharmaceutical applications.

Background of the invention

[0002] Schlee et al., Immunity, 2009, 31, 25-34 describe blunt-ended double stranded RNAs carrying a 5'-O-triphosphate moiety on one of the strands that act as potent stimulators of the immune system by binding the RIG-I helicase. Thus, there is a need to provide a simple and efficient method for preparing triphosphate-modified oligonucleotides in high purity, suitable for pharmaceutical applications.

[0003] The coupling of triphosphate groups or analogues thereof to the 5'-OH group of nucleosidic compounds is well known in the art. Ludwig J. et al., J. Org. Chem., 1989, 54, 631-635 disclose a solution triphosphorylation method for preparing 5'-O-triphosphates of nucleosides and analogues using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one as the phosphitylating agent. Gaur R.K. et al., 1992, Tetrahedron Letters, 33, 3301-3304 describe the use of said method on solid-phase for the synthesis of 2'-O-methylribonucleoside 5'-O-triphosphates and their P_{α}-thio analogues. US-Patent 6,900,308 B2 discloses the solid-phase synthesis of modified nucleoside 5'-O-triphosphates as potential antiviral compounds and US-Patents 7,285,658, 7,598,230 and 7,807,653 disclose triphosphate analogues of nucleosides with modifications in the sugar, nucleobase and in the triphosphate entity. WO96/40159 describes a method for producing capped RNA or RNA analogue molecules, wherein an RNA or RNA analogue oligonucleotide is reacted with a phosphitylating agent such as 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one or a ring-substituted derivative thereof. The resulting intermediate is reacted with a phosphate or pyrophosphate or salt thereof, oxidized or hydrolyzed. The di- or triphosphorylated RNA or RNA analogue is capped by reacting with an activated m^7G tri-, di- or monophosphate or analogue.

[0004] WO 2009/060281 describes immune stimulatory oligoribonucleotide analogues containing modified oligophosphate moieties and methods for the preparation of such compounds. This method includes the synthesis of the oligonucleotide on a solid support, reacting a nucleotide at a 5'-end of the oligonucleotide with a phosphitylating agent such as 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in a suitable solvent and in the presence of a base, reacting the phosphitylated oligonucleotide with a pyrophosphate or pyrophosphate analogue, oxidizing the oligonucleotide with an oxidizing agent and deprotecting the oligonucleotide to give a triphosphate- or triphosphate analogue-modified oligonucleotide.

[0005] Polyacrylamide gel-electrophoresis as employed in WO 96/40159 is applicable only for small scale separations. The resolution power of ion exchange chromatography for 5'-mono-, di-, triphosphorylated products of longer oligoribonucleotides is limited. The required denaturing conditions make separation a tedious task (Sproat, 1999; Zlatev et al., Organic Latters, 2010, vol. 12(10), 2190-2193; WO 2009/060281), moreover, products are usually contaminated with n-1, n-2 sequences and their mono- and diphosphates resulting in insufficient purity. Given the sensitivity for precise terminal structures of the RIG-I ligands, these purification methods are suboptimal for pharmacological applications.

[0006] Dual targeting strategies (siRNA and RIG ligand) require general sequence independent purification methods.

Summary of the invention

[0007] The invention is defined in the appended claims.

[0008] It is highly desirable to produce 5'-O-triphosphorylated oligonucleotides and their analogues in large scale for potential clinical use, and a convenient preparation method would be highly desirable. In the present application it is shown that the 5'-O-cyclotriphosphate intermediate of a solid-phase bound fully protected oligonucleotide (see Figure 1) can be ring opened with a capture tag, e.g. decylamine to give a linear P_{γ} tagged species that is stable to the deprotection of the RNA. The nature of the tag is such as to impart a specific retention of the capture tagged triphosphate species on a capture tag specific reagent, enabling easy separation from the impurities that do not contain the tag. The tag can be subsequently removed if desired. The method can be extended to encompass analogues of the triphosphate moietity, e.g. analogues containing for instance β,γ-methylene, fluoromethylene, difluoromethylene and imino groups replacing an oxygen atom.

[0009] Advantages of the capture tagging method are simple purification and improved recovery of the desired species, e.g. at room temperature by RP-HPLC or affinity chromatography, optionally followed by cleavage of the capture tag under suitable conditions.

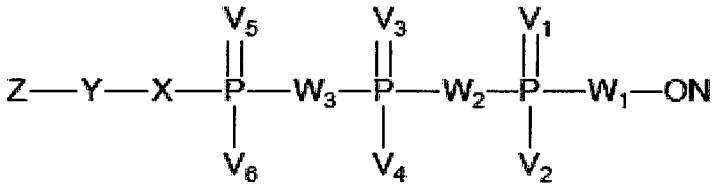
Detailed description of the preferred embodiments

[0010] The present invention describes the synthesis and purification of oligonucleotide triphosphates, including an-

alogues thereof that contain capture tags, as defined in the claims. The most widely employed method for the HPLC purification of standard 5'-OH oligonucelotides is reversed phase chromatography of trityl-ON oligonucleotides.

[0011] The method described in this invention offers a practical solution with similar efficacy for 5'-triphosphorylated oligonucleotides.

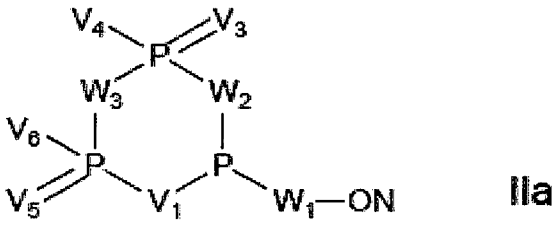
[0012] Thus, a subject-matter of the present invention is a method of preparing an oligonucleotide of formula (I),



I

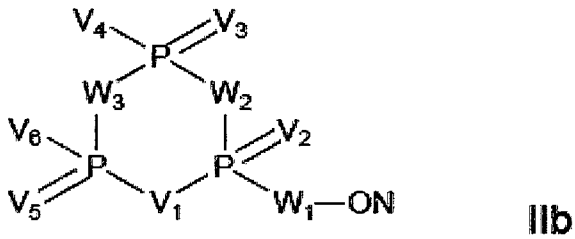
wherein V_1, V_3 and V_5 are independently in each case selected from O, S and Se;
V_2, V_4 and V_6 are independently in each case selected from OH, OR^1, SH, SR^1, F, NH_2, NHR^1, $N(R^1)_2$ and $BH_3^-M^+$,
W_1 is O or S,
W_2 is O, S, NH or NR^2,
W_3 is O, S, NH, NR^2, CH_2, CHHal or $C(Hal)_2$,
R^1, R^2 and R^3 are selected from C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{2-6} acyl or 1a cyclic group, each optionally substituted,
or wherein two R^1 may form a ring together with an N-atom bound thereto,
M^+ is a cation,
X is NH, NR^3, O or S,
Z represents a capture tag, which is a C_{8-24} alkyl residue, a perfluoroalkyl entity, an azide or an alkynyl group,
Y represents a bond or a linker connecting the capture tag to X, and
ON represents an oligonucleotide comprising at least 4 nucleotide or nucleotide analogue building blocks,
comprising the steps:

(a) reacting a compound of formula (IIa)



IIa

wherein V_1, V_3, V_5, V_4, V_6, W_1, W_2, W_3, and ON are as defined above, with an oxidizing agent to obtain a compound of formula (IIb)



IIb

wherein V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 and ON are as defined above,

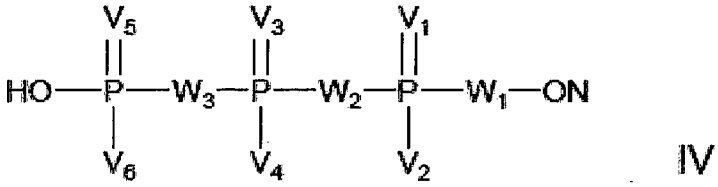
(b) reacting the oxidized compound with a capture tag agent of formula (III),

Z-Y-XH (III)

wherein X, Z, and Y are as described above to obtain a reaction product comprising the oligonucleotide of formula (I), and

(c) contacting the reaction product of step (b) with a reagent capable of interacting with the capture tag wherein the capture reagent is selected from the group consisting of a solid phase of a standard reverse phase chromatography such as RP-HPLC, a chromatographic material with affinity for hydrophobic groups, a chromatographic material with affinity for fluorinated groups such as a fluorous affinity support, a capture reagent containing an alkynyl moiety, and a capture reagent containing an azido moiety, wherein the contacting takes place under conditions which allow separation of the oligonucleotide (I) from other species contained in said reaction product.

[0013] Optionally, the method further comprises the step (d) removing the capture tag to obtain an oligonucleotide of formula (IV),



IV

wherein V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 and ON are as described above. This step is carried out under conditions which do not cause degradation of the triphosphate moiety, e.g. as described in detail below.

[0014] In further embodiments, the capture tag is not or not completely removed. In these embodiments, the tagged oligonucleotide as such may have utility, e.g. utility as pharmaceutical agent.

[0015] The term "oligonucleotide" in the context of the present application encompasses compounds comprising a plurality, e.g. at least 4 nucleotide or nucleotide analogue building blocks. Preferably, the oligonucleotide comprises 6-100, e.g. 20-40 building blocks. The nucleotide or nucleotide analogue building blocks may comprise nucleoside or nucleoside analogue subunits connected by inter-subunit linkages. The nucleoside subunits include deoxyribonucleoside subunits, ribonucleoside subunits and/or analogues thereof, particularly sugar- and/or nucleobase-modified nucleoside analogues. Further, the oligonucleotides may comprise non-nucleotidic building blocks and/or further terminal and/or side-chain modifications.

[0016] In preferred sugar-modified subunits the 2'-OH of a ribonucleoside subunit is replaced by a group selected from OR, R, halo, SH, SR, NH_2, NHR, NR_2 or CN, wherein R is C_{1-6} alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl and halo is F, Cl, Br or I. In further preferred sugar-modified subunits, the ribose may be substituted, e.g. by another sugar, for example a pentose such as arabinose. This sugar modification may be combined with 2'-OH modifications as described above, such as in 2'-fluoroarabinonucleoside subunits. Still further preferred sugar-modified subunits include locked nucleosides (LNA) or 2',3'-seco-nucleosides (UNA). In preferred nucleobase-modified nucleosidic building blocks, a non-standard, e.g. non-naturally occurring nucleobase, is used instead of a standard nucleobase. Examples of non-standard nucleobases are uracils or cytosines modified at the 5-position, e.g. 5-(2-amino)propyl uracil or 5-bromouracil; hypoxanthine; 2,6-diaminopurine; adenines or guanines modified at the 8-position, e.g. 8-bromoguanine; deazanucleosides, e.g. 7-deazaguanine or 7-deazaadenine; or O- and N-alkylated nucleobases, e.g. N^6-methyladenine, or N^6,N^6-dimethyladenine. Further suitable nucleobase analogues may be selected from universal nucleobase analogues such as 5-nitroindole.

[0017] The inter-subunit linkage between subunits may be a phosphodiester linkage or a modified linkage, e.g. a phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, boranophosphate, or another modified linkage known to a skilled person in the art.

[0018] The oligonucleotide may be selected from deoxyribonucleotides, ribonucleotides and oligonucleotide analogues. Analogues of desoxyribonucleotides or ribonucleotides may comprise at least one desoxyribonucleoside or ribonucleoside subunit and at least one modified nucleosidic subunit and/or at least one modified inter-subunit linkage, e.g. as described above. Oligonucleotide analogues may also consist in their entirety of modified nucleosidic subunits.

[0019] The oligonucleotide may be a single-stranded molecule or a double-stranded molecule. Double-stranded oligonucleotides may comprise completely or partially complementary strands. Double-stranded molecules may be blunt-ended or comprise at least one overhang, e.g. a 5'- or 3'-overhang. Overhangs, if present, are preferably located at the distal end of the molecule (with regard to the triphosphate/triphosphate analogue group). Double-stranded oligonucleotides may also comprise a hairpin-structure, wherein the duplex is closed by a loop at the distal end thereof (with regard to the triphosphate/triphosphate analogue group). The loop may comprise nucleotide and/or non-nucleotide building blocks, for example diol-based building blocks such as ethylene glycol moieties, e.g. tri(ethylene)glycol or hexa(ethylene)glycol; propane-1,3-diol; dodecane-1,12-diol; or 3,12-dioxa-7,8-dithiatetradecane-1,14-diol.

[0020] In a preferred embodiment, double-stranded molecules are blunt-ended, particularly at the proximal end thereof (with regard to the triphosphate/triphosphate analogue group).

[0021] The oligonucleotide may comprise further terminal and/or side-chain modifications, e.g. cell specific targeting entities covalently attached thereto. Those entities may promote cellular or cell-specific uptake and include, for example lipids, vitamins, hormones, peptides, oligosaccharides and analogues thereof. Targeting entities may e.g. be attached to modified nucleobases or non-nucleotidic building blocks by methods known to the skilled person.

[0022] The oligonucleotide of formula (I) or (IV) comprises a triphosphate/triphosphate analogue group. In this group, V_1, V_3 and V_5 are independently selected from O, S and Se. Preferably, V_1, V_3 and V_5 are O. V_2, V_4 and V_6 are in each case independently selected from OH, OR^1, SH, SR^1, F, NH_2, NHR^1, $N(R^1)_2$ and $BH_3^-M^+$. Preferably, V_2, V_4 and V_6 are OH. R^1 may be C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{2-6} acyl or a cyclic group, e.g. a C_{3-8} cyclo(hetero)alkyl group, a C_{3-8} cyclo(hetero)alkenyl group, phenyl or C_{5-6} heteroaryl group, wherein heteroatoms are selected from N, O and S. Further, two R^1 may form a ring, e.g. a 5- or 6-membered ring together with an N-atom bound thereto. R^1 may also comprise substituents such as halo, e.g. F, Cl, Br or I, O(halo)C_{1-2} alkyl and - in the case of cyclic groups - (halo)C_{1-2} alkyl. M^+ may be an inorganic or organic cation, e.g. an alkali metal cation or an ammonium or amine cation.

[0023] W_1 may be O or S. Preferably, W_1 is O. W_2 may be O, S, NH or NR^2. Preferably, W_2 is O. W_3 may be O, S, NH, NR^2, CH_2, CHHal or $C(Hal)_2$. Preferably, W_3 is O, CH_2 or CF_2. R^2 may be selected from groups as described for R^1 above. Hal may be F, Cl, Br or I.

[0024] The triphosphate/triphosphate analogue group is preferably attached to a terminus of the oligonucleotide. Preferably, the group is attached to the 5'-terminus of the oligonucleotide, particularly to the 5'-OH-group of the 5'-terminal sugar thereof.

[0025] Step (a) of the method of the invention comprises the reaction of cyclic P(V)-P(V)-P(III) species of formula (IIa) with an oxidizing agent. The compound of formula (IIa) may be obtained according to standard methods as described by Ludwig et al, 1989, supra and Gaur et al., 1992, supra, namely by reacting the 5'-terminal OH-group of an oligonucleotide with a trifunctional phosphitylating agent, e.g. 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one under suitable conditions, e.g. in the presence of base (pyridine or diisopropylmethylamine) in a suitable solvent such as dioxane or dichloromethane, and subsequent reaction with pyrophosphate (W_3=O) or a modified pyrophosphate (W_3 is different from O, e.g. CH_2, CCl_2, NH or CF_2). Preferably, a tri-n-butylammonium salt of the pyrophosphate or modified pyrophosphate in DMF is used. The resulting cyclic P(III)-P(V) intermediate (IIa) is then oxidized under anhydrous conditions, e.g. with a peroxide, such as t-butyl hydroperoxide, cumene hydroperoxide, (10-camphorsulfonyl)oxaziridine. Alternatively, phenylacetyldisulfide (V_2=S), or borane-diisopropylethylamine complex (V_2=BH_3) can also be employed respectively, to give the corresponding cyclic 5'-triphosphate/triphosphate analogue of formula (IIb). Reference in this context is also made to WO 96/40159 or WO 2009/060281.

[0026] Reaction step (a) may take place with an oligonucleotide in solution or with an oligonucleotide bound to a solid phase, e.g. an organic resin or glass, such as CPG. The oligonucleotide may further comprise protecting groups, e.g. sugar- or nucleobase protecting groups that are well known to the skilled person. Preferred examples of protecting groups are 2-cyanoethyl for the internucleoside phosphodiester or phosphorothioate, tert-butyldimethylsilyl, triisopropylsilyloxymethyl or bis(acetoxyethoxy)methyl for the ribose 2'-hydroxyl group, 4-t-butylphenoxyacetyl or phenoxyacetyl, acetyl, isobutyryl, benzoyl for the exocyclic amino groups of the nucleobases. More preferably, step (a) is carried out with a solid-phase bound oligonucleotide.

[0027] According to step (b) of the method of the invention, compound (IIb) is reacted with a capture tag agent of formula (III)

Z-Y-XH (III)

wherein X is a group selected from NH, NR^3, O or S. R^3 is defined as described above for R^1. Preferably, X is NH or S.

[0028] The capture tag is functionally defined below by a series of plausible Examples. A general rule may be:

Z has to allow a convenient purification, and it should be removable under conditions which are compatible with pppRNA stability requirements.

Y represents a chemical bond or a linker, e.g. an alkylene, preferably a C_{1-6}-alkylene linker, more preferably a C_{2-5}-alkylene linker, or aralkylene linker, optionally comprising heteroatoms or heteroatom-containing groups, such as O, S, NH, C=O or C=S, and/or optionally comprising C=C or C≡C bonds.

[0029] In another preferred embodiment the linker is a polyalkylene oxide, preferably a poly-C_2-C_6-alkylene oxide, more preferably a poly-C_2-C_3-alkylene oxide. The number average molecular weight of the linker may be in the range from 30-800 g/mol, preferably from 40-450 g/mol, more preferably from 40-250 g/mol. The linker may be $[-CH_2CHR^4-O-]_n$ with n = 1-10, preferably n = 1-7, more preferably n = 2-5, and even more preferably n = 3. R^4 may be H or C_{1-6}-alkyl.

[0030] In a preferred embodiment R^4 is H.

[0031] In an especially preferred embodiment the linker has the formula $-CH_2-CH_2-[(O-CH_2CH_2)]_3-$.

[0032] Reaction step (b) may take place with an oligonucleotide in solution or with an oligonucleotide bound to a solid phase, e.g. an organic resin or glass. The oligonucleotide may further comprise protecting groups as described above. More preferably, step (b) is carried out with a solid phase-bound oligonucleotide.

[0033] The capture tag Z according to the present invention is a moiety as defined in the claims, capable of non-covalently or covalently interacting with a capture reagent under conditions which allow separation for compounds comprising the capture tag, e.g. the oligonucleotide (I) from other species, which do not contain the capture tag. Preferably, the capture reagent is an immobilized reagent or a reagent capable of being immobilized, as defined in the claims.

[0034] Suitable capture tags are for instance long-chain, e.g. C_{8-24}, preferably C_{13-24} aliphatic alkyl residues such as decyl or octadecyl or other lipidic/lipophilic residues such as e.g. cholesteryl or tocopheryl. In this case, the tagged triphosphate entity can be captured and purified on a solid phase by standard reversed phase chromatography, e.g. RP-HPLC, or by hydrophobic interaction chromatography (HIC). The capture tag may also be a perfluoroalkyl entity, e.g. a 4-(1H,1H,2H,2H-perfluorodecyl)benzyl or a 3-(perfluorooctyl)propyl residue for specific capture of the modified oligo-triphosphate on a Fluorous Affinity support such as is commercially available from Fluorous Technologies, Inc.

[0035] The capture tag other than Z may be a first partner of a non-covalent high-affinity binding pair, such as biotin, or a biotin analogue such as desthiobiotin, a hapten or an antigen, which has a high affinity (e.g. binding constant of 10^{-6} l/mol or less) with the capture reagent, which is a second complementary partner of the high-affinity binding pair, e.g. a streptavidin, an avidin or an antibody.

[0036] In yet another embodiment, the capture tag may be a first partner of a covalent binding pair, which may form a covalent bond with the capture reagent, which is a second complementary partner of the covalent binding pair, wherein the covalent bond may be a reversible or an irreversible bond. In this embodiment, the capture tag component Z may be a reactive chemical entity such as an azide or alkynyl group enabling covalent reaction with a capture reagent that contains a complementary reactive group, e.g. an alkynyl or azido moiety, respectively, in the case of the Husigen 3+2 cycloaddition reaction (the so-called "click-reaction" that is Cu(I) catalyzed or a variant thereof that proceeds without Cu(I) ions via release of severe ring strain in e.g. cyclooctyne derivatives). A specific example for Z-Y-X in such a case would be propargylamino.

[0037] The capture tag component other than Z may be a chemical entity which contains an additional nucleophilic group, for instance a second amino group in an NH_2-Y-XH type reagent. A wide range of suitable electrophilic reagent such as cholesterol, chloroformiate or biotin N-hydroxy succinimide active esters may then be used to introduce the tagging group while the oligonucleotide is attached to the solid phase, thus significantly extending the scope of the tagging reaction.

[0038] In the present invention, the capture tag Z is a long-chain alkyl residue, a perfluoroalkyl entity, an azide or an alkynyl group.

[0039] Moreover, Y may optionally contain a disulfide bond to enable recovery of the modified triphosphorylated oligonucleotide with a free sulfhydryl moiety connected via part of the linker through X to the γ-phosphorus.

[0040] In a further embodiment of the present invention, the oligonucleotide may carry a second capture tag at a different position, e.g. at the 3'-terminus. The first and the second capture tags are preferably selected as to allow purification by two orthogonal methods to enable recovery of extremely high purity material. For example the first capture tag may be a lipophilic group, which interacts with a suitable chromatographic support and the second capture tag may be biotin, which interacts with streptavidin.

[0041] The second capture tag may be conveniently introduced by performing the synthesis using a modified CPG (controlled glass support) for oligoribonucleotide synthesis.

[0042] Step (c) of the method of the present invention comprises contacting the reaction product of step (b), with a capture reagent capable of interacting with the capture tag Z under conditions which allow separation of the capture tag containing oligonucleotide (I) from other species contained in the reaction product. Before step (c), the solid phase bound oligonucleotide (I) is cleaved from the solid phase and deprotected, i.e. the protection groups are partially or completely removed. The capture reagent is preferably immobilized on a suitable support, e.g. a chromatographic support. In order to provide separation of capture tag containing oligonucleotide (I) from non-capture tag-containing species, the reaction products from step (b) are cleaved from a solid phase and deprotected, if necessary, and subjected to a separation procedure, preferably a chromatographic separation procedure based on the interaction of the capture tag Z with the capture reagent. During the separation step, the purity of the oligonucleotide (I), which is generally in the range of 25-70% for the crude material depending upon the length and complexity of the sequence, may be increased to 90%, 91%, 92%, 93%, 94%, 95% or more. For toxicity studies a purity of > 85% is desirable, whereas in late stage clinical trials the purity should be in the range of at least 90-95%. Thus, the present invention provides a way to obtain a high purity pppRNA as would be required for human clinical trials.

[0043] In step (c), the capture tag and the capture reagent capable of interacting therewith are preferably selected from (i) a hydrophobic or fluorinated group and a chromatographic material with affinity for hydrophobic or fluorinated

groups, e.g. a reversed phase material or a fluorous affinity support; (ii) a first partner of a non-covalent high-affinity binding pair and a second complementary partner of a non-covalent high-affinity binding pair, (iii) a first partner of a covalent binding pair and a second complementary partner of a covalent binding pair, where the first and second partner form covalent bonds.

[0044] After the purification step (c), capture tag Z may be cleaved from the triphosphate-modified oligonucleotide in a further step (d) resulting in an untagged oligonucleotide (IV).

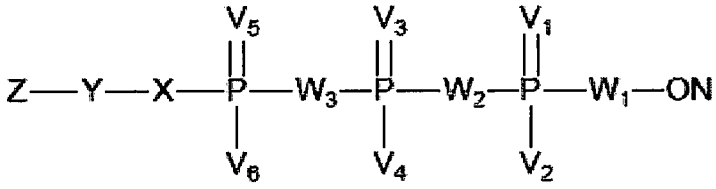
[0045] Step (d) has to be compatible with stability requirements of the triphosphate end product and with stability requirements of the interribonucleotide bond. It may comprise cleavage by mildly acidic conditions when X is NH, cleavage with silver ions when X is S, cleavage by a thiol such as dithiothreitol leading to elimination of thiirane when Y-X-P contains -S-S-CH_2-CH_2-O-P.

[0046] In further embodiments, the capture tag set remains completely or partially on the triphosphate-modified oligonucleotide, particularly when the tagged oligonucleotide is suitable for pharmaceutical applications. In these embodiments, the reagent Z-Y-XH has to be selected from a subgroup of Z-residues, which are functionally compatible with the structural requirements of the RIG-I sensor. For instance, the Z=decyl-octadecyl, Y=link XH=NH combination is known to fulfill these requirements.

[0047] The triphosphate/triphosphate analogue modified oligonucleotides produced according to the present invention are particularly suitable for pharmaceutical applications due to their high purity. In an especially preferred embodiment, the oligonucleotide (I) or (IV) is an activator of RIG-1 helicase. Specific examples of suitable RIG-1 activators are disclosed in Schlee et al., 2009, supra.

[0048] Further disclosed are oligonucleotides of Formula (I), obtainable by a method according to the present invention.

[0049] Still another subject-matter of the invention is the use of a kit for preparing an oligonucleotide of formula (I)



I

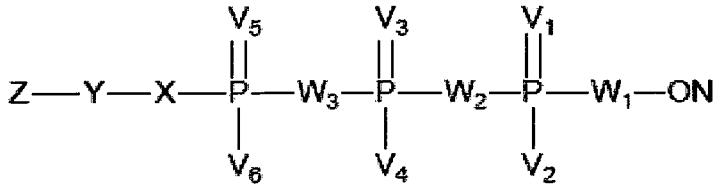
wherein V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3, X, Y, Z and ON are defined as above,
wherein the kit comprises (a) a capture tag agent of formula (III)

Z-Y-XH (III)

wherein X, Z and Y are defined as above, and

(b) a capture reagent capable of interacting with the capture tag, as defined in the claims.

[0050] Further disclosed is a modified oligonucelotide of formula (I)



I

wherein

X is NH, O, R-O-$[P(V_1)V_2$-$W_1]_n$ or R-O-$P(V_3)V_4$-W_2-P-$(V_1)V_2$-W_1,
n is 1-12, preferably 1 or 2,
Y is a bond,
Z is C_{13}-C_{24} alkyl, Q or $QNHC_2$-C_{24} alkyl,
Q is selected from H, aminoacids, aminoacid analogues, C_1-C_{24} alkyl, preferably C_{12}-C_{24} alkyl, peptides and lipids,
R is C_1-C_{24} alkyl, C_2-C_{24} alkenyl, C_2-C_{24} alkynyl and lipids,
R is C_1-C_{24} alkyl, C_2-C_{24} alkenyl, C_2-C_{24} alkynyl, C_2-C_{24} acyl or a cyclic group, and optionally substituted,

and V_1, V_2, V_3, V_4, V_5, V_6, W_1, W_2, W_3 and ON are defined as above, wherein V_1, V_2, V_3, V_4, V_5, V_6, W_1, W_2 and W_3 are preferably O.

[0051] According to a preferred embodiment a modified oligonucleotide of formula (I) has X being NH. This embodiment preferably has Z being Q or Z being $QNHC_2$-C_{24} alkyl, wherein in a particularly preferred embodiment C_2-C_{24} alkyl is C_2 alkyl and/or Q is H. Particularly preferred embodiments of the identified oligonucleotide according to the invention are shown in Fig. 8.

[0052] Further, the present invention shall be explained in more detail by the following Figures and Examples.

Fig. 1 shows a schematic overview of the method of the invention using a decyl residue as capture tag Z

Fig. 2 shows RP-HPLC purification of pppRNA via n-decyl-NH-pppRNA intermediate

(A) crude reaction mixture containing 65 % n-decyl-NH-pppRNA (peak at 14 min);
(B) isolated n-decyl-NH-pppRNA;
(C) pppRNA; the pH=3.8 60 min hydrolysis product from B

In Fig. 2 the x-axis means time [min] and the y-axis means absorbance at 260 nm [mAu].
The broad peak at 10 min retention time in A contains the nonphosphorylated 24-mer, shorter synthesis failure sequences, the minor pppRNA hydrolysis product and the 5'-H-phosphonate derivative of the 24-mer. The insert shows the position of pppRNA and 5'-OH RNA in this system.
Column: Hamilton PRP-1 4.1 x 250 mm, 10 μm
Gradient: 1-100 % B in 18 min, A= 0.05 M TEAB ; B= 80% Methanol 0.05 M TEAB

Fig. 3 shows MALDI -TOF spectra (x-axis: mass [Da]) corresponding to HPLC traces A, B and C in Fig 2 respectively.

(A) spectrum recorded from the crude reaction mixture after desalting showing the presence of n-decyl-NH-ppp RNA (24d), pppRNA (24c), 5'-H-phosphonate RNA(24b) and 5'-OH -RNA(24a) and shorter synthesis failure sequences indicated as peaks 12-23 ;
(B) spectrum recorded from HPLC isolated n-decyl-NHpppRNA (B),
(C) spectrum of pure pppRNA as obtained from the direct EtOH precipitation of the pH= 3.8 hydrolysis product of n-decyl-NH-pppRNA

Fig. 4 shows a reaction scheme explaining the generation of side products **24 a-c**

Fig. 5 shows the time course for the conversion of n-decyl-NH-pppRNA to pppRNA via acidic hydrolysis of the phosphoramidate bond.

Fig. 6 shows typical MALDI spectra (x-axis: mass [Da]) of 21-mer, 24-mer, 27-mer pppRNA products as obtained after capture tag removal and EtOH precipitation as Na+ salt. The correct mass peak is observed at m/z 6911.6 (A), m/z 7828 (B), m/z 8808.1 (C) and the peaks at m/z 3462 (A), m/z 3918 (B), 4408 (C) are due to the doubly charged pppRNA, respectively. Similar quality spectra have been obtained in more than 50 examples with a variety of sequences containing nucleoside analogs and 3' modifications in the 15-42-mer range.

Fig. 7A shows a semipreparative scale reversed phase HPLC purification of a 1 μmol scale reaction of decyl-NHpppRNA 21 mer on a 7 mm Hamilton PRP-1 column
Column: Hamilton PRP-1 7 x 250 mm, 10 μm Flow rate 3 mL/min.
Gradient: 1-80 % B in 50 min, A= 0.1M TEAB ; B= 80% Methanol 0.1 M TEAB

Fig. 7B and **Fig. 7C** show semipreparative scale reversed phase HPLC purifications, in particular showing how the inventive method is able to deal with sub-optimal synthesis and/or 5'-phosphorylation conditions.
In all figures the x-axis is volume [ml] and the y-axis is absorbance at 260 nm [mAu].

Fig. 8 shows especially preferred modified oligonucleotides of formula (I).

Fig. 9 shows the synthesis of compounds F-TAG-pppRNA and N3-TAG-pppRNA (A) and the strategy for reversible covalent immobilisation using N3-TAG RNA (B)

Fig.10 shows MALDI spectra of F-TAG-pppRNA (A) N3-TAG-pppRNA (B)

Fig.11 shows the RP-HPLC analysis of pppRNA and n-alkyl-NH-pppRNAs with alkyl residues of increasing chain length:

A. pppRNA, RT= 9.3 min
B. n-decyl-NH-pppRNA, RT=13.8 min,
C. n-dodecyl-NH-pppRNA, RT= 15.5 min
D. n-tetradecyl-NH-pppRNA, RT=17.3 min
E. n-octadecyl-NH-pppRNA, RT=19.7 min

Example 1

Preparation of a 5'-triphosphate modified oligonucleotide using a decyl amine capture tag purification step.

[0053] An overview of the reaction scheme described in Example 1 is shown in Fig. 1.

[0054] **Step 1:** Dissolve 203 mg (1 mmol) of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in 1 mL of dry dioxane in a 10 mL septum vial under argon.

[0055] **Step 2:** Dry the synthesis column containing the fully protected RNA that has been detitrylated and thoroughly washed with acetonitrile, in vacuum for 12 h. Wash the column contents thoroughly by repeatedly drawing in and expelling 2 mL of anhydrous dioxane/pyridine solution, 3:1 (v/v) in an argon atmosphere.

[0056] **Step 3:** Add into a vial first 2 mL of pyridine/dioxane, 3:1 v/v followed by 100 μL of 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one solution in dry dioxane to give a 50 mM solution of the phosphitylating reagent, e.g. 2-chloro-4H-1,3,2-benzodioxaphosphorin-2-one, in dioxane/pyridine, 3:1 (v/v). Homogenize the solution by gently shaking. Start the reaction by drawing the 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one solution through the synthesis column from the vial.

[0057] During the reaction, repeatedly draw in and expel the 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one containing solution from the synthesis column, in order to allow thorough contact and good mixing with the solid phase supported RNA. A 30 min reaction time usually gives near quantitative reaction of the free 5'-OH group of the support bound oligomer in the 20-40 nt range.

[0058] **Step 4:** After a 30 min reaction time expel the dioxane/pyridine solution containing the excess phosphitylating agent into a waste container, fill a new syringe with a vortexed mixture of 1 mL of 0.5 M $(Bu_3NH)_2$ pyrophosphate in dry DMF and 238 μL (1 mmol) of dry Bu_3N to give a 0.5 M $(Bu_3N)_4$ pyrophosphate solution. Push this solution through the column thereby replacing the dioxane/pyridine solution. The large excess of the pyrophosphate ensures a quantitative conversion of the intermediate to the P(III)-P(V) cyclic anhydride IIa.

[0059] **Step 5:** Wash the column with 3 mL of CH_3CN to remove the DMF and excess PP_i, and to fill the column reactor with dry CH_3CN.

[0060] **Step 6:** Dissolve 300 μL of t-BuOOH (5.5 M solution in decane, Sigma-Aldrich) in 2 mL of anhydrous CH_3CN to give an approximately 0.7 M homogeneous solution. Contact the synthesis support with this solution for 15 min in order to obtain the oxidized P(V) cyclic anhydride IIb.

[0061] **Step 7:** Wash the column with 3 mL of dry CH_3CN to remove the excess peroxide and fill it with dry CH_3CN.

[0062] **Step 8:** Dissolve 300 μL of dry decylamine in 1 mL of dry CH_3CN under argon and bring the solution in contact with the support in the column. Move the decylamine solution through the support. The contact time of the CPG with the amine solution should be **3 min.**

[0063] **Step 9:** Wash the column thoroughly with 9 mL acetonitrile, then dry the column contents by flushing argon through it.

[0064] **Step 10- First stage of the deprotection:** Pass 1 mL of deprotection solution (40% aq. methylamine/conc. aq. ammonia 1:1 v/v. AMA reagent) through the support for 2-3 times. After a contact of 30 min transfer the solution into a new vial. Wash the support with same volume of AMA deprotection solution and combine the washings. Heat the combined solution and washings for 10 min at 65°C. After cooling on ice, concentrate the solution to a volume of 300-500 μL, then evaporate to dryness.

[0065] **Step 11 - Removal of the 2'-O-TBDMS protecting groups:** Dry the residue by addition and coevaporation of 300 μL of dry EtOH, add 1 mL of dry 1 M TBAF (tetra-n-butylammonium fluoride) in THF, seal tightly and put on a shaker for 16 h. Quench the reaction with 1 mL of sterile aqueous 1 M TEAB (triethylammonium bicarbonate), and desalt it on a NAP™-25 (Nucleic Acid Purification) column using sterile water as eluent. Filtration through a sterile 2 μm filter may be necessary at this step. Combine and evaporate the UV-absorbing fractions to a volume of 150 μL, add 100 mL of 1 M TEAB pH8 and store the solution frozen at -20°C until the HPLC purification can be performed. The decyl-NHpppRNA product is stable at -20°C for weeks at pH 7-8.

[0066] **Step 12 - HPLC purification:** The reaction product from an 1 μmol scale reaction mixture from step 11 was loaded into a 7x25 mm PRP-1 column (Hamilton). Purification was performed using a linear gradient buffer B from 0 to 80% in 50 min at a flow rate of 3 mL/min. Buffer A is 100 mM TEAB and buffer B is 100 mM TEAB in methanol/water 8:2 v/v. A typical example of a 27-mer purification is shown in Figure 7A.

[0067] Fractions 5 and 6 are collected, evaporated on a rotary evaporator and desalted by several coevaporations with dry methanol, The residue (approx. 200-250 nmol of decyl-NHpppRNA) was dissolved in water and transferred into a screw cap Eppendorf vial.

[0068] **Step 13 - Removal of the decylamine tag:** 100 nmol of decyl-NHpppRNA was dissolved in 400 μL of pH 3.8 deprotection buffer in a 2 mL Eppendorf tube, and the sealed tube was heated at 60°C for 70 min. These conditions result in quantitative cleavage of the phosphoramidate bond with no degradation of the triphosphate moiety. Then the reaction mixture was cooled on ice and 25 μL of sterile 5 M NaCl solution and 1.2 mL of absolute EtOH were added. After thorough mixing the solution was kept at -20°C overnight to precipitate the pppRNA. The precipitate was collected by centrifugation, washed with cold ethanol, dried on a SpeedVac, then dissolved in 500 mL of sterile water and stored frozen at -20°C.

Table 1: Summary of the reaction conditions for introduction of the 5'-terminal decyl-NHppp-residue.

1 μmol scale synthesis column containing support bound detitrylated RNA			
↔ bidirectional movements of reagents,			
→ unidirectional washing step			
Step	**Reagent**	**Time**	
1	3 mL dioxane/pyridine, 3:1 v/v	wash	→
2	50 mM 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in 2 mL of dioxane/pyridine, 3:1 v/v	30 min	←→
3	1 mL of 0.5 M $(Bu_3NH)_2PP_i$ in DMF plus 2.38 μL of Bu_3N	10 min	←→
4	3 mL of dry acetonitrile	wash	→
5	300 μL of *t*-BuOOH (5,5 M in decane) in 2 mL of CH_3CN	15 min	←→
6	3 mL of dry acetonitrile	Wash	→
7	300 μL of n-decylamine in 1 mL of dry acetonitrile (1.1 M decylamine)	3 min	←→
8	10 mL of acetonitrile	wash	→

[0069] In analogous manner, a 5'-triphosphate modified oligonucleotide was also synthesized and purified using an octadecyl or a cholesteryl capture tag.

Example 2

Preparation of triphosphate oligonucleotides using non-lipophilic capture tags

(F-TAG-pppRNA and N_3-TAG-pppRNA)

[0070] In order to demonstrate the utility of non-lipophilic interaction based purification strategies the pppRNA derivatives **F-TAG-RNA** and **N3-TAG-RNA** were prepared (see Fig.9). All steps of the synthesis are identical with the procedure described in Example 1 except that in step 8 of Fig. 1, 2 mL of a 0.1 M solution of 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-Heptadecafluoroundecylamine in anhydrous acetonitrile was used for the ring opening of the solid phase bound cyclotriphosphate with an increased 3 h reaction time to give F-TAG-RNA; and 2 mL of a 0.1 M solution of 11-azido-3,6,9-trioxaundecan-1-amine in dry acetonitrile for 3 h was used to give N_3-TAG-pppRNA. The following deprotection steps are identical with those given in the detailed description for DecNHpppRNA in Example 1.

[0071] **F-TAG-RNA** and **N3-TAG-RNA** analytical data (see Fig 10) :

(the RNA sequence in these examples is 5'-GACGCUGACCCUGAAGUUCAUCUU)

	HPLC retention time*	Calculated Mass, Da	Mass measured by MALDI, Da	Time required for complete P-N cleavage at pH 3.8 at 60°C
F-TAG-pppRNA	15.1 min	8287.74	8290.30	70 min
N3-TAG-ppRNA	11 min	8033.20	8033.92	70 min
* PRP-1 column 0-100% B in 20 min (A = 100 mM Triethylammoniumbicarbonate (TEAB), B = 100 mM TEAB 80% MeOH)				

[0072] pppRNA oligonucleotides containing fluorous tags (F-TAG-pppRNA) can be purified using commercial "fluorous" cartridges, or fluorous HPLC columns which enable the exploitation of the strong nonconvalent interaction between perfluorinated alkyl chains. The gamma azide modified pppRNA derivatives (N3-TAG-pppRNA) can be covalently bound to commercially available propyne modified solid phases by RNA compatible versions of the copper(I)-catalysed-alkyne-azide cycloaddition reaction (click chemistry). This procedure enables the purification of highly structured pppRNA sequences because in the resin bound form denaturing conditions can be applied to remove non-triphosphorylated by-products.
Upon acid hydrolysis both F-TAG-RNA and N3-TAG-RNA release the pppRNA end product with comparable kinetics to the simple P-N alkyl amide as described in Fig 5.

Example 3

Variation of the RP-HPLC elution position of Tag-pppRNA by n-alkyl capture tags of increasing chain length

[0073] Besides the n-decyl-tag described in Example 1, aliphatic n-alkyl residues with longer chain lengths (C_{12}, C_{14}, C_{18}) can be used to increase the retention time of the Tag-pppRNA product during RP-HPLC purification enabling an efficient separation from impurities that do not contain the tag. N-dodecyl-NH-pppRNA, n-tetradecyl-NH-pppRNA and n-octadecyl-NH-pppRNA can be prepared following the procedure described in example 1 by variation of step 8: A 0.1 M solution of n-alkylamine (n-dodecylamine, n-tetradecylamine or n-octadecylamine) in dry CH_2Cl_2 is prepared and 2 mL of the solution is brought in contact with the support in the column. The alkylamine solution is pushed to and fro through the support. After a contact time of 3 h an additional washing step with 2 mL of CH_2Cl_2 is required prior to continuing with the next workup steps.

Analytical data:

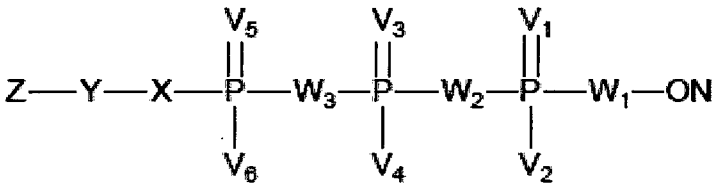
[0074]

	RP-HPLC* retention time (min)	Calculated Mass (Da)	Mass measured by MALDI (Da)	Time for complete P-N cleavage at pH 3.8 at 60°C
C_{12}-NH-pppRNA	15.5	7995.7	7999.2	70 min
C_{14}-NH-pppRNA	17.3	8023.7	8028.1	70 min
C_{18}-NH-pppRNA	19.7	8079.8	8082.2	70 min gives > 80% product
* PRP-1 column 0-100% B in 20 min (A = 100 mM Triethylammoniumbicarbonate, B = 100 mM TEAB 80% MeOH)				

[0075] **Figure 11** shows the RP-HPLC analysis of pppRNA and n-alkyl-NH-pppRNAs with alkyl residues of increasing chain length.

Claims

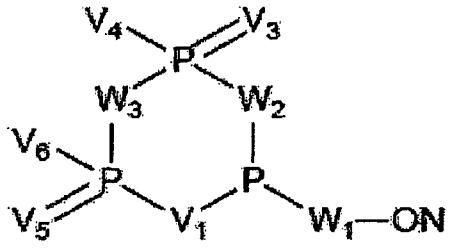
1. A method of preparing an oligonucleotide of formula (I),



I

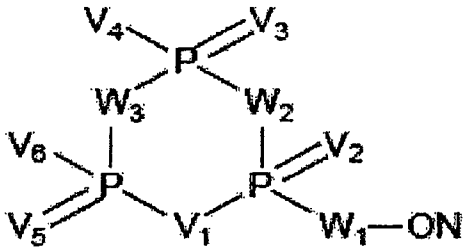
wherein V_1, V_3 and V_5 are independently in each case selected from O, S and Se;
V_2, V_4 and V_6 are independently in each case selected from OH, OR^1, SH, SR^1, F, NH_2, NHR^1, $N(R^1)_2$ and $BH_3^-M^+$,
W_1 is O or S,
W_2 is O, S, NH or NR^2,
W_3 is O, S, NH, NR^2, CH_2, CHHal or $C(Hal)_2$,
R^1, R^2 and R^3 are selected from C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{2-6} acyl or a cyclic group, each optionally substituted,
or wherein two R^1 may form a ring together with an N-atom bound thereto,
M^+ is a cation,
X is NH, NR^3, O or S,
Z represents a capture tag, which is a C_{8-24} alkyl residue, a perfluoroalkyl entity, an azide or an alkynyl group,
Y represents a bond or a linker connecting the capture tag to X, and
ON represents an oligonucleotide comprising at least 4 nucleotide or nucleotide analogue building blocks, comprising the steps:

(a) reacting a compound of formula (IIa)



IIa

wherein V_1, V_3, V_5, V_4, V_6, W_1, W_2, W_3, and ON are as defined above, with an oxidizing agent to obtain a compound of formula (IIb)



IIb

wherein V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 and ON are as defined above,
(b) reacting a compound of formula (IIb) with a capture tag agent of formula (III),

Z-Y-XH (III)

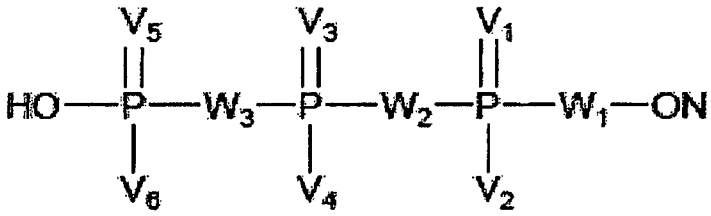
wherein X, Z, and Y are as defined above to obtain a reaction product comprising the oligonucleotide of formula (I), and
(c) contacting the reaction product of step (b) with a capture reagent capable of interacting with the capture tag, wherein the capture reagent is selected from the group consisting of a solid phase of a standard reverse phase chromatography such as RP-HPLC, a chromatographic material with affinity for hydrophobic groups,

a chromatographic material with affinity for fluorinated groups such as a fluorous affinity support, a capture reagent containing an alkynyl moiety, and a capture reagent containing an azido moiety, wherein the contacting takes place under conditions which allow separation of the oligonucleotide (I) from other species contained in said reaction product.

2. The method of claim 1, wherein the triphosphate/triphosphate analogue group is attached to the 5'-terminus of the oligonucleotide, particularly to the 5'-OH-group of the 5'-terminal sugar thereof.

3. The method of any one of claims 1 or 2, further comprising the step:

(d) removing the capture tag to obtain an oligonucleotide of formula (IV):



IV

wherein V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 and ON are as defined in claim 1.

4. The method of any one of claims 1-3, wherein the oligonucleotide is selected from desoxyribonucleotides, ribonucleotides and oligonucleotide analogues.

5. The method of any one of claims 1-4, wherein the oligonucleotide is single-stranded or double stranded.

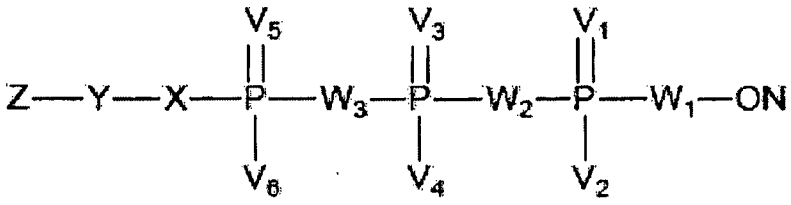
6. The method of claim 5, wherein the oligonucleotide is double-stranded and the duplex is closed by a loop at the distal end thereof, wherein the loop comprises nucleotide and/or non-nucleotide building blocks.

7. The method of claim 5 or 6, wherein the oligonucleotide is double-stranded and the duplex is blunt-ended at the proximal end thereof.

8. The method of any one of claims 1-7, wherein the oligonucleotide comprises a cell-specific targeting entity covalently attached thereto.

9. The method of any one of claims 1-8, wherein the oligonucleotide (I) or (IV) is an activator of the RIG-1.

10. Use of a kit for preparing an oligonucleotide of formula (I)



I

wherein V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3, X, Y, Z and ON are defined as in any one of claims 1-9, wherein the kit comprises:

(a) a capture tag agent of formula (III)

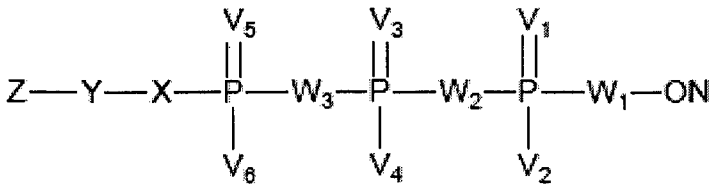
Z-Y-XH (III)

wherein X, Z and Y are defined as in any one of claims 1-9, and
(b) a capture reagent capable of interacting with the capture tag, wherein the capture reagent is selected from

the group consisting of a solid phase of a standard reverse phase chromatography such as RP-HPLC, a chromatographic material with affinity for hydrophobic groups, a chromatographic material with affinity for fluorinated groups such as a fluorous affinity support, a capture reagent containing an alkynyl moiety, and a capture reagent containing an azido moiety.

Patentansprüche

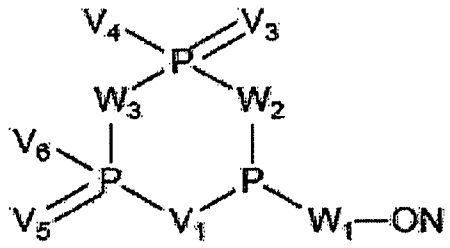
1. Verfahren zum Herstellen eines Oligonukleotids der Formel (I),



I

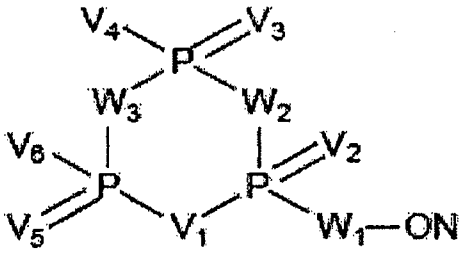
wobei V_1, V_3 und V_5 in jedem Fall unabhängig ausgewählt sind aus 0, S und Se; V_2, V_4 und V_6 in jedem Fall unabhängig ausgewählt sind aus OH, OR^1, SH, SR^1, F, NH_2, NHR^1, $N(R^1)_2$ und $BH_3^-M^+$,
W_1 O oder S ist,
W_2 0, S, NH oder NR^2 ist,
W_3 O, S, NH, NR^2, CH_2, CHHal oder $C(Hal)_2$ ist,
R^1, R^2 und R^3 ausgewählt sind aus C_{1-6}-Alkyl, C_{2-6}-Alkenyl, C_{2-6}-Alkinyl, C_{2-6}-Acyl oder einer cyclischen Gruppe, jeweils gegebenenfalls substituiert,
oder wobei zwei R^1 zusammen mit einem daran gebundenen N-Atom einen Ring bilden können,
M^+ ein Kation ist,
X NH, NR^3, O oder S ist,
Z einen Affinitäts-Marker darstellt, der ein C_{8-24}-Alkylrest, eine Perfluoralkyleinheit, eine Azid- oder eine Alkinylgruppe ist,
Y eine Bindung oder einen Linker darstellt, die/der den Affinitäts-Marker mit X verbindet, und
ON ein mindestens 4 Nukleotid- oder Nukleotidanaloga-Bausteine umfassendes Oligonukleotid darstellt,
umfassend die Schritte:

(a) Reagieren einer Verbindung der Formel (IIa)



IIa

wobei V_1, V_3, V_5, V_4, V_6, W_1, W_2, W_3, und ON wie oben definiert sind, mit einem Oxidationsmittel, um eine Verbindung der Formel (IIb) zu erhalten



IIb

wobei V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 und ON wie oben definiert sind,
(b) Reagieren einer Verbindung der Formel (IIb) mit einem Affinitätsmarkierungsmittel der Formel (III),

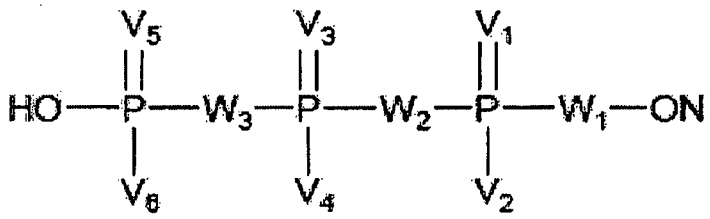
Z-Y-XH (III)

wobei X, Z, und Y wie oben definiert sind, um ein das Oligonukleotid der Formel (I) umfassendes Reaktionsprodukt zu erhalten, und
(c) In-Kontakt-Bringen des Reaktionsprodukts von Schritt (b) mit einem Affinitätsreagenz, das zum Wechselwirken mit der Affinitätsmarkierung fähig ist, wobei das Affinitätsreagenz ausgewählt ist aus der Gruppe bestehend aus einer festen Phase einer Standard-Umkehrphasenchromatographie wie RP-HPLC, einem chromatographischen Material mit Affinität für hydrophobe Gruppen, einem chromatographischen Material mit Affinität für fluorierte Gruppen wie einem Fluor-Affinitätsträger, einem eine Alkinyleinheit enthaltenden Affinitätsreagenz, und einem eine Azideinheit enthaltenden Affinitätsreagenz, wobei das In-Kontakt-Bringen unter Bedingungen stattfindet, die Trennung des Oligonukleotids (I) von anderen in dem Reaktionsprodukt enthaltenen Spezies erlaubt.

2. Verfahren nach Anspruch 1, wobei das Triphosphat / die Triphosphatanalog-Gruppe an den 5'-Terminus des Oligonukleotids angehängt ist, insbesondere an die 5'-OH-Gruppe des 5'-terminalen Zuckers davon.

3. Verfahren nach einem beliebigen der Ansprüche 1 oder 2, des Weiteren umfassend den Schritt:

(d) Entfernen der Affinitätsmarkierung, um ein Oligonukleotid der Formel (IV) zu erhalten:



IV

wobei V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 und ON wie in Anspruch 1 definiert sind.

4. Verfahren nach einem beliebigen der Ansprüche 1-3, wobei das Oligonukleotid ausgewählt ist aus Deoxyribonukleotiden, Ribonukleotiden und Oligonukleotidanaloga.

5. Verfahren nach einem beliebigen der Ansprüche 1-4, wobei das Oligonukleotid einzelsträngig oder doppelsträngig ist.

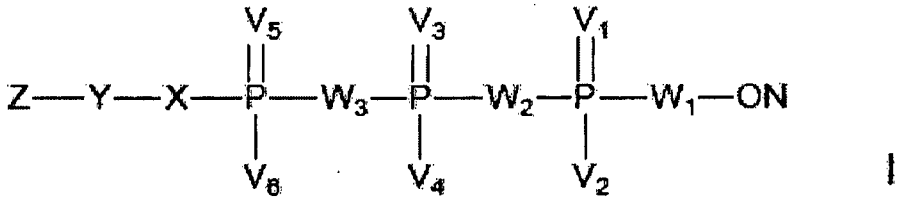
6. Verfahren nach Anspruch 5, wobei das Oligonukleotid doppelsträngig ist und der Duplex durch eine Schleife an dem distalen Ende davon geschlossen ist, wobei die Schleife Nukleotid- und/oder Nicht-Nukleotid-Bausteine umfasst.

7. Verfahren nach Anspruch 5 oder 6, wobei das Oligonukleotid doppelsträngig ist und der Duplex an dem proximalen Ende davon ein stumpfes Ende aufweist.

8. Verfahren nach einem beliebigen der Ansprüche 1-7, wobei das Oligonukleotid eine zellspezifische Zielführungseinheit umfasst, die daran kovalent angehängt ist.

9. Verfahren nach einem beliebigen der Ansprüche 1-8, wobei das Oligonukleotid (I) oder (IV) ein Aktivator des RIG-1 ist.

10. Verwendung eines Kit zum Herstellen eines Oligonukleotids der Formel (I)



wobei V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3, X, Y, Z und ON wie in einem beliebigen der Ansprüche 1-9 definiert sind, wobei das Kit umfasst:

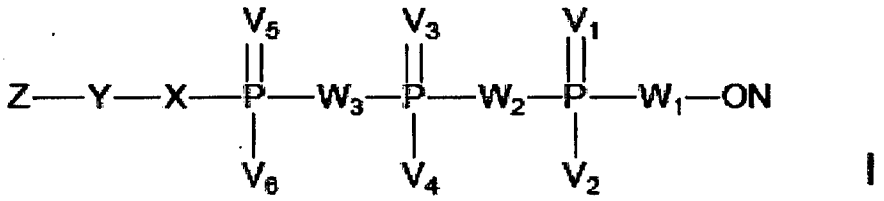
(a) ein Affinitätsmarkermittel der Formel (III)

Z-Y-XH (III)

wobei X, Z und Y wie in einem beliebigen der Ansprüche 1-9 definiert sind, und
(b) ein Affinitätsreagenz, das zum Wechselwirken mit dem Affinitätsmarker fähig ist, wobei das Affinitätsreagenz ausgewählt ist aus der Gruppe bestehend aus einer festen Phase einer Standard-Umkehrphasenchromatographie wie RP-HPLC, einem chromatographischen Material mit Affinität für hydrophobe Gruppen, einem chromatographischen Material mit Affinität für fluorierte Gruppen wie einem Fluor-Affinitätsträger, einem eine Alkinyleinheit enthaltenden Affinitätsreagenz, und einem eine Azideinheit enthaltenden Affinitätsreagenz.

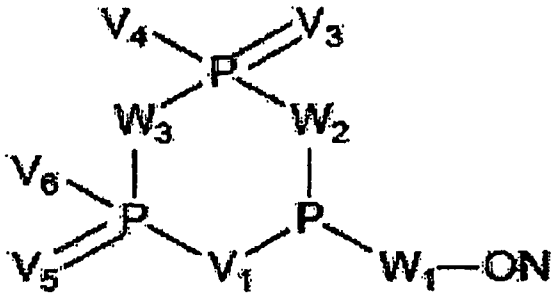
Revendications

1. Procédé de préparation d'un oligonucléotide de formule (I),



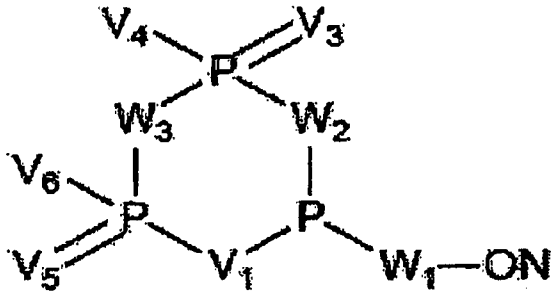
dans lequel V_1, V_3 et V_5 sont, dans chaque cas, indépendamment choisis parmi O, S et Se ;
V_2, V_4 et V_6 sont, dans chaque cas, indépendamment choisis parmi OH, OR^1, SH, SR^1, F, NH_2, NHR^1, $N(R^1)_2$ et $BH_3^-M^+$,
W_1 est O ou S,
W_2 est O, S, NH ou NR^2,
W_3 est O, S, NH, NR^2, CH_2, CHHal ou $C(Hal)_2$,
R^1, R^2 et R^3 sont choisis parmi un alkyle en C_{1-6}, alcényle en C_{2-6}, alcynyle en C_{2-6}, acyle en C_{2-6} ou un groupe cyclique, chacun étant éventuellement substitué,
ou dans lequel deux R^1 peuvent former un cycle conjointement avec un atome d'azote lié à ceux-ci,
M^+ est un cation,
X est NH, NR^3, O ou S,
Z représente un marqueur de capture, qui est un résidu alkyle en C_{8-24}, une entité perfluoroalkyle, un azide ou un groupe alcynyle,
Y est une liaison ou un lieur reliant le marqueur de capture à X, et
ON représente un oligonucléotide comprenant au moins 4 blocs de construction nucléotidique ou d'analogue nucléotidique,
comprenant les étapes :

(a) réaction d'un composé de formule (IIa)



IIa

dans lequel V_1, V_3, V_5, V_4, V_6, W_1, W_2, W_3 et ON sont tels que définis ci-dessus, avec un agent oxydant pour obtenir un composé de formule (IIb)



IIa

dans lequel V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 et ON sont tels que définis ci-dessus,
(b) réaction d'un composé de formule (IIb) avec un agent marqueur de capture de formule (III),

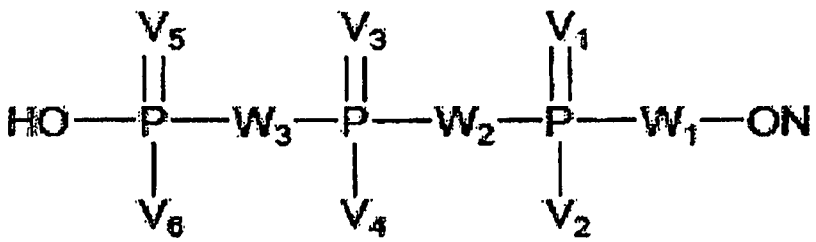
Z-Y-XH (III)

dans lequel X, Z et Y sont tels que définis ci-dessus pour obtenir un produit de réaction comprenant l'oligonucléotide de formule (I), et
(c) mise en contact du produit de réaction de l'étape (b) avec un réactif de capture apte à interagir avec le marqueur de capture, dans laquelle le réactif de capture est choisi dans le groupe constitué par une phase solide d'une chromatographie standard en phase inverse telle qu'une RP-HPLC, un matériau chromatographique ayant une affinité pour les groupes hydrophobes, un matériau chromatographique ayant une affinité pour les groupes fluorés tel qu'un support d'affinité fluoré, un réactif de capture contenant un groupement alcynyle et un réactif de capture contenant un groupement azido, dans laquelle la mise en contact a lieu dans des conditions qui permettent la séparation de l'oligonucléotide (I) d'autres espèces contenues dans ledit produit de réaction.

2. Procédé selon la revendication 1, dans lequel le groupe triphosphate/analogue de triphosphate est attaché à l'extrémité 5' de l'oligonucléotide, en particulier au groupe 5'-OH du sucre terminal en 5' de celui-ci.

3. Procédé selon l'une quelconque des revendications 1 ou 2, comprenant en outre l'étape :

(d) élimination du marqueur de capture pour obtenir un oligonucléotide de formule (IV) :



IV

dans lequel V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 et ON sont tels que définis dans la revendication 1.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel l'oligonucléotide est choisi parmi les désoxyribonucléotides, les ribonucléotides et les analogues d'oligonucléotide.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel l'oligonucléotide est simple brin ou double brin.

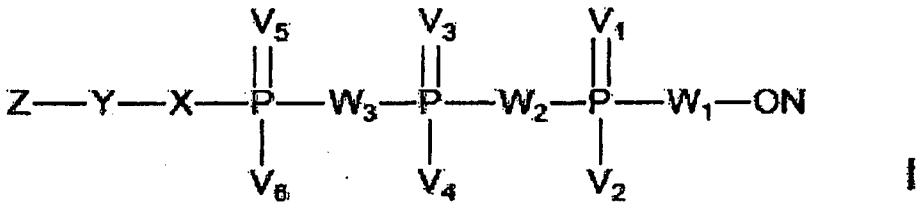
6. Procédé selon la revendication 5, dans lequel l'oligonucléotide est double brin et le duplex est fermé par une boucle à son extrémité distale, dans lequel la boucle comprend des blocs de construction nucléotidique et/ou non nucléotidique.

7. Procédé selon la revendication 5 ou 6, dans lequel l'oligonucléotide est double brin et le duplex est à extrémités franches à son extrémité proximale.

8. Procédé selon l'une quelconque des revendications 1 à 7, dans lequel l'oligonucléotide comprend une entité ciblant spécifiquement une cellule attachée de manière covalente à celui-ci.

9. Procédé selon l'une quelconque des revendications 1 à 8, dans lequel l'oligonucléotide (I) ou (IV) est un activateur de RIG-1.

10. Utilisation d'un kit pour préparer un oligonucléotide de formule (I)



I

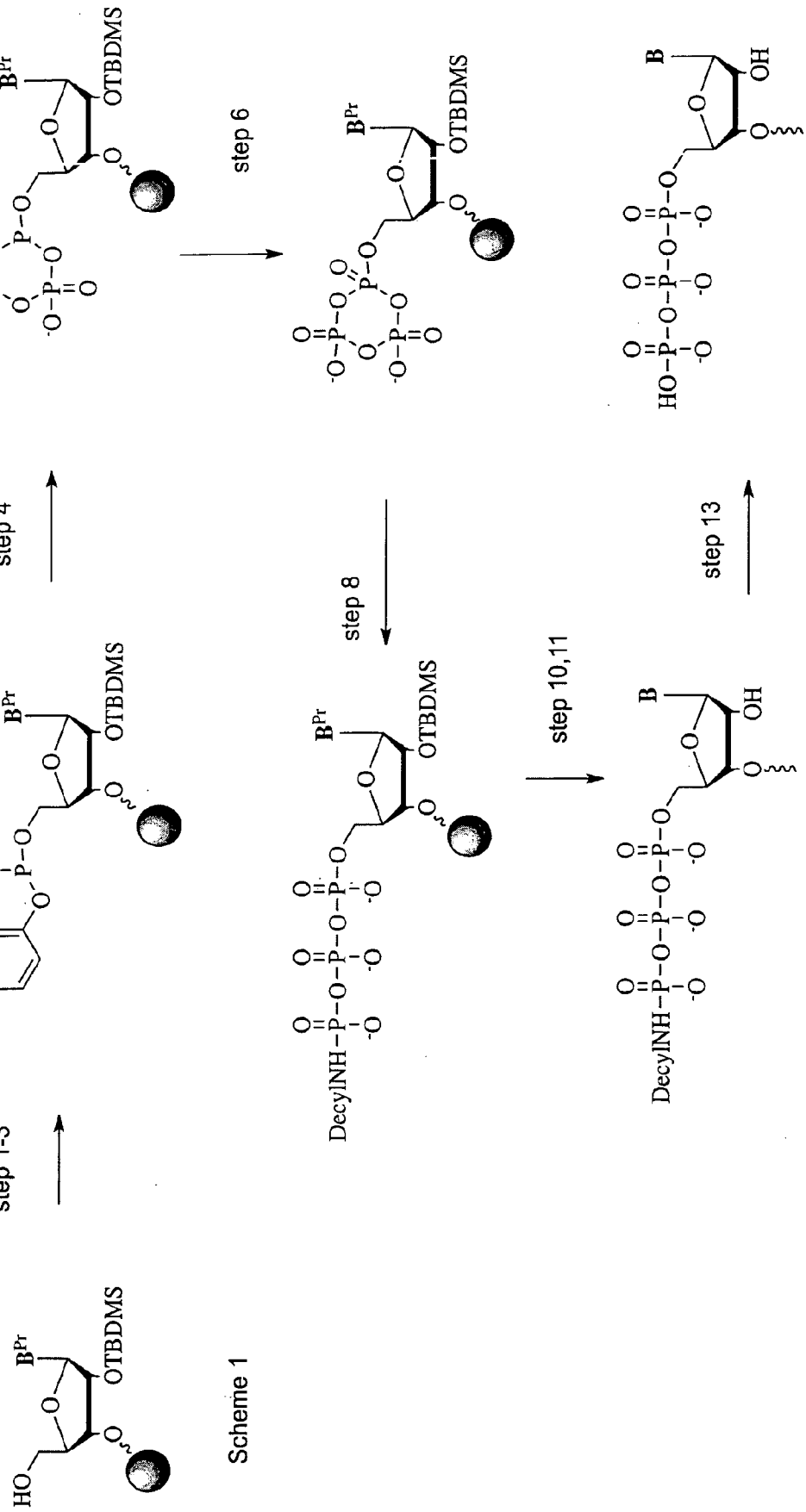
dans lequel V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3, X, Y, Z et ON sont tels que définis dans l'une quelconque des revendications 1 à 9,
dans laquelle le kit comprend :

(a) un agent marqueur de capture de formule (III)

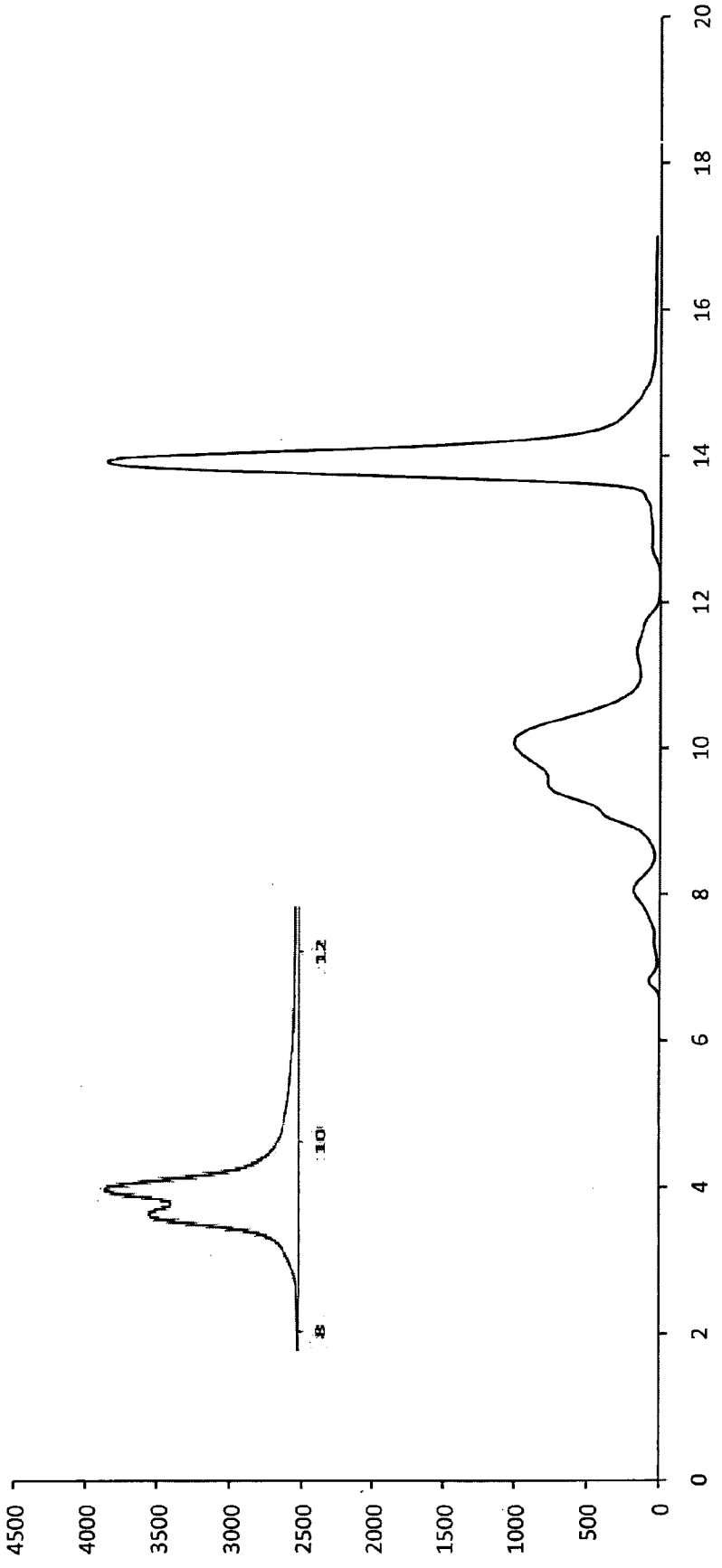
Z-Y-XH (III)

dans lequel X, Z et Y sont tels que définis dans l'une quelconque des revendications 1 à 9, et
(b) un réactif de capture apte à interagir avec le marqueur de capture, dans lequel le réactif de capture est choisi dans le groupe constitué par une phase solide d'une chromatographie standard en phase inverse telle qu'une RP-HPLC, un matériau chromatographique ayant une affinité pour les groupes hydrophobes, un matériau chromatographique ayant une affinité pour les groupes fluorés tel qu'un support d'affinité fluoré, un réactif de capture contenant un groupement alcynyle et un réactif de capture contenant un groupement azido.

Figure 1



Scheme 1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12

Figure 2A

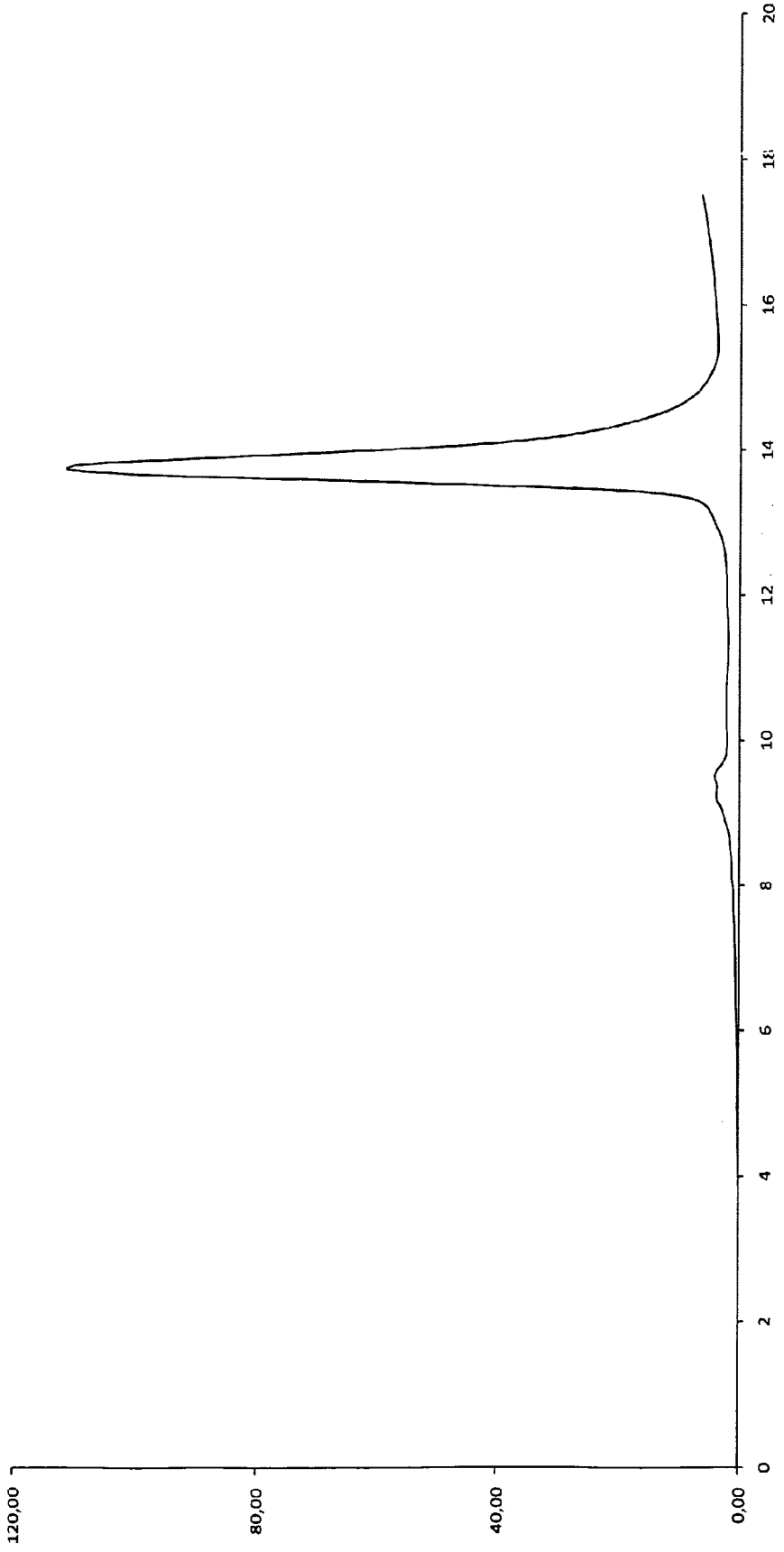


Figure 2B

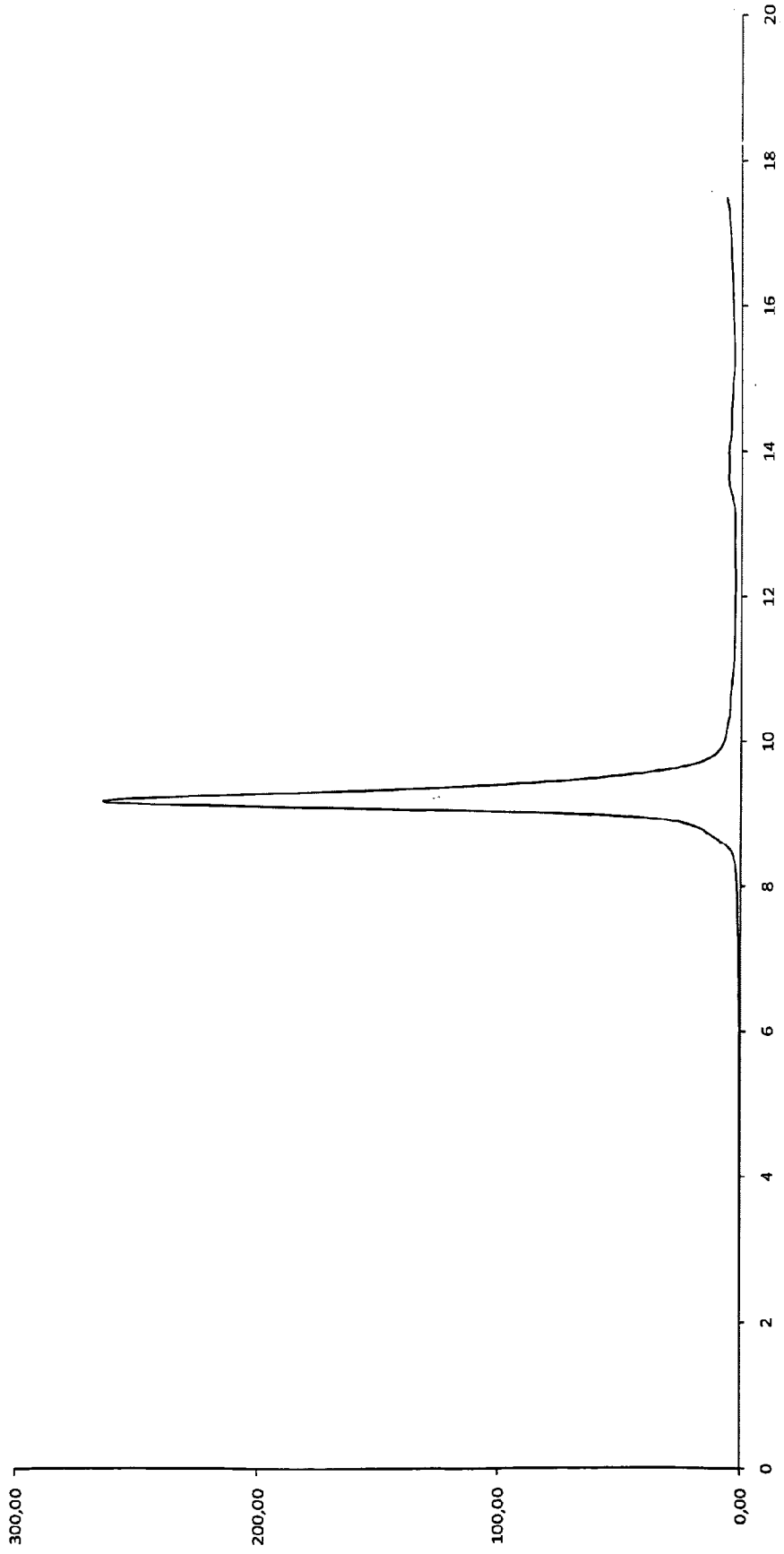
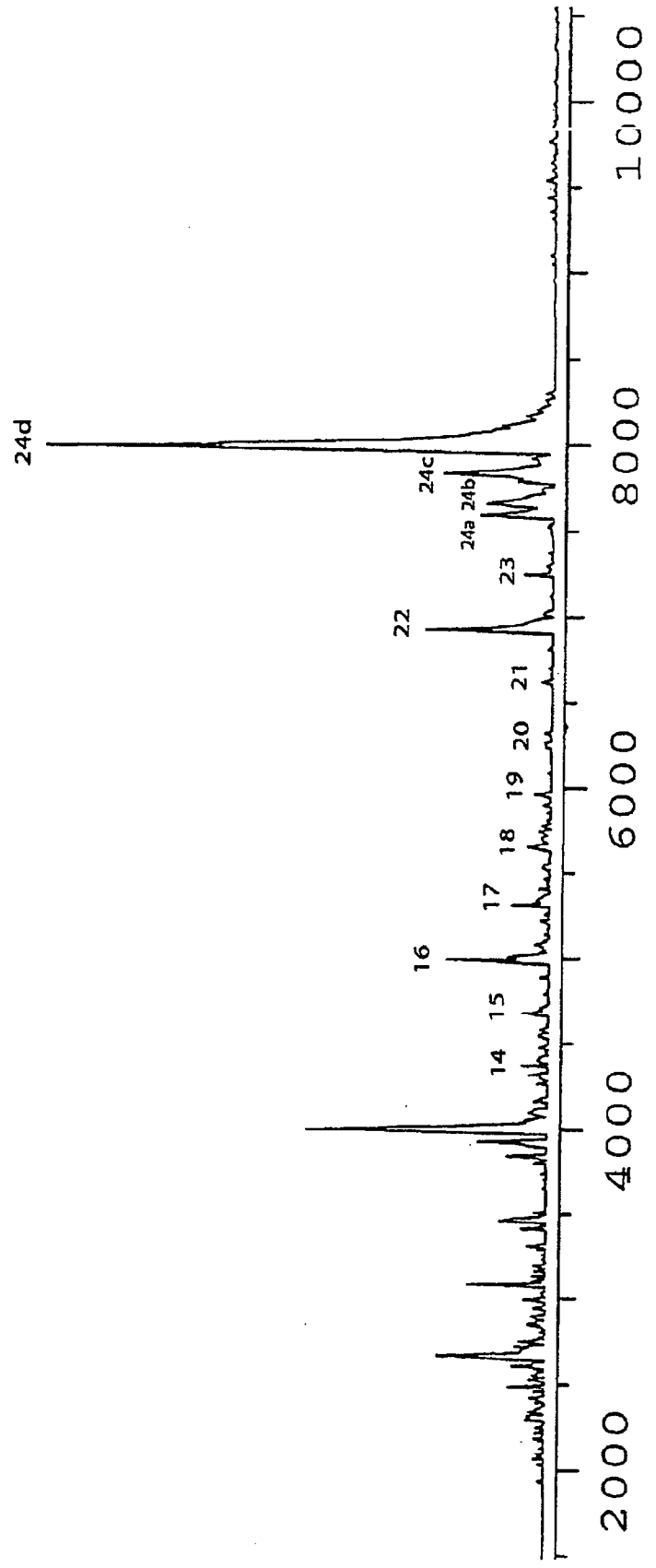


Figure 2C


24d
24c
24b
24a
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19
18
17
16
15
14
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4000
6000
8000
10000

Figure 3A

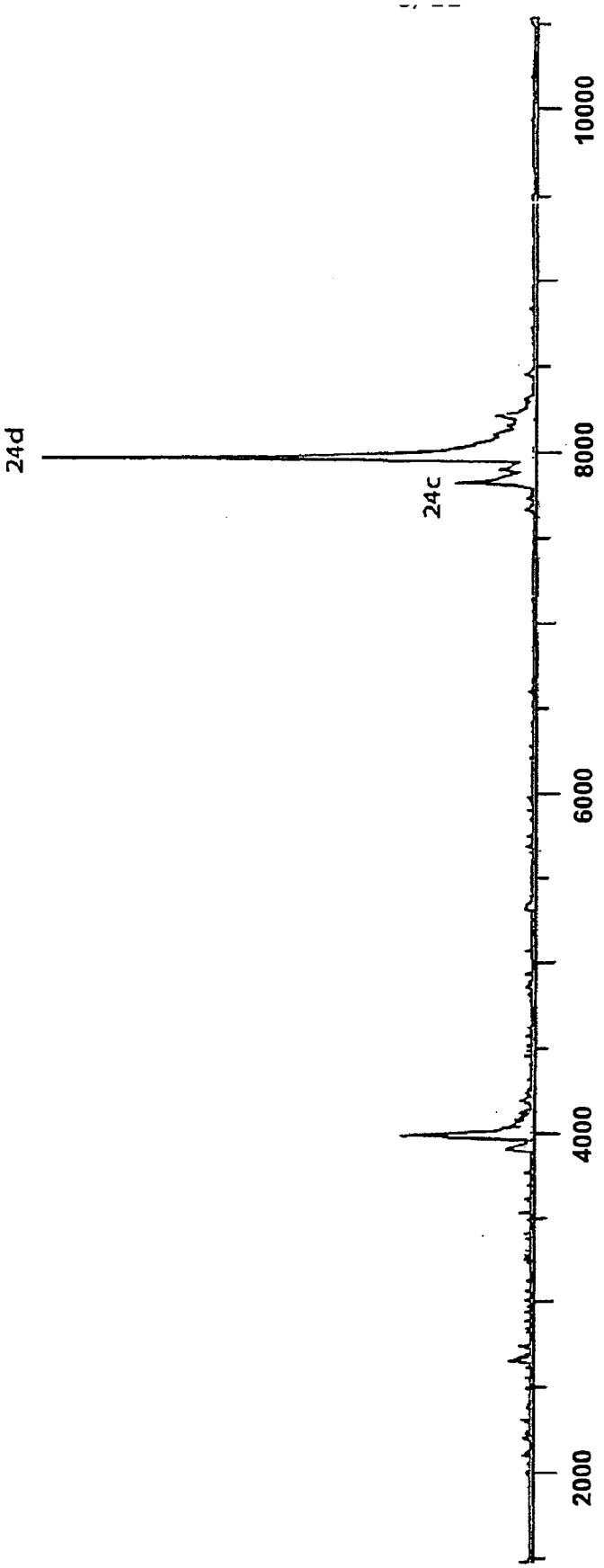


Figure 3B

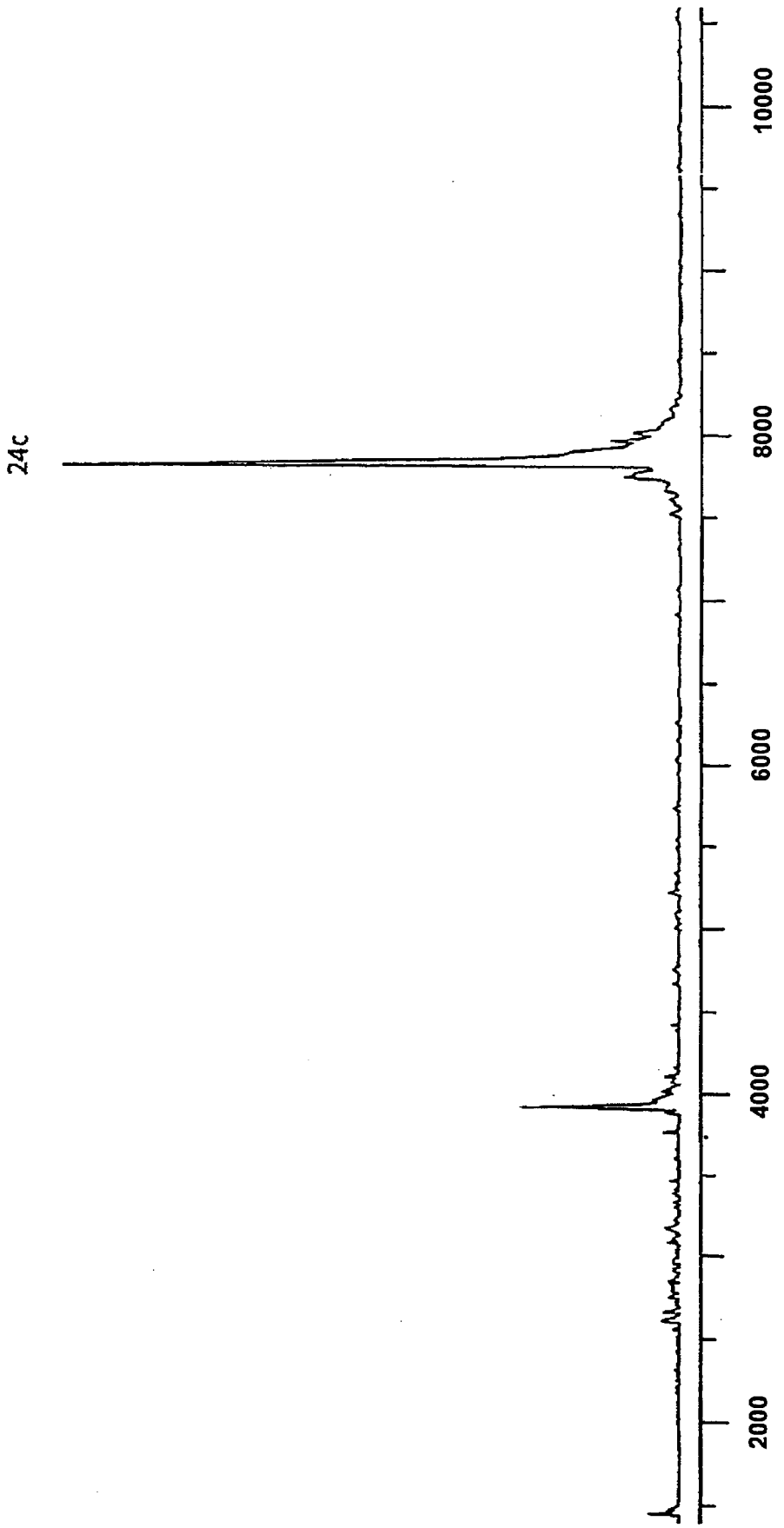
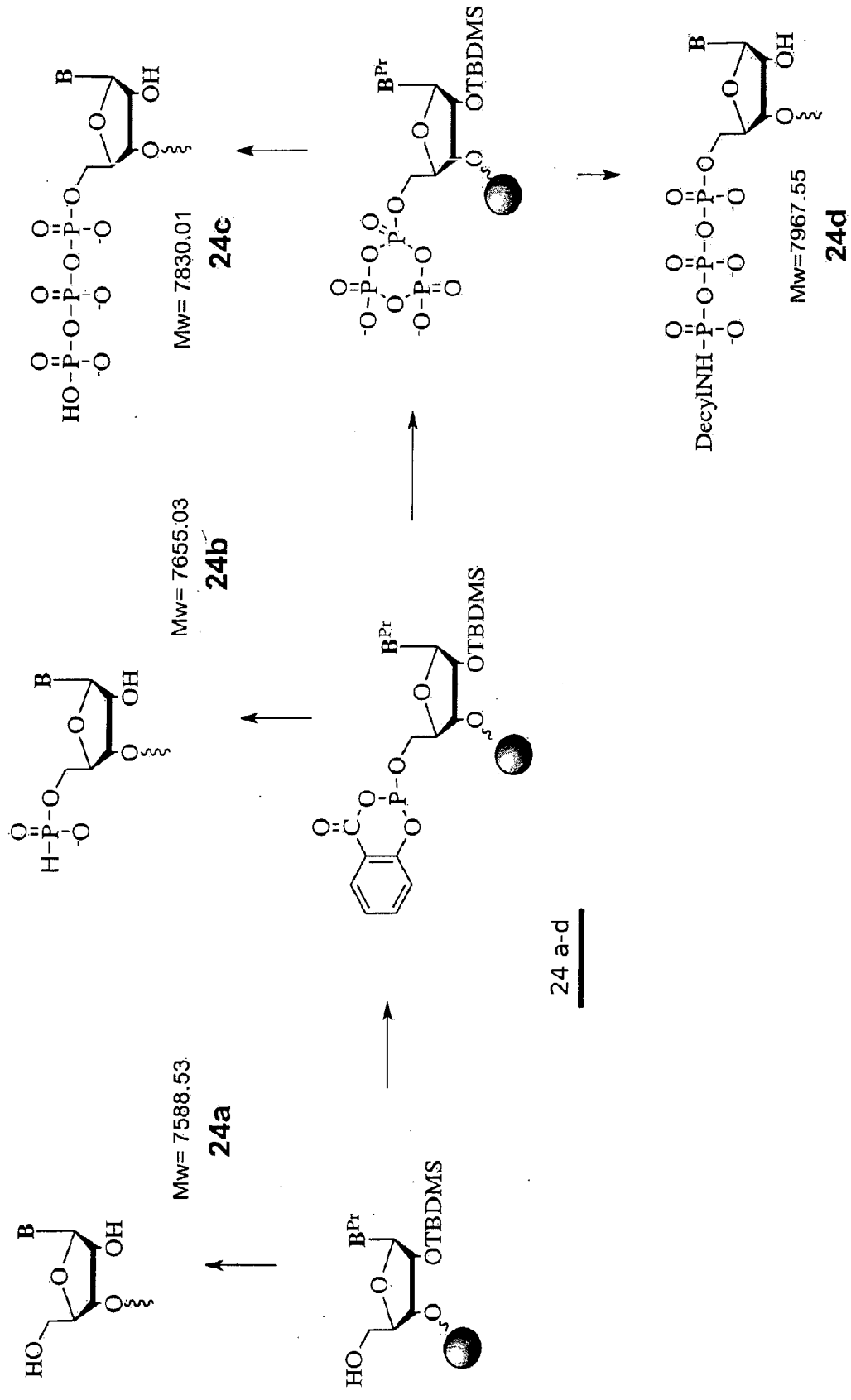


Figure 3C

Figure 4a


Mw= 7588.53
24a
Mw= 7655.03
24b
Mw= 7830.01
24c
Mw=7967.55
24d
24 a-d
OTBDMS
OH
DecylNH

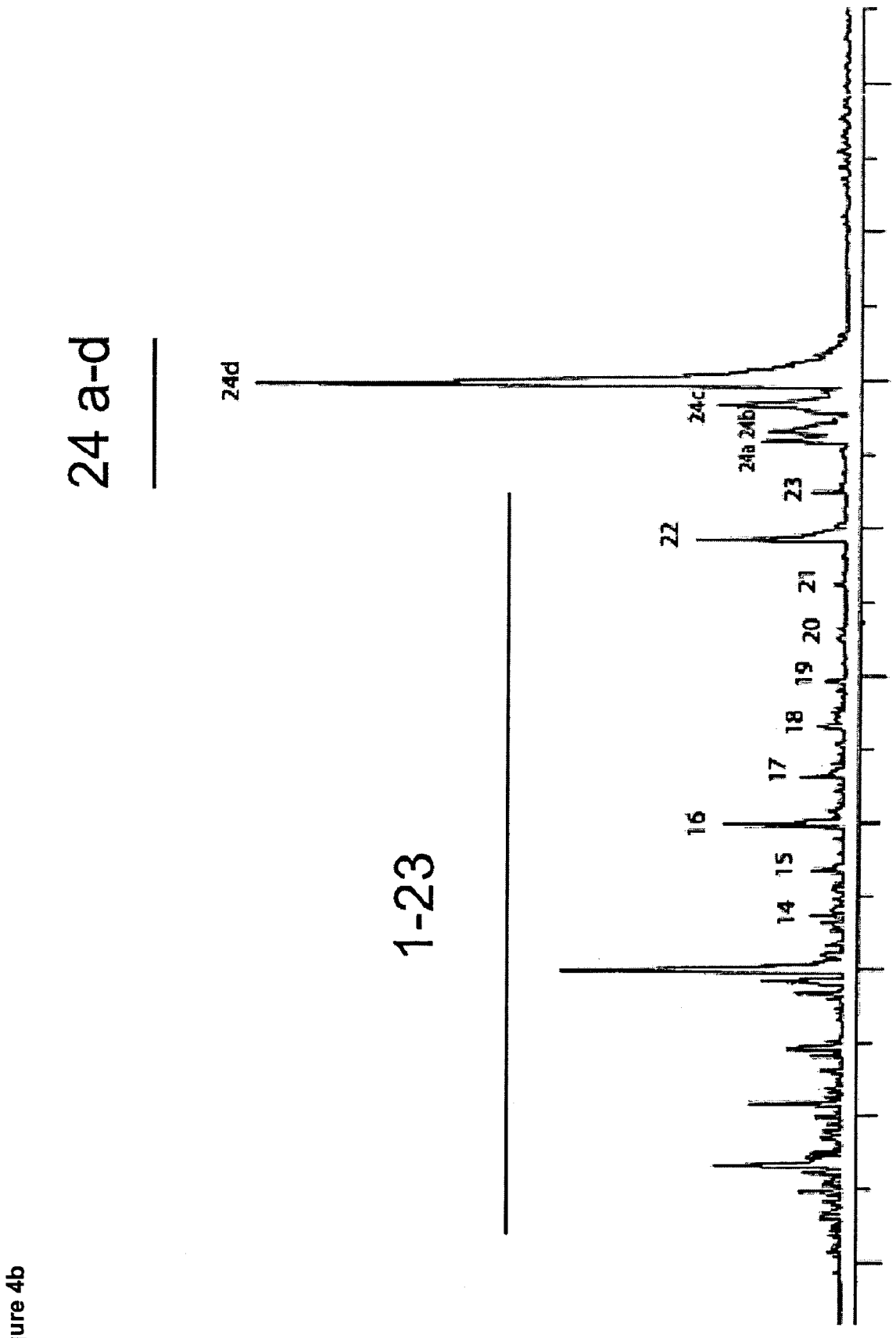


Figure 4b

Figure 5

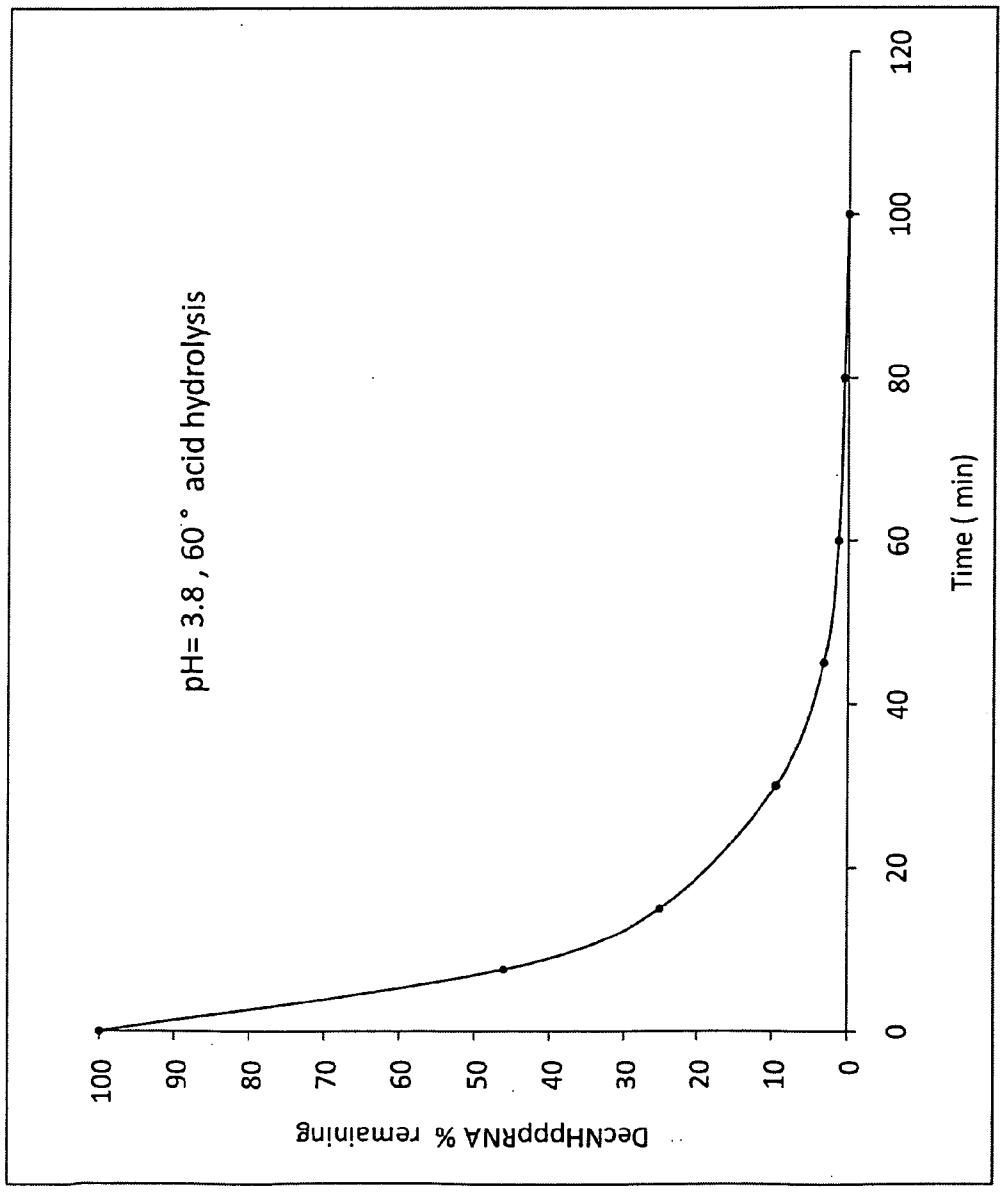

pH= 3.8 , 60° acid hydrolysis
DecNHpppRNA % remaining
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Time (min)

Figure 6A

pppRNA 21 mer

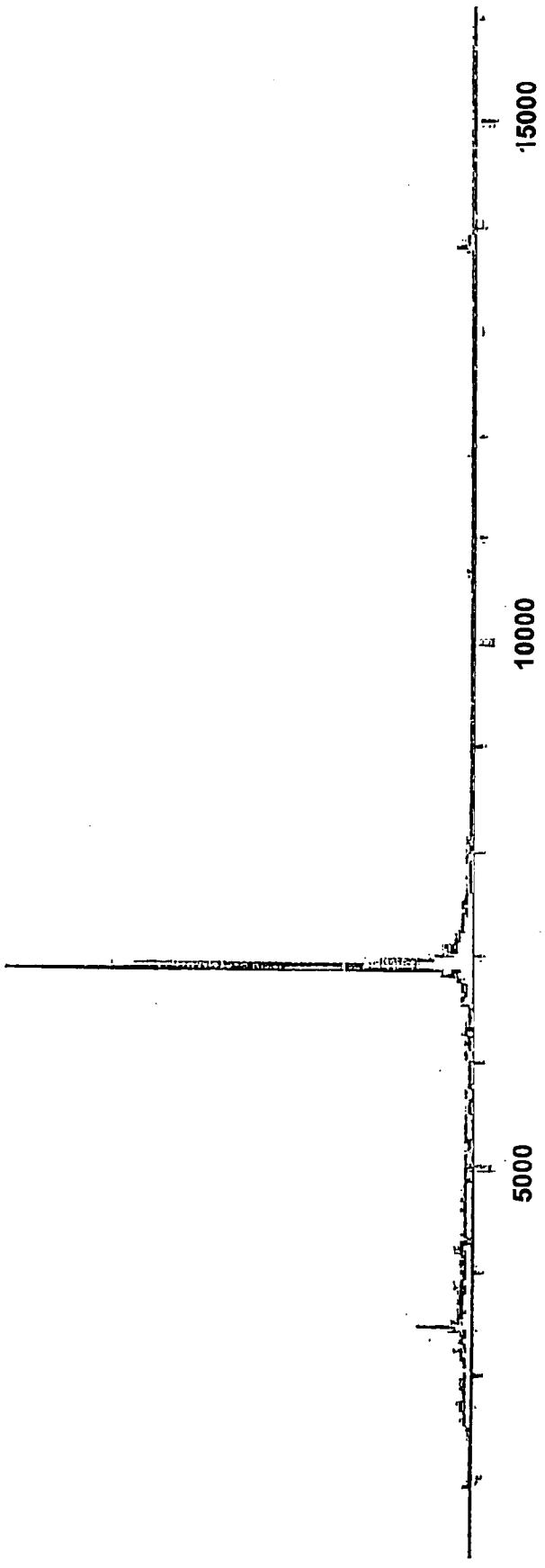


Figure 6B

pppRNA 24 mer

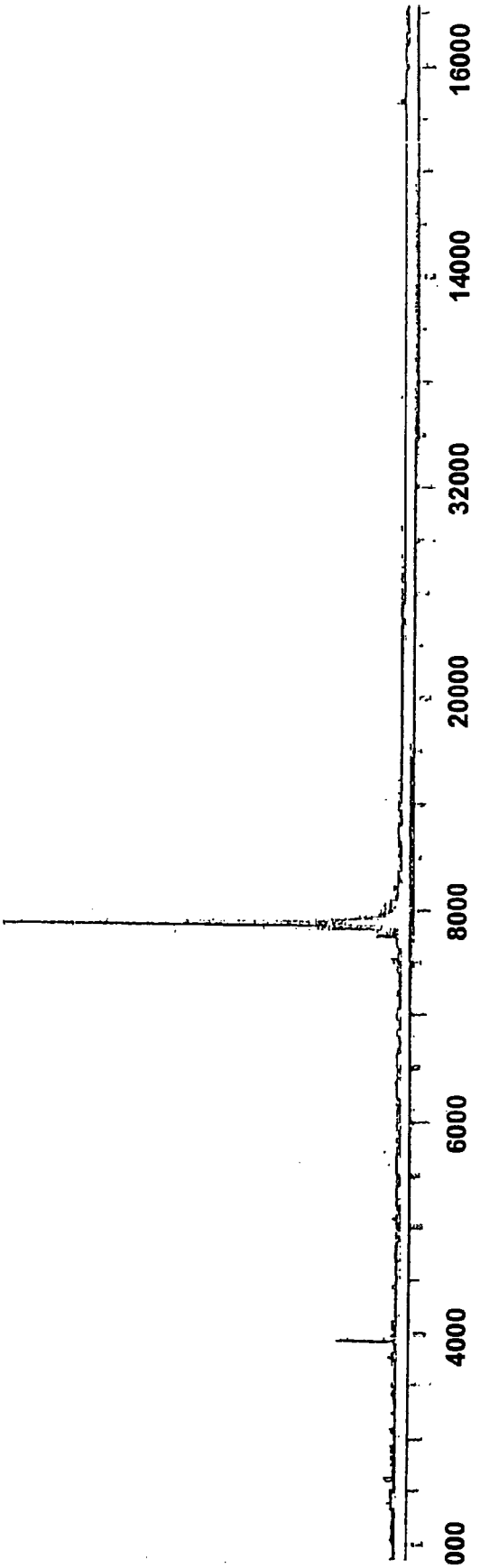
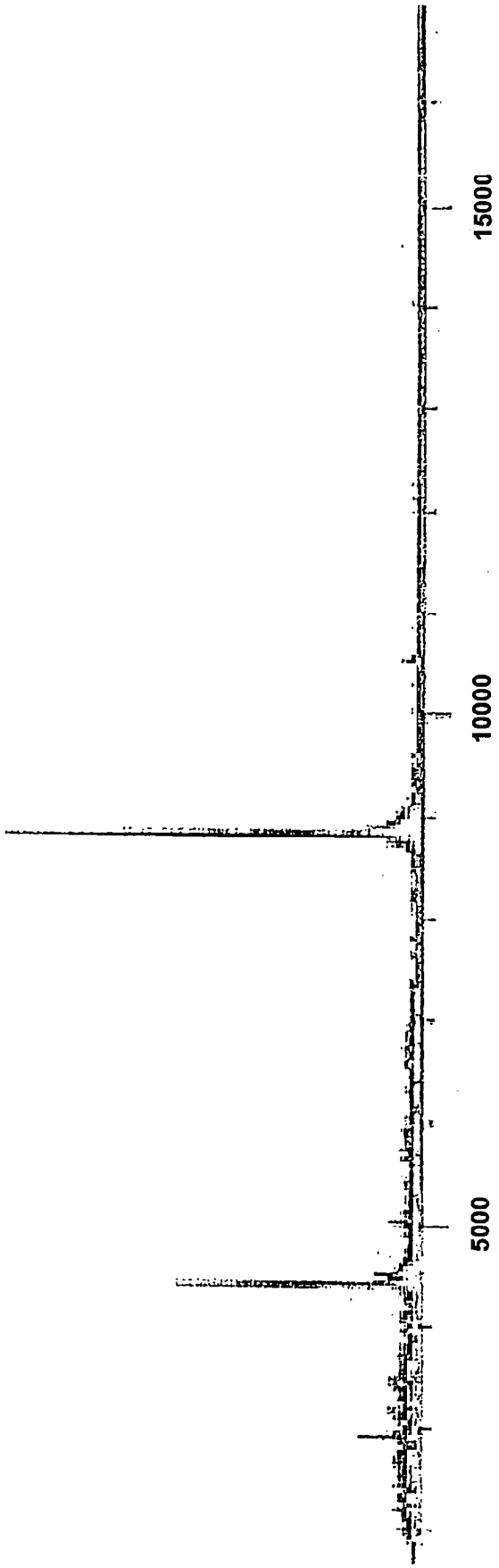
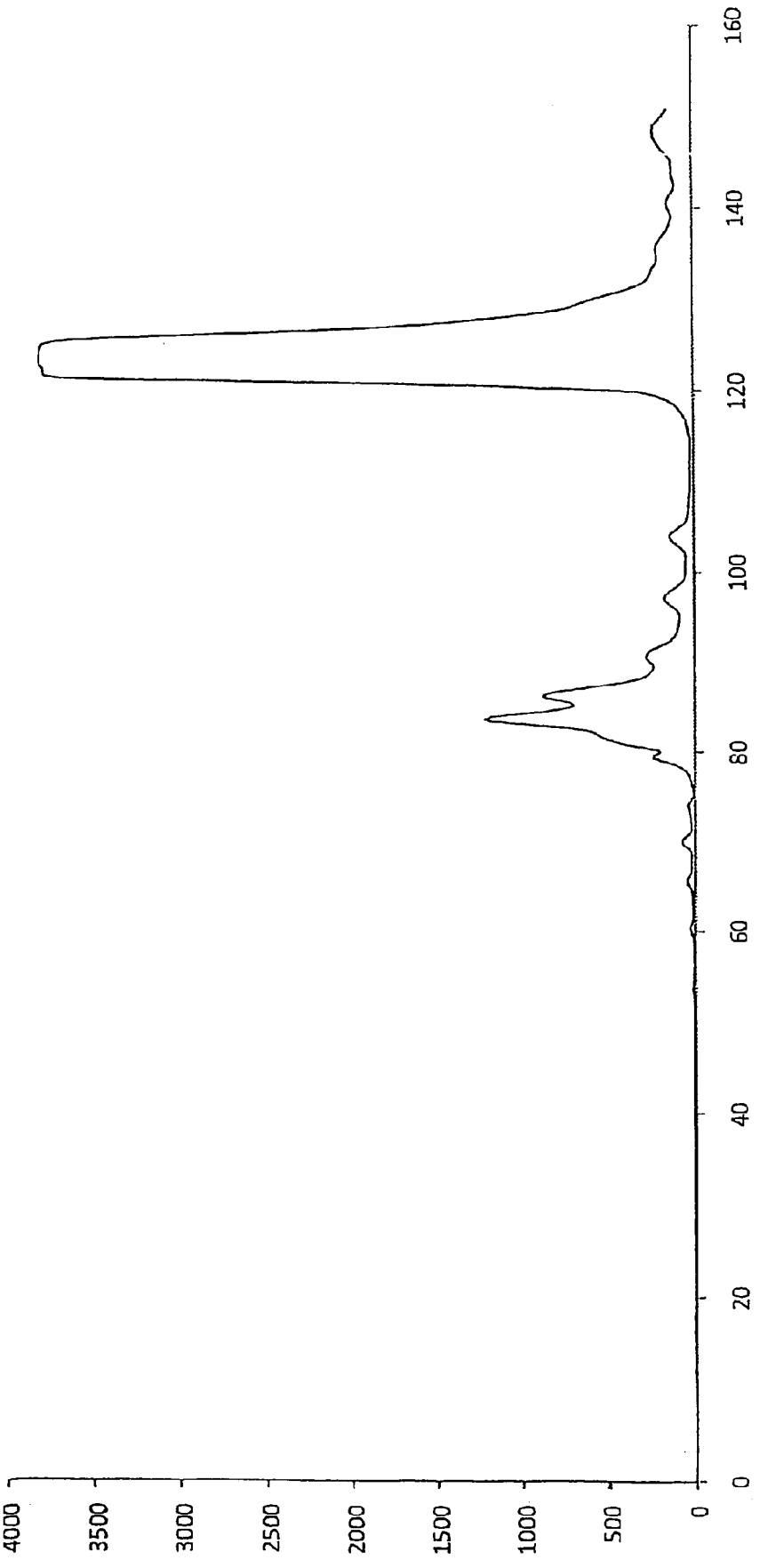


Figure 6C

pppRNA 27 mer




4000
3500
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1500
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0
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40
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100
120
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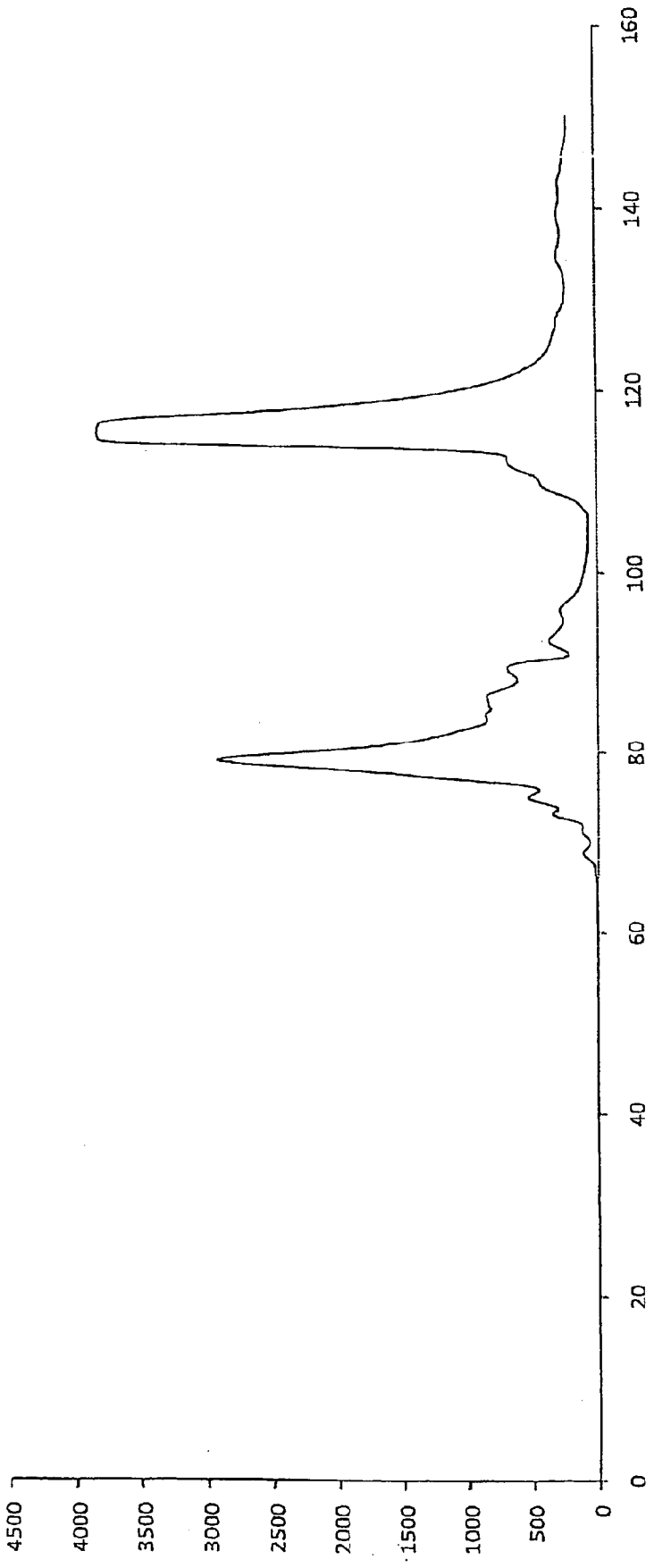
160

Figure 7A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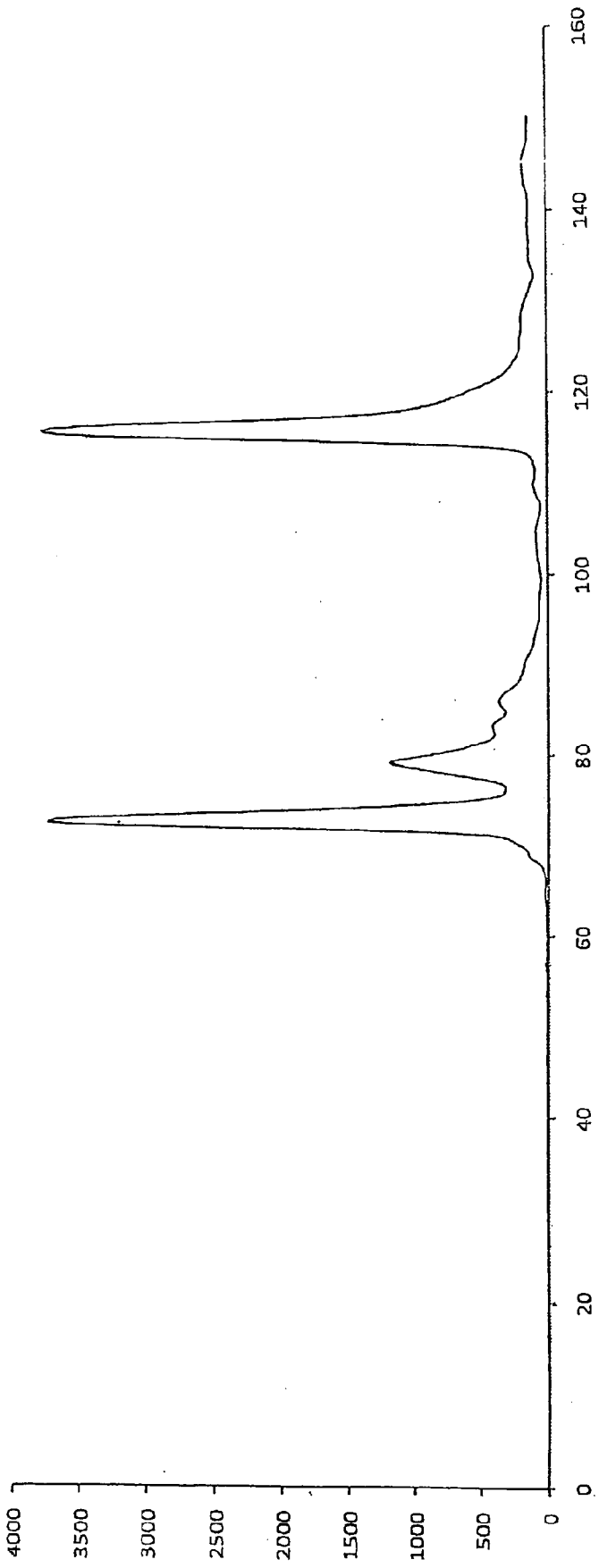
100
120
140
160

Figure 7B


4000
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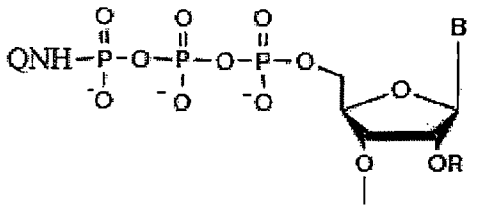
140
160

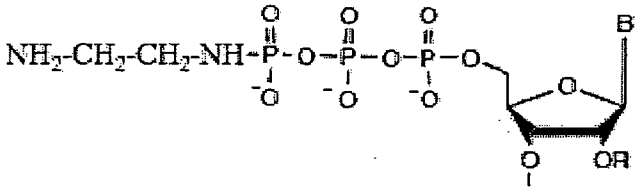
Figure 7C

Figure 8

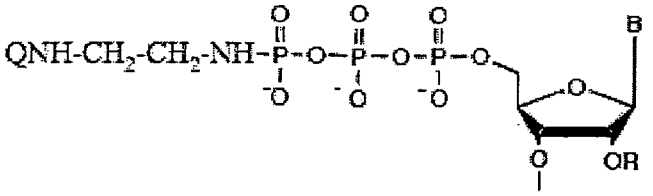
gamma modification



A



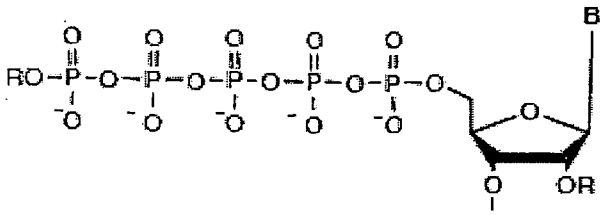
B1



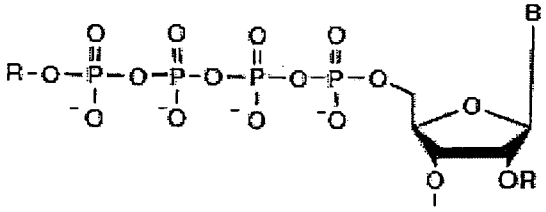
B

Q=alkyl R>12, aminoacids , peptids ,aminoacid analogs , lipids , phospholipids

extended chain



D1



D2

Figure 9A

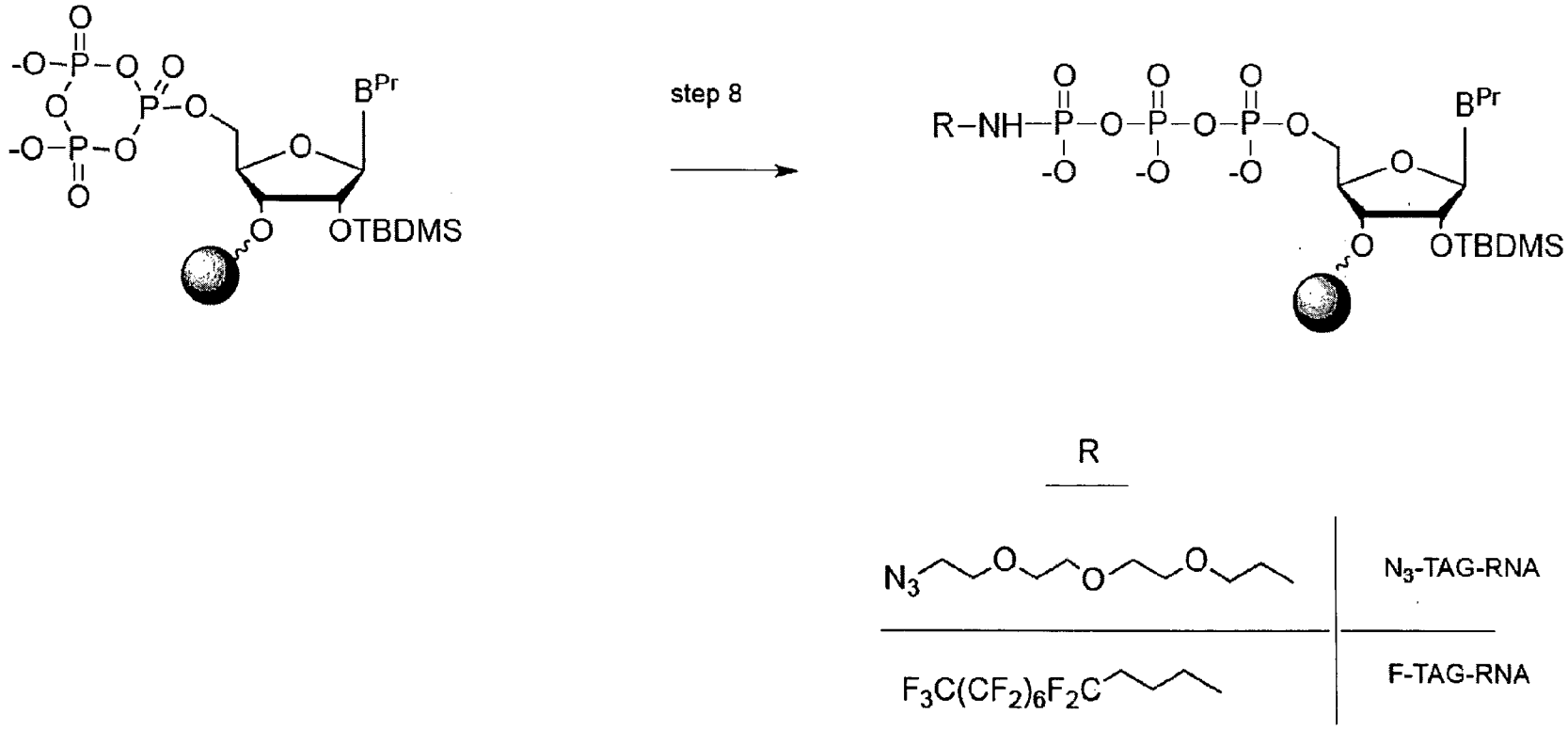


Figure 9B

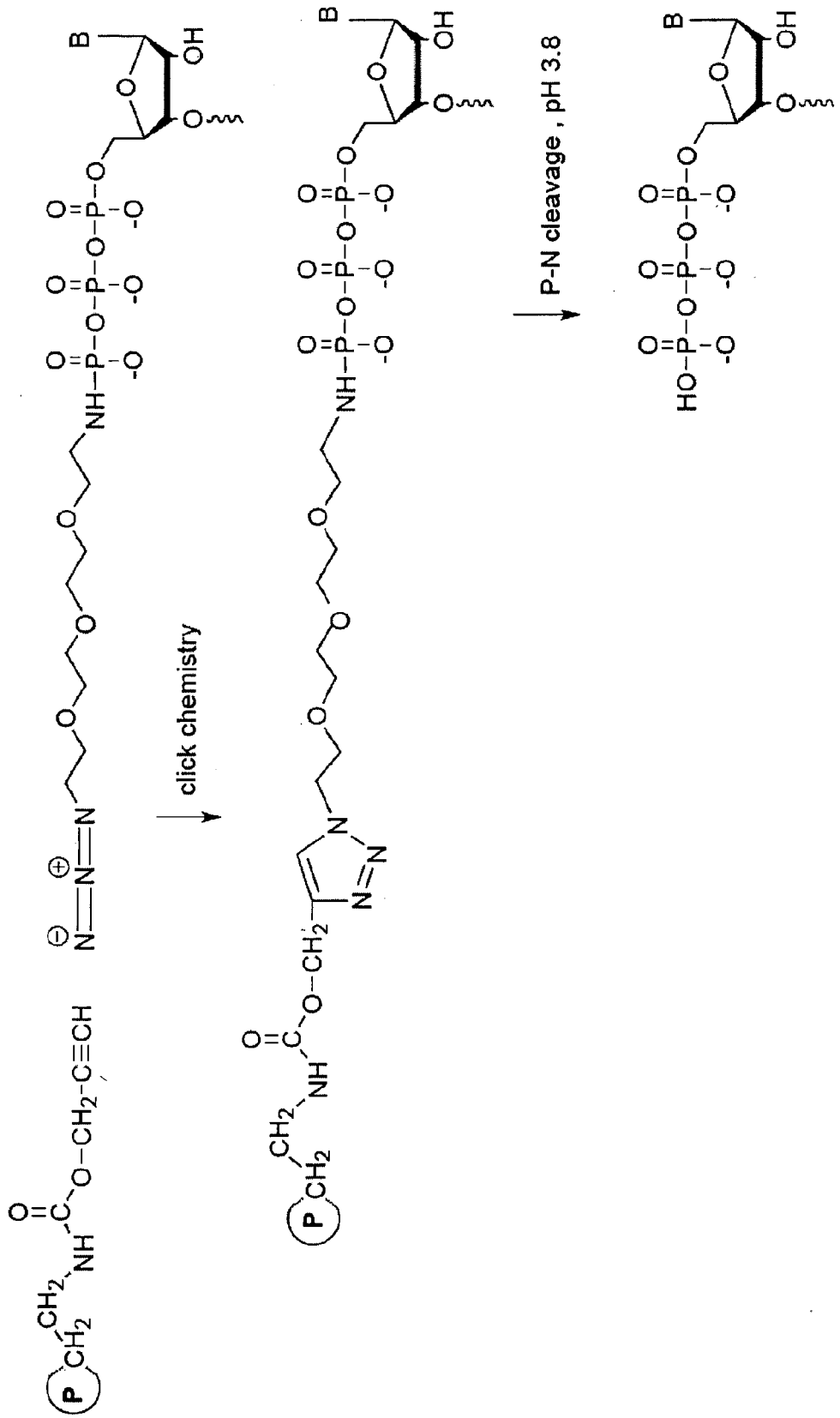
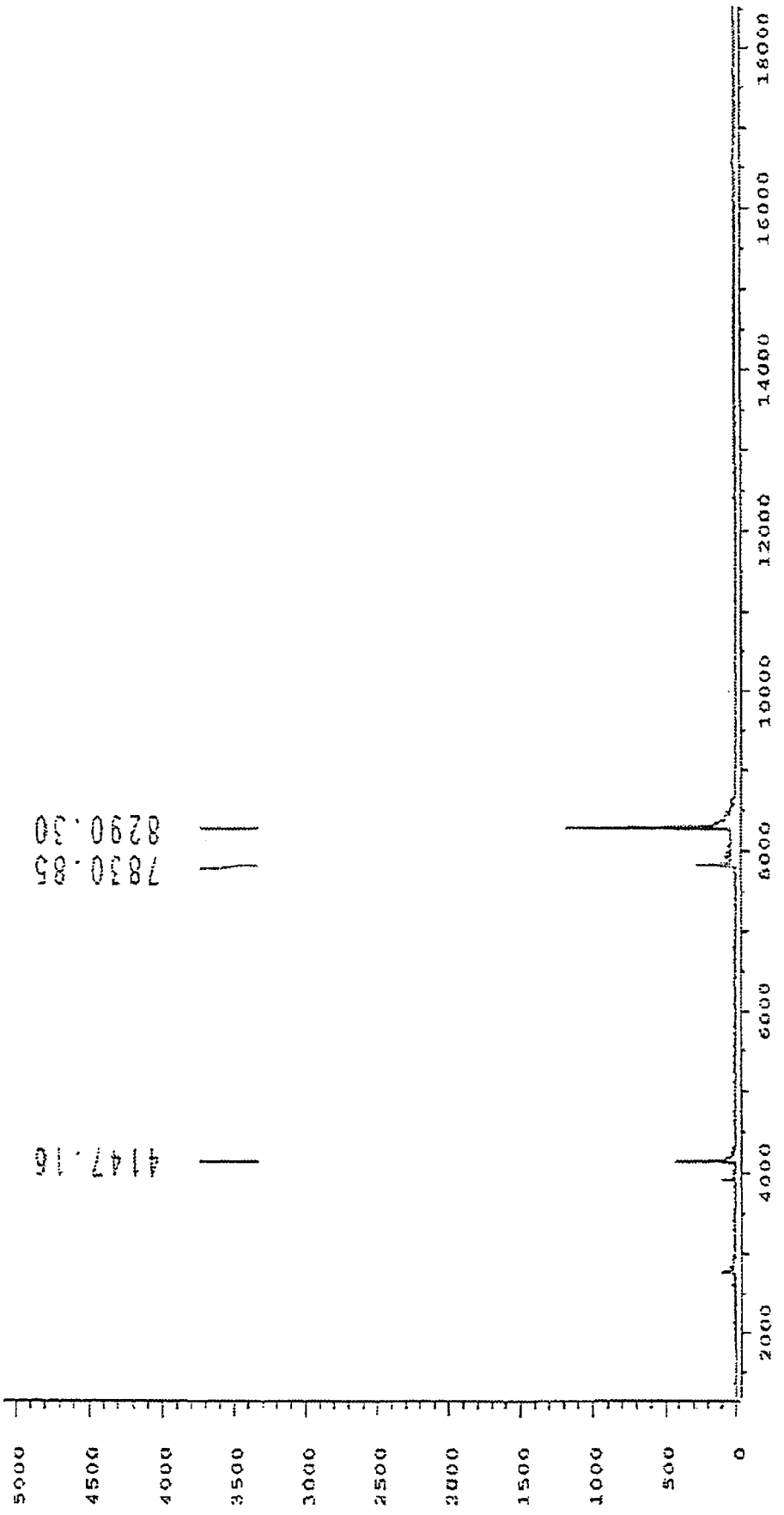


Figure 10A


4147.16
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8290.30
0
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1500
2000
2500
3000
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4000
4500
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8000
10000
12000
14000
16000
18000
mass [Da]

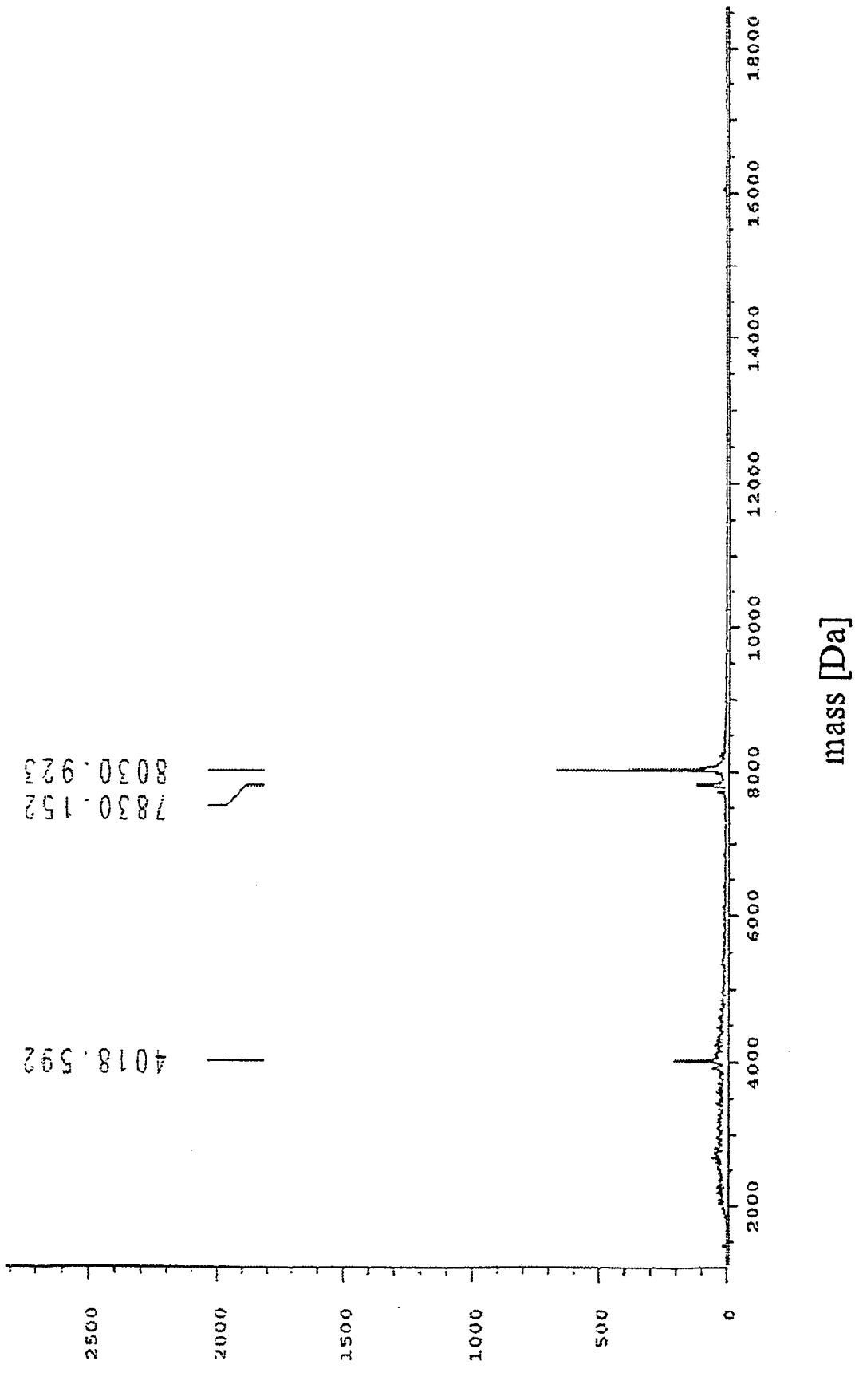
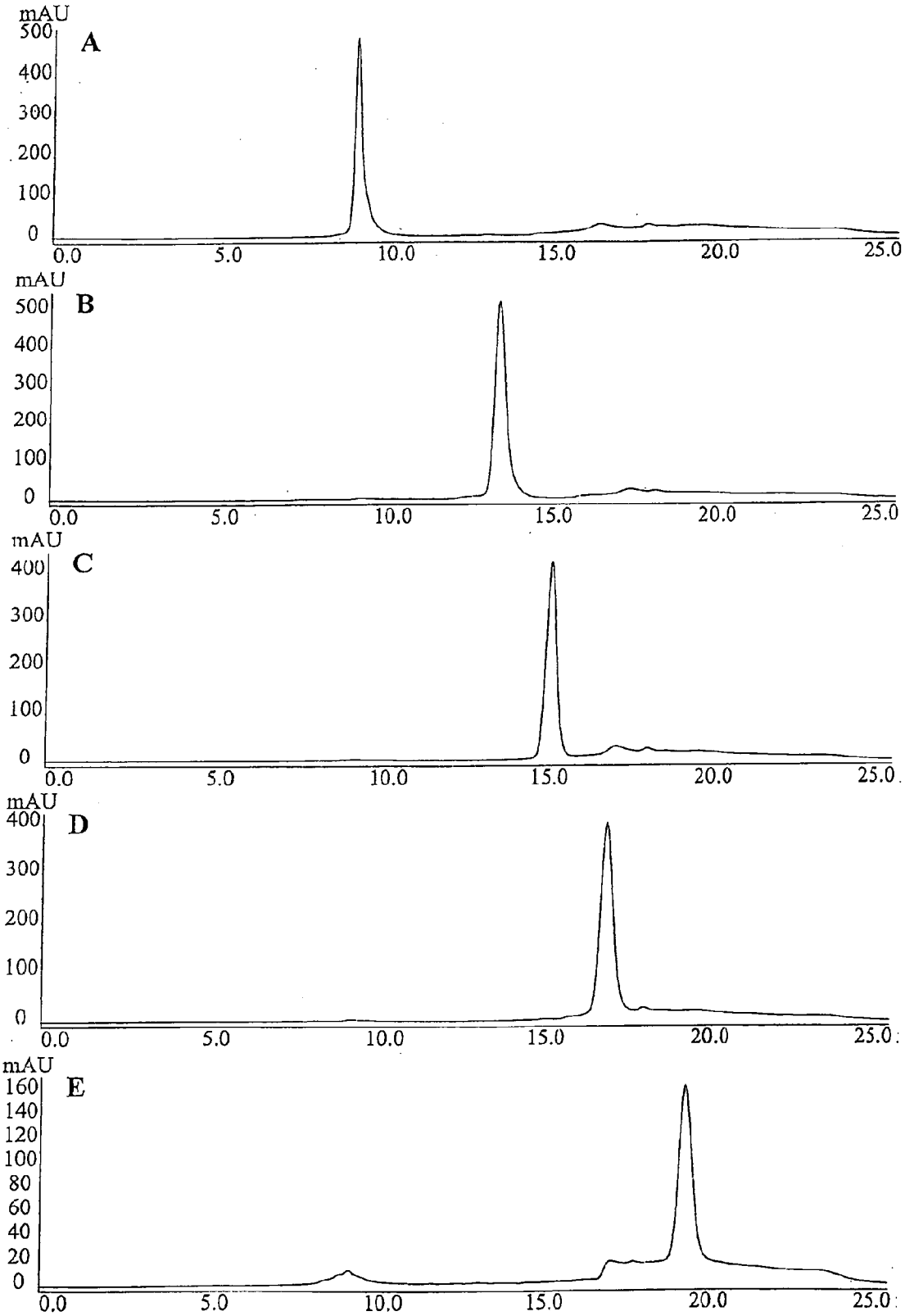

4018.592
7830.152
8030.923
2500
2000
1500
1000
500
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
mass [Da]

Figure 10B

Figure 11


mAU
A
500
400
300
200
100
0
0.0
5.0
10.0
15.0
20.0
25.0
mAU
B
500
400
300
200
100
0
0.0
5.0
10.0
15.0
20.0
25.0
mAU
C
400
300
200
100
0
0.0
5.0
10.0
15.0
20.0
25.0
mAU
D
400
300
200
100
0
0.0
5.0
10.0
15.0
20.0
25.0
mAU
E
160
140
120
100
80
60
40
20
0
0.0
5.0
10.0
15.0
20.0
25.0
time (min)

REFERENCES CITED IN THE DESCRIPTION

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- US 7598230 B **[0003]**
- US 7807653 B **[0003]**
- WO 9640159 A **[0003] [0005] [0025]**
- WO 2009060281 A **[0004] [0005] [0025]**

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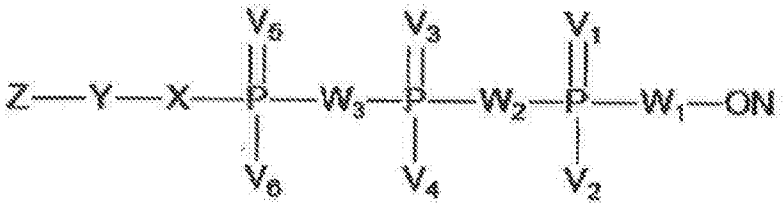
- **GAUR R.K. et al.** *Tetrahedron Letters,* 1992, vol. 33, 3301-3304 **[0003]**
- **ZLATEV et al.** *Organic Latters,* 2010, vol. 12 (10), 2190-2193 **[0005]**

Trifoszforilezett oligonukleotidok tisztítása affinitás-markerek alkalmazásával

SZABADALMI IGÉNYPONTOK

1. Eljárás egy



I

képletű oligonukleotid előállítására,

ahol a V_1, V_3 és V_5 csoportokat egymástól függetlenül az O, S és Se közül választjuk;

a V_2, V_4 és V_6 csoportokat egymástól függetlenül az OH, OR^1, SH, SR^1, F, NH_2, NHR^1, $N(R^1)_2$ és a $BH_3^-M^+$ közül választjuk,

W_1 jelentése O vagy S,

W_2 jelentése O, S, NH vagy NR^2,

W_3 jelentése O, S, NH, NR^2, CH_2, CHHal vagy $C(Hal)_2$,

az R^1, R^2 és R^3 csoportokat az 1-6 szénatomos alkil-, 2-6 szénatomos alkenil-, 2-6 szénatomos alkinil-, 2-6 szénatomos acil-csoport vagy egy ciklusos csoport közül választjuk ki, amelyek mindegyike adott esetben szubsztituált,

vagy ahol két R^1 csoport egy hozzájuk kötődő N atommal egy gyürüt képezhet,

M^+ jelentése kation,

X jelentése NH, NR^3, O vagy S,

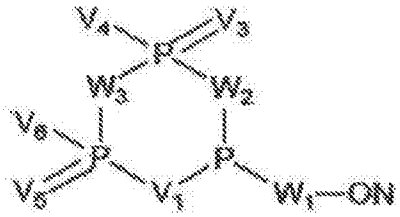
Z jelentése affinitás-marker, amely egy 8-24 szénatomos alkil-maradék, egy perfluor-alkil-egység, egy azid- vagy egy alkinil-csoport,

Y jelentése kötés vagy a kötőcsoport, amely összeköti az affinitás-markert az X csoporttal, és

ON jelentése oligonukleotid, amely tartalmaz legalább 4 nukleotidot vagy nukleotid-analóg építőelemet,

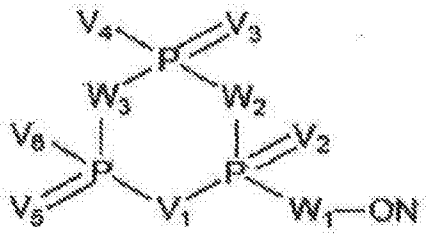
amely magában foglalja a következő lépéseket:

(a) egy



IIa

képletű vegyületnek, amelyikben V_1, V_3, V_5, V_4, V_6, W_1, W_2, W_3 és ON a fentiek szerint van meghatározva, egy oxidálószerrel való reakcióját, azzal a céllal, hogy egy



IIb

képletű vegyületet, amelyikben V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 és ON a fentiek szerint van meghatározva, kapjunk

(b) egy (IIb) képletű vegyületnek egy

Z-Y-XH (III)

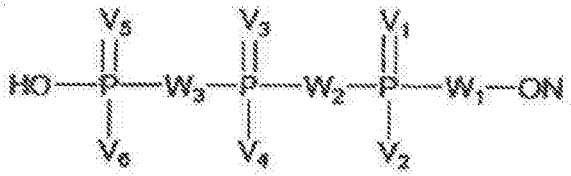
képletű affinitás-markerrel való reagáltatását, ahol X, Y és Z jelentései a fentiek szerint vannak meghatározva, amellyel egy (I) képletű oligonukleotidot tartalmazó reakcióterméket kapunk, és

(c) a (b) lépés szerinti reakcióterméknek egy olyan affinitás-reagenssel való érintkeztetését, amely képes az affinitás-markerrel való kölcsönhatásra, ahol a affinitás-markert egy standard reverz fázisú kromatográfia, például RP-HPLC-nek egy szilárd fázisából, egy hidrofób csoportokra affinitást mutató kromatográfiás anyagból, egy fluorozott csoportokra affinitást mutató kromatográfiás anyagból, például egy fluoros-affinitás hordozóból, egy alkinil-csoportot tartalmazó affinitás-reagensből valamint az, egy azid-csoportot tartalmazó affinitás-reagensből alkotott csoportból választjuk ki, ahol az érintkeztetés olyan körülmények között valósul meg, amely lehetővé teszi az (I) oligonukleotidnak a reakcióterméken található más anyagoktól való elválasztását.

2. Az 1. igénypont szerinti eljárás, amelyben a trifoszfát/trifoszfát-analóg csoport az oligonukleotid 5'-végéhez, különösen ennek az 5'-terminális cukorjának az 5'-OH-csoportjához kapcsolódik.

3. Az 1. vagy 2. igénypont szerinti eljárás, amely továbbá magában foglalja a következő lépést:

(d) az affinitás-marker eltávolítása, amellyel egy



IV

képletű oligonukleotidot kapunk, amelyikben V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3 és ON az 1. igénypont szerint van meghatározva.

4. Az 1-3. igénypontok egyike szerinti eljárás, amelyben az oligonukleotidot a dezoxiribonuleotidok, ribonukleotidok és az oligonukleotid-analógok közül választjuk ki.

5. Az 1-4. igénypontok egyike szerinti eljárás, amelyben az oligonukleotid egyszálas vagy kétszálas.

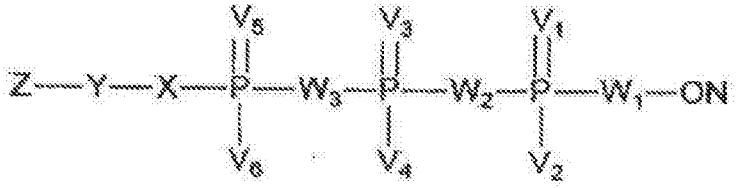
6. Az 5. igénypont szerinti eljárás, amelyben az oligonukleotid kétszálas és a duplex ennek disztális végén egy hurok által zárt, ahol a hurok tartalmaz nukleotid és/vagy nem-nukleotid építőelemeket.

7. Az 5. vagy 6. igénypont szerinti eljárás, amelyben az oligonukleotid kétszálas és a duplex, ennek a proximális végén blunt-végű.

8. Az 1-7. igénypontok egyike szerinti eljárás, amelyben az oligonukleotid tartalmaz egy sejt-specifikus célentitást, amely ehhez kovalensen kapcsolódik.

9. Az 1-8. igénypontok egyike szerinti eljárás, amelyben az (I) vagy (IV) oligonukleotid a RIG-1-nek egy aktivátora.

10. Egy



I

képletű oligonukleotid,

amelyben V_1, V_3, V_5, V_2, V_4, V_6, W_1, W_2, W_3, X, Y, Z és ON az 1-9. igénypontok egyike szerint van meghatározva,

előállítására szolgáló készlet alkalmazása, ahol a készlet tartalmaz:

(a) egy

Z-Y-XH (III)

képletű affinitás-marker anyagot, ahol X, Y és Z az 1-9. igénypontok egyike szerint van meghatározva, és

(b) affinitás-reagenst, amely képes az affinitás-markerrel való kölcsönhatásra, ahol az affinitás-reagenst egy standard reverz fázisú kromatográfia, például RP-HPLC-nek

egy szilárd fázisából, egy hidrofób csoportokra affinitást mutató kromatográfiás anyagból, egy fluorozott csoportokra affinitást mutató kromatográfiás anyagból, például egy fluoros-affinitás hordozóból, egy alkinil-csoportot tartalmazó affinitás-reagensből valamint az, egy azid-csoportot tartalmazó affinitás-reagensből alkotott csoportból választjuk ki.

A meghatalmazott:

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