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- (71) Applicant (for all designated States except US): **RHEINISCHE FRIEDRICH-WILHELMS-UNIVERSITÄT BONN** [DE/DE]; Regina-Pacis-Weg 3, 53113 Bonn (DE).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **LUDWIG, Janos** [DE/DE]; Tuckermannweg 6, 37085 Göttingen (DE). **GOLDECK, Marion** [DE/DE]; Wenzelgasse 44, 53111 Bonn (DE). **SPROAT, Brian** [GB/BE]; Jaarmarktstraat 48, B-2221 Booischot (BE).
- (74) Agent: **HEUBECK, Christian**; Weickmann & Weickmann, Postfach 860 820, 81635 München (DE).
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(54) Title: PURIFICATION OF TRIPHOSPHORYLATED OLIGONUCLEOTIDES USING CAPTURE TAGS

(57) Abstract: The present invention relates to a method of preparing triphosphate-modified oligonucleotides using a capture tag. The method allows the synthesis and purification of triphosphate-modified oligonucleotides in high yield and purity suitable for pharmaceutical applications.

Purification of triphosphorylated oligonucleotides using capture tags

5

Description

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

Background of the invention

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.

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.

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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
5 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 m⁷G tri-, di- or monophosphate or analogue.

10 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-
15 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.

20 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
25 make separation a tedious task (Sproat, 1999; Zlatev, 2010; 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.

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

Summary of the invention

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 moiety, e.g. analogues containing for instance β,γ-methylene, fluoromethylene, difluoromethylene and imino groups replacing an oxygen atom.

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

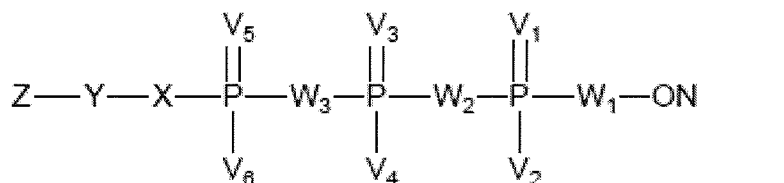
The present invention describes the synthesis and purification of oligonucleotide triphosphates, including analogues thereof that contain capture tags. The most widely employed method for the HPLC purification of standard 5'-OH oligonucleotides is reversed phase chromatography of trityl-ON oligonucleotides.

30

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

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



5 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 , $\text{N}(\text{R}^1)_2$ and $\text{BH}_3\cdot\text{M}^+$,

W_1 is O or S,

10 W_2 is O, S, NH or NR^2 ,

W_3 is O, S, NH, NR^2 , CH_2 , CHHal or $\text{C}(\text{Hal})_2$,

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,

15 M^+ is a cation,

X is NH, NR^3 , O or S,

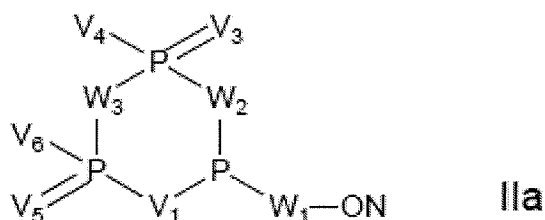
Z represents a capture tag,

Y represents a bond or a linker connecting the capture tag to X, and

20 ON represents an oligonucleotide comprising at least 4 nucleotide or nucleotide analogue building blocks,

comprising the steps:

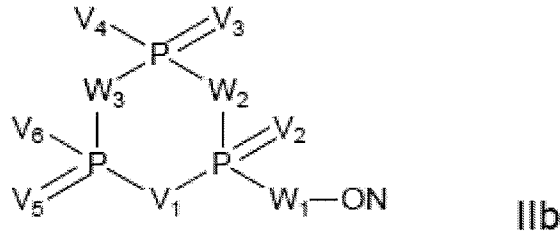
(a) reacting a compound of formula (IIa)



- 5 -

wherein V₁, V₃, V₅, V₄, V₆, W₁, W₂, W₃, and ON are as defined above, with an oxidizing agent to obtain a compound of formula (IIb)

5



wherein V₁, V₃, V₅, V₂, V₄, V₆, W₁, W₂, W₃ and ON are as defined above,

10

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



15

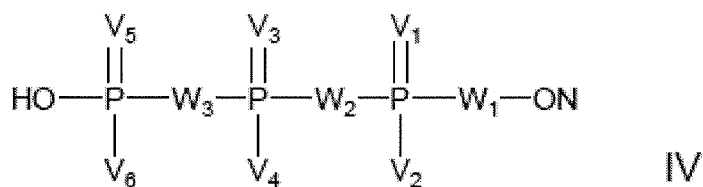
wherein X, Z, and Y are as described above to obtain a reaction product comprising the oligonucleotide of formula (I), and

20

(c) contacting the reaction product of step (b) with a reagent capable of interacting with the capture tag under conditions which allow separation of the oligonucleotide (I) from other species contained in said reaction product.

25

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



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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.

5 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.

The term "oligonucleotide" in the context of the present application
10 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
15 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.

20

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,
25 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
30 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

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5 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. N⁶-methyladenine, or N⁶,N⁶-dimethyladenine. Further suitable nucleobase analogues may be selected from universal nucleobase analogues such as 5-nitroindole.

10 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.

15 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.

20 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).
25 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
30 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-dioxo-7,8-dithiatetradecane-1,14-diol.

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

5 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
10 nucleobases or non-nucleotidic building blocks by methods known to the skilled person.

The oligonucleotide of formula (I) or (IV) comprises a triphosphate/triphosphate analogue group. In this group, V_1 , V_3 and V_5 are
15 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 $BH_3 \cdot M^+$. 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}
20 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, $O(\text{halo})C_{1-2}$ alkyl and - in the case of cyclic groups - $(\text{halo})C_{1-2}$ alkyl. M^+ may be an inorganic or organic cation, e.g. an alkali metal
25 cation or an ammonium or amine cation.

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
30 described for R^1 above. Hal may be F, Cl, Br or I.

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.

Step (a) of the method of the invention comprises the reaction of cyclic P(V)-
5 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
10 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).
15 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
20 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, the contents of which are herein incorporated
by reference.

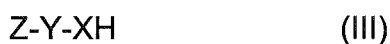
25 Reaction step (a) may take place with an oligonucleotide in solution or with
an oligonucleotide bound to a solid phase, e.g. an organic resin or glass,
such as CPG. The oligonucleotide may further comprise protecting groups,
e.g. sugar- or nucleobase protecting groups that are well known to the skilled
person. Preferred examples of protecting groups are 2-cyanoethyl for the
30 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,

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step (a) is carried out with a solid-phase bound oligonucleotide.

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

5



wherein X is a group selected from NH, NR³, O or S. R³ is defined as described above for R¹. Preferably, X is NH or S.

10

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.

15

Y represents a chemical bond or a linker, e.g. an alkylene, preferably a C₁₋₆-alkylene linker, more preferably a C₂₋₅-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.

20

In another preferred embodiment the linker is a polyalkylene oxide, preferably a poly-C₂-C₆-alkylene oxide, more preferably a poly-C₂-C₃-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 [-CH₂CHR⁴-O-]_n with n = 1-10, preferably n = 1-7, more preferably n = 2-5, and even more preferably n = 3. R⁴ may be H or C₁₋₆-alkyl.

25

30 In a preferred embodiment R⁴ is H.

In an especially preferred embodiment the linker has the formula -CH₂-CH₂-[(O-CH₂CH₂)]₃-.

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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
5 oligonucleotide.

The capture tag Z according to the present invention is a moiety 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.
10

Suitable capture tags are for instance long-chain, e.g. C₈₋₂₄, preferably C₁₃₋₂₄ 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
15 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.
20

In another embodiment, the capture tag 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
25 streptavidin, an avidin or an antibody.
30

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

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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
5 with a capture reagent that contains a complementary reactive group, e.g. an alkynyl or azido moiety, respectively, in the case of the Huisgen 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
10 case would be propargylamino.

In another embodiment, the capture tag component may be a chemical entity which contains an additional nucleophilic group, for instance a second amino group in an NH₂-Y-XH type reagent. A wide range of suitable electrophilic Z
15 reagent such as cholesterol, chloroformate 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.

20 In a preferred embodiment the capture tag is a long-chain alkyl residue, a perfluoroalkyl entity, an azide or an alkynyl group.

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

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
30 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.

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

5 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
10 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
15 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
20 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.

25 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
30 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.

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

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
10 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-CH₂-CH₂-O-P.

In further embodiments, the capture tag set remains completely or partially on the triphosphate-modified oligonucleotide, particularly when the tagged
15 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.

20 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
25 helicase. Specific examples of suitable RIG-1 activators are disclosed in Schlee et al., 2009, supra, the content of which is herein incorporated by reference.

In another embodiment the present invention refers to oligonucleotides of
30 Formula (I), obtainable by a method according to the present invention.

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

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claims 1-11, wherein V_1 , V_2 , V_3 , V_4 , V_5 , V_6 , W_1 , W_2 and W_3 are preferably O.

According to a preferred embodiment of the present invention a modified oligonucleotide of formula (I) has X being NH. This embodiment preferably
5 has Z being Q or Z being QNHC₂-C₂₄ alkyl, wherein in a particularly preferred embodiment C₂-C₂₄ alkyl is C₂ alkyl and/or Q is H. Particularly preferred embodiments of the identified oligonucleotide according to the invention are shown in Fig. 8.

10 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

15

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);

20 (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].

25 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 μ m

30 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

- 17 -

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].

5

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)

10

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:

15

- 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

20

Example 1

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

25

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

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.

30

- 19 -

Step 2: Dry the synthesis column containing the fully protected RNA that has been detitrated 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.

Step 3: Add into a vial first 2 mL of pyridine/dioxane, 3:1 v/v followed by 100 μ L 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.

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.

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 $(\text{Bu}_3\text{NH})_2$ pyrophosphate in dry DMF and 238 μ L (1 mmol) of dry Bu_3N to give a 0.5 M $(\text{Bu}_3\text{N})_4$ 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.

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 .

- 20 -

Step 6: Dissolve 300 μ L of t-BuOOH (5.5 M solution in decane, Sigma-Aldrich) in 2 mL of anhydrous CH₃CN 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.

5

Step 7: Wash the column with 3 mL of dry CH₃CN to remove the excess peroxide and fill it with dry CH₃CN.

Step 8: Dissolve 300 μ L of dry decylamine in 1 mL of dry CH₃CN 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**.

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

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 μ L, then evaporate to dryness.

Step 11 - Removal of the 2'-O-TBDMS protecting groups: Dry the residue by addition and coevaporation of 300 μ L 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 NAPTM-25 (Nucleic Acid Purification) column using sterile water as eluent. Filtration through a sterile 2 μ m filter may be necessary at this step. Combine and evaporate the UV-absorbing fractions to a volume of 150 μ L, add 100 mL of 1 M TEAB pH8 and store the solution frozen at -20°C until the HPLC purification can be

30

- 21 -

performed. The decyl-NHpppRNA product is stable at -20°C for weeks at pH 7-8.

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 .

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.

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 µL 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 detitrated RNA

↔ bidirectional movements of reagents,
→ unidirectional washing step

- 22 -

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 ₃ NH) ₂ PP _i in DMF plus 238 μL of Bu ₃ N	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 ₃ CN	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	→

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

5 Example 2

Preparation of triphosphate oligonucleotides using non-lipophilic capture tags (F-TAG-pppRNA and N₃-TAG-pppRNA)

10

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 N₃-TAG-pppRNA. The following deprotection steps are identical with those given in the detailed description for DecNHpppRNA in Example 1.

20

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

(the RNA sequence in these examples is 5'-

25

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-pppRNA	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)

- 5 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 noncovalent interaction between perfluorinated alkyl chains. The gamma azide modified pppRNA derivatives (N3-TAG-pppRNA) can be covalently bound to commercially available
- 10 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.
- 15 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.

20 Example 3

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

- Besides the n-decyl-tag described in Example 1, aliphatic n-alkyl residues
- 25 with longer chain lengths (C₁₂, C₁₄, C₁₈) 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
- 30 variation of step 8: A 0.1 M solution of n-alkylamine (n-dodecylamine, n-

- 24 -

tetradecylamine or n-octadecylamine) in dry CH₂Cl₂ 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 CH₂Cl₂ is required prior to continuing with the next workup steps.

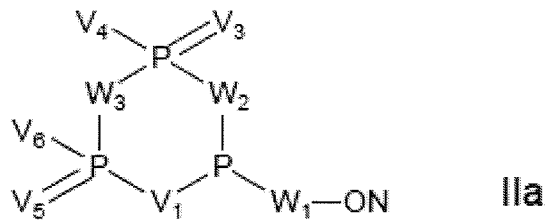
Analytical data:

	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)

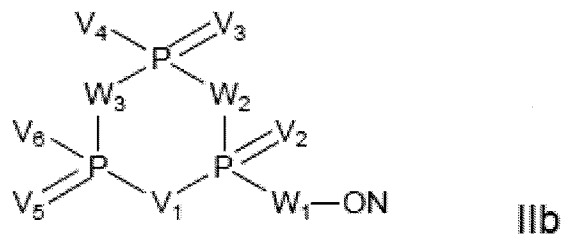
Figure 11 shows the RP-HPLC analysis of pppRNA and n-alkyl-NH-pppRNAs with alkyl residues of increasing chain length.

- 26 -



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

5 with an oxidizing agent to obtain a compound of formula (IIb)



10 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),



15

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 contacting takes place under conditions which allow separation
 20 of the oligonucleotide (I) from other species contained in said reaction product.

25 2. The method of claim 1, wherein the capture tag and the capture reagent capable of interacting therewith are selected from:

(i) a hydrophobic or fluorinated group and a chromatographic

- 27 -

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 binding pair and a second partner of a non-covalent binding pair, and
- 5 (iii) a first partner of a covalent binding pair and a second partner of a covalent binding pair, where the first and second partner form covalent bonds.

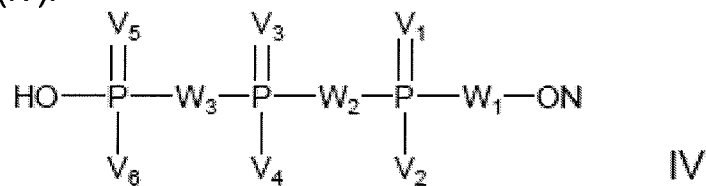
3. The method of claim 1 or 2,

10 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.

4. The method of any one of claims 1-3, further comprising the step:

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

(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.

20

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

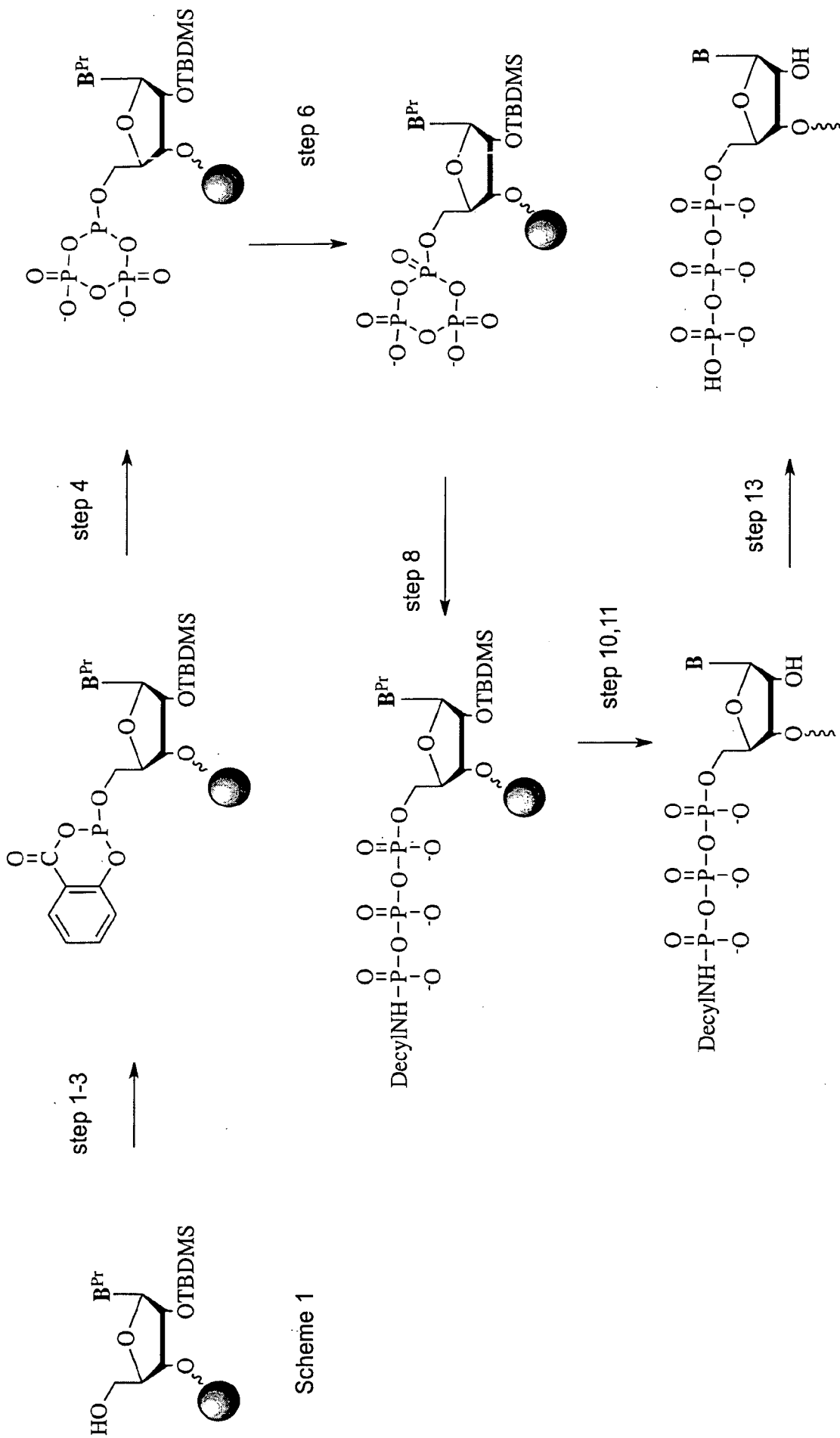
25

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

30

7. The method of claim 6, wherein the oligonucleotide is double-stranded and the duplex is closed by a loop at the distal end thereof, wherein the

Figure 1



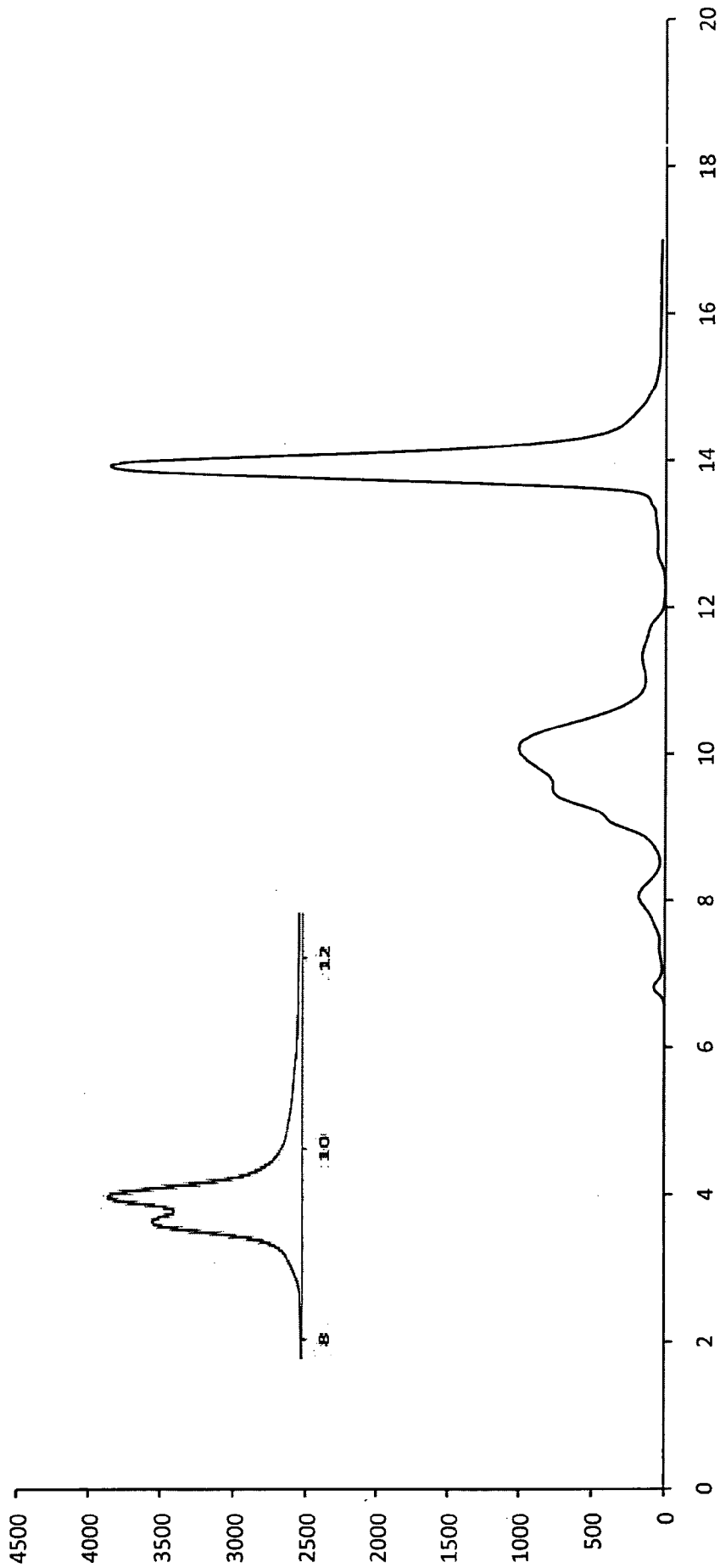


Figure 2A

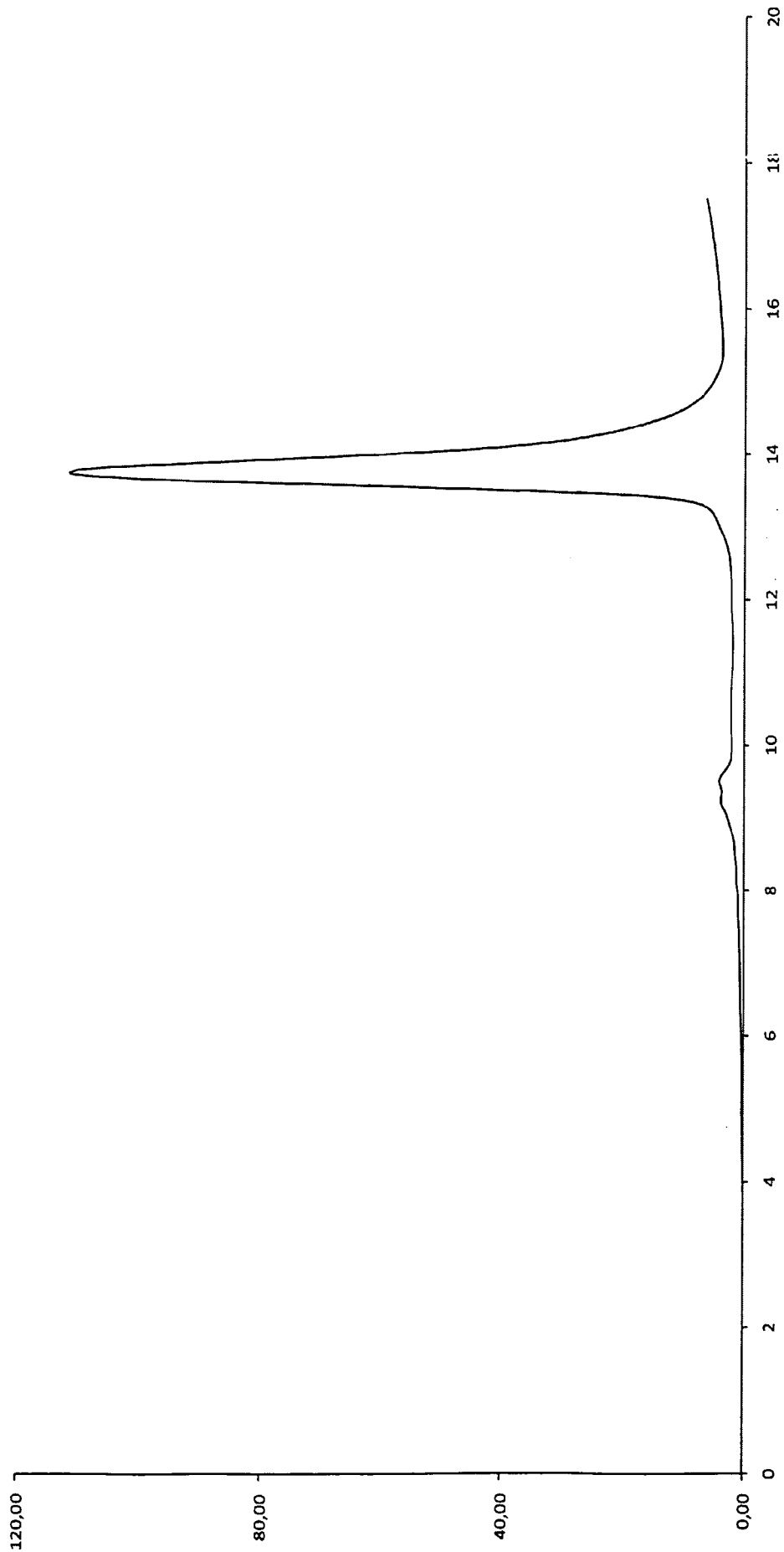


Figure 2B

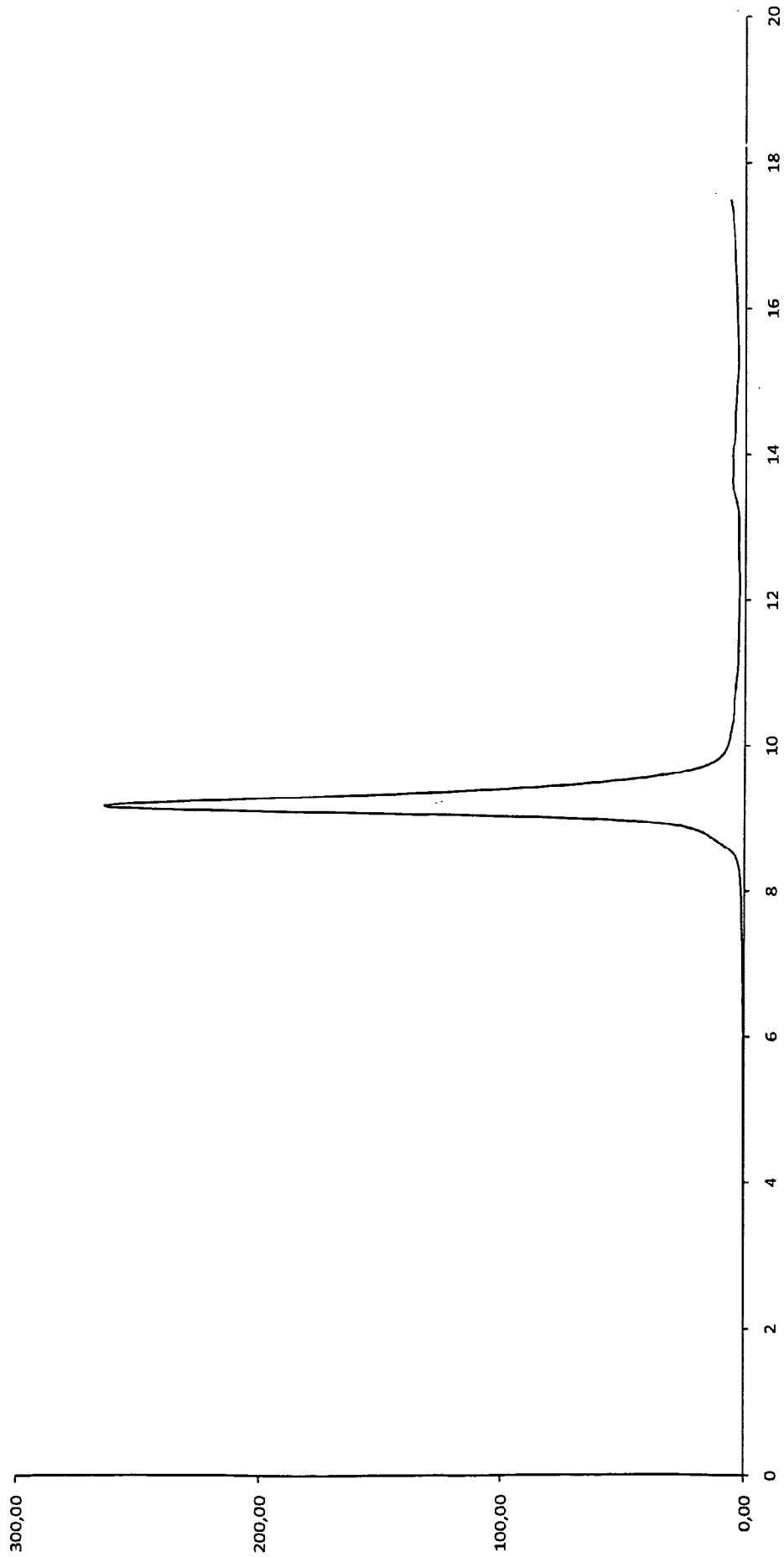


Figure 2C

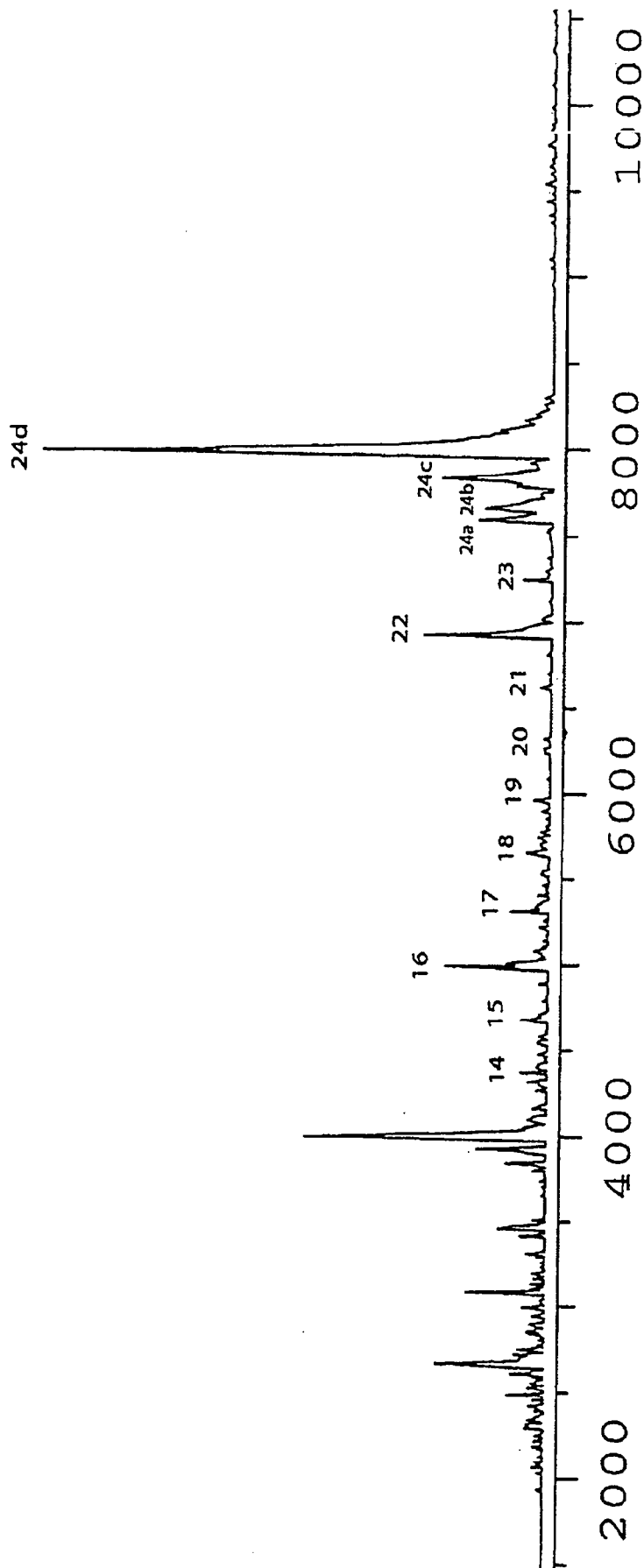


Figure 3A

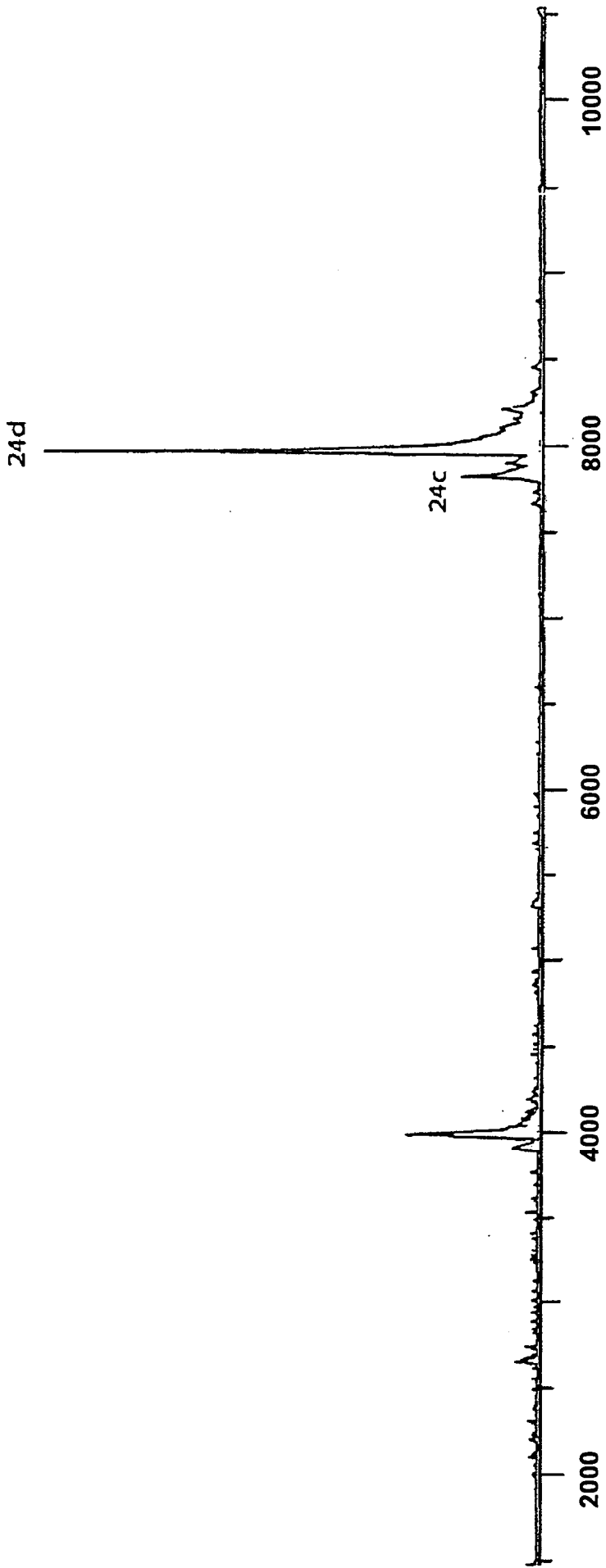


Figure 3B

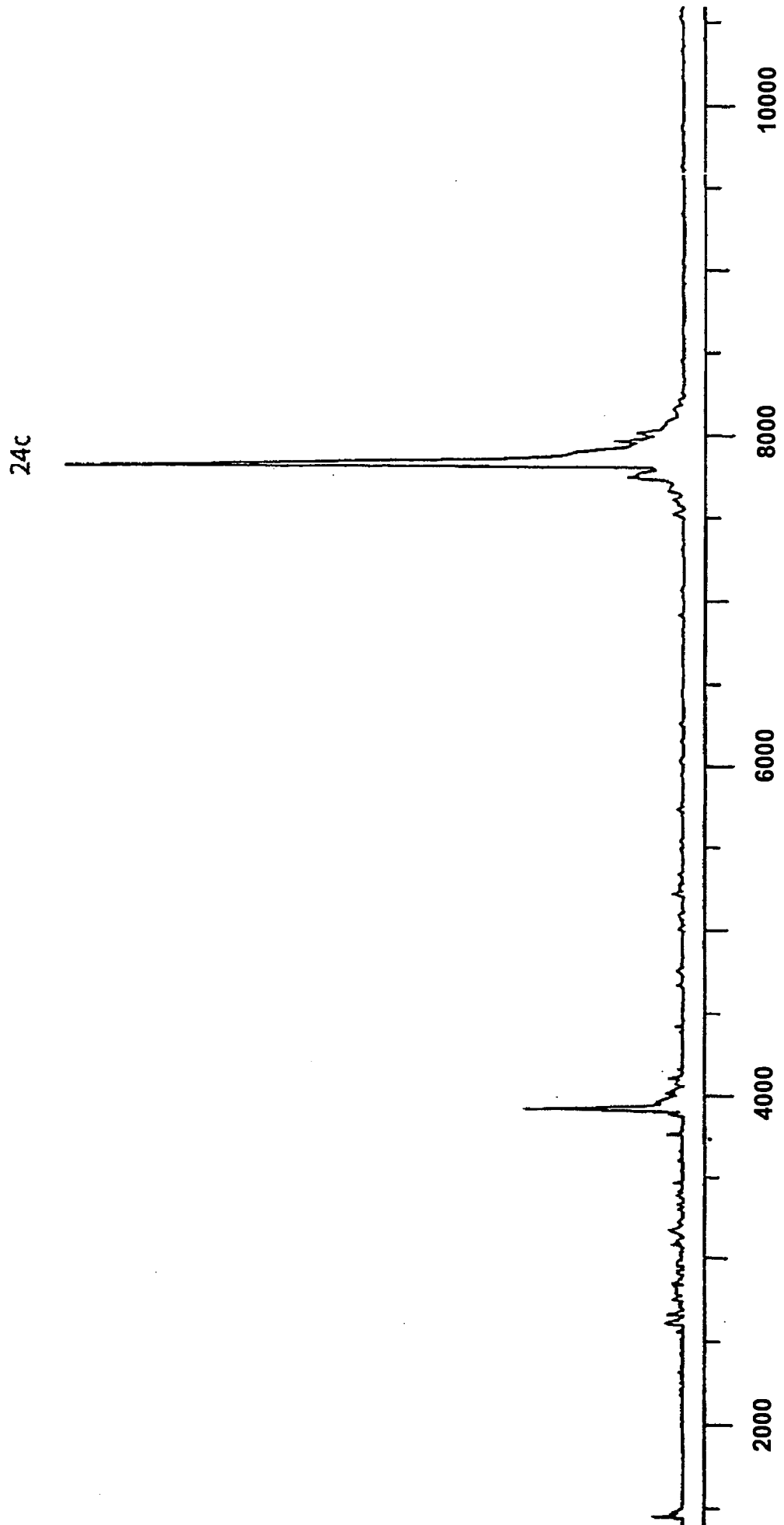


Figure 3C

Figure 4a

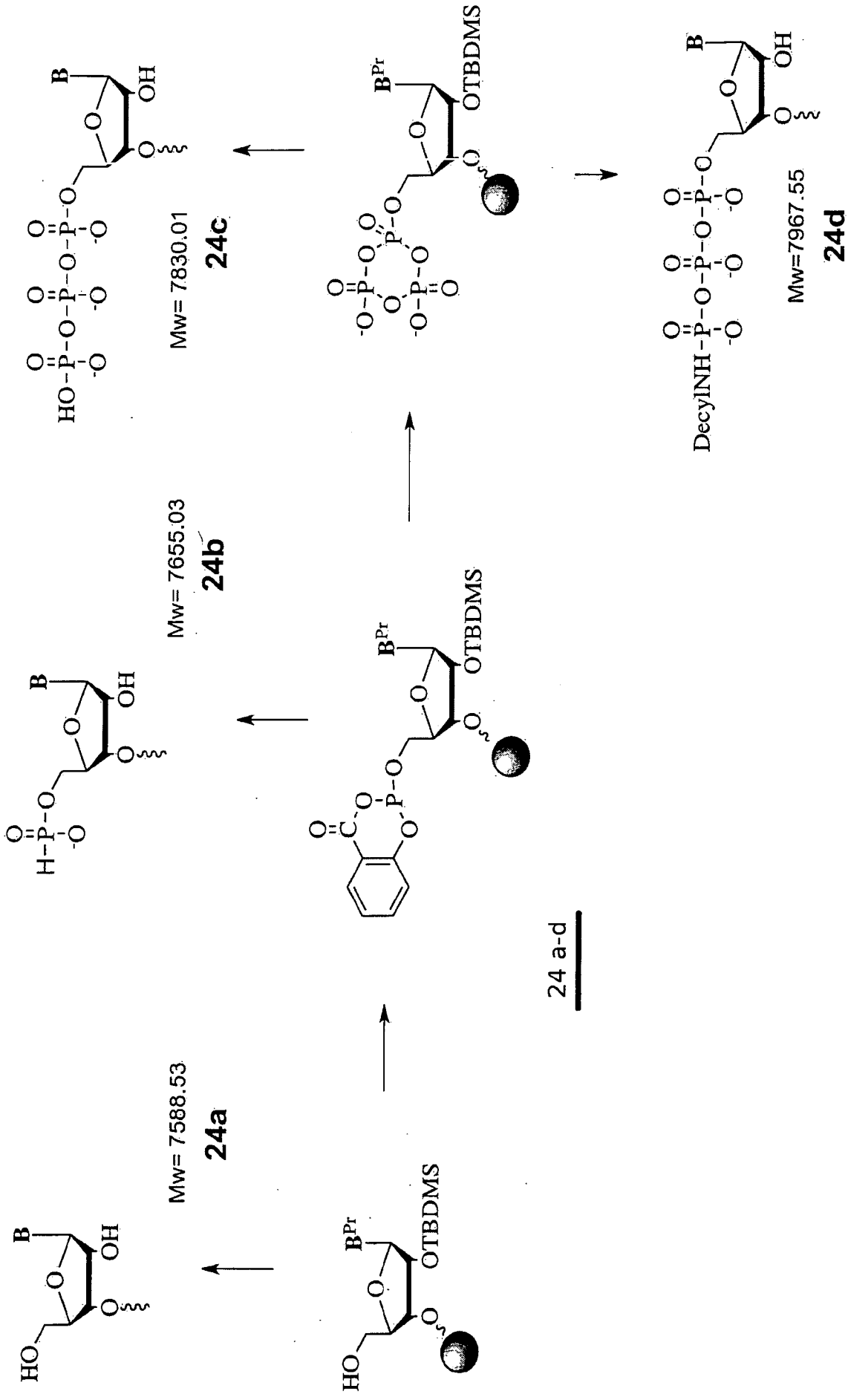
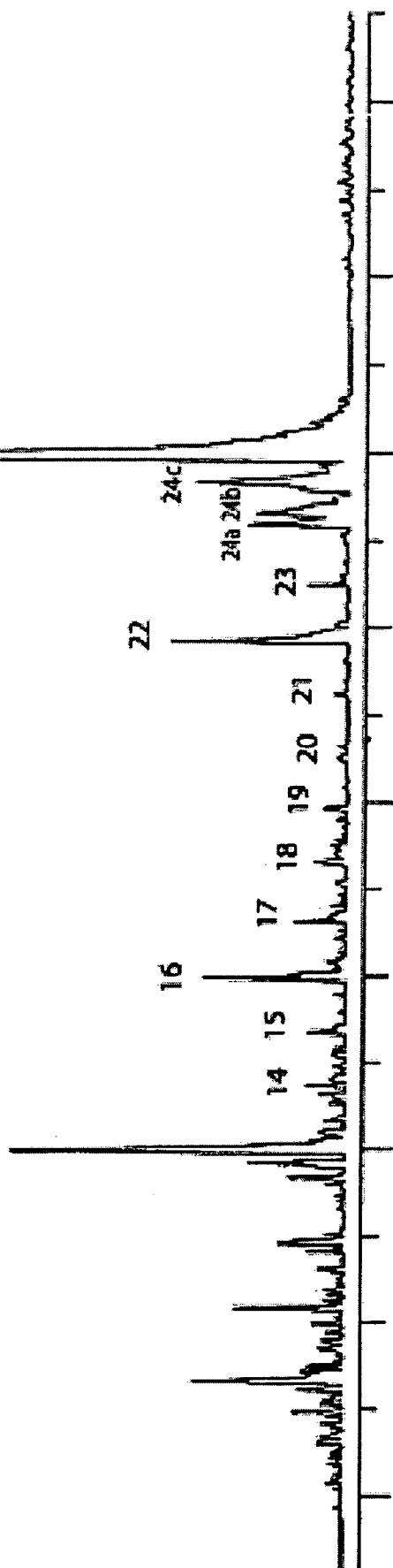


Figure 4b

24 a-d

24d

1-23



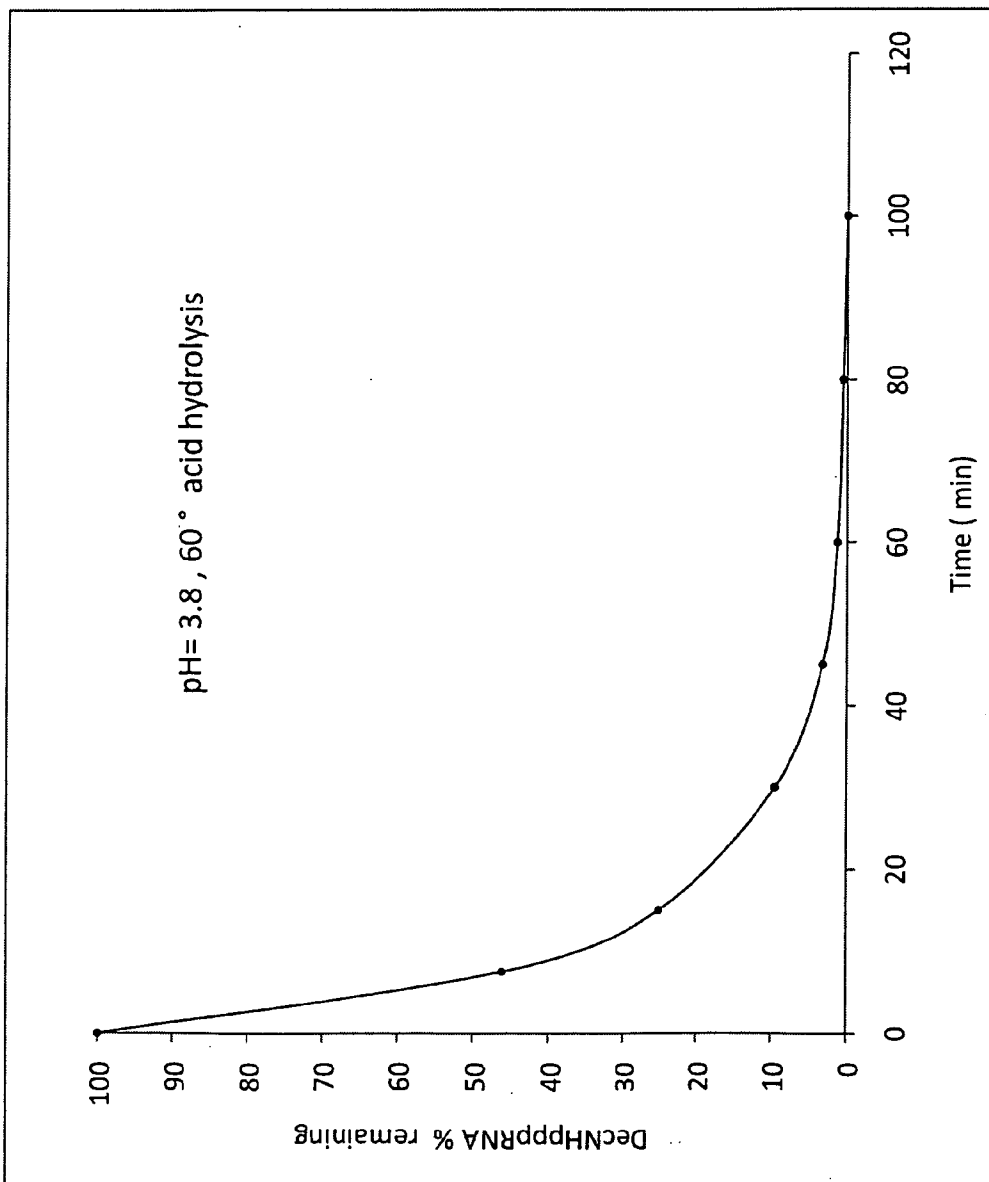


Figure 5

Figure 6A

pppRNA 21 mer

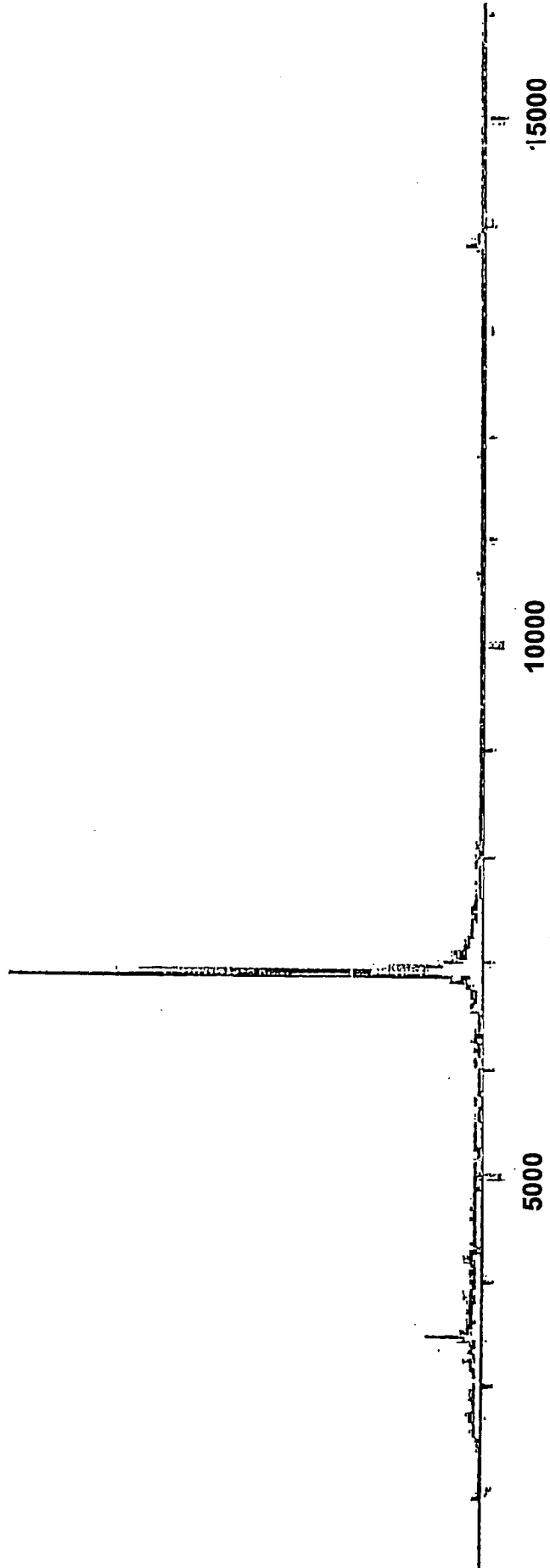


Figure 6B

pppRNA 24 mer

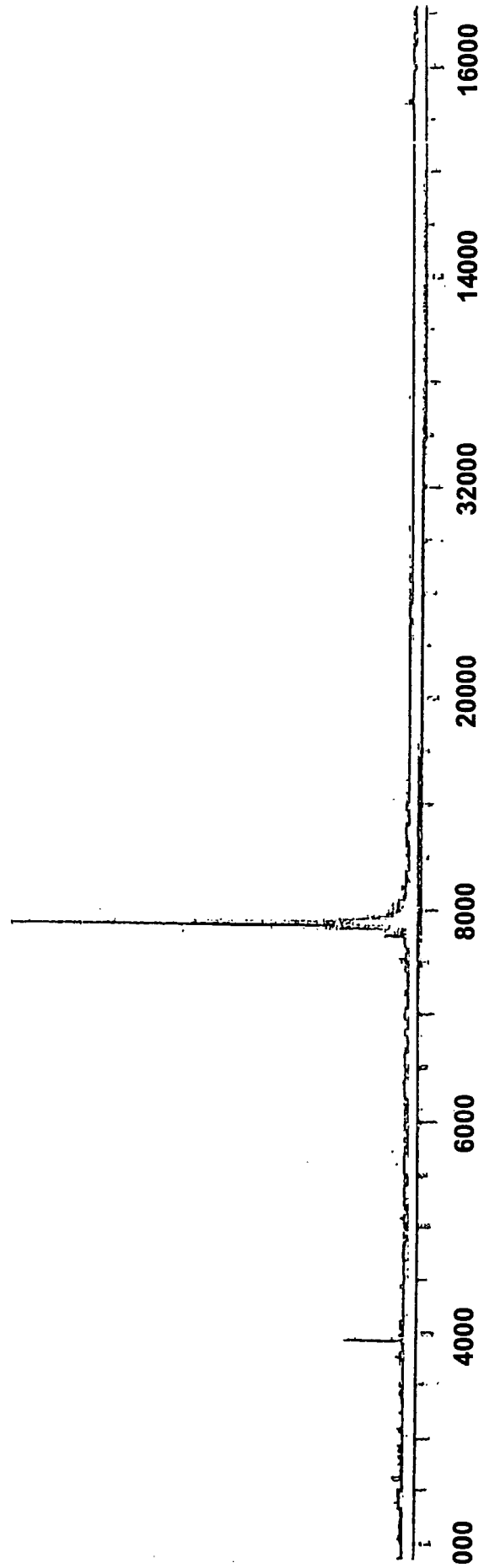
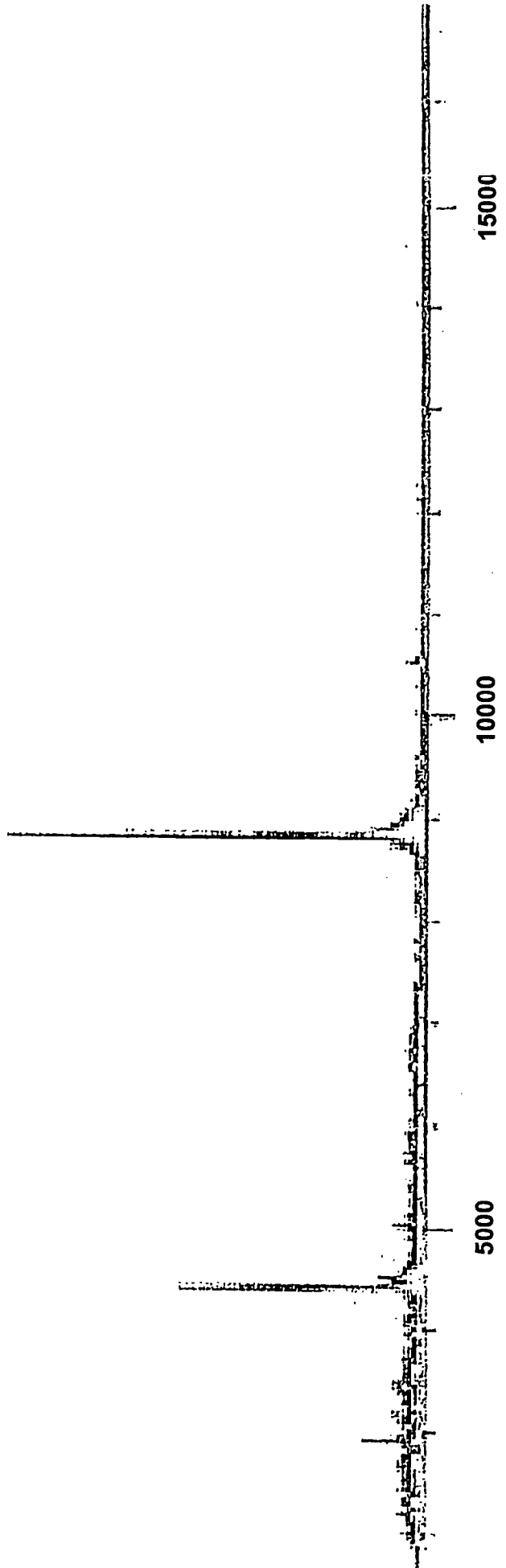


Figure 6C

pppRNA 27 mer



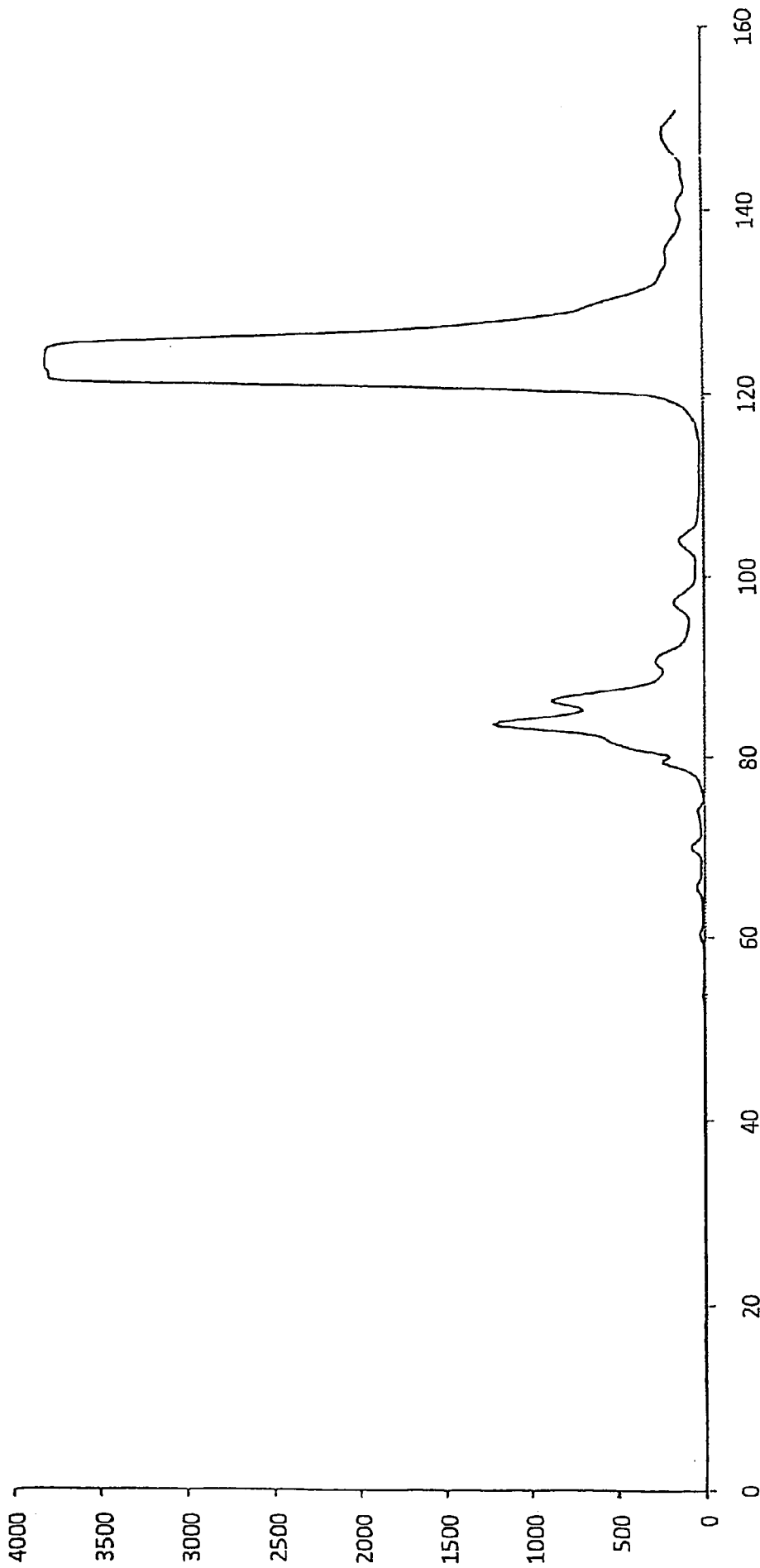


Figure 7A

Figure 7B

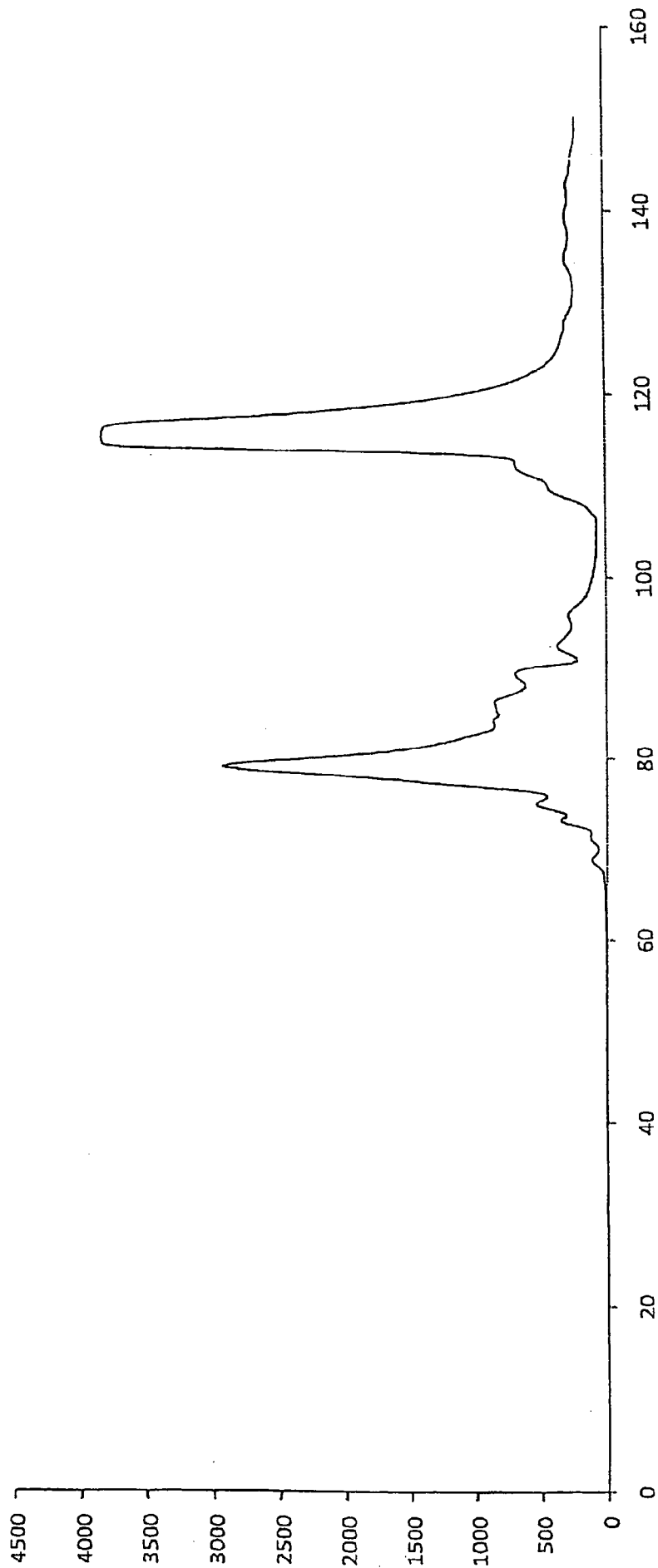


Figure 7C

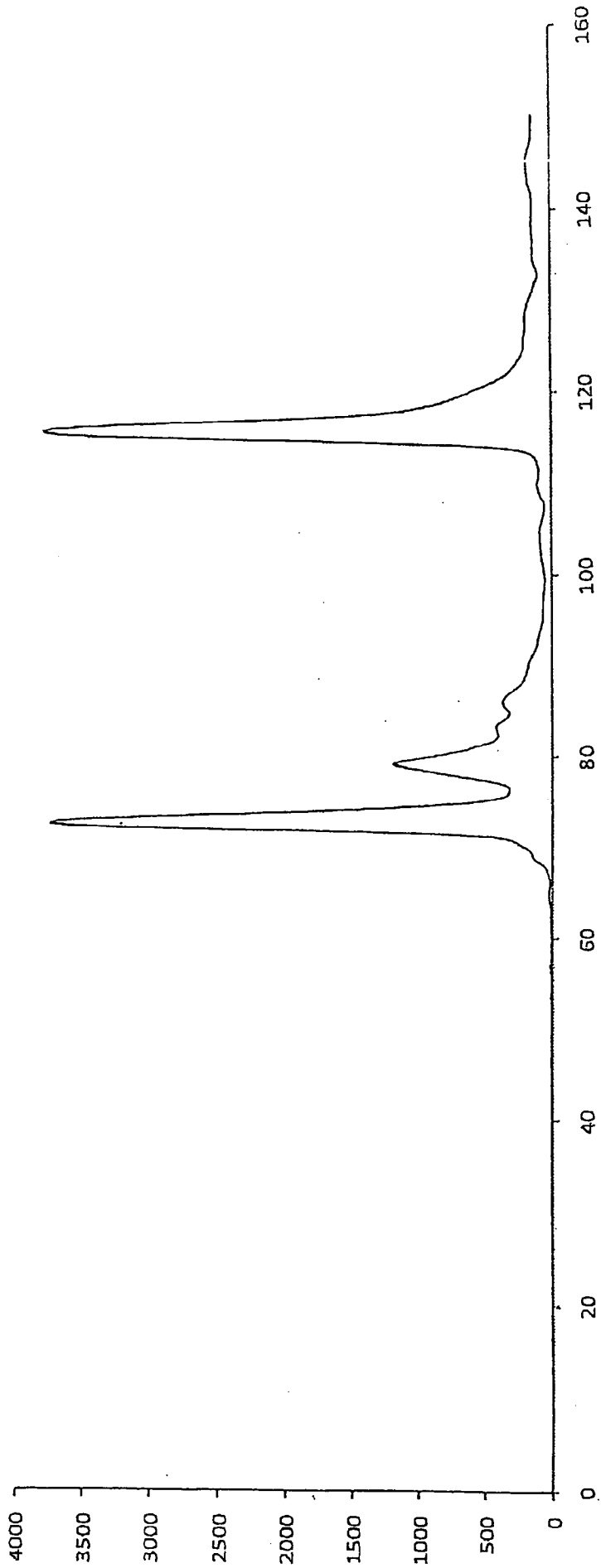
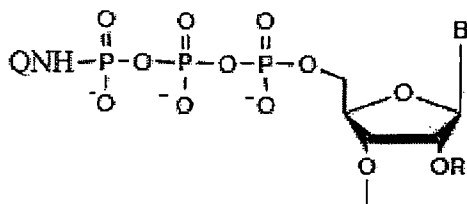
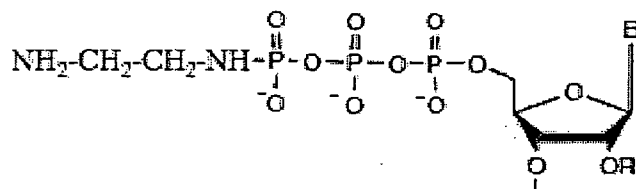


Figure 8

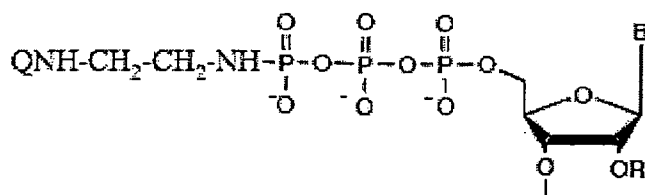
gamma modification



A



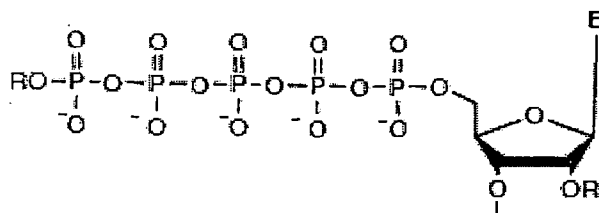
B1



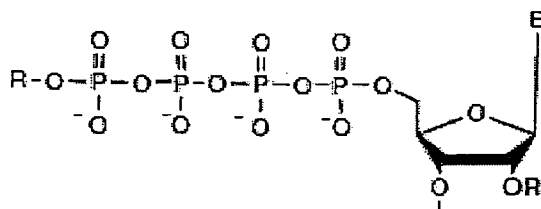
B

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

extended chain



D1



D2

Figure 9B

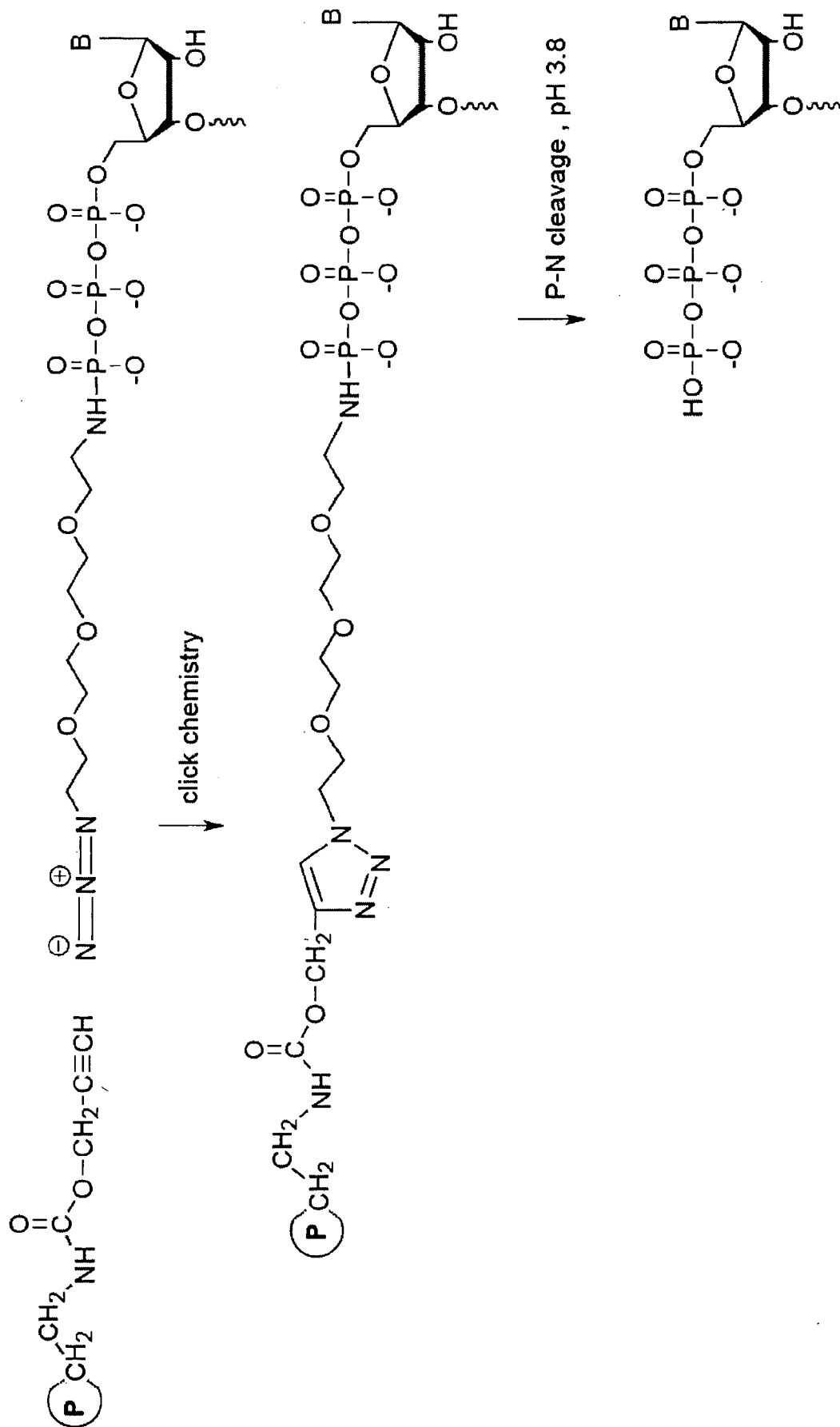


Figure 10A

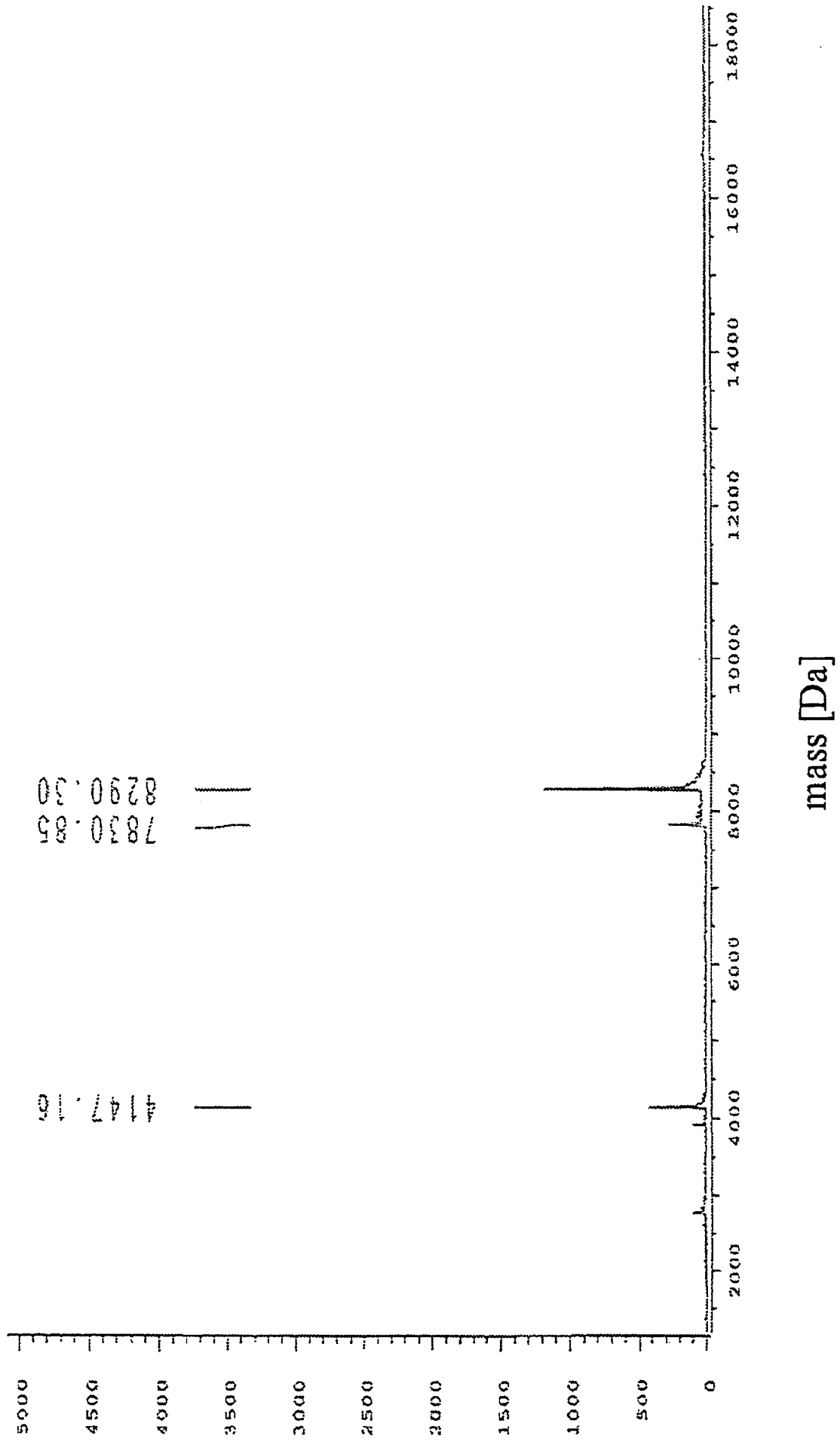


Figure 10B

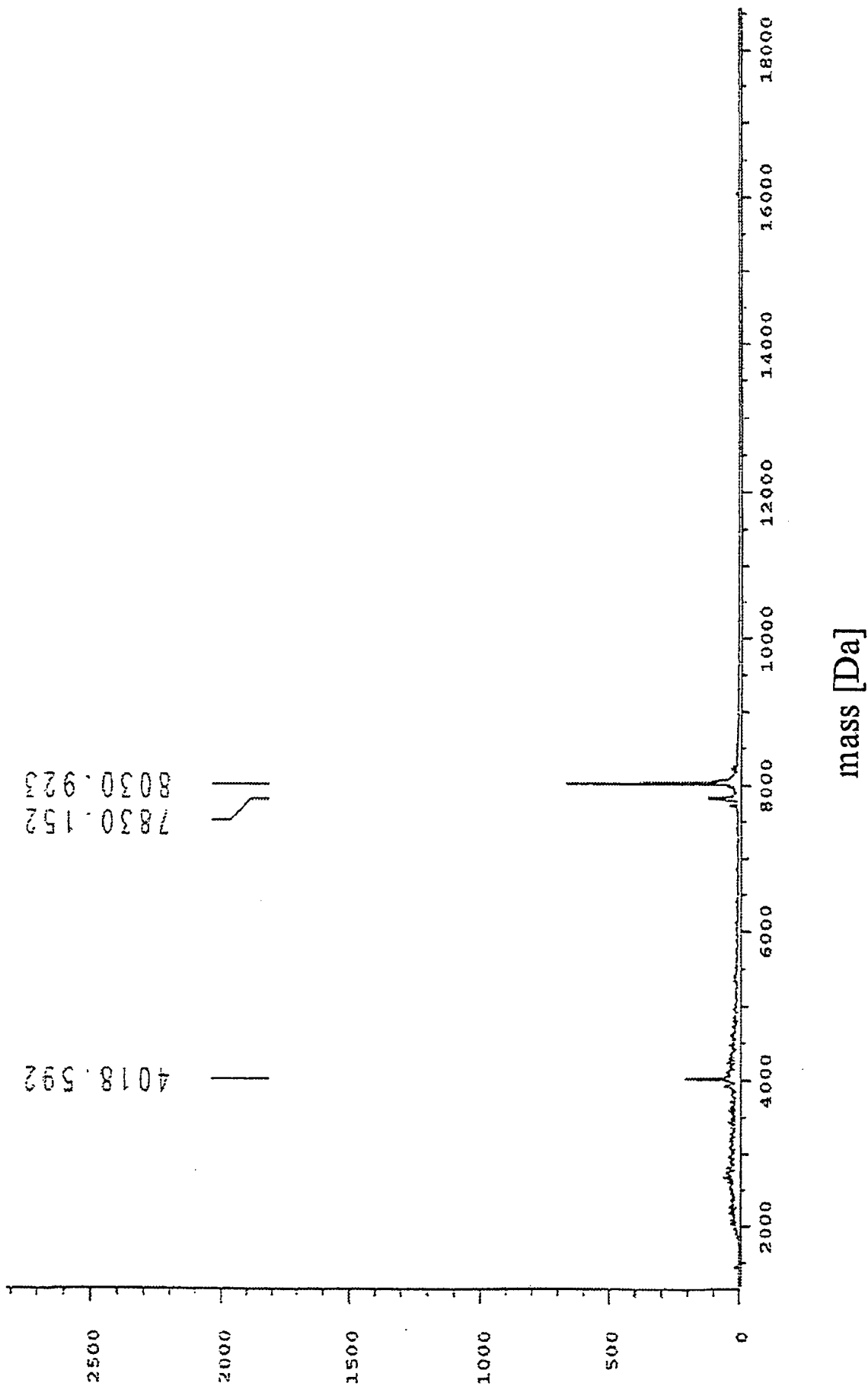
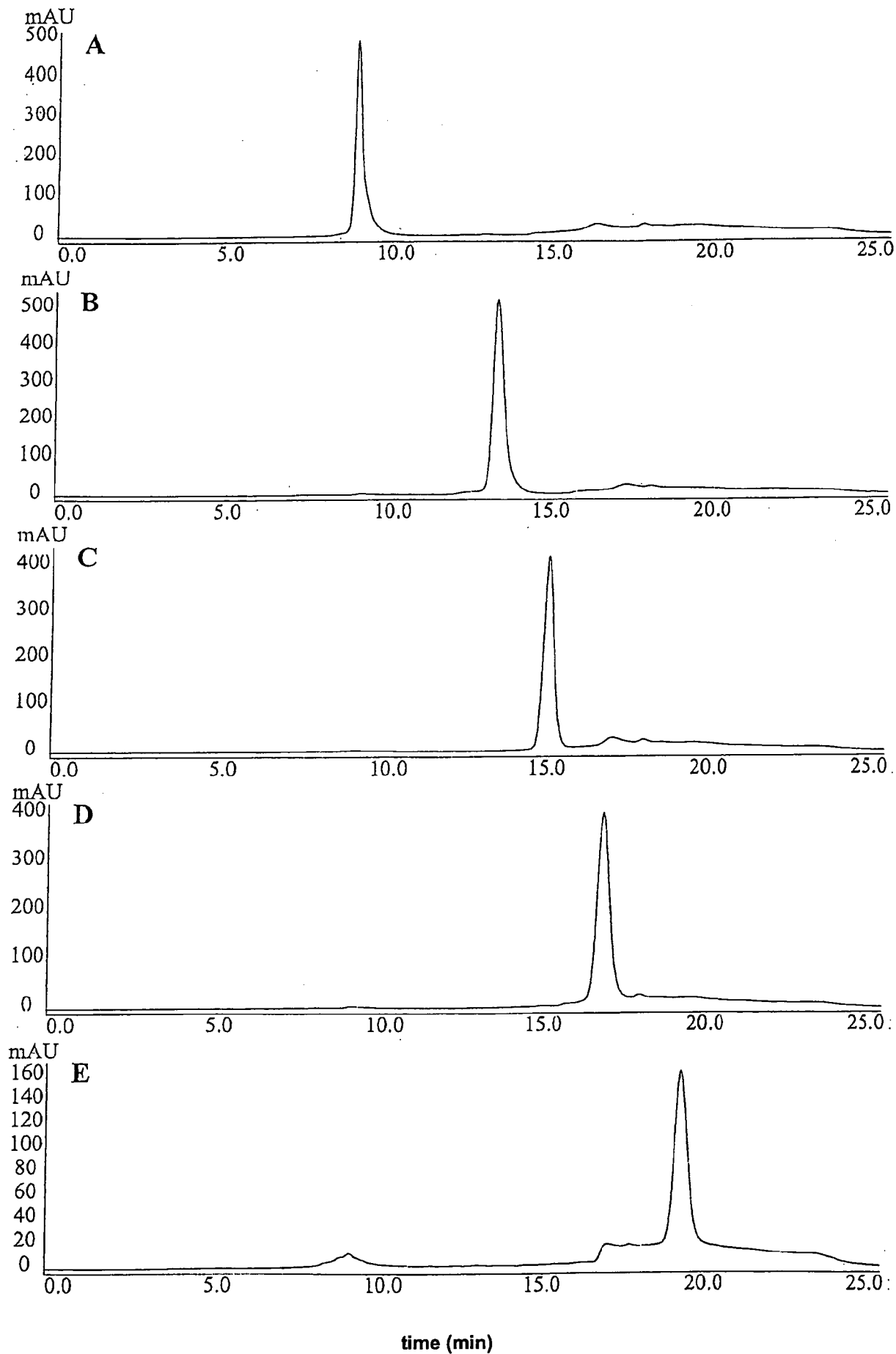


Figure 11



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/055520

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H21/02
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C07H
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/060281 A2 (COLEY PHARM GMBH [DE]; DEBELAK HARALD [DE]; UHLMANN EUGEN [DE]) 14 May 2009 (2009-05-14) cited in the application	13-15
A	pages 1,43 claim 1 figure 2 ----- -/--	1-12

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 23 July 2012	Date of mailing of the international search report 27/07/2012
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Nikolai, Joachim
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/055520

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEBEDEV A V ET AL: "PREPARATION OF OLIGODEOXYNUCLEOTIDE 5'-TRIPHOSPHATES USING SOLID SUPPORT APPROACH", NUCLEOSIDES, NUCLEOTIDES AND NUCLEIC ACIDS, TAYLOR & FRANCIS, PHILADELPHIA, PA, vol. 20, no. 4-7, 1 January 2001 (2001-01-01), pages 1403-1409, XP009081703, ISSN: 1525-7770, DOI: 10.1081/NCN-100002565	13,14
A	the whole document	1-12,15
X	IVAN ZLATEV ET AL: "Efficient Solid-Phase Chemical Synthesis of 5'-Triphosphates of DNA, RNA, and their Analogues", ORGANIC LETTERS, vol. 12, no. 10, 21 May 2010 (2010-05-21), pages 2190-2193, XP055005331, ISSN: 1523-7060, DOI: 10.1021/ol1004214	13,14
A	the whole document	1-12,15
X	WO 2011/028218 A1 (ALNYLAM PHARMACEUTICALS INC [US]; CENTRE NAT RECH SCIENT [FR]; ZLATEV) 10 March 2011 (2011-03-10)	13,14
A	page 31 - page 33 pages 37,44 figure 1	1-12,15
X	MARTIN SCHLEE ET AL: "Recognition of 5' Triphosphate by RIG-I Helicase Requires Short Blunt Double-Stranded RNA as Contained in Panhandle of Negative-Strand Virus", IMMUNITY, vol. 31, no. 1, 1 July 2009 (2009-07-01), pages 25-34, XP55032801, ISSN: 1074-7613, DOI: 10.1016/j.immuni.2009.05.008	13,14
A	cited in the application the whole document	1-12,15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/055520

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-12

Method of preparing an oligonucleotide of formula (I),
oligonucleotide of formula (I) obtainable by a method
according to claim 1 and a kit for preparing an
oligonucleotide of formula (I).

2. claims: 13-15

Modified oligonucleotide of formula (I).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/055520

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009060281	A2	14-05-2009	
		AU 2008326187 A1	14-05-2009
		CA 2704853 A1	14-05-2009
		EP 2207787 A2	21-07-2010
		JP 2011502979 A	27-01-2011
		US 2010260788 A1	14-10-2010
		WO 2009060281 A2	14-05-2009

WO 2011028218	A1	10-03-2011	NONE
