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Description

[0001] The subject of the present invention is a method of synthesis and purification of a nucleotide, a modified nucleotide, a DNA molecule and an oligonucleotide library comprising said modified nucleotide and use of said oligonucleotide library. The objects of the invention can be applied in *in vitro* selection of aptamers used as therapeutic molecules and also as basic components of molecular diagnostic tools.

[0002] Aptamers are oligonucleotides - single-stranded fragments of ribonucleic acid or deoxyribonucleic acid (RNA or DNA), usually from over ten to several tens of nucleotides in length, whose defined sequence allows them to conform a three-dimensional structure fitting to the molecular structure of the ligand and to bind said ligand with high sensitivity and selectivity. Aptamers present very numerous applications in biotechnology and medicine, both as therapeutic molecules and as basic components of molecular diagnostic tools. This leads to aptamers being often compared with widely used antibodies. However, the scope of potential ligands, towards which working aptamers have already been selected, is much broader than mostly protein-based partners of antibodies. Aptamers ligands also include low molecular weight compounds of almost any structure, such as e.g. metal ions, alkaloids, organic dyes, amino acids, nucleosides, nucleotides, porphyrins or sugars [1-3]. Other aptamers advantages, which place them above antibodies in many applications, include: small size (the smallest selected working aptamer is 15 nucleotides in length[4], which corresponds to the mass of about 4600 g/mol); low or zero immunogenicity; cheap and efficient chemical synthesis, which also provides 100% homogenic preparation; the possibility of introducing various chemical modifications; strong resistance to non-physiological conditions and the ability of spontaneous renaturation, and relatively fast process of aptamers selection towards chosen molecular target.

[0003] Aptamers are obtained in a process of "*in vitro* evolution" called the SELEX method (Systematic Evolution of Ligands by EXponential enrichment). It is based on repetitive binding of a pool of oligonucleotide molecules with different sequences (usually 10^{12} - 10^{14} molecules in the first round of selection) with a chosen molecular target, and subsequent physical partitioning of the molecules which have bound the target from the unbound molecules. After the partitioning the oligonucleotides binding the target are enzymatically amplified and the process is repeated, usually with more stringent conditions in order to select molecules of desired target binding parameters[5,6]. Aptamers obtained in such a way are subjected to sequencing - knowledge of the nucleotide sequence allows to chemically synthesize a pure aptamer for further use or for further optimization, comprising for example of introducing modifications or trimming of the oligonucleotide.

[0004] The libraries are sets of single-stranded nucleic acid molecules comprising in their sequences a region of random nucleotide sequence. During the SELEX process the sequences (clones) selected from the library are those sequences which exert the ability to bind a chosen molecular target, towards which the selection is being performed. Each nucleic acid molecule in the library has the same structure - two primer regions at both ends which enable the amplification of the library with Polymerase Chain Reaction (PCR), and a region with random sequence in the middle.

[0005] Both DNA and RNA are susceptible to digestion by nucleolytic enzymes. The use of aptamers *in vivo* or even *in vitro* in samples comprising nucleases (e.g. cell lysates) is hindered due to the degradation of nucleic acids by those enzymes. To overcome this problem many modifications to sugar-phosphate backbone of aptamers have been proposed, for example substituting the hydroxide group at 2' carbon with an amine group, or a substitution of one of the oxygen atoms in the phosphodiester bond with a sulfur atom (thioaptamers). Another examples of methods reducing nucleic acids susceptibility to enzymatic digestion can be the introduction of "spiegelmers" - oligonucleotides whose sugar backbone is composed of L-ribose isomer instead of naturally occurring D-isomer; or the employment of LNA (Locked Nucleic Acid) - an analogue in which ribose molecules have an additional bond connecting 4' carbon and 2' oxygen (thus closing the ribose in 3'-endo conformation, which also influences the hybridization properties of the oligonucleotide).

[0006] A separate problem is a poor repertoire of monomers (nucleotides) of which the nucleic acids strands are formed in nature. The composition of naturally occurring nucleic acids includes only four different nucleobase molecules: adenine, guanine, cytosine and thymine (or uracil in the case of RNA), having only a few chemical groups which can take part in potential binding of the molecular target. It has generated a need of enriching the oligonucleotide libraries used for SELEX process with new unnatural nucleotides and chemical groups - analogues of naturally occurring nucleotides, which can help in selecting aptamers with better target binding properties. To meet this challenge many research groups have worked on enzymatic incorporation of modified nucleotides into the libraries and their use for selection[7-9].

[0007] In the state of art modified nucleotides are obtained by advanced organic synthesis methods, often long and multistep processes with poor yield, not accessible for laboratories without special organic synthesis equipment[8-11]. Therefore, there is a need for easier, more efficient and quicker method of synthesis of modified nucleotides which may be further incorporated into nucleic acids using enzymatic methods, and said method will be useful in selection of new modified aptamers with better parameters than aptamers comprising only naturally occurring nucleotides.

[0008] Huisgen's Copper(I)-catalyzed Azide-Alkyne Cycloaddition (CuACC) enables a highly selective, efficient (often yields over 90%), quick (conjugation time can be less than an hour), run in mild-conditions (room temperature, normal pressure, broad selection of possible solvents including water, pH around 4 to 12), one-step conjugation of two com-

pounds, one of which comprises a free azide group ($-N_3$), and the other a terminal alkyne ($-C\equiv CH$). The reaction is performed in the presence of copper (I) ions (a catalizator) and the resulting stable covalent triazol bond has always the same regioisometry[12,13]. For these reasons this reaction is classified as an example of so called "Click Chemistry" - a simple and "click"-like efficient conjugation chemistry with a broad application spectrum[14,15].

[0009] Authors of US patent 6,175,001 (Barbas and Kandasamy, 2001) [10] describe preparation and use for DNA synthesis of nucleoside triphosphates bearing a modification in position 5 of deoxyuracil pyrimidine ring - it comprises a linker in the form of allylamine (prop-2-ene-1-amine) and a functional group attached to the linker through an amid bond. The functional groups used included benzoic acid, imidazole, pyridine, benzylamine or phenol. A series of organic synthesis reactions, in which from the initial compound 5-iododeoxyuridine the authors obtained a precursor for other modifications - 5-(3-aminepropene)-deoxyuridine triphosphate, reached a yield of 24%. Next, the authors used this precursor compound to prepare modified nucleoside triphosphates with reactions, whose yield was 58-73%.

[0010] A publication "Expanding the chemistry of DNA for in vitro selection", Vaught et al., JACS 2010, 132, 4141-4151[9] discloses structures and a method of preparation of modified nucleoside triphosphates. The authors used 5-iododeoxyuridine in a palladium-catalyzed carboxyamidation reaction to prepare derivatives, whose functional groups (comprising benzyl, isobutane, methyl-naphthalene, imidazol-4-ethane or (1H-indole-3-)-ethane) are attached to carbon number 5 of pyrimidine ring with an amid bond. The conjugation reaction itself requires one step but lasts for 48-72 hours. It further requires prior substrate manipulation by introducing protective groups for hydroxyl groups in several reactions. The yield of the process has been presented by the authors as medium to good - from 30 to 60%. Next the authors have performed tests, in which they have shown that modified nucleotides are incorporated into DNA in a Primer Extension Reaction (PER) by DNA-dependent DNA polymerases (KOD XL, Pfu (exo-), D. Vent (exo-), Tth, Taq, KF (exo-)), and that the oligonucleotides comprising said modified nucleotides in their sequence can be also used as a template for PER. Modified nucleotides however couldn't be successfully used in a PCR.

[0011] In another publication "Synthesis of Deoxynucleoside Triphosphates that Include Proline, Urea, or Sulfonamide Groups and Their Polymerase Incorporation into DNA", Marcel Hollenstein, Chem. Eur. J. 2012, 18, 13320-13330[16] the author presents synthesis and subsequent incorporation into DNA molecules of nucleotides enriched with complex functional groups (comprising proline residues, urea group, sulfamid group) attached to carbon number 5 of pyrimidine ring with a linker in the form of allylamine (prop-2-ene-1-amine) and an amid bond. Preparation of intermediate precursor from 5-iodouridine was performed with 31% yield. Next the precursor was used to synthesize five different nucleoside triphosphates - the series of synthesis reactions with the highest yield provided app. 23% conversion of the precursor into the product.

[0012] In the PCT application WO2009012363A2 were disclosed oligonucleotide aptamers selectively binding a target glycosylated polypeptide or protein, and having biased affinity for the glycan through a boronic acid linked to a nucleosidic base of a nucleotide(s). The disclosure encompasses methods for isolating an aptamer(s) selectively binding a target glycosylated polypeptide, where, from a population of randomized oligonucleotides that have at least one nucleotide having a boronic acid label linked to a base, is selected a first subpopulation of aptamers binding to the target glycosylated polypeptide or protein. This subpopulation is then amplified without using boronic acid-modified TTP, and amplification products not binding to a target glycosylated polypeptide or protein are selected. The second subpopulation of aptamers is then amplified using boronic acid-modified TTP to provide a population of boronic acid-modified aptamers capable of selectively binding to a glycosylation site of a target polypeptide or protein. Other aspects of the disclosure encompass methods for the use of the modified aptamers to detect glycosylated species of a polypeptide or protein.

[0013] A publication "Design, Synthesis, and Polymerase-Catalyzed Incorporation of Click-Modified Boronic Acid- TTP Analogues", CHEMISTRY - AN ASIAN JOURNAL, vol. 6, 2011, pages 2747-2752, XPO55160640 discloses the synthesis of four boronic acid-modified thymidine triphosphate (TTP) analogues. The synthesis of certain analogues was through the use of a single dialkyne tether for both the Sonogashira coupling with thymidine and the later Cu-mediated [3+2] cycloaddition for linking the boronic acid moiety. All analogues showed very good stability under polymerase chain reaction (PCR) conditions and were recognized as a substrate by DNA polymerase, and thus incorporated into DNA.

[0014] In a publication "A Versatile Approach Towards Nucleobase-Modified Aptamers", ANGEWANDTE CHEMIE, INTERNATIONAL EDITION, vol. 54, no. 37, 23 July 2015, pages 10971-10974, XPO55589969 was presented a novel and versatile method for modular expansion of the chemical space of nucleic acid libraries, thus enabling the generation of nucleobase-modified aptamers with unprecedented recognition properties. Reintroduction of the modification after enzymatic replication gives broad access to many chemical modifications.

[0015] Another publication "Site-Specifically Arraying Small Molecules or Proteins on DNA Using An Expanded Genetic Alphabet", CHEMISTRY - A EUROPEAN JOURNAL, vol. 19, no. 42, 11 October 2013 (2013-10-11), pages 14205-14209, XP055590293 & "Supporting Information", XPO55590282 disclosed a class of replicable unnatural DNA base pairs formed between d5SICS and either dMMO2, dDMO, or dNaM, which were. The synthesis of a variety of derivatives bearing propynyl groups, an analysis of their polymerase-mediated replication, and subsequent site-specific modification of the amplified DNA by Click chemistry is disclosed. With the d5SICS scaffold a propynyl ether linker is accommodated better than its aliphatic analogue, but not as well as the protected propargyl amine linker explored previously. It was also

found that with the dMMO2 and dDMO analogues, the dMMO2 position *para* to the glycosidic linkage is best suited for linker attachment and that although aliphatic and ether-based linkers are similarly accommodated, the direct attachment of an ethynyl group to the nucleobase core is most well tolerated. To demonstrate the utility of these analogues, a variety of them were used to site-selectively attach a biotin tag to the amplified DNA. Finally, the use of d5SICS^{co}-dNaM to couple one or two proteins to amplified DNA, with the double labeled product was visualized by atomic force microscopy. The ability to encode the spatial relationships of arrayed molecules in PCR amplifiable DNA should have important applications, ranging from SELEX with functionalities not naturally present in DNA to the production, and perhaps "evolution" of nanomaterials.

[0016] Following publication "C5-Modified nucleosides exhibiting anticanceractivity", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, vol. 19, no.16, 15 August 2009 (2009-08-15), pages 4688-4691, XP026419057 describes a simple method for the synthesis of C5-modified nucleosides from 5-iodo-2'-deoxyuridine and (ii) their activity against six types of human cancer cell lines (HCT15, MM231, NCI-H23, NUGC-3, PC-3, ACHN). It shows nitrile oxides in situ from oximes using a commercial bleaching agent; their cycloadditions with 5-ethynyl-2'-deoxyuridine yielded isoxazole derivatives possessing activity against the cancer cell lines. Several azides was synthesized from benzylic bromides and their click reactions with 5-ethynyl-2'-deoxyuridine provided triazole derivatives.

[0017] In another publication "A 2'-deoxycytidine long-linker click adduct forming two conformers in the asymmetric unit", ACTA CRYSTALLOGRAPHICA, SECTION C: CRYSTAL STRUCTURE COMMUNICATIONS, vol. 68, no. 4, 2012, pages 1-17, XPO55519073 disclosed a compound, which systematic name is: 4-amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-5-[6-(1-benzyl-1*H*-1, 2, 3-triazol-4-yl) hex-1-ynyl]pyrimidin-2 (1*H*)-one, C₂₄H₂₈N₆O₄, shows two conformations in the crystalline state, viz. (I-1) and (I-2). The pyrimidine groups and side chains of the two conformers are almost superimposable, while the greatest differences between them are observed for the sugar groups. The N-glycosylic bonds of both conformers adopt similar *anti* conformations, with $\chi = -168.02 (12)^\circ$ for conformer (I-1) and $\chi = -159.08 (12)^\circ$ for conformer (I-2). The sugar residue of (I-1) shows an N-type (C3'-*endo*) conformation, with $P = 33.1 (2)^\circ$ and $\tau_m = 29.5 (1)^\circ$, while the conformation of the 2'-deoxyribofuranosyl group of (I-2) is S-type (C3'-*exo*), with $P = 204.5 (2)^\circ$ and $\tau_m = 33.8 (1)^\circ$. Both conformers participate in hydrogen-bond formation and exhibit identical patterns resulting in three-dimensional networks. Intermolecular hydrogen bonds are formed with neighbouring molecules of different and identical conformations (N-H...N, N-H...O, O-H...N and O-H...O).

[0018] In a publication "AZIDOPROPYL VINYL SULFONAMIDE AS A NEW BIFUNCTIONAL CLICK REAGENT FOR BIOORTHOGONAL CONJUGATIONS: APPLICATION FOR DNA-PROTEIN CROSS-LINKING", CHEMISTRY-A EUROPEAN JOURNAL, vol. 21, no. 45, 2 November 2015 (2015-11-02), pages 16091-16102, XP055519078 was presented *N*-(3-Azidopropyl)vinylsulfonamide as a new bifunctional bioconjugation reagent suitable for the cross-linking of biomolecules through copper (I)-catalyzed azide-alkyne cycloaddition and thiol Michael addition reactions under biorthogonal conditions. The reagent is easily clicked to an acetylene-containing DNA or protein and then reacts with cysteine-containing peptides or proteins to form covalent cross-links. Several examples of bioconjugations of ethynyl- or octadiynyl-modified DNA with peptides, p53 protein, or alkyne-modified human carbonic anhydrase with peptides are given.

[0019] A publication "Cross-Linked DNA: Site-Selective "Click" Ligation in Duplexes with Bis-Azides and Stability Changes Caused by Internal Cross-Links", BIOCONJUGATE CHEMISTRY, vol. 23, no. 6, 1 January 2012 (2012-01-01), pages 1230-1243, XP055519081 showed a heterodimeric interstrand cross-linked DNA constructed by the "bis-click" reaction carried out on preformed oligonucleotide duplexes with the bis-azide 1. For this, alkynylated 8-aza-7-deazapurine or corresponding 5-substituted pyrimidine nucleosides were synthesized. Cross-linking resulted in chemoselective formation of heterodimeric duplexes while homodimers were suppressed. For product identification, heterodimeric DNA was prepared by the "stepwise click" reaction, while noncomplementary homodimers were accessible by "bis-click" chemistry, unequivocally. Studies on duplex melting of complementary cross-linked duplexes (heterodimers) revealed significantly increased T_m values compared to the non-cross-linked congeners. The stability of this cross-linked DNA depends on the linker length and the site of modification. Cross-linked homodimers hybridized with single-stranded complementary oligonucleotides show much lower stability.

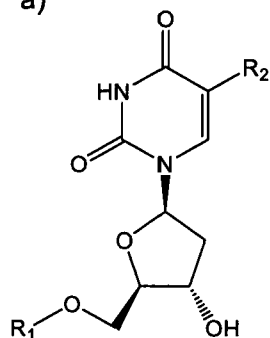
[0020] Presented methods of preparation of modified nucleotides require multiple reagents, specialized equipment for organic synthesis, they also characterize in low yield and multiple steps, which elongates the time of the preparation of the desired final compound.

[0021] The technical problem to be solved by the present invention is to propose such a method of synthesis of modified nucleotides, which would provide modified nucleotides that can be incorporated into nucleic acids by enzymatical methods, and thus they would be useful for selection of new modified aptamers with better parameters than aptamers comprising only naturally occurring nucleotides, wherein said method would not require using complicated and expensive specialized laboratory equipment and would be a process of high yield not requiring a prior substrate manipulation, and wherein said method would be relatively simple and quick, and the obtained product would be characterized by high purity level. Surprisingly, aforementioned technical problems and aims were provided by the present invention.

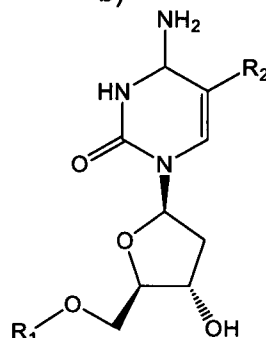
[0022] The first object of the invention is a method of synthesis and purification of a nucleotide, of which the substrate of structure 1 is a mono-, di- or triphosphate, characterized in that that a copper - catalyzed Huisgen's azide-alkyne

cycloaddition reaction is being performed using a compound of structure 1:

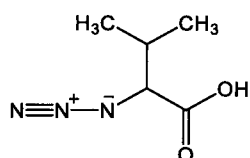
a)



b)

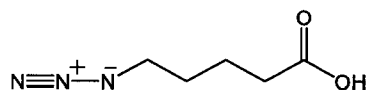


with a compound of structure 2



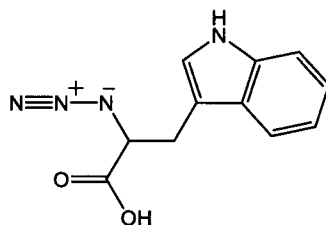
Structure 2

or with a compound of structure 3



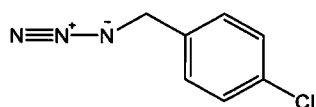
Structure 3

or with a compound of structure 4



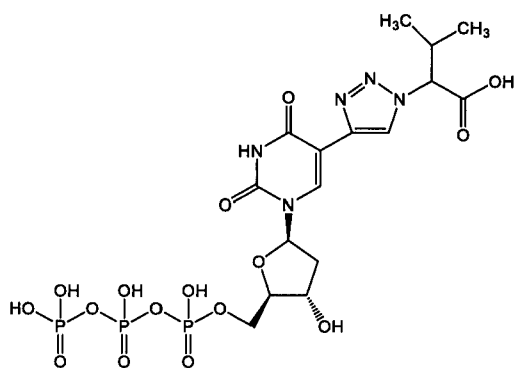
Structure 4

or with a compound of structure 5



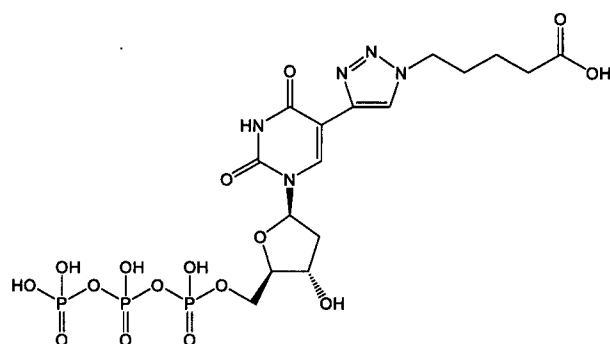
Structure 5

wherein reaction mixture solvents comprise TEAA buffer ie triethylamine-acetic acid, sodium ascorbate and DMSO, wherein the synthesis is performed at temperature of 40°C for 2 hours is essential for obtaining compound with a structure 6:



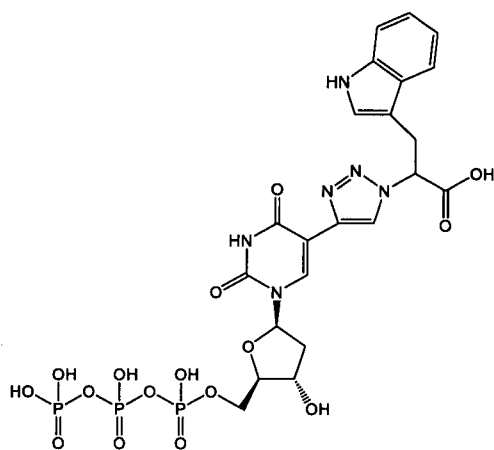
from structure 2 and temperature 40-55°C for 1-6 hours is essential for obtaining compound with a:

structure 7

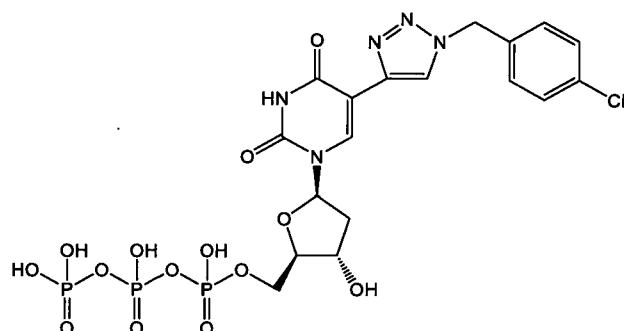


or

structure 8

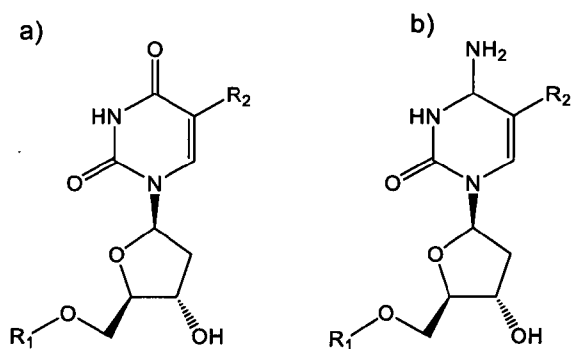


or structure 9



from the corresponding azides of structures 3-5, and wherein the direct one-step purification of the synthesis product is performed using reversed-phase chromatography.

[0023] The second object of the invention is a modified a nucleotide being a mono-, di- or triphosphate, comprising cytosine or uracil as its nucleobase, which at position 5 of the heterocyclic ring has a 1,2,3-triazol group or an alkane or alkyne chain having a terminal 1,2,3-triazol group, and has a substituent at position 1 of said 1,2,3-triazol group which is a derivative of one of the compounds from the group of structures 2 to 5, and said modified nucleotide being of structure 10:



Structure 10

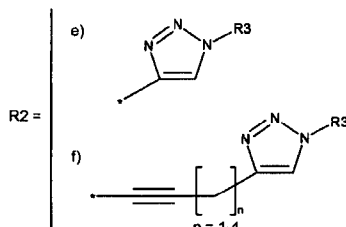
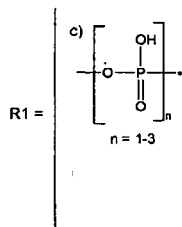
where:

5

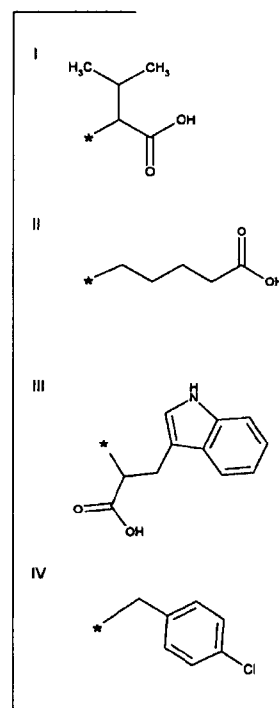
10

15

20



R3 =



[0024] The third object of the invention is a DNA molecule comprising a single- or double-stranded DNA chain, characterized in that it comprises in one or more positions of the sequence of any or of both strands one or more modified nucleotides, as described in the second object of the invention. In a preferred embodiment of the invention the position of one or more modified nucleotides in the sequence is unrestricted. In another preferred embodiment the DNA molecule has been prepared by an enzymatic reaction (PCR, PER) or by chemical synthesis of oligonucleotides.

[0025] The fourth object of the invention is an oligonucleotide library having in its sequence a random sequence region of at least 10 nucleotides in length, and two flanking regions of constant sequences of at least 10 nucleotides in length, or deprived of flanking regions, characterized in that it comprises in one or more positions of the sequence one or more modified nucleotides, as described in the second object of the invention. In a preferred embodiment of the invention the position of one or more modified nucleotides in the sequence is unrestricted. In another embodiment of the invention the oligonucleotide library has been prepared by an enzymatic reaction (PCR, PER) or by chemical synthesis of oligonucleotides.

[0026] The fifth object of the invention is the use of the oligonucleotide library, as described in the fourth object of the invention, to obtain aptamers by SELEX technique and its derivatives.

[0027] The method of synthesis and purification of modified nucleotides according to the present invention allows preparing modified nucleotides, which can be incorporated into nucleic acids by enzymatic methods, thanks to which it is possible to use them in selection of new modified aptamers with better parameters than aptamers comprising only naturally occurring nucleotides. Presented method allowed to limit the number of steps of synthesis and purification in order to prepare modified nucleotides, wherein it didn't require the use of complex laboratory equipment. Synthesis and purification are characterized by high efficiency of the process, don't require prior substrate manipulation, are quick, and obtained products are characterized by high purity level. The enzymatic incorporation of the modified nucleotides into oligonucleotides is characterized by high efficiency, similar to the incorporation of naturally occurring nucleotides. Oligonucleotides described in the third object of the invention comprising modified nucleotides in their sequence are also characterized in that they can act as a template for PER or PCR, during which by the use of naturally occurring nucleotides there occurs a synthesis of unmodified oligonucleotides with sequences complementary to that of the template modified oligonucleotides - this requirement is necessary to be able to use the modified library for the selection of aptamers by SELEX technique and its derivatives, as described in the fifth object of the invention.

[0028] Example embodiments of the invention are presented by Examples and in the Figures, wherein Fig. 1 presents an overall scheme of the Huisgen's copper-catalyzed azide-alkyne cycloaddition reaction, Fig. 2 presents schemes of reactions according to the present invention, Fig. 3 presents a chromatogram of the nucleotide of structure 6 prepared by reversed-phase chromatography, Fig. 4 presents a representative mass spectrum of the nucleotide of structure 6 obtained by mass analysis with MS-TOF (Mass Spectrometry - Time Of Flight), Fig. 5 presents the electrophoretic analysis result (an electropherogram) of the product of the enzymatic synthesis of an oligonucleotide comprising modified nucleotides of structure 9 in its sequence, and Fig. 6 presents the electrophoretic analysis result of the product of the

enzymatic synthesis of an unmodified oligonucleotide, for which the template during the synthesis reaction was an oligonucleotide comprising modified nucleotides of structure 6 in its sequence.

Example 1 - Synthesis and purification of a modified nucleotide of structure 6

[0029] The synthesis reaction of nucleotide of structure 6 was prepared in 200 μ l. To 4.0 μ l of 100 mM EdUTP (5-ethynyl 2'-deoxyuridine 5'-triphosphate - structure 1.a).c).e); 400 nmol), 20 μ l 10x TEAA buffer (triethylamine-acetic acid 500 mM pH 7.0) and 30 μ l of DMSO-dissolved (S)-2-azido-3-methylbutyric acid (structure 2) at 200 mM concentration (6.0 μ mol, 15 molar equivalents of EdUTP) were added. Next, 10 μ l of previously prepared Cu-TBTA mixture (10 mM CuSO₄, 25 mM TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine), 50% DMSO, 16,6% *tert*-butanol) was added to final copper concentration in the reaction of 0.5 mM (0.25 molar equivalents of EdUTP). The mixture was filled up to 180 μ l using DMSO, mixed, and 20 μ l of sodium ascorbate at 200 mM concentration was added (to final concentration of 20 mM, 10 molar equivalents of EdUTP), thus initiating the reaction by reducing the copper to I oxidation state, and the tube cap was tightly closed. A parallel negative control was prepared in 20 μ l volume while maintaining the concentrations as in the positive sample, but with sodium ascorbate being replaced with deionized water. The reaction was conducted in a closed tube for 2 hours in 40°C with vigorous shaking.

[0030] Next the product was purified by reversed-phase chromatography, using TEAA 100 mM buffer and methanol as mobile phase (Bio-Rad's NGC chromatography system, Water's column AccQ-Tag, 60Å, 4 μ m, 3,9 mm x 150 mm). The chromatogram of the analysis both from the negative control and the positive sample is presented in Figure 3. Collected fractions containing the purified nucleotide of structure 6 were combined, dried in a vacuum concentrator (Labconco's CentriVap Benchtop Centrifugal Vacuum Concentrator) and dissolved in known volume of 25% DMSO. Next the sample was analyzed by MS-TOF (Water's Xevo G2-XS QToF spectrometer) in negative mode electrospray ionization: detected mass 634.04 Da, calculated mass 634.30 Da. A representative mass spectrum is presented in Figure 4.

Example 2 - Synthesis and purification of modified nucleotides of structure 7-9

[0031] The synthesis reaction of nucleotides of structures 7-9 were performed analogically to the synthesis of compound of structure 6 (Example 1) using corresponding azides of structures 3-5 instead of compound of structure 2. Reaction temperature 40-55°C, reaction time 1-6 hours.

[0032] Mass analysis of products of structures 7-9: nucleotide of structure 7: detected mass 634.04 Da, calculated mass 634.30 Da; nucleotide of structure 8: detected mass 721.0 Da, calculated mass 721.38 Da; nucleotide of structure 9: detected mass 658.50 Da, calculated mass 658.75 Da.

Example 3 - Enzymatic synthesis of single-stranded DNA using modified nucleotide

[0033] The enzymatic synthesis of an oligonucleotide comprising modified nucleotides of structure 9 in its sequence was performed in 10 μ l volume using single-stranded template D56 (SEQ ID NO: 1) and primer LHAb (SEQ ID NO: 2; a polyA chain attached to 5'-end of the primer allows to discriminate the template from the product by mass) in a PER process. The reaction mixture was prepared, containing D56 at 2 μ M concentration, LHAb at 4 μ M concentration, Pwo polymerase 0.5 U, 1x concentrated buffer with magnesium supplied by the polymerase vendor, deionized water, and nucleotide triphosphates: dATP, dCTP and dGTP at final 200 μ M concentration each. The negative control (sample N) did not contain any other nucleotides, the positive control (sample P) contained additionally TTP at 100 μ M concentration, and the test sample (sample T) contained additionally the nucleotide of structure 9 (prepared as in Example 2) in 10x dilution. The reaction mixtures were incubated for one minute in 95°C, two minutes in 50°C and 30 minutes in 70°C. After the reaction the samples were subjected to electrophoresis in a denaturing (7 M urea) 10% polyacrylamide gel (acrylamide 19:1 bis-acrylamide) heated to 55°C with constant voltage of 300 V. The gel was visualized using Midori Green stain (Nippon Genetics) - Figure 5.

[0034] Analogical experiments were performed for nucleotides of structures 6-8 confirming their proper incorporation into DNA by PER reaction. Obtaining of the product was also confirmed with other polymerase - DeepVent(exo-), as well as with different templates than D56 oligonucleotide.

[0035] The capability of DNA synthesis by Pwo polymerase on a biotinylated template immobilized on magnetic microbeads coated with streptavidin was also confirmed, accordingly with previous reports[9].

Example 4 - Enzymatic synthesis of single-stranded DNA library comprising modified nucleotides in its random region

[0036] The synthesis of single-stranded DNA library was performed with PER reaction analogically to Example 3, with explicit changes: unmodified library (SEQ ID NO: 3) was used as template, L4 primer was used as primer (SEQ ID NO: 4).

[0037] Single-stranded library comprising modified nucleotide or nucleotides of structures 6, 7, 8, or 9 was prepared by separating the strands of double-stranded product using magnetic microbeads coated with streptavidin, accordingly with previous reports[9], and its purity was assessed by electrophoresis (as in Example 3).

[0038] Another method to obtain single-stranded library comprising modified nucleotide or nucleotides of structures 6, 7, 8, or 9, is to separate the strands of double-stranded product in a denaturing (7 M urea) 10% polyacrylamide gel (acrylamide 19:1 bis-acrylamide) heated to 55°C with constant voltage of 300 V. The gel was visualized using Midori Green stain, next the bands corresponding to the modified strand were cut out and they were subjected to purification procedure according to standard protocols.

Example 5 - Enzymatic synthesis of unmodified single-stranded DNA using a template comprising modified nucleotides in its sequence

[0039] Using a purified single-stranded oligonucleotide complementary to SEQ ID NO: 1 as a template which comprised modified nucleotides of structure 6 in its sequence (prepared as in Example 3 and purified from a polyacrylamide gel according to standard protocols), enzymatic synthesis of unmodified single-stranded DNA was performed (the product of the reaction is a reconstructed oligonucleotide D56 of SEQ ID NO: 1). The reaction was performed in 20 µl using a template described above and L4 primer (SEQ ID NO: 4) in a PER process. The reaction mixture was prepared, containing template at 0.1-2.0 µM concentration, L4 at 2-5 µM concentration, DeepVent polymerase 1 U or Pwo polymerase 1 U, 1x concentrated buffer supplied by the polymerase vendor (adequate to the polymerase used), magnesium sulfate at 2.0 mM, nucleotide triphosphates TTP, dATP, dCTP and dGTP at final 400 µM concentration each, and deionized water. The negative control (sample N) was not subjected to incubation (incubated at 4°C). The test sample 1 (sample T1) was incubated for one minute in 95°C, one minute in 50°C and 60 minutes in 70°C. The test sample 2 (sample T2) was subjected to 10 incubation cycles: for one minute in 95°C, one minute in 50°C and 20 minutes in 70°C.

[0040] After the reaction the samples were subjected to electrophoresis in a denaturing (7 M urea) 10% polyacrylamide gel (acrylamide 19:1 bis-acrylamide) heated to 55°C with constant voltage of 300 V. The gel was visualized using Midori Green stain - Figure 6.

[0041] Analogical experiments were performed with oligonucleotide templates comprising modified nucleotides of structure 7, 8, or 9 in their sequences.

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[0042]

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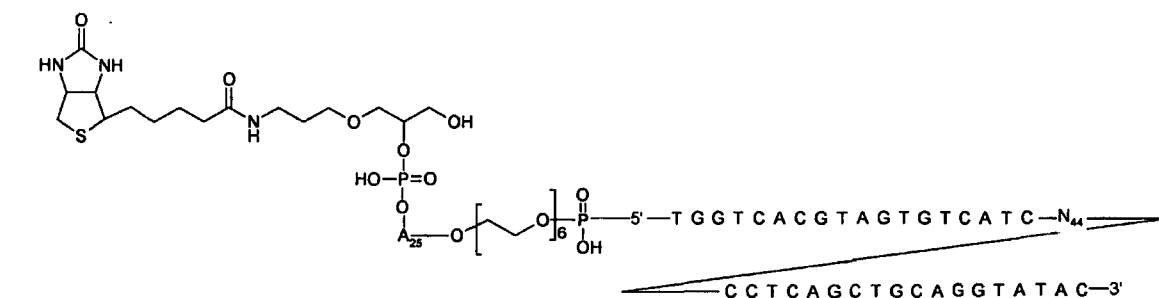
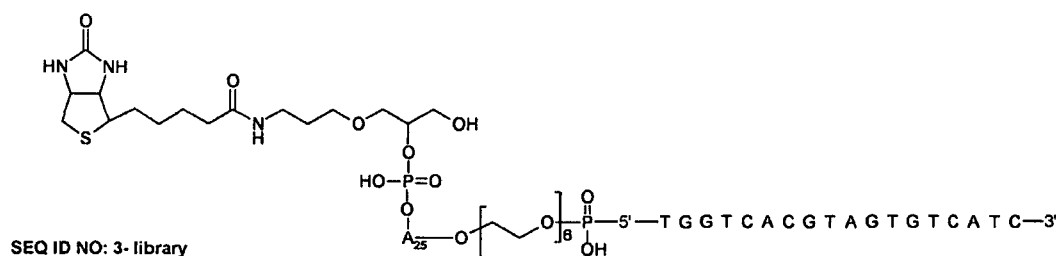
Sequence list:

[0043]

SEQ ID NO: 1 - D56

5'-GTATACCTGCAGCTGAGGTACAGACTTG-
CACGAGTTTTCGATGACACTACGTGACCA-3'

SEQ ID NO: 2 - LHAb primer



SEQ ID NO: 4 - L4 primer
5'-GTATACCTGCAGCTGAGG-3'

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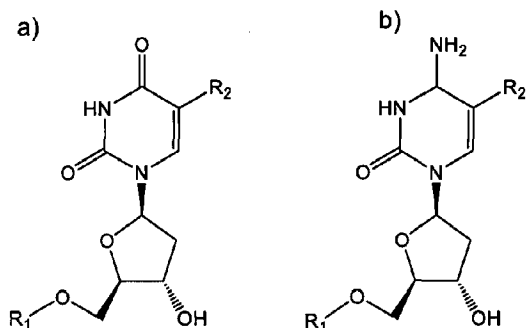
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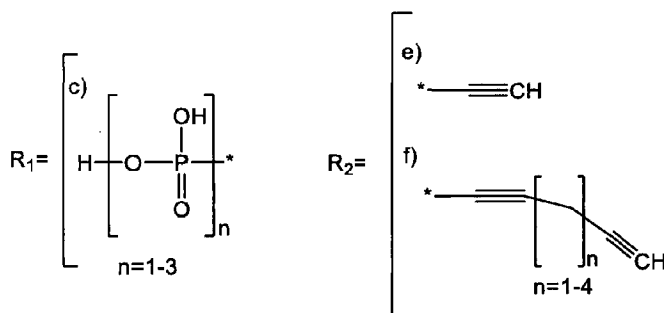
P A T E N T K R A V

1. Fremgangsmåde til syntese og oprensning af et nukleotid som er et mono-, di- eller triphosphat, **kendetegnet ved, at** en kobber-katalyseret Huisgen's azid-alkyn-cycloaddition-reaktion udføres omfattende anvendelse af forbindelse ifølge struktur 1:

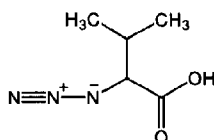


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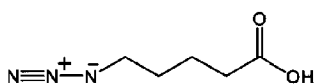
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Struktur 2

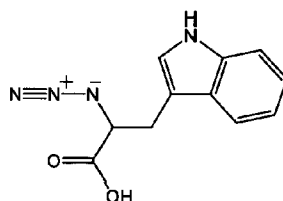
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eller med en forbindelse ifølge struktur 3:



Struktur 3

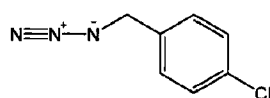
eller med en forbindelse ifølge struktur 4:



Struktur 4

15

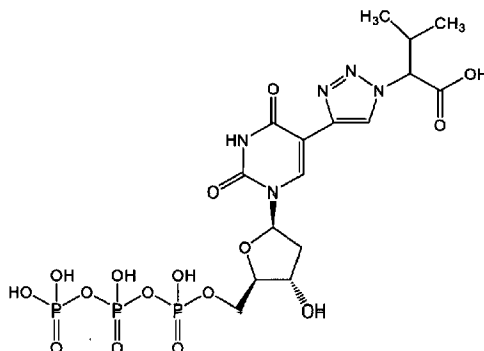
eller med en forbindelse ifølge struktur 5:



Struktur 5

hvor reaktionsblandingsopløsningsmidler omfatter TEAA-buffer, dvs. triethylamin-eddikesyre, natriumascorbat og DMSO,

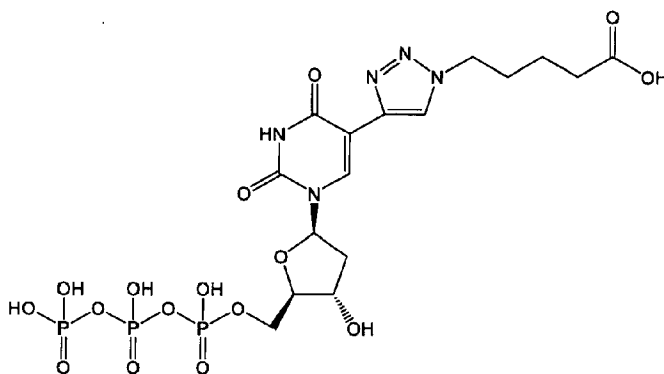
hvor syntesen udføres ved en temperatur på 40°C i 2 timer er essentiel for at opnå forbindelse med en struktur 6:



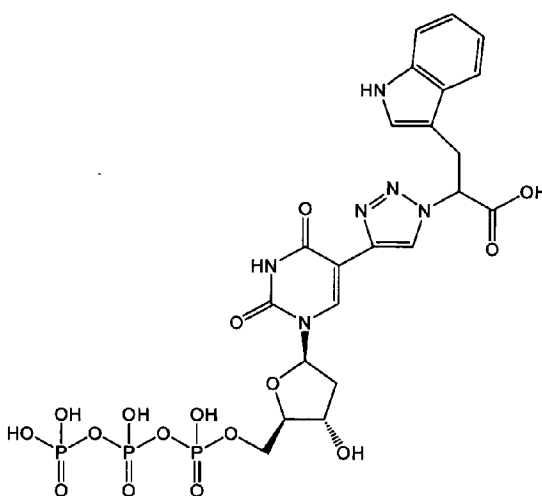
5

fra struktur 2 og en temperatur 40-55°C i 1-6 timer er essentiel for at opnå forbindelse med en:

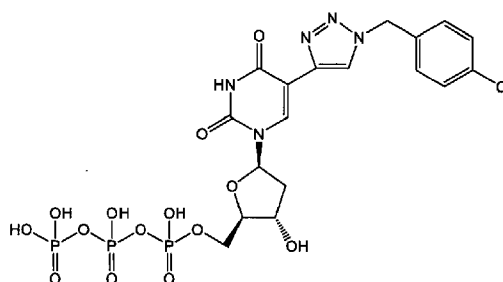
Struktur 7



eller struktur 8



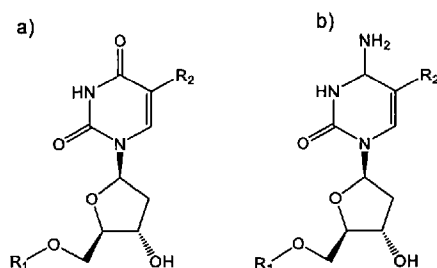
eller struktur 9



fra de tilsvarende azider ifølge strukturer 3-5,

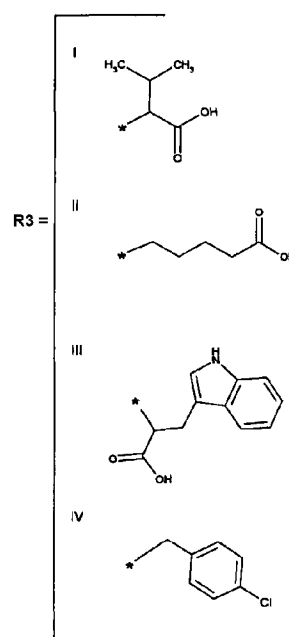
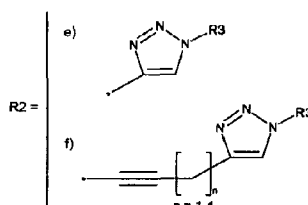
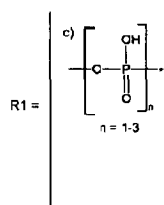
og hvor den direkte ét-trins-oprensning af synteseproduktet udføres ved anvendelse af omvendt-fase-kromatografi.

- 5 **2.** Modificeret nukleotid som er et mono-, di- eller triphosphat, omfattende cytosin eller uracil som sin nukleobase, som ved position 5 af den heterocykliske ring har en 1,2,3-triazolgruppe eller en alkan- eller alkynkæde som har en terminal 1,2,3-triazolgruppe, og har en substituent R3 ved position 1 af 1,2,3-triazolgruppen som er et derivat af én af forbindelserne fra gruppen af strukturer 2 til 5 som defineret i krav 1, og det modificerede
- 10 nukleotid er ifølge struktur 10:



Struktur 10

hvor:



3. DNA-molekyle udgjort af en enkelt- eller dobbeltstrenget DNA-kæde, **kendetegnet ved, at** det ved én eller flere positioner af sekvensen af en hvilken som helst af eller begge af strengene omfatter ét eller flere modificerede nukleotider ifølge krav 2.

5 4. DNA-molekyle ifølge krav 3, **kendetegnet ved, at** positionen af ét eller flere modificerede nukleotider er ubegrænset.

5. DNA-molekyle ifølge krav 3 eller 4, **kendetegnet ved, at** det er blevet fremstillet ved en enzymatisk reaktion PCR, PER, eller ved hjælp af kemisk syntese af oligonukleotider.

10 6. Bibliotek af oligonukleotider som i sin sekvens omfatter et område med en tilfældig sekvens som er mindst 10 nukleotider i længde, og to flankerende områder med konstante sekvenser som er mindst 10 nukleotider i længde, eller frataget de flankerende områder, **kendetegnet ved, at** det ved én eller flere positioner af sekvensen omfatter ét eller flere modificerede nukleotider ifølge krav 2.

15 7. Bibliotek ifølge krav 6, **kendetegnet ved, at** positionen af ét eller flere modificerede nukleotider i sekvenser er ubegrænset.

8. Bibliotek ifølge krav 6 eller 7, **kendetegnet ved, at** det er blevet fremstillet ved en enzymatisk reaktion PCR, PER, eller ved hjælp af kemisk syntese af oligonukleotider.

20 9. Anvendelse af oligonukleotidbiblioteket ifølge krav 6 eller 7 til at fremstille aptamerer ved SELEX-teknikken eller derivater deraf.

DRAWINGS

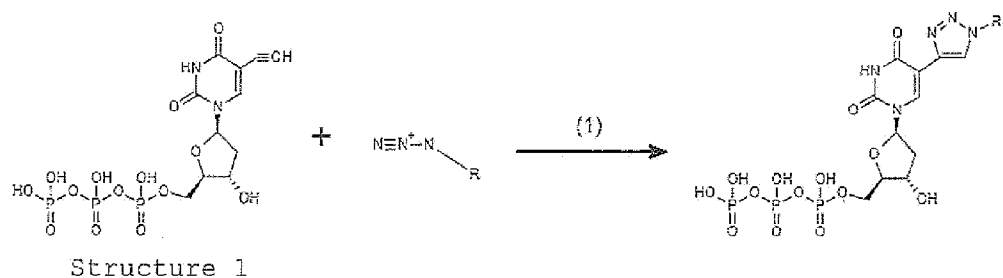


Fig. 1

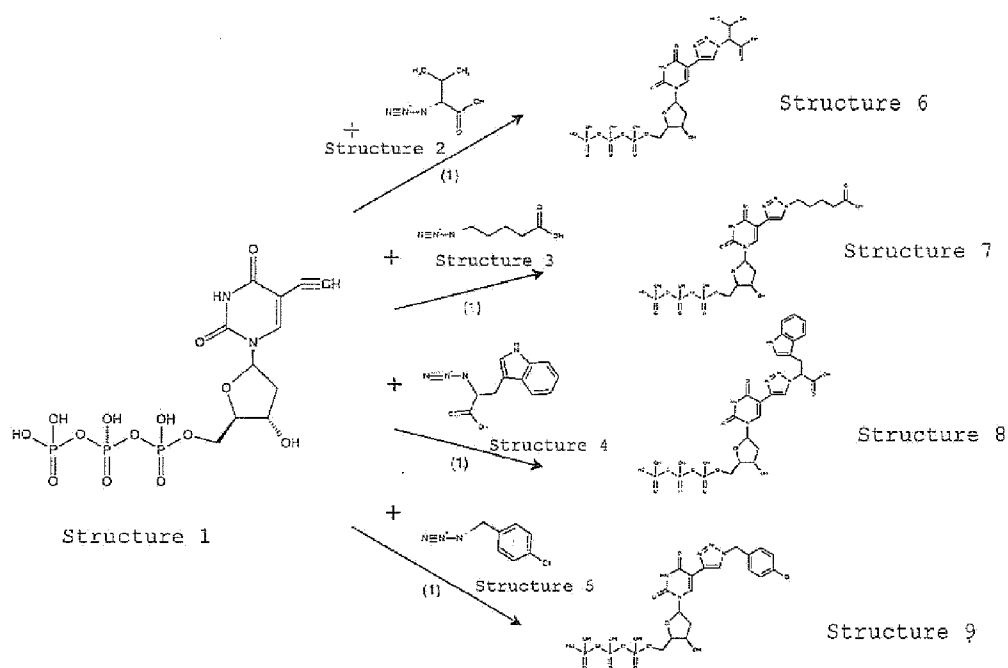


Fig. 2

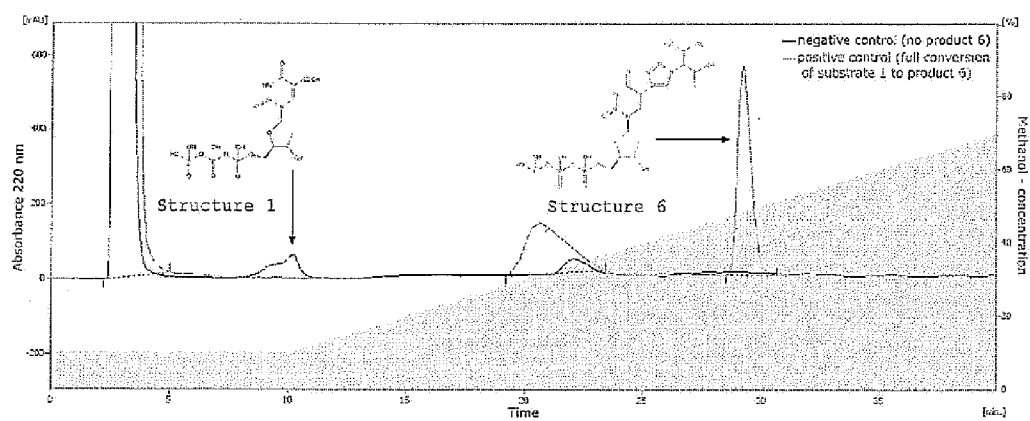


Fig. 3

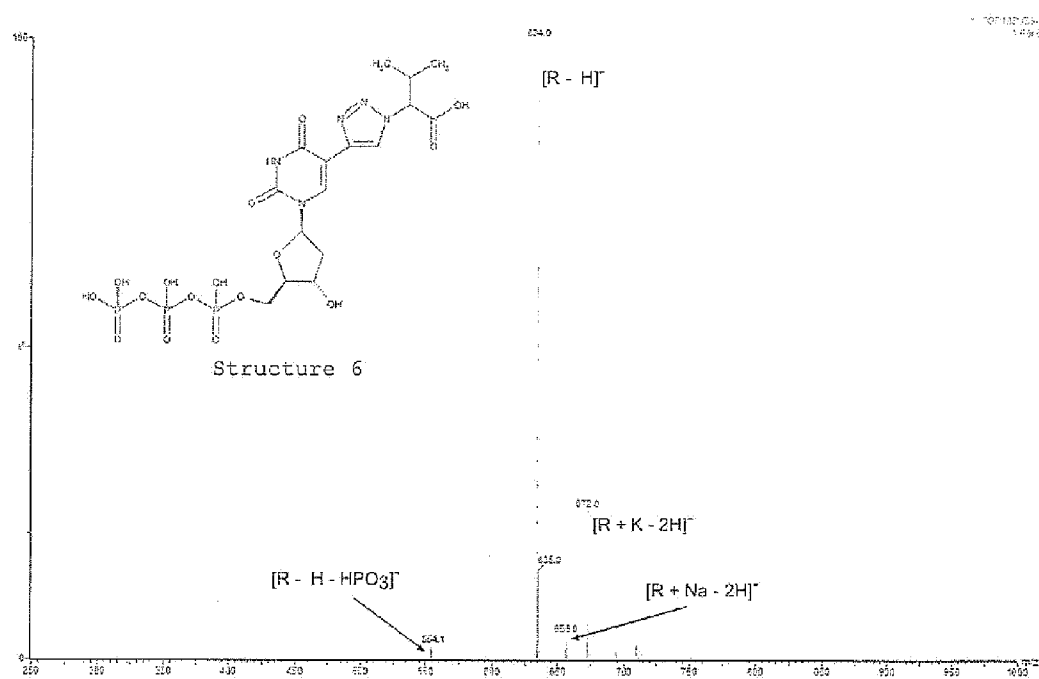


Fig. 4

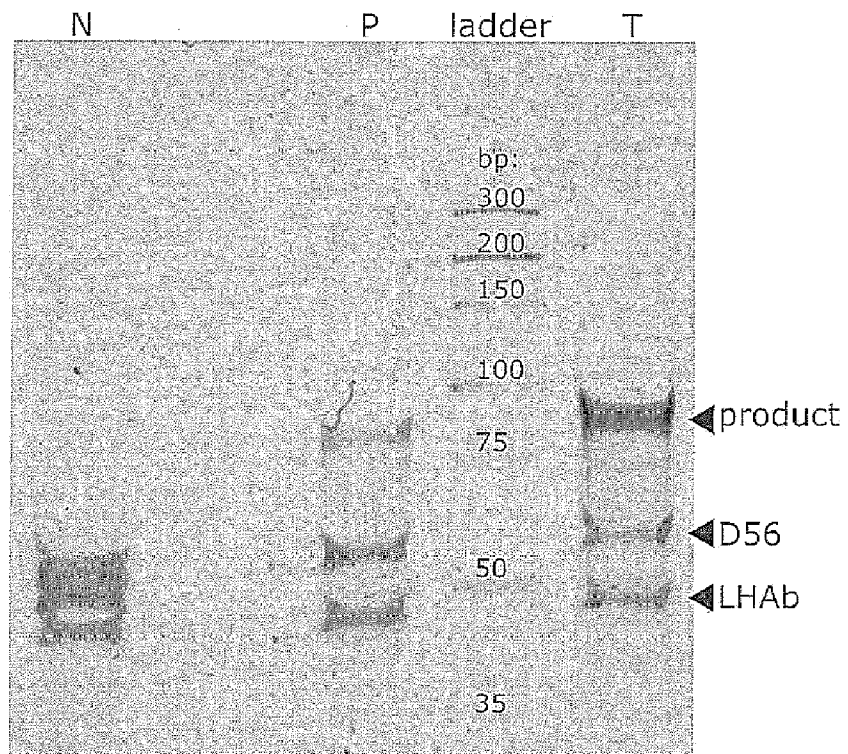


Fig. 5

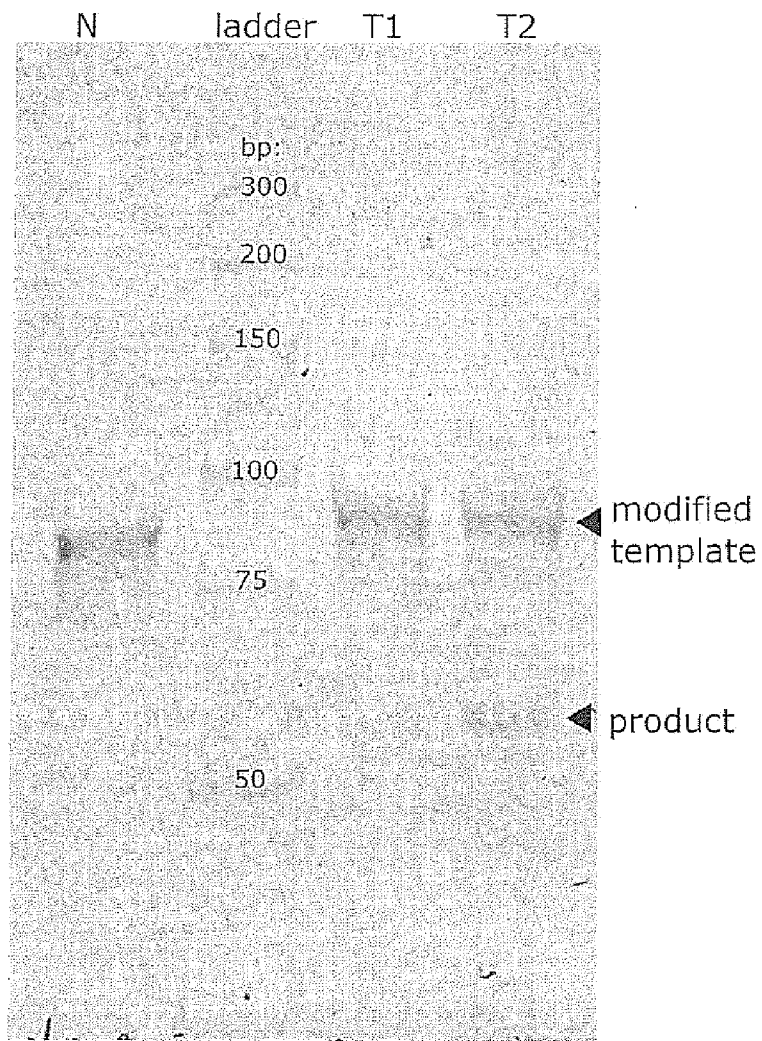


Fig. 6