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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
61/191,065	09/06/2008		105	ChG 00108		

CONFIRMATION NO. 5235

Indu M. Anand, Esq.
15 Greem Way
Chelmsford, MA 01824

FILING RECEIPT

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Date Mailed: 10/10/2008

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Applicant(s)

Suresh C. Srivastava, Burlington, MA;
Divya Pandey, Lucknow (U.P.), INDIA;
Neveen P. Srivastava, Burlington, MA;
Satya P. Bajpai, Lucknow (U.P.), INDIA;

Power of Attorney:
Indu Anand--52557

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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 61/191,065**
Projected Publication Date: None, application is not eligible for pre-grant publication
Non-Publication Request: No
Early Publication Request: No
**** SMALL ENTITY ****
Title

Reverse direction synthesis of high purity RNA, and applications of resulting synthetic RNA

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 1 of 2

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EB 739855248 US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Suresh C.	Srivastava	Burlington, Massachusetts
Divya	Pandey	Lucknow (U.P.), India
Naveen P.	Srivastava	Burlington, Massachusetts
Satya P.	Bajpai	Lucknow (U.P.), India

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max):

REVERSE DIRECTION SYNTHESIS OF HIGH PURITY RNA, AND APPLICATIONS OF RESULTING SYNTHETIC RNA

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☐ Application Data Sheet. See 37 CFR 1.76

☑ Drawing(s) *Number of Sheets* Nine

☑ Specification (e.g. description of the invention) *Number of Pages* 29

☐ CD(s), Number of CDs _____

☐ Other (specify) _____

Fees Due: Filing Fee of $210 ($105 for small entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is also due, which is $260 ($130 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

METHOD OF PAYMENT OF THE FILING FEE AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fee and application size fee (if applicable).

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TOTAL FEE AMOUNT ($) $105

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SIGNATURE IMAnand Date 9/6/08

TYPED or PRINTED NAME Indu M. Anand, Esq. REGISTRATION NO. 52,557 *(if appropriate)*

TELEPHONE (978) 250-9003 Docket Number: ChG 00108

Attorney Docket No.: ChG_00108

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors:	Suresh C. Srivastava, Divya Pandey, Naveen P. Srivastava and Satya P. Bajpai
Application:	Provisional Application
Filed:	September 6, 2008
Title:	REVERSE DIRECTION SYNTHESIS OF HIGH PURITY RNA, AND APPLICATIONS OF RESULTING SYNTHETIC RNA

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Sir:

Please find enclosed for filing the Provisional Patent Application pursuant to 37 CFR 1.53(c), with the Title and Inventors named above.

Please find enclosed in this mailing the following:

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- Specification in accordance with 35 U.S.C. 112, 29 pages
- Drawings in accordance with 35 U.S.C. 113, 9 pages
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Dated: September 6, 2008

Respectfully submitted,

By _____

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REVERSE DIRECTION SYNTHESIS OF HIGH PURITY RNA, AND APPLICATIONS OF RESULTING SYNTHETIC RNA

Suresh C. Srivastava, Divya Pandey, Naveen P. Srivastava and Satya P. Bajpai
ChemGenes Corp. 33 Industrial Way, Wilmington, MA 01803

FIELD OF THE INVENTION

This invention relates to synthesis of novel RNA monomer phosphoramidites, corresponding solid supports that are suitable for novel method of RNA oligonucleotide synthesis in reverse 5' → 3' direction. This approach leads to very clean oligonucleotide synthesis allowing for introduction of various modifications at the 3'- end cleanly and efficiently in order to produce high purity and therapeutic grade RNA oligonucleotides.

BACKGROUND OF THE INVENTION

Defined sequence RNA synthesis in the 3' → 5' direction is now well established and currently in use for synthesis and development of vast variety of therapeutic grade RNA aptamers, tRNA's, si RNA and biologically active RNA molecules. This approach utilizes a ribonucleoside with suitable N- protecting group, 5'- Protecting group, generally and most popular being dimethoxytriphenyl, commonly called DMT group, 2'- protecting group, out of which most popular being t-Butyldimethylsilyl ether and a 3'- phosphoramidite, the most popular being cyanoethyl diisopropyl (component 1). This component is then coupled with a nucleoside with a suitable N- protecting group, 2' or 3' succinate of a ribonucleoside attached to a solid support (component 2). The coupling of component 1 and 5'- OH-n-protected –2',3'-protected-nucleoside (component 3) are also achieved in solution phase in presence of an activator to lead to dimers and oligoribonucleotides, followed by oxidation (3'→5' direction synthesis), also lead to protected dinucleoside having a 3'-5'- internucleotide linkage, Ogilvie, K.K., Can. J. Chem., 58, 2686, 1980 (scheme 1). However synthesis of RNA in reverse direction (5'- 3' direction) has not been achieved so far to the best of our knowledge. The 2'- silyl ethers of component 1 have been developed extensively and they are known to have remarkable stability. Solvolysis of silyl ethers have been extensively studied and that bulky alkyl silyl ethers have high degree of stability, Bazani, B and Chvalowski, V Chemistry of Organosilicon compounds, Vol. 1, Academic Press, New York, 1965. Extensive research work was subsequently done by Ogilvie and coworkers as 2'- hydroxy protecting group for oligo ribonucleotide synthesis (Ogilvie, K.K., Sadana, K.L, Thompson, E.A., Quilliam, M.A., and Westmore, J.B *Tetrahedron Letters*, 15, 2861-2864, 1974; Ogilvie, K.K., Beaucage, S.L, Entwistle, D.W., Thompson, E.A., Quilliam, M.A., and Westmore, J.B. *J. Carbohydrate Nucleosides Nucleotides*, 3, 197-227, 1976 ; Ogilvie, K.K. Proceedings of the 5th International Round Table on Nucleosides,

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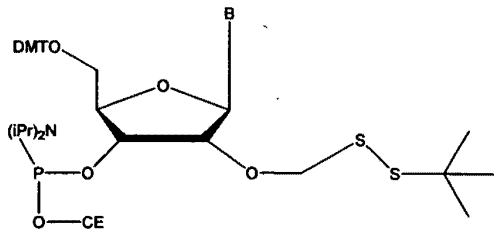
Nucleotides and Their Biological Applications, Rideout, J.L., Henry , D.W., and Beacham L.M., III, eds., Academic, London, pp. 209-256,1983). These studies subsequently led to continued developments of methods which were amenable to both solution and solid phase oligonucleotide synthesis, and the first chemical synthesis of RNA molecules of the size and character of tRNA (Usman, N., Ogilvie, K.K., Jiang, M.-Y.,and Cedergren, R.J. *J. Am. Chem. Soc.* 109, 7845-7854, 1987; Ogilvie, K.K., Usman, N., Nicoghosian, K, and Cedergren, R.J. *Proc. Natl. Acad. Sci. USA*, 85, 5764-5768, 1988 ; Bratty, J., Wu, T., Nicoghosian, K., Ogilvie, K.K., Perrault, J.-P., Keith, G. and Cedergren, R., *FEBS Lett.* 269, 60-64, 1990). The literature has been amply reviewed in subsequent excellent publication: Gait, M.J., Pritchard, C. and Slim, G., Oligonucleotides and Their Analogs: A Practical Approach (Gait, M.J., ed.), Oxford University Press Oxford, England, pp 25-48, 1991. Other protecting groups which have been lately employed for RNA synthesis are ; bis (2- acetoxyethyl-oxy) methyl (ACE), Scaringe, S.A., Wincott, F.E., Caruthers, M.H., J. Am. Chem. Soc., 120: 11820-11821, 1998; triisopropylsilyloxy methyl (TOM), Pitsch, S., Weiss, P.A., Jenny, L., Stutz, A., Wu, X., Helv. Chim. Acta. 84, 3773-3795, 2001 and t-butyldithiomethyl (DTM) (structure 1), Semenyuk, A., Foldesi, A., Johansson, T., Estmer-Nilsson , C., Blomgren, P., Brannvall, M., Kirsebom, L.A., Kwiatkowski, M., J.Am. Chem. Soc., 128: 12356-12357, 2006 have been introduced. However none of these are amenable to carry out the synthesis in reverse direction (5'→3'direction), and hence lack the capability of introduction of many ligands and chromophores conveniently and efficiently at the 3'- end of RNA molecules.

Chemically modified RNA have been synthesized having modified arabino sugars, 2'-deoxy-2'- fluoro- beta-D_arabinonucleic acid (FANA; structure 2)) and 2'-deoxy-4'- thio-2'- fluoro- beta-D_arabinonucleic acid (4'-Thio-FANA; structure 3) into sequences for SiRNA activities, Dowler, T., Bergeron, D., Tedeschi, Anna-Lisa, Paquet, L., Ferrari, N., Damha, M.J., Nucl. Acids Res., 34, 1669-1675, 2006. Amongst several new 2' –protecting group chemistry which have been developed , the 2'- protecting 2-cyanoethoxymethyl (CEM) (structure 4) has been shown for producing very long RNA also carries out RNA synthesis in conventional (3'→5'direction). Furthermore the quality of RNA produced remains in question.



(2'- DTM – RNA amidites)
Structure 1.

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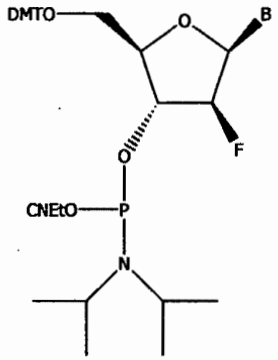
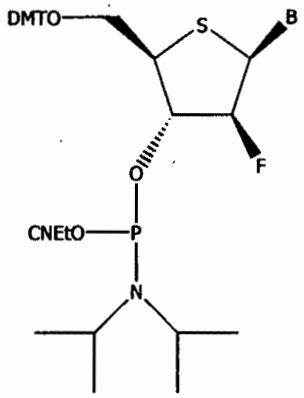
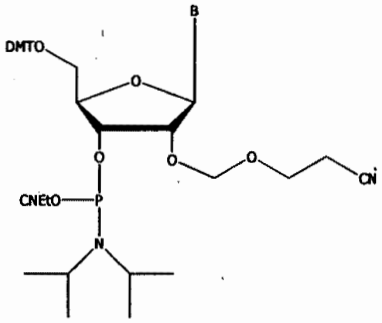


Structure 2.
2'- F-ANA modified phosphoramidites



Structure 3.
4'-Thio-2'- F-ANA modified phosphoramidites



Structure 4.
2'- CEM Protected RNA Intermediates

Chemical synthesis of RNA is desirable because it avoids the inefficiencies and limitation of scale of synthesis such as by in vitro transcription by T7 RNA polymerase, Helm, M., Brule, H., Giege, R., Florence, C., RNA, 5:618-621, 1999. Chemical synthesis of RNA is desirable for studies of RNA structure and function, and many useful modifications can be achieved selectively, such as site specific introduction of functional groups;viz., disulphide cross linking as a probe of RNA tertiary structures, Maglott, E.J., Glick, G.D., Nucl. Acids Res., 26: 1301-1308, 1999. Synthesis of long RNA is very important for biologically active molecules such as tRNA, and such synthesis have been achieved, Persson, T., Kutzke, U., Busch, S., Held, R., Harmann, R.K., Bioorgan. Med. Chem., 9:51-56, 2001; Oglvie, K.K., Usman, N., Nicoghosian, K., Cedrgren, R.J., Proc. Natl. Acad. Sci., USA, 85:5764-5768, 1988; Bratty, J., Wu, T., Nicoghosian, K., Ogilvie, K.K., Perreault, J.-P., Keith, G., Cedergren, R.J., F.E.B.S. Lett., 269:60-64 , 1990; Gasparutto, D., Livache, T., Bazin, H., Duplaa, A.M., Guy, A., Khorlin, A., Molko, D., Roget, A., Teoule, R., Nucl. Acids. Res., 20:5159-5166, 1992; Goodwin, J.T., Stanick, W.A., Glick, G.D., J.Org. Chem., 59:7941-7943, 1994. None of the techniques mentioned in this paragraph, however employ synthesis of RNA in reverse direction (5'→3'direction), and hence introduction of a number of groups required for selective introduction at 3'- end is practical and convenient. We have observed higher coupling efficiency per step during automated oligo

synthesis with our reverse RNA amidites, and therefore they have greater ability to get higher purity and produce very long oligos. We also demonstrated that the process of our invention leads oligonucleotide free of M+1 species. Such species lead to closer impurities as shoulder of desired peak during HPLC analysis or purification or Gel purification.

The t-butyldimethyl silyl protecting group on 2'- hydroxyl of ribonucleosides has been the group of choice for making 3'- phosphoramidites and utilizing them for oligonucleotide synthesis. shown to migrate to 3'- hydroxyl position rather easily. This has been documented amply and in detail (Ogilvie, K.K., and Entwistle, D.W. *Carbohydrate Res.*, 89, 203-210, 1981; Wu, T., and Ogilvie, K.K. *J. Org. Chem.*, 55, 4717-4734, 1990). That fact complicates the synthesis of the desired phosphoramidites and requires an efficient method of purification that clear resolves corresponding isomers and prevents any contamination of the final monomer.

Our present invention is directed towards the synthesis of high purity RNA's and specifically to introduce groups at 3'- end of oligonucleotides of synthetic RNA's. Such RNA's have vast application in therapeutics, diagnostics, drug design and selective inhibition of an RNA sequence within cellular environment, blocking a function of different types of RNA present inside cell. Silencing gene expression at mRNA level with nucleic acid based molecules is fascinating approach. Among these RNA interference (RNAi) has become a proven approach which offers great potential for selective gene inhibition and showing great promise for application in control and management of various biochemical and pharmacological processes. Early studies by Fire etal., Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C, Nature, 391, 806-811, 1998, showed that RNA interference in Caenorhabditis elegans is mediated by 21 and 22 nucleotide RNA sequences. This was further confirmed as general phenomenon of specific inhibition of gene expression by small double stranded RNA's being mediated by 21 and 22 nucleotide RNA's, Genes Dev., 15, 188-200, 2001. Simultaneous studies by Capie, N.J., Parrish, S., Imani, F., Fire, A., and Morgan , R.A., confirmed such phenomenon of specific gene expression by small double stranded (dS) RNAs in invertebrates and vertebrates alike. Subsequently a vast amount of research led to confirmation of above studies and established RNAi as a powerful tool for selectively and very specific gene inhibition and regulation, Nishikura, K., Cell, 107, 415-418, 2001; Nykanen, A., Haley, B., Zamore, P.D., Cell, 107, 309-321, 2001; Tuschl, T., Nat. Biotechnol., 20, 446-448, 2002; Mittal, V., Nature Rev., 5, 355-365, 2004; Proc. Natl. Acad. Sci. USA, 99, 6047-6052, 2002; Donze, O. & Picard, D., Nucl. Acids. Res., 30, e46,2002; Sui, G.,Soohoo, C., Affar el, B., Gay, F., Shi, Y., Forrester, W.c., and Shi, Y., Proc. Natl. Acad. Sci. USA, 99, 5515-5520, 2002; Paddison, P.J.,Caudy, A.A., Bernstein, E., Hannon, G.J., and Conklin, D.S., Genes Dev., 16, 948-959, 2002.

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Besides the natural double stranded (ds) RNA sequences, chemically modified RNA have been shown to cause similar or enhanced RNA interference in mammalian cells
using 2'-deoxy-2'- fluoro- beta-D_arabinonucleic acid (FANA) into sequences for SiRNA activities, Dowler, T., Bergeron, D., Tedeschi, Anna-Lisa, Paquet, L., Ferrari, N., Damha, M.J., Nucl. Acids Res., 34, 1669-1675, 2006.

Various other modifications to improve SiRNA properties have been persued, which include alteration in backbone chemistry, 2'- sugar modifications, nucleobase modifications, some of which have been recently reviewed ; Nawrot, B, and Sipa, K., Curr. Top. Med. Chem., 6,913-925, 2006; Manoharan, M. Curr. Opin. Chem. Biol., 8, 570-579, 2004; The PS modifications of Si RNA are well tolerated , although some reports indicate increased toxicity and some reduced efficacy, Harborth, J., Elbasir, S.M., Vandenburgh, K., Manninga, H., Scaringe, S.A., Weber, K., Tuschl, T., Antisense Nucleic Acid Drug Dev., 13, 83-105, 2003. The 2'- Omethyl modification , although maintains A form (RNA like) helix, and has been shown to be either retaining SiRNA activity or in reduction depending in a number of such modifications within a sequence, Chiu, Y.L., Rana, T.M., RNA, 9, 1034-1048, 2003. It has also been shown that extensive 2'- O-Methyl modification of a sequence can be made in the sense strand without loss of SiRNA activity, Kraynack,B.A., Baker, B.F., RNA, 12, 163-176, 2006. Bicyclic locked nucleic acids (LNA's) that confer high binding affinity have been introduced in SiRNA sequences, especially when central region of SiRNA sequence is avoided, Braash, D.A., Jensen, S., Liu, Y., Kaur, K., Arar, K., White, M.A., and Corey , D.R., Biochemistry, 42, 7967-7995, 2003. Similarly altritol sugar modified oligonucleotides (ANA) have recently been reported. Altrilol sugar offer a rigid conformation and shown to form very bstble duplexes with RNA in a sequence sprecific manner, and shown to stay in A (RNA type) conformation. It was shown that ANA modified SiRNAs targeting MDR1 gene exhibited improved efficacy as compared to unmodified controls, specifically effective when this modification was near the 3'- end of sense or antisense strand, Fisher, M., Abramov, M., Aerschot, A.V., Xu, D., Juliano, R.L., Herdewijn, P., Nucl. Acids Res.,35, 1064-1074, 2007.

Among various requirements for effective RNA interference (RNAi) to take place, there are a number of observations and facts have been established. Thus the RNA has to be double stranded (ds) in the region of identity to the target. For the chemical requirement, besides capability of 5'- end to be able to be converted to triphosphates, modifications of A, C, G were found to be fully compatible with interference activity. Backbione modification of RNA , such as 2'- fluoro, 2'- amino uracil, 2'- deoxy thymidine, 2'- deoxycytidine appear to stabilize the modified RNA in the resulting double strand. Amongst the nucleoside base modification 5- bromouracil, 4- thiouracil, 5-iodouracil, 5-(3-aminoallyl)- uracil, inosine are readily incorporated in the RNA intereference complex (RNAi & RISC complex). Similarly inosine could be substituted for guanosine.

It has been shown that cholesterol – conjugated siRNA can achieve delivery into cells and silence gene expression. Further it has been shown that lipid conjugated SiRNA, bile acids, long chain fatty acids can mediate SiRNA uptake into cells and silence gene expression *in vivo.* Efficient and selective uptake of siRNA conjugates in tissues is dependent on the maximum association with lipoprotein particles, lipoprotein / receptor interactions and transmembrane protein mediated uptake. It has been shown that high density lipoproteins direct the delivery of of SiRNA into liver, gut , kidney and steroidal containing organs. It has been further shown that LDL directs siRNA primarily to the liver. It has been shown that LDL receptor is involved in the delivery of siRNA. These results show great promise for SiRNA uptake by one of the delivery system and can be exploited in development of therapeutics. (article by Marcus Stoffel, OTS, 2007), p.64.

It has been proposed that siRNA can be designed with chemical modifications to protect against nuclease degradation, abrogate inflammation, reduce off target gene silencing , and thereby improve effectiveness for target genes. Delivery vehicles or conjugates of lipids and other lipophilic miolecules which allow enhanced cellular uptake are essential for therapeutic developments. Such siRNA's are presently being developed for human target validation and interfering with diseases pathways and developing new frontier for drug development. Alan Sachs, Merck, Oligonucleotide Therapeutics Conference (OTS), page 80, 2007.

The 3'- end of sense strand of siRNA can be modified and has been shown to tolerate modification, and attachment of ligands is most suited at this end (figure 1 &2), as detailed in a number of key publications; siRNA function in RNAi: a chemical modification, Ya-Lin Chiu and Tariq Rana, RNA, 9, 1034-1048, 2003; M. Manoharan, Curr. Opin. Chem.Biol, 6, 570-579,2004; Nawrot, B. and Sipa, K., Curr.Top.Med. Chem., 6, 913-925, 2006; Scaringe, S., Marshall, W.S., Khvorova, A., Naty. Biotechnol., 22, 326-30, 2004.

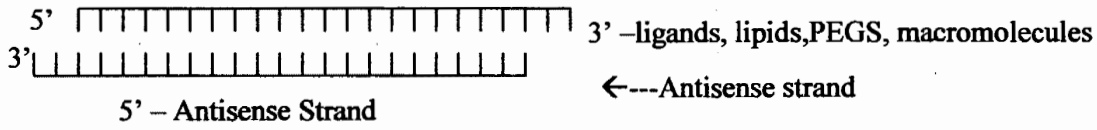

Fig. 1. (SiRNA duplex)

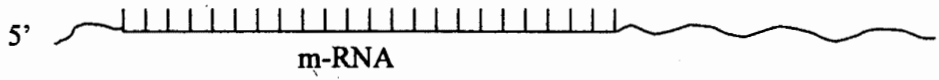

Fig. 2. (m-RNA Complementary to antisense strand)

The introduction of lipophilic or hydrophobic groups and enhancing of siRNA delivery and optimization of targets has been addressed and achieved through bio-conjugation (fig 1). Generally the attachment is done, preferably at the 3'- end of senses strand, and occasionally on the 3'- end of the antisense strand. The design of nuclease resistant siRNA has been subject of intense research and development recently to develop effective therapeutics. Thus base modifications such as , 2-thiouridine, pseudouridine, dihydrouridine have revealed the

effect on conformations of RNA molecules and the associated biological activity; Sipa, K., Sochacka, E., Kazmierczak-Baranska, J., Maszewska, M., Janicka, M., Nowak, G., Nawrot, B., RNA, 13, 1301-1316, 2007. It was shown that 2'- modified RNA's especially 2'- Fluoro have great resistance towards nuclease and are biological active *in- vivo*, Layzer, J.M., McCaffrey, A.P., Tanner, A.K., Huang, Z., Kay, M.A., and Sullenger, B.A., RNA, 10, 766-771, 2004. 2'- O-Alkyl-modification , such as 2'- Omethyl's and 2'- O-MOE, Prakash, S., Allerson, CV.R., Dande,P., Vickers, T.A., Siofi, T.A., Jarres, R., Baker, B.F., Swayze, E.E., Griffey, R.H., and Bhat, B., J.Med. Chem., 48, 4247, 4253, 2005. The same authors used 4'-thio modified sugar nucleosides in combination of 2'- O alkyl modification for improving SiRNA propertie s and RNAi enhancement, Dande, P., Prakas, T.P., Sioufi, N., Gaus, H., Jarres, R., Berdeja, A., Swayne, E.E., Griffey, R.H., Bhat, B.K, J. Med. Chem., 49, 1624-1634, 2006. The Replacement of internucleotide phosphate with phosphorothioate and boranophosphates of SiRNAs show promise *in -vivo*, Li, Z.Y., Mao, H., Kallick, D.A., and Gorenstein, D.G., Biochem. Biophys. Res. Comm., 329, 1026-1030, 2005; Hall, A.H.S., Wan, J., Shaughnessy, E.E., Ramsay Shaw, B., Alexander, K.A., Nucl. Acids Res., 32, 5991-6000, 2004.

Bioconjugation of siRNA molecules, biologically RNA molecules , aptamers and synthetic DMNA molecules require a key feature for cell membrane permeability, in addition to in vivo stability and of appropriate modification of nucleoswides. Insufficient cross-membrane cellular uptake limits the utility of siRNA's, other single stranded RNA's or even various DNA molecules. Thus cholesterol attached at 3'- end of siRNA has been shown to improve *in-vivo* cell trafficking and therapeutic silencing f gene, Soutschek, J., Akine, A., Bramlage, B., Charisse, K., Constein, R., Donoghue, M., Elbasir, S., Geickk, A., Hadwiger,P., Harborth, J., Nature, 432, 173-0178, 2004.

Among various conjugations, besides cholesterol, which have been developed are;

(a) Natural and synthetic protein transduction domains (PTDs), also called cell permeating peptides (CPPs) or membrane permeant peptides (MPPs) which are short amino acid sequences that are able to interact with the plasma membrane. The uptake of MPP-SiRNA conjugates takes place rapidly. Such peptides can be conjugated preferably to the 3'- of stand strand.
(b) Other polycationinc molecules can be conjugated at the 3'- end of either sense or antisense strand of RNA.
(c) PEG (polyethylene glycols-oligonucleotide conjuagates) have been used in various complex pssess significant gene silencing effect after uptake in target cells, Oishi, M., Nagasaki, Y., Itaka, K., Nishiyama, N., and Kataoka, K., J. Am. Chem. Soc., 127, 1624-1625, 2005.
(d) Aptamers have been used for site specific delivery of SiRNA's. Since Aptamers have high affinity for their targets, the conjugates with SiRNA act as excellent delivery system , which result in efficient inhibition of the target gene expression, Chu, T.C., Twu, K.Y., Ellington, A.D. and Levy, M., Nucl. Acids Res., 34(10), e73, 2006. These molecues can once again be conjugated at the 3'- end of Si RNA or other biologically active oligonucleotides.
(e) Various lipid conjugations at the 3'- end can be achieved through our invention and can be utilized for efficient internalization of oligonucleotides.

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The lipophilic moiety can consuist of a hydroxyl function to synthesis a phopsphoramidite. Similarly the lipophilic moiety can have carboxylic function at the terminus. The later can be coupled to a 3'- amino group having a spacer, synthesized by last addition of amino linkers such as C-6 amino linker amidite, of the reverse synthesized oligonucleotide, to the carboxylic moiety using DCC (dicyclohexyl cabodiimide) or similar coupling reagent.

These research have been reviewed elegantly by Paula, De. D., Bentley, M.V.L.B., Mahao, R.L.,RNA, 13, 431-456,2007.

Another class of RNA, closely related to siRNA are microRNA, commonly referred as miRNA.These are a large class of non coding RNA's have a big role in gene regulation, Bartel, D.P. Cell, 116,281-297, 2004; He, L., Hannon, G.J. Nat. Rev. Genet, 5:522-531, 2004; Lagos-Quintana, M., Rauhut, R., Lendeckel, W., Tuschl, T., Science, 204:853-858,2001. In human genome there are at least 1000 miRNA scattered across the entire genome. A number of these micro RNA's down regulate large number of target mRNAs, Lim, L.P., Lau, N.C., Garrett- Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsey, P.S., Johnson, J.M., Nature, 433:769-773, 2005. Different combination of miRNAs are possibily involved in regulation of target gene in mammalian cell. It has also been shown that siRNA can function as miRNAs, Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., da Piedade, I., Gunsalus, K.C., Stoffel, M., Nat. Genet., 37: 495-500, 2005; Doench, J.G., Petersen, C.P., Sharp, P.A., Genes Dev., 17:438-442, 2003. miRNA have great potential as therapeutics and in gene regulation, Hammond, S.M., Trends Mol. Med. 12:99-101, 2006. A vast amount of efforts are being currently devoted towards understanding miRNA pathways, role in development and diseases and focusing on cancer. miRNA targets are being developed for therapeutic and diagnostics development. A great number of miRNA are being identified and their role is being determined through microiarrays, PCR and informatics. Synthesis of RNA designed to target miRNA also require RNA synthesios and similar modification as required for siRNA's, for stability of RNA and bioconjugation for better cellular uptakes. Our invention of reverse synthesis will greatly accelerate the pace of this research and development.

Synthesis of vast variety of therapeutic grade RNA and siRNA requires a modification or labeling of 3'- end of an oligonucleotide. In the case of SiRNA, generally it is the 3'- end of sense strand. The synthesis of 3'- end modified RNA requiring lipophilic, long chain ligands or chromophores, using 3' → 5' synthesis methodology is challenging, requires corresponding solid support and generally results in low coupling efficiency and lower purity of the final oligonucleotide in general because of large amount of truncated sequences containing desired hydrophobic modification. We have approached this problem by developing reverse RNA monomer phosphoramidites for RNA synthesis in 5'→ 3'- direction. They lead to very clean oligonucleotide synthesis allowing for introduction of various modifications at the 3'- end cleanly and efficiently. In order to enhance stability and additional eliciting additional favorable biochemical properties, 2'-5'- linked DNA (Structure 5) and RNA (Structure 6) have been developed in the past.

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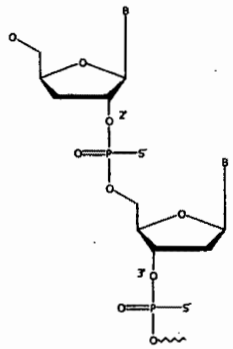
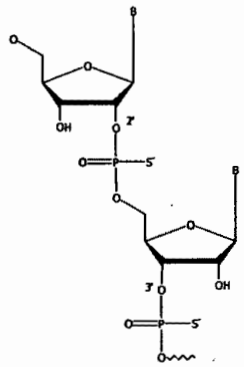
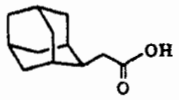


Structure 5. (2'-5'- linked DNA unit)

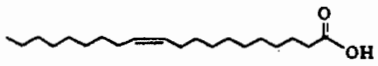


Structure 6. (2'-5'- linked RNA unit)

For efficient delivery of RNA and to increase cellular concentration of oligonucleotides, lipid containing oligonucleotides are generally synthesized. Since lipid containing synthetic nucleosides, enhance uptake of many synthetic nucleoside drugs, like AZT. Lipipoid nucleic acids and are expected to reduce the hydrophilicity of oligonucleotides. Similarly hydrophobic molecules such as cholesterol can bind to LDL particles and lipoproteins and activate a delivery process involving these proteins to transport oligonucleotides. It has also been shown that lipidoic nucleic acids improve the efficacy of oligonucleotides. Shea, R.G., Marsters, J.C., Bischofberger, N., Nucleic Acids Res., 18, 3777, 1990; Letsinger, R.L., Zhang, G., Sun, D.K., Ikeuchi, T., Sarin, P.S., Proc. Natl. Acad. Sci. USA 86, 6553, 1989; Oberhauser, B., and Wagner, E., Nucleic Acids Res., 20, 533, 1992; Saison,-Behmoaras, T., Tocque, B., Rey, I., Chassignol, M., Thuong, N.T., Helene, C. The EMBO Journal , 10, 1111, 1991; Reed, M.W., Adams, A.D., Nelson, J.S., Meyer, R.B., Jr., Bioconjugate Chem., 2, 217, 1991; Polushin,N.N., Cohen, J., J. Nucleic Acids Res., 22, 5492, 1994; Vu, H., Murphy, M., Riegel, Joyce, M., Jayaraman, K., Nucleosides & Nucleotides , 12, 853, 1993; Marasco, Jr., Angelino, N.J., Paul, B., Dolnick, B.J., Tetrahedron Lett., 35, 3029, 1994. The Tm of a series of hydrophobic groups; such as adamantane (structure 7) , eicosenoic acid (structure 8), cholesterol were attached to oligodeoxy nucleotide sequences at the 3'- end and were hybridized to complemetary RNA sequences and the Tm was found to be unaffected, which indicate that such groups do not interefere with oligo hybridization properties; Manoharan, M., Tivel, K.L., Andrade, L.K., Cook, P.D., Tetrahedron Lett., 36, 1995; Manoharan, M., Tivel, K.L., Cook, P.D., Tetrahedron Lett., 36, 3651-3654, 1995; Gerlt, J.A. Nucleases, 2 nd Edition, Linn, S.M., Lloyd, R.S., Roberts, R.J., Eds. Cold Spring Harbor Laboratory Press, p-10, 1993..



Structure 7. Adamantane acetic acid



Structure 8.Cis-11-eicosenoic acid

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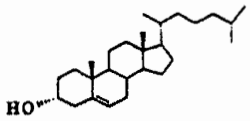
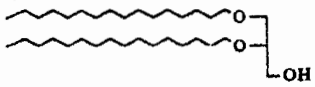


Structure 9. Cholesterol

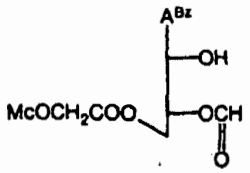


Structure 10. 2,3- Di hexadexyl-rac-glycerol

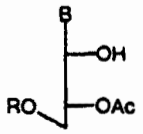
Besides the preceeding lipidoic molecules other class of molecules, which have shown high promise are short and long chain polyethyene glycols (PEG), Bonora, G.M., Burcovich, B., Veronese, F.M., Plyasunova, O., Pokrovsky, A. and Zarytova, V., XII International Round Table Conference, Nucleosides, Nucleotides and their Biological Applications, La Jolla, CA, Sept., 15-19, PPI 89, 1996. For efficient delivery of synthetic RNA, and DNA molecules PEG attachment to various oligonucleotides have shown to very favorable properties. PEG – oligomers have shown nice enzymatic stability by preventing fast digestion. The thermal melting behaviour was not effected, there by still retaining properties of double strand formation.

Our approach to peroduce reversed RNA phosphoramidites required selective introduction of 5'- esters, such as 5'- benzouyl, acetyl, levulinyl or substituted- 5'- benzoyl –n-protected ribonucleoside. Subsequently introduction of an appropriate protecting group at 2'- position, such as 2'- tBDsilyl or 2'- TOM (triisopropyl silyl methoxy; TOM) was required. In our scheme we show synthesis of selected molecules to achieve this purpose.

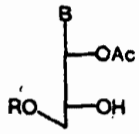
In order to produce target compounds, structure 28a-d, the key intermediate required was 2'-silylether-5'- O acylated-N-protected ribonucleoside, compound 23a-e. The first intermediate required for this was 5'- acylated-N-protected ribonucleoside compound (22a-e). To the best of our knowledge, the compounds 21a-e have not been reported. The compounds, which are reported for RNA synthesis in the past utilized intermediates, structure 11, 12 and 13. Various 2' and 3' acetates with 5'- acetate; viz., 5'- benzoyl protected nucleosides, Reese, B.E., Jarman, M., Reese, C.B., Tetrahedron, 24, 639, 1968; Neilson, T., Werstiuk, E.S., Can. J. Chem. 49, 493, 1971; Neilon, T., Wastrodowski, E.V., Werstiuk, E.S., Can. J. Chem., 51, 1068, 1973; Eckstein, F., Cramer, F., Chem.Ber., 98, 995, 1965; Zemlicka, J., Chladek, S., Tet. Lett., 3057, 1965, Amarnath,V. & Broom, A.D., Chemical Reviews, 77, 183-219,1977. In our present invention, however we require free 2' and 3' hydroxyl groups, such as in structures 22a-e.



Structure 11.



Structure 12



Structure 13

In the present invention, comparative synthesis and purification of RNA's were carried out , both by conventional method (3'→5') and reverse Direction (5'→3'). High Purity of RNAs, Smooth 3'- Conjugation- Cholesterol, HEG and PEG (Polyethylene glycols) & Demonstration of Absence of M+1 in Reverse RNA Synthesis

Salient Features as observed from the figures 9-18:

From the data presented in the discussions and in the figures, following observations are made;

1. The crude RNA's have much closer impurities (N-1) in the conventional method (3'-5'-direction), as compared to reverse RNA synthesis (5'- 3' direction). Therefore after purification RNA synthesized by reverse RNA synthesis are purer.
2. The feature mentioned above is much more visible in the synthesis of cholesterol attached to 3'- end of RNA (see fig 11 vs. fig. 12). Therefore it is easier to purify RNA with cholesterol at 3'- end synthesized by reverse RNA synthesis (see fig. 12).
3. M+1 impurities are essentially absent in the RNA's synthesized by reverse RNA synthesis method.

   It is postulated that in the molecule, ribonucleoside- 3'- DMT-2'- tBDsilyl-5'- phosphoramidites, the 3'- DMT is not cleaved by 5-ethylthiotetrazole or similar activators during oligonucleotide coupling step and within the coupling time of oligonucleotide chain extension.

4. RNA containing macromolecules at the 3'- end which are generally inaccessible by conventional methods (3'→5') are easily synthesized by reverse RNA synthesis (5'→3' direction). These RNA's can be produced in high purity.
5. 3'- PEG RNA (21- mer) was synthesized, and after purification was essentially 100% pure (see figs. 15 &16).

SUMMARY OF THE INVENTION

The invention provides a novel RNA monomer phosphoramidites shown below in Structure 14. The synthetic route that has been developed allows obtaining desired phosphoramidites without any contamination with unwanted isomers.

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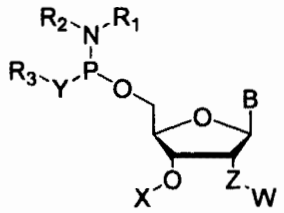
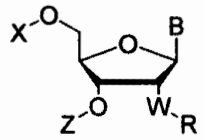


Structure 14.

The RNA phosphoramidite monomers in our invention contain 3'- DMT group in ribonucleosides, carrying 5'-cyanoethylphosphoramidite (CED) and various methyloxy or silyl protecting groups at 2'- position of the ribose moiety (Structure 14). The solid support having protected RNA nucleosides containing 3'- DMT group and 5' terminus is attached to solid support (Structure 15).



Structure 15.

The RNA phosphoramidites can be used in reverse 5'→ 3'- directed oligonucleotide synthesis. A large number of RNA synthesis, Conventional (3'→5') and Reverse Direction (5'→3') have been performed. The modification or labeling at 3'-terminus of RNA oligomer can be attached by using corresponding phosphoramidite or active ester at the end of the synthesis and don't require special solid support. Moreover, that approach leads to a very clean 3'-labeled oligonucleotide and don't require expensive methods of purification.

High Purity of RNAs were consistently obtained in the RNA synthesis by Reverse Direction (5'→3'), Smooth 3'- Conjugation of molecules such as Cholesterol, HEG (hexaethyloxyglycol) and PEG (Polyethylene glycols). We have further demonstrated that in the RNA synthesis in the reverse Direction (5'→3'), there is absence of M+1 oligonucleotide impurities.

BRIEF DESCRIPTION OF THE DAWINGS

1. HPLC- Chromatogram of N^4-Benzoyl-*2'O*-TBDMS-3'-*O*-DMT-adenosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite **(10a)**, 98.6% purity.
2. NMR ^{31}P Spectrum of N^4-Benzoyl-*2'O*-TBDMS-3'-*O*-DMT-adenosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite **(10a)**, sharp doublet at 149.35 ppm., 98.6% purity.

3. HPLC- Chromatogram of N^4-Acetyl-2'-*O*-TBDMS-3'-*O*-DMT-cytidine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (**10c**), 96.9% purity.
4. NMR ^{31}P Spectrum of N^4-Acetyl-2'-*O*-TBDMS-3'-*O*-DMT-cytidine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (**10c**), sharp doublet at 149.33 ppm., 100% purity.
5. HPLC- Chromatogram of N^2-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (**10d**), 98.3% purity.
6. NMR ^{31}P Spectrum of N^2-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (**10d**), sharp doublet at 149.35 ppm., 100% purity.
7. Diagrammaic representation of 5'→3' RNA synthesis and application to 3'-modifications.
8. Coupling Efficiency Trityl Histogram Notes (21 mer RNA synthesis):
   Coupling Efficiency (step wise yield by base leveling) : 99.6%
   Final Yield (by rolling average): 100% ; Stepwise Yield for monomer G: 100%
   Stepwise Yield for monomer A: 99.2% ; Stepwise Yield for monomer C: 99.6%
   Stepwise Yield for monomer C: 99.6% ; Stepwise Yield for monomer U :100.0%

9. Electropherogram of the crude oligonucleotide SEQ ID No 1 made by conventional method
10. Electropherogram of the crude oligonucleotide SEQ ID No 1 made by reverse synthesis.
11. Electropherogram of the crude oligonucleotide SEQ ID No 2 made by conventional method.
12. Electropherogram of the crude oligonucleotide SEQ ID No 2 made by reverse synthesis.
13. 21- Mer RNA with 3'- Cholesterol-TEG linker. Reverse direction (5'→3') synthesis and HPLC purification. 1 umole scale. Purity; 99.9%.
14. Electropherogram of the crude oligonucleotide SEQ ID No 3 made by reverse synthesis.
15. 21- mer RNA synthesized by reverse direction (5'→3') , followed by HEG (Hexa - ethyloxyglycol) attachment. After HPLC purification, purity by CE; 94.39%.
16. Electropherogram of the crude oligonucleotide SEQ ID No 4 made by reverse synthesis.
17. Electropherogram of the oligonucleotide SEQ ID No 4 made by reverse synthesis and purified by reverse-phase HPLC.
18. ESI Mass Spectral analysis of 21- mer RNA with 3'- PEG -2000 attachment, purified RNA as shown in Fig. 15. The synthesis was carried out in reverse direction (5' → 3' direction) . The PEG-2000 was attached as last step via the corresponding phosphoramidite, ChemGenes catalog ;CLP-3119 Calculated Molecular Weight: 8684.1 Observed Molecular Weight: 8681.1 Note: There is a distribution of at least 14 PEG species of the RNA on both sides of the Calculated molecular weight with PEG-2000. Thus species from 8417.1 to

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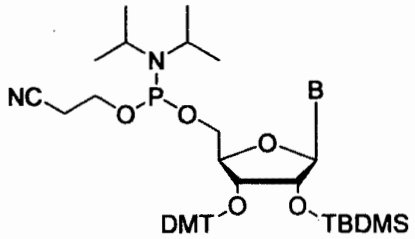
8945.3 are present with a molecular weight difference of a glycol unit (+/- 44).

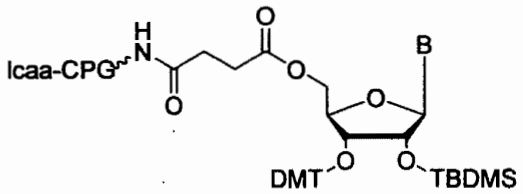
DETAILED DESCRIPTION OF THE INVENTION

The reverse RNA monomer phosphoramidites in our invention carry a 3'- DMT group in ribonucleosides, carrying 2'-tBDsilyl (tBDSi) -5'-cyanoethylphosphoramidite (CED) (Structure 16), 3'-DMT-2'-tBDsilyl-5'-succinyl-Icaa CPG-n-protected nucleosides (Structure 17) or 3'- DMT-2'-triisopropylsiloloxymethyl (TOM)- 5'- CED phosphoramidite group (Structure 18).



Where B = A (*N*-Bz), C (*N*-Bz), C (*N*-Ac), G (*N*-iBu),
A (*N*-*t*BPac), C (*N*-*t*BPac), C (*N*-tBPac), G (*N*-tBPac),
A (*N*-Pac), C (*N*-Pac), C (*N*-Pac), G (*N*-Pac), U.

Structure 16. 3'-DMT-2'-tBDSilyl-5'-Amidites
(Reverse RNA-tBDsilyl- amidites)



Where B = A (*N*-Bz), C (*N*-Bz), C (*N*-Ac), G (*N*-iBu),
A (*N*-*t*BPac), C (*N*-*t*BPac), C (*N*-tBPac), G (*N*-tBPac),
A (*N*-Pac), C (*N*-Pac), C (*N*-Pac), G (*N*-Pac), U.

Structure 17. 3'-DMT-2'-tBDSilyl-5'-CPG
(Reverse RNA-tBDsilyl- 5'-Icaa CPG)

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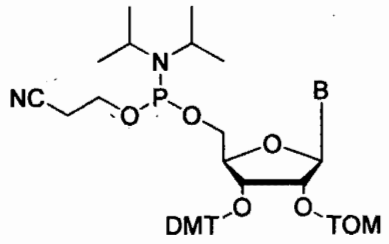
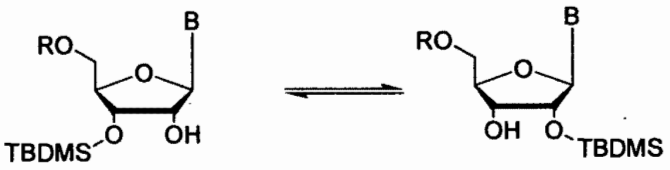


Where B = A (*N*-Ac), C (*N*-Ac), G (*N*-Ac), U.

Structure 18. 3'-DMT-2'-TOM (triisopropylsilyl oxymethylene) -5'-amidites
(Reverse RNA- TOM -5'-amidites)

The invention also contemplates method for preparing the disclosed compositions. The starting base protected nucleoside **19** affording isopropyliden protected nucleoside **20**. Benzoylation followed by isopropyliden group removal yields 5'-benzolylated nucleoside **22**. Consecutive silylation reaction with TBDMS chloride in pyridine provides mixture of 2'- and 3'- TBDMS protected nucleosides (**23** and **24**) in the ratio of 3:2 respectively. After column chromatography isomers have been resolved and isolated in % yield. Further reaction of the isomer **23** afforded 3'-DMT-2'-TBDMS protected nucleoside **26**.

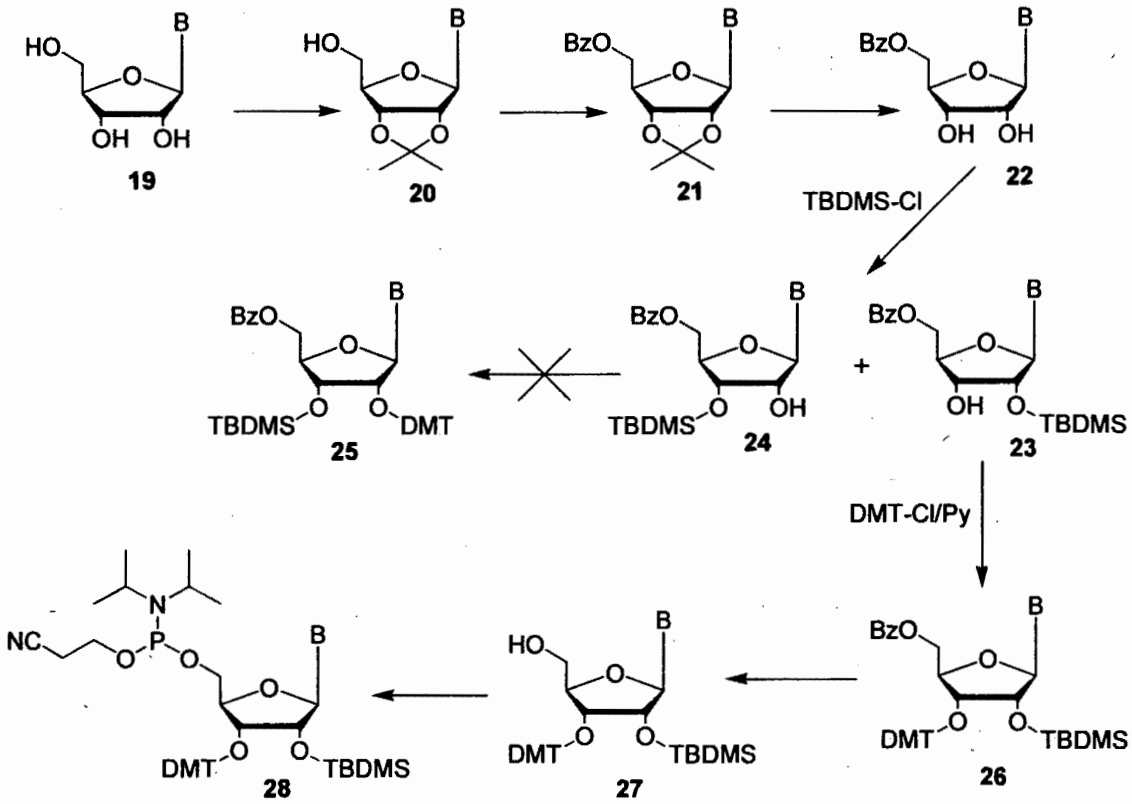
It is therefore conceivable that during subsequent functionalization of 3'– hydroxyl group, there will be significant migration of 2'– tBdsilyl group.



Scheme 1.

During our functionalization of 3'– hydroxyl group with DMT- (4,4-dimethoxytrityl), we observed no significant migration. Moreover, the 3'-TBDMS protected isomer **24** also was involved in the same tritylation reactionas isomer **23** with DMT chloride in pyridine, however nucleoside **25** was not observed in that reaction. Therefore in case of contamination of the 2'-TBDMS protected nucleoside **23** with its isomer **24**, unwanted isomer **25** cannot be formed in the tritylation conditions and desired nucleoside **26** can be isolated in high purity. The 3'-TBDMS protected nucleoside **24** can be utilized in the synthesis of the desired product and converted into **23** due to isomerization process outlined in the scheme 1.

Removal of 5'-benzoyl group with sodium hydroxide in methanol followed by phosphitilation reaction using CEDP and DIPA tetrazolate affording final reverse phosphoramidite **28**.



Where B = a) A (*N*-Bz), b) C (*N*-Bz), c) C (*N*-Ac), d) G (*N*-iBu)
e) A (*N*-tBPac), f) C (*N*-tBPac), g) G (*N*-tBPac),
h) A (*N*-Pac), i) C (*N*-Pac), j) G (*N*-Pac), k) U.

Scheme 2.

Oligonucleotide synthesis using reverse phosphoramidites was performed in the direction from 5'→ 3'. The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope. In particular the following examples demonstrate synthetic methods for obtaining the compounds of the invention. Starting materials useful for preparing the compounds of the invention and intermediates thereof, are commercially available or can be prepared from commercially available materials using known synthetic methods and reagents. All oligonucleotide sequences are written from the 5'-terminus on the left to the 3'-terminus on the right. The coupling efficiency of the 3'- DMT -5'- CED phosphoramidites indicated per step coupling surpassing 99%, leading to high purity RNA. A large number of homopolymers and 20-21 mers

oligonucleotides have been synthesized using these monomer phosphoramidfites. A typical data is presented in the figure 8.

Our data show that there is no difference in coupling efficiency during oligo synthesis using our reverse RNA monomers (for 5'→3'- direction) as compared to standard 3'- CED phosphoramidites in synthesis 3'→5' direction (see figures 9 and 10).

In another embodiment the invention provides method for synthesis of ribonucleic acid oligomers with modification or labeling of 3'- end of an oligonucleotide. The synthesis of 3'- end modified RNA requiring lipophilic, long chain ligands or chromophores fluorophores and quenchers can be performed using corresponding phosphoramidites. Our data show that 5'→3'- direction synthesis has very distinct advantage compared to conventional method (see figure 11 and 12). Moreover the 3'-modifications that not available on solid support such as HEG or PEG-2000 can be easily introduced by using 5'→3'- direction synthesis and purified by reverse-phase HPLC. The oligonucleotide SEQ ID No 4 has been purified by RP HPLC affording 95-98 % pure products (see figure 15).

Experimental: Examples

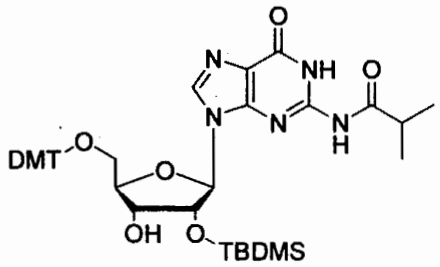
EXAMPLE 1

Synthesis of ***N*²-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28d)** as shown in the scheme 2.

***N*²-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine (27d)**: To the solution of 14 g (16.0 mmol) of the compound **26d** in 196 mL of pyridine and 21 mL of methanol mixture were added 16 mL of 2 M aqueous solution of sodium hydroxide (32.0 mmol) dropwise with stirring at 0-5 °C during the course of 25 min. The reaction mixture was neutralized with 15 mL of 2 M HCl. The solvent was removed under diminished pressure and residue was extracted with two portions of 25 mL of chloroform. Organic layer was combined, washed with 50 mL of brine and dried over anhydrous Na_2SO_4. Flash chromatography with 5:2:3 chloroform/hexanes/acetone provided 10.4 g (84.3%) of the compound **27d**. ^{1}H NMR ($CDCl_3/D_2O$) δ -0.54 (s, 3H), 0.01 (s, 3H), 088 (s, 9H), 1.21 (d, 3H, J=7.0 Hz), 1.23 (d, 3H, J=7.0), 2.66 (qq, 1H, J=7.0), 2.89 (d, 1H, J=12 Hz), 3.28 (s, 1H), 3.37 (dd, 1H, $J_{5a,5b}$=15 Hz, $J_{5,4}$=2.5Hz) 3.80 (s, 6H), 4.24 (d, 1H, J=5 Hz), 4.83 (dd, 1H, $J_{2,1}$=8 Hz, $J_{2,3}$=5 Hz), 5.97 (d, 1H, J=8 Hz), 6.84 (dd, 4H, J=9 Hz, J=2 Hz), 7.23 (t, 1H, J=7.5 Hz), 7.30 (t, 2H, J=7 Hz), 7.45 (m, 4H), 7.59 (d, 2H, J=7.5 Hz), 7.77 (s, 1H). ESMS 792.8 [$C_{41}H_{51}N_5O_8Si$ $(M+Na)^+$ requires 792.9].

***N*²-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28d)**: To the solution of 10.4 g (13.5 mmol) of the compound **27**, 3.4 g (26.12 mmol) of ethylthiotetrazole, 4.7 mL (27 mmol) of DIPEA and 1.08 mL (13.5 mmol) of *N*-methylimidazole in 104 mL of acetonitrile

were added 8.4 mL (26.12 mmol) of 2-cyanoethyl-*N,N,N,N*-tetraisopropylphosphane dropwise with stirring under Ar at room temperature. After 3 hrs the reaction mixture was diluted with 100 mL of ethylacetate and washed with 200 mL of saturated sodium bicarbonate and 200 mL of brine. The organic layer was separated and dried over 2 g of anhydrous Na_2SO_4. Flash chromatography with 7:2:1 chloroform/hexanes/triethylamine provided 12 g (92.1%) of the compound **28d**. ^{31}P NMR ($CDCl_3$) δ 149.05 and 149.73. ESMS 993.3 [$C_{50}H_{68}N_7O_9PSi$ $(M+Na)^+$ requires 993.2].



Formula 6.

Table 1. Comparative ^1H NMR data of structures **27d, 28d** and formula 6.

Structure	H-1'	H-2'	H-3'	H-4'	H-5'ab	H-8	CH	CH_3
27d	5.97, d. J=8.0	4.83, dd J=8.12	4.24, d. J=5	3.28, br.s.	3.37, dd. J=15.0, 2.5 2.98, d. J=15.0	7.76, s.	2.66, qq. J=7	1.31, d., J=7 1.27, d., J=7
Formula 6	5.78, d. J=7 and 5.72, d. J=6.7	5.13, dd. J=7, 5 and 4.87, dd. J=7, 5.5	4.8, br.s.	4.356-4.353, m. and 4.242-4.223, m.	3.89, dd. J=10, 2.5 3.54, dd. J=10, 2.5 3.11 J=10, 2.5	7.84, s.	2.78, qq. J=7	1.192, d. J=7 1.179, d. J=7
28d	6.22, d., J=7,5 and 5.96, d., J=5					8.21, s. and 8.08, s.		

EXAMPLE 2

Synthesis of ***N*⁴-Acetyl-2'-*O*-TBDMS-3'-*O*-DMT-cytidine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28c).**

***N*⁴-Acetyl-2'-*O*-TBDMS-3'-*O*-DMT-cytidine (27c)**: Was prepared analogously to N^2-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine (9d). Yield is %. ^1H NMR ($CDCl_3/D_2O$) δ 0.07 (s, 3H), 0.16 (s, 3H), 0.97 (s, 9H), 2.24 (s, 3H), 3.16 (br.d, 1H, $J_{5a,5b}$=12 Hz), 3.55 (br.d, 1H, $J_{5a,5b}$=12 Hz) 3.79 (s, 6H), 4.08 (t, 1H, J=4.5 Hz), 4.44 (br.s., 1H), 4.78 (br.s., 1H), 5.61 (d, 1H, J= 4.1), 6.80 (dd, 4H, J=7 Hz, J=3 Hz), 7.22 (t, 1H, J=7.5 Hz), 7.27 (t, 2H, J=7 Hz), 7.38 (m, 4H), 7.52 (d, 2H, J=7.3 Hz), 8.08 (br.s, 1H). ESMS 724.8 [$C_{38}H_{47}N_3O_8Si$ $(M+Na)^+$ requires 724.3].

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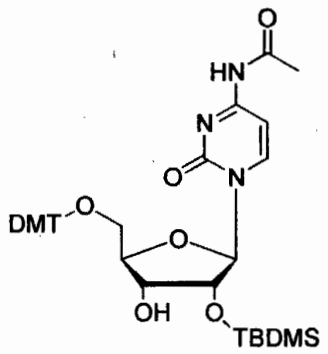
N^4-Acetyl-2'-*O*-TBDMS-3'-*O*-DMT-cytidine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28c): Was prepared analogously to N^2-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (10d). Yield is 62.6 %. ^{31}P NMR ($CDCl_3$) δ 149.1 and 149.5. ESMS 925.0 [$C_{47}H_{64}N_5O_9PSi$ $(M+Na)^+$ requires 924.4].



Formula 7.

Table 2. Comparative ^{1}H NMR data of structures **27c**, **28c** and formula 7.

Structure	H-1'	H-2'	H-3'	H-4'	H-5'ab	H-5	H-6	-$COCH_3$
27c	5.61, d. J=4.1	4.78, br.s.	4.43, br.s.	4.08, t., J=4.6	3.55, br.d. J=12.5 3.16, br.d. J=11.5	6.81, d. J=7	7.21, d. J=7	2.24, s.
Formula 7	5.90, s. and 5.62, d. J=5	4.34-436, m.	4.20-428, m.	4.10, br.d. J=5	3.58, d.d. J=11.5, 2 3.52, dd. J=11.5, 2	7.12, d. J=7	7.16, d. J=7	2.25, s.
28c	5.83, d., J=2 6.32, d., J=5							

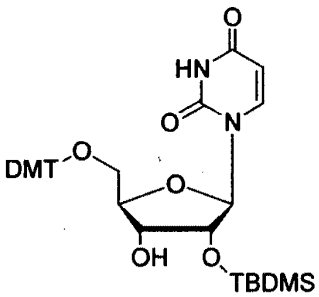
EXAMPLE 3

Synthesis of ***2'-O*-TBDMS-3'-*O*-DMT-Uridine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28k).**

2'-*O*-TBDMS-3'-*O*-DMT-Uridine (27k): Was prepared analogously to N^2-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine (27d). Yield is %. ^{1}H NMR ($CDCl_3$) δ 0.06 (s, 3H), 0.14 (s, 3H), 0.97 (s, 9H), 2.12 (br.d, 1H, J=4 Hz), 3.16

(br.dd, 1H, $J_{5a,5b}$=12.7 Hz, J_{5a4}=7.6 Hz), 3.55 (br.d, 1H, $J_{5a,5b}$=12.7 Hz J_{5b4}=4.3 Hz) 3.65-3-64 (m, 1H) 3.79 (s, 6H), 4.07 (t, 1H, J=4.3 Hz), 4.28 (t, 1H, J=4.3 Hz), 5.66 (d, 1H, J=4.9 Hz), 5.69 (d, 1H, J= 8.1 Hz), 6.82 (dd, 4H, J=7 Hz, J=3 Hz), 7.22 (t, 1H, J=7.5 Hz), 7.28 (t, 2H, J=7 Hz), 7.38 (d, 4H, J=8.9 Hz), 7.54 (d, 2H, J=8.8 Hz), 7.68 (d, 1H, J=8.2), 8.74 (br.s, 1H). ESMS [$C_{36}H_{44}N_2O_8Si$ $(M+Na)^+$ requires 683.3].

***2'-O*-TBDMS-3'-*O*-DMT-Uridine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28k)**: Was prepared analogously to N^2-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28d). Yield is %. ^{31}P NMR ($CDCl_3$) δ 148.9 and 149.6. ESMS 884.1 [$C_{45}H_{61}N_4O_9PSi$ $(M+Na)^+$ requires 884.0].



Formula 8.

Table 3. Comparative ^1H NMR data of structures **27k**, **28k** and formula 8.

Structure	H-1'	H-2'	H-3'	H-4'	H-5'ab	H-5	H-6
27k	5.66, d. J=4.9	4.28, t. J=4.7	4.07, t., J=4.3	3.64-3.65, br.m.	3.53, br.dd. J=13.7, 4.4 3.19, br.dd. J=13.7, 7.6	5.68, d. J=8.1	7.68, d. J=8.1
Formula 8	5.95, d. J=2.8 and 5.69, d. J=4.6	4.49, t. J=5 and 4.19, t. J=5	4.05-4.08, m.	4.10-4.11, br.s.	3.49-3.51, m. 3.90-3.93, m.	5.30, d. J=8.2 5.73, d. J=8.1	7.95, d. J=8.2 7.75, d, J=7.2
28k	6.00, d., J=4.5 and 6.38, d., J=7.1					7.75, d. J=8.2 7.92, d. J=8.2	

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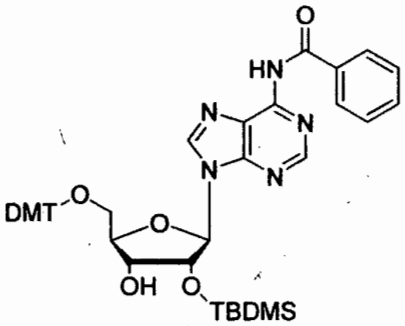
EXAMPLE 4

Synthesis of N^4-Benzoyl-*2'O*-TBDMS-3'-*O*-DMT-adenosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28a).

N^4-Benzoyl-2'-*O*-TBDMS-3'-*O*-DMT-adenosine (27a): Was prepared analogously to N^2-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine (27d). Yield is %. ^{1}H NMR ($CDCl_3/H_2O$) δ –0.75 (s, 3H), -0.01 (s, 3H), 0.86 (s, 9H), 3.03 (t, 1H, $J_{5a,5b}$=12.8 Hz), 3.29 (s, 1H), 3.47 (br.d, 1H, $J_{5a,5b}$=12.8 Hz) 3.80 (s, 6H), 4.35 (d, 1H, J=4.9 Hz), 5.17 (dd, 1H, J=8 Hz, J=5 Hz), 5.93 (dd, 1H, J= 12 Hz, J=2 Hz), 6.15 (d, 1H, J=8 Hz), 6.85 (dd, 4H, J=7 Hz, J=3 Hz), 7.23 (t, 1H, J=7.5 Hz), 7.30 (t, 2H, J=7 Hz), 7.48 (m, 4H), 7.52 (t, 2H, J=7.3 Hz), 7.62 (d, 3H, J=7.5 Hz), 8.03 (d, 2H, J=7.5 Hz), 8.14 (s, 1H), 8.77 (s, 1H), 9.07 (s, 1H). ESMS [$C_{44}H_{49}N_5O_7Si$ $(M+Na)^+$ requires 787.3].

N^4-Benzoyl-*2'-O*-TBDMS-3'-*O*-DMT-adenosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28a): Was prepared analogously to N^2-Isobutyryl-2'-*O*-TBDMS-3'-*O*-DMT-guanosine-5'-cyanoethyl-*N,N*-diisopropyl-phosphoramidite (28d). Yield is %. ^{31}P NMR ($CDCl_3$) δ 143.97 and 144.14. ESMS 987.2 [$C_{53}H_{66}N_7O_8PSi$ $(M+Na)^+$ requires 987.45].



Formula 9.

Table 4. Comparative ^{1}H NMR data of structures **27a, 28a** and formula 9.

Structure	H-1'	H-2'	H-3'	H-4'	H-5'ab	H-2	H-8
27a	6.15, d. J=8	5.17, dd. J=8, 4.8	4.35, d. J=4.8	3.29, br.s.	3.47, br.dd. J=11.5, 2 3.04, t. J=12.3	8.14, s.	8.76, s.
Formula 9	5.82, d. J=7	5.00, t. J=5.5	4.37-4.35, m.	4.27, dd. J=7, 3.4	3.54, dd. J=10.7, 3 3.40, dd. J=10.7, 3.8	8.21, s.	8.71, s.
28a	6.53, d., J=7 and 6,24, d., J=4.5					8.54, s. and 8.48, s.	8.87, s. and 8.79, s.

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EXAMPLE 5

Synthesis of **2'-*O*-TBDMS-3'-*O*-DMT-uridine-5'-succinyl-CPG.**
2'-*O*-TBDMS-3'-*O*-DMT-Uridine-5'-succinate: To the solution of 2.0 g (3.03 mmol) of the compound **27k** and 0.11 g (0.91 mmol) of DMAP in 20 mL of pyridine were added 0.9 g (9.1 mmol) of succinic anhydride with stirring at 37 °C. After 12 hrs the reaction mixture was diluted with 30 mL of chloroform and washed with 50 mL of brine. The organic layer was separated and dried over anhydrous Na_2SO_4. Flash chromatography with 5:3:2:0.01:0.05 chloroform/hexanes/acetone/pyridine/methanol provided 1.8 g (75.9 %) of 2'-*O*-TBDMS-3'-*O*-DMT-uridine-5'-succinate. ^{1}H NMR ($CDCl_3/D_2O$) δ 0.05 (s, 3H), 0.07 (s, 3H), 0.96 (s, 9H), 2.21 (dt, 1H, J=17 Hz, J=6 Hz), 2.40-2.46 (m, 1H), 2.51 (dt, 1H, J=17 Hz, J=6 Hz), 2.60-2.66 (m, 1H), 3.42 (t, 1H, J=3.3 Hz), 3.77 (s, 6H), 3.95-3.98 (m, 2 H), 4.11 (br.d, 1H, J=12.8 Hz), 4.16 (m, 1H), 5.72 (d, 1H, J=2.8 Hz), 5.74 (d, 1H, J= 8.1 Hz), 6.79 (dd, 4H, J=9 Hz, J=1.9 Hz), 7.21 (t, 1H, J=7 Hz), 7.25 (t, 2H, J=7 Hz), 7.38 (d, 4H, J=8.9 Hz), 7.48 (d, 2H, J=7.2 Hz), 7.61 (d, 1H, J=8.2 Hz), 7.72 (t, 1H, J=5.9 Hz), 8.61 (br.d, 1H, J=4.4 Hz). ESMS 784.2 [$C_{40}H_{48}N_2O_{11}Si$ $(M+Na)^+$ requires 783.9].

2'-*O*-TBDMS-3'-*O*-DMT-uridine-5'-succinyl-CPG: To the suspension of 18 g of amino-lcca-CPG and 3.6 mL of triethylamine in 60 mL of DMF was added the solution of 1.8 g (2.3 mmol) of *O*-TBDMS-3'-*O*-DMT-uridine-5'-succinate, 0.408 g (3.55 mmol) *N*-hydroxysuccinimide and 0.586 g (2.76 mmol) of DCC in 4 mL of DMF. The reaction mixture was warmed to 37 °C. After 16 hrs CPG was filtered, washed with 3x20 mL portions of acetonitrile, capped with acetic anhydride in pyridine/*N*-methylimidazole mixture and washed 3x20 mL portions of acetonitrile. The solid support was dried under diminished pressure and nucleoside loading was measured by DMT removal procedure yielding 18 g of final product with 44.2 μmol/g loading.

EXAMPLE 6

Oligonucleotide Synthesis: The following oligonucleotides (Table 5) were synthesized using 3' → 5' directed standard phosphoramidite chemistry in 1 mmole scale. The standard dT and cholesterol corresponding solid supports suitable for oligonucleotide synthesis have been used.

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Table 5. Oligonucleotide sequences synthesized by conventional method.

SEQ ID No. 1	rCrArGrGrUrGrCrArGrArGrCrCrUrUrGrCrCrCTT
SEQ ID No. 2	rCrArGrGrUrGrCrArGrArGrCrCrUrUrGrCrCrCTT-Cholesterol

The following oligonucleotides (Table6) were synthesized using 5' → 3' directed reverse phosphoramidite chemistry in 1 mmole scale. The same synthesis cycle and ancillary reagents as in standard process have been used for reverse synthesis. The reverse rC-lcaa-CPG was used in all oligonucleotide syntheses. The 3'-modifications of the oligonucleotides SEQ ID No. 2-4 have been introduced by using cholesterol, PEG-2000 or HEG phosphoramidites respectively.

Table 6. Oligonucleotide sequences synthesized by reverse method.

SEQ ID No. 1	rCrArGrGrUrGrCrArGrArGrCrCrUrUrGrCrCrCTT
SEQ ID No. 2	rCrArGrGrUrGrCrArGrArGrCrCrUrUrGrCrCrCTT-Cholesterol
SEQ ID No. 3	rCrArGrGrUrGrCrArGrArGrCrCrUrUrGrCrCrCTT-PEG/2000
SEQ ID No. 4	rCrArGrGrUrGrCrArGrArGrCrCrUrUrGrCrCrCTT-HEG

Crude oligonucleotides were analyzed by CE and ESI mass-spectrometry.
A vast number of applications are possible for easy attachment at 3'- End of an oligonucleotide. Some of the examples are outlined in figure 7:
1. For attachment of bulky molecules at the 3'- end of the RNA, such as cholesterol, long chain aliphatic chains such as C-18, triethylene glycols, hexaethylene glycols. Direct coupling with these amidites can be achieved easily.
2. Attachment of Polyethylene Glycols such as PEG 2000 amidie and PEG 4000 amidites at the 3'- end of the RNA molecule.
3. For Easy attachment of 3'- thiol modification. 3'-Disulfides from readily available amidites, viz., C-3 disulfide, C-6 disulfide.
4. 3'- Biotin attachment *via* biotin amidite in a single step and avoiding biotin CPG for this purpose.

6. Modification of 3'- End of the Sense Strand of SiRNA. The modification of the overhang of the sense strand (3'- End) of SiRNA is not expected to affect targeted mRNA recognition , as the antisense SiRNA strand guides target recognition. Useful modification for improvement of delivery of SiRNA can be easily designed.

We claim:

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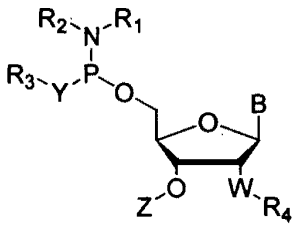
CLAIMS

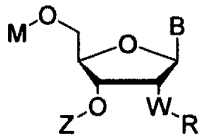
1. Derivatized nucleoside and phosphoramidites of general formula 1.



Formula 1.

wherein
Y is oxygen or sulfur;
W is oxygen, nitrogen, sulfur or fluorine;
R_4 is silyl ether such as TBDMS, triisopropylsilyl oxymethylene, Fmoc, alkyl, aryl, or acetyl;
Z is DMT, MMT, TMT protecting group;
R_1 and R_2 are independently selected from an alkyl or aryl group;
R_3 is cyanoethyl, alkyl or aryl.

2. Derivatized nucleoside attached to solid support of general formula 2.

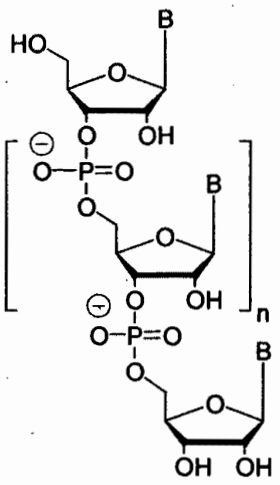


Formula 2.

wherein
M is a proton or not proton and it comprises of formula Y-CO-;
Y is a chain of atoms from 2 to 20 in length, consisting essentially of a hydrocarbon chain optionally substituted by one or more heteroatoms independently selected from the group consisting of oxygen, nitrogen and sulfur; connected to CPG, polystyrene or any other solid support suitable for oligonucleotide synthesis;
W is oxygen, nitrogen, sulfur or fluorine;
R is silyl ether such as TBDMS, triisopropylsilyl oxymethylene, Fmoc, alkyl, aryl, amino or acetyl;
Z is DMT, MMT, TMT protecting group;

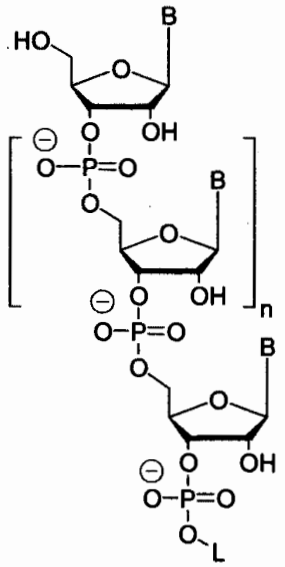
3. A method for reverse, *via* 5' to 3' direction of oligonucleotide bond formations shown in formula 10 in synthetic RNA oligomers. The RNA could consist of

natural of modified nucleo bases, gapmers, phosphodiesters, phosphorothiates, phosphoselenate. The synthesis could be performed on automated, semi automated DNA/RNA or other synthesizers or manually. The synthesis can be performed at various scales from microgram to kilogram scales.



Formula 10.

4. A method of attachment of modifications to 3'-terminus of RNA molecules using corresponding phosphoramidites (Formula 11), wherein L is modification such as biotin, cholesterol, selected from the group consisting of fluorophore, quencher dyes, polyethylene glycols, peptides.



Formula 11.

5. Synthesis of automated high purity RNA using Reverse Direction (5'→3') RNA synthesis resulting in high purity RNA.
6. 3'- Conjugation of RNA with macromolecules such as Cholesterol, HEG (hexaethyloxyglycols and PEG (Polyethylene glycols).
7. Application of the automated RNA synthesis in the reverse Direction (5'→3'), resulting in absence of M+1 oligonucleotide impurities.
8. The modified nucleosides incorporated by this method could consists of one or more of purine or pyrimidine modifications such as but not limited to , 5-Fluoro-U, 5-Fluoro dU, 5-fluoro-dC, 5-Fluro-rC, pseudouridine, 5-methyl-dU, 5-methyl-rU, 5-methyl-dC, 5-methyl-rC, 5-bromo-dU, 5-bromo-rU, 5-bromo-dC, 5-bromo-rC, 5-iodo-dU, 5-iodo-rU, 5-vinyl-dU, 5-vinyl-rU, 5-vinyl thymidine, N-3 methyldeoxy uridine, N-3 methyl-ribouridine, N-3 methyl thymidine, 4-thio uridine, 4-thio-2'-deoxyuridine, 2,6-diaminopurine deoxy riboside, N-3 methyl ribothymidine, 2, 6-diaminopurine riboside, 8-bromo 2'-deoxy adenosine, 8-bromo-r-adenosine, 8-oxo-deoxy adenosine, 8-oxo-riboadenosine, 8-oxo-2'-deoxy- adenosine, 8-oxo-riboadenosine, 8-oxo-deoxy inosine, 8-oxo-ribo inosine, 8-bromo-deoxy inosine, 8-bromo-ribo-inosine, N-1 methyl-riboadenosine, N-1 methyl-2'- deoxy adenosine, N-1 methyl 2'-deoxy inosine, N-1 methyl riboadenosine, N-1 methyldeoxy guanosine, N-1-methyl- riboguanosine, etheno adenosine, etheno 2'- deoxy adenosine, purine 2'- deoxy riboside, purine-ribonucleoside, 2-aminopurine-2'-deoxyriboside, 2-aminopurine-ribonucleoside,
9. Labelling of internal positions of an RNA synthesized by this methods with chromophores such as , but not limited to Fluoroscein-C-5 dT, Dabcyl- C-5 thymidine, internal carboxyl group 5-dU-methylacrylate, biotin dT (biotin wattached via spacer to C-5 of dU), amino-dT (terminal amino attached via C-6 spacer to C-5 dU).
10. The sugar modification of modified nucleosides could consist of 2'- deoxy-2'-fluoro ribo nucleosides (2'-F-ANAs) such as A, C, G, U , Inosine and modified nucleosides containing 2'- Fluoro, in one or more positions of an RNA or DNA sequence synthesized by this method.
11. The sugar modification of modified nucleosides could consist of 2'- deoxy-2'-methoxy ribo nucleosides (2'-OMe-) such as A, C, G, U , Inosine and modified nucleosides containing 2'- methoxy, in one or more positions of an RNA or DNA sequence synthesized by this method.
12. The sugar modification of modified nucleosides could consist of 2'- deoxy-2'-amino ribo nucleosides (2'-NH2) such as A, C, G, U , Inosine and modified nucleosides containing 2'- amino, in one or more positions of an RNA or DNA sequence synthesized by this method.
13. The sugar modification of modified nucleosides could consist of 2'- deoxy-2'-terminal amino ribo nucleosides (2'-terminal NH2), attached via spacer from 2-10 atoms on nucleosides such as A, C, G, U , Inosine and modified nucleosides containing 2'- terminal amino, in one or more positions of an RNA or DNA sequence synthesized by this method.
14. The sugar modification of modified nucleosides could consist of 2'- deoxy-2'-methoxy ethoxy ribo nucleosides (2'-MOE), such as A, C, G, U , Inosine and

modified nucleosides containing 2'- MOE, in one or more positions of an RNA or DNA sequence synthesized by this method.

15. The sugar modification of modified nucleosides could consist of other 2'- O-alkyl groups, such as 2'- deoxy-2'-ethoxy, propargyl, butyne ribo nucleosides (2'-OEt, O-Propargyl, 2'-O-Butyne), such as A, C, G, U , Inosine and modified nucleosides containing 2'- 2'-OEt, O-Propargyl, 2'-O-Butyne, in one or more positions of an RNA or DNA sequence synthesized by this method.
16. The sugar modification of modified nucleosides could consist of 2'- deoxy-2'-fluoro arabino nucleosides (2'-F-ANAs) such as A, C, G, U , Inosine and modified nucleosides containing 2'-F-ANAs), in one or more positions of an RNA or DNA sequence synthesized by this method.
17. The sugar modification of modified nucleosides could consist of 2'- deoxy-2'-fluoro 4'-thioarabino nucleosides (4'-S-FANAs) such as A, C, G, U, Inosine and modified nucleosides containing 4'-S-FANAs in one or more positions of an RNA or DNA sequence synthesized by this method.
18. The RNA comprising one or more 2'-5'- linkage within the sequence , at the 3'- end of the sequence or at the 5'- end of the sequence.
19. The RNA having a 3'- end, synthesized by the method of this invention containing reverse attached deoxy nucleosides such as dT, dC, dG , thymidine, attached via their 3'- hydroxyl function.
20. The RNA having a 3'- end synthesized by the method of this invention containing reverse attached ribonucleosides such as rA, rC, rG, rU, attached via their 2' or 3'- hydroxyl function.
21. The reverse RNA synthesis comprising 2'- triisopropylsilyloxy methyl (TOM), protecting group.
22. The reverse RNA synthesis comprising 2'- t- butyldithiomethyl (DTM) protecting group.
23. The reverse RNA synthesis comprising the modified base comprising 2'-deoxy-2'- fluoro- beta-D_arabinonucleic acid (FANA).
24. The reverse RNA synthesis comprising the modified base comprising 4'-thio-2'- deoxy-2'- fluoro- beta-D_arabinonucleic acid (4'-Thio- FANA).
25. The reverse RNA synthesis comprising the modified sugar comprising 2'-OMethyl modification.
26. The reverse RNA synthesis comprising the modified sugar comprising Bicyclic locked nucleic acids (LNA's).
27. The reverse RNA synthesis comprising the modified sugar comprising altritol sugar modified oligonucleotides (ANA).
28. The reverse RNA synthesis comprising the step of conjugation of lipophilic or hydrophobic groups at the 3'- end of the RNA either through a amidite function on the hydrophobic moiety or through an amino linker at the 3'- end of reverese synthesized oligonucleotide having a terminal amino group. The later synthesis involving a coupling step between amino at the 3'- terminal of oligonucleotide and carboxylic function on the lipophilic moiety. The lipophilic moieties consist of various glycol, such as triethylene glycol, hexaethylene glycol, polyethylene glycols, various lipids.

29. The reverse RNA synthesis comprising the step of conjugation of peptides, such as cell penetrating peptides (CPPs) or membrane permeant peptide (MPPs) utilizing either the free amine function of such peptides and a 3'-terminal carboxylic function on the reverse synthesized RNA. The CPPs and MPPs having an appropriate carboxyl function can be coupled to the free terminal amino function of a 3'- end of the reverse synthesized RNA.
30. The reverse RNA synthesis comprising the 2'-5'- linked DNA units or 2'-5'-RNA units within the sequence , at the 3'- end of the sequence or at the 5'- end of the sequence.

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ABSTRACT

The present invention relates to novel phosphoramidites, A-n-bz, C-n-bz, C-n-ac, G-n-ac and U that are produced with an HPLC purity of greater than 98% and ^{31}P NMR purity greater than 99%. A novel process of reverse 5' → 3' directed synthesis of RNA oligomers has been developed and disclosed. Using that method it demonstrates high quality RNA synthesis with coupling efficiency approaching 99%.

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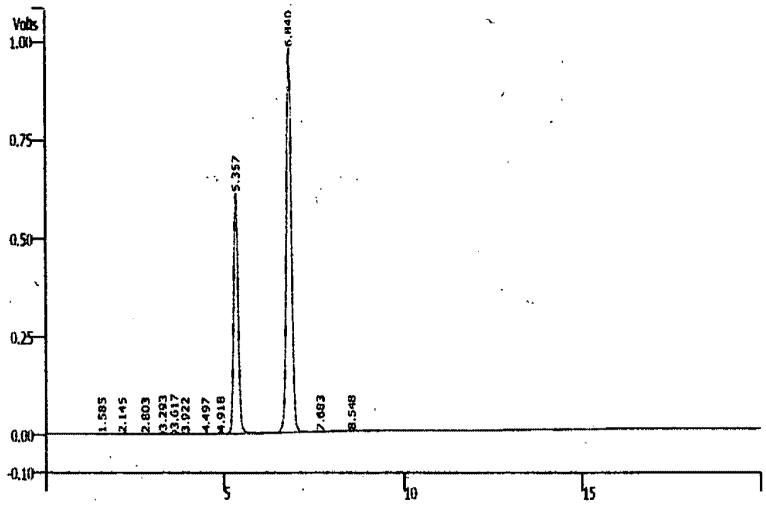


Figure 1. HPLC Chromatogram of the compound **10a**.

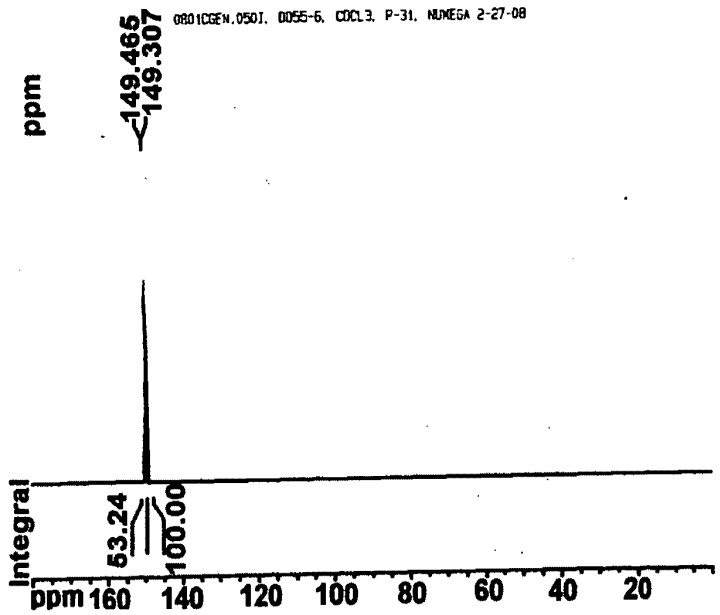


Figure 2. ^{31}P NMR Spectrum of the compound **10a**.

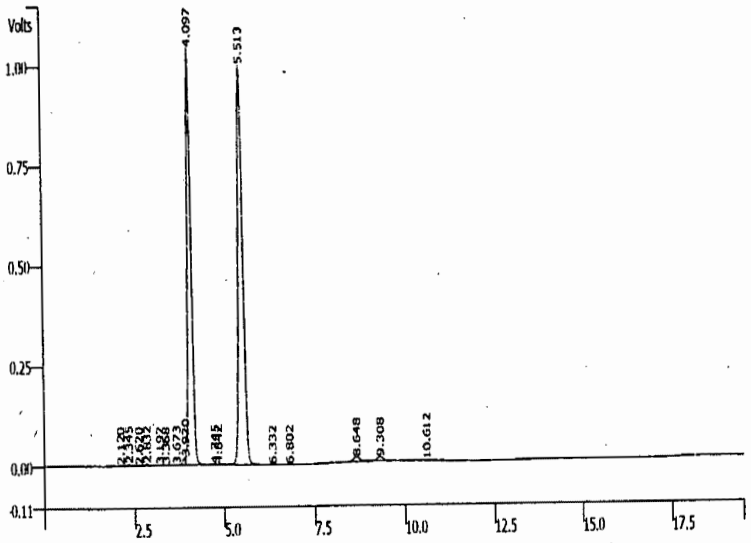


Figure 3. HPLC Chromatogram of the compound **10c**.

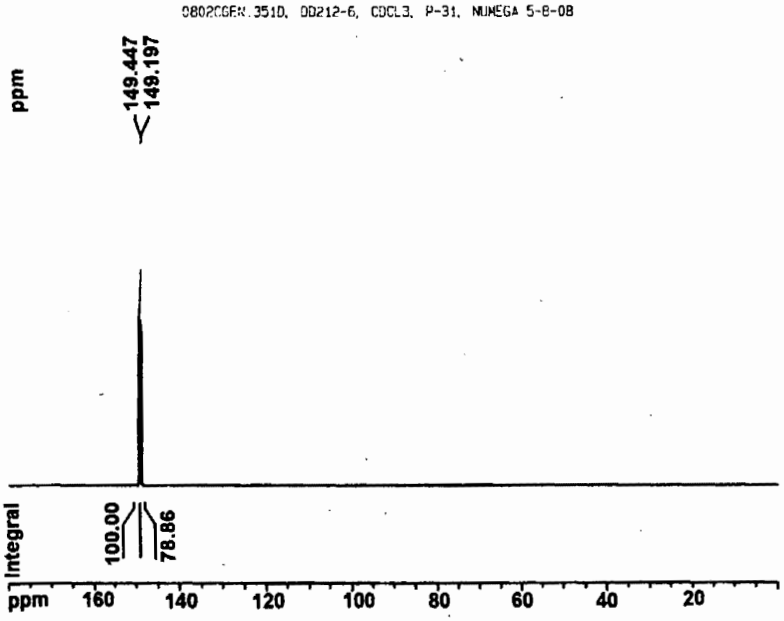


Figure 4. ^{31}P NMR Spectrum of the compound **10c**.

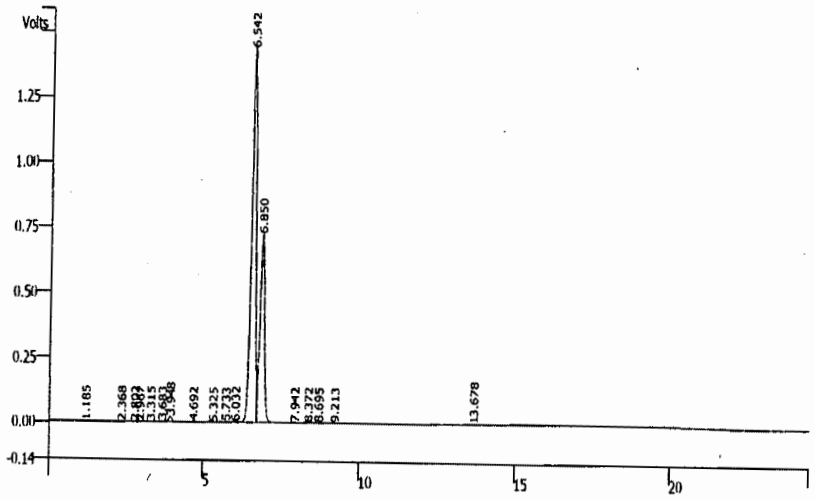


Figure 5. HPLC Chromatogram of the compound **10d**.

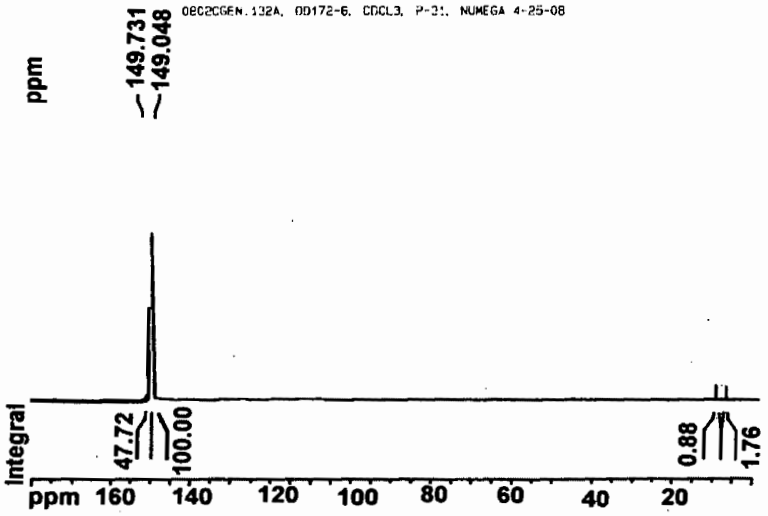


Figure 6. ^{31}P NMR Spectrum of the compound **10d**.

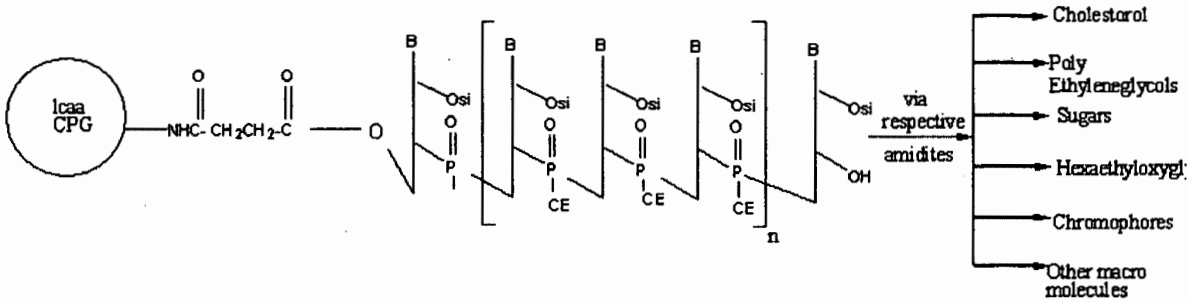


Figure 7. Diagrammaic Representation of 5'→3' RNA synthesis and application to 3'- modifications.

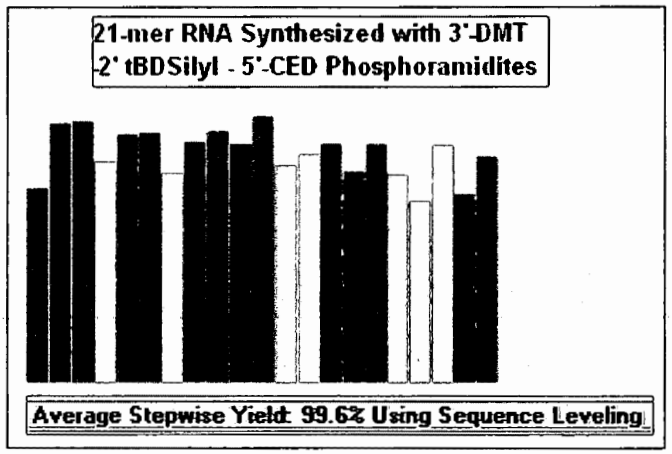


Figure 8. Coupling Efficiency Trityl Histogram Notes (21 mer RNA synthesis):

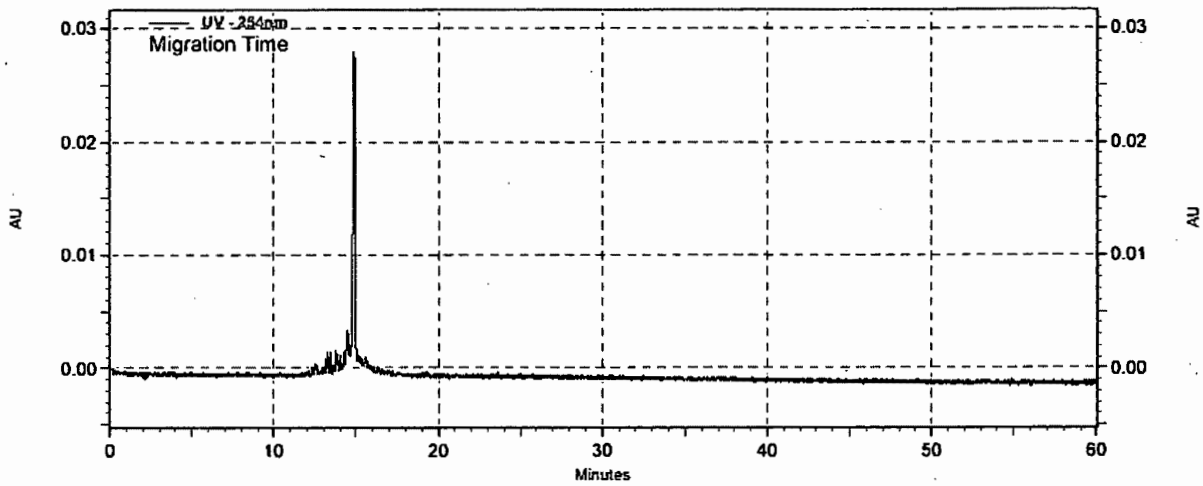


Figure 9.

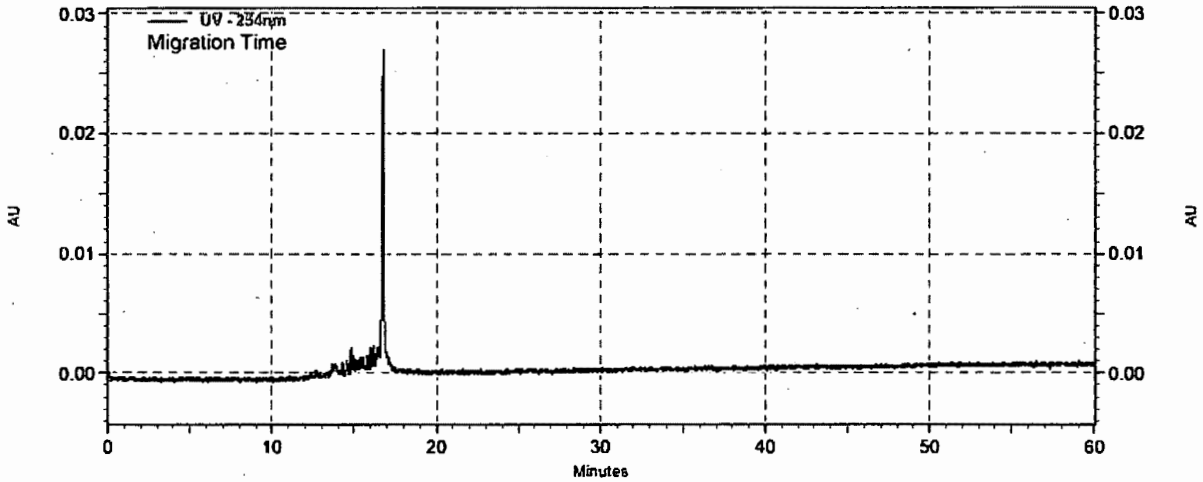


Figure 10.

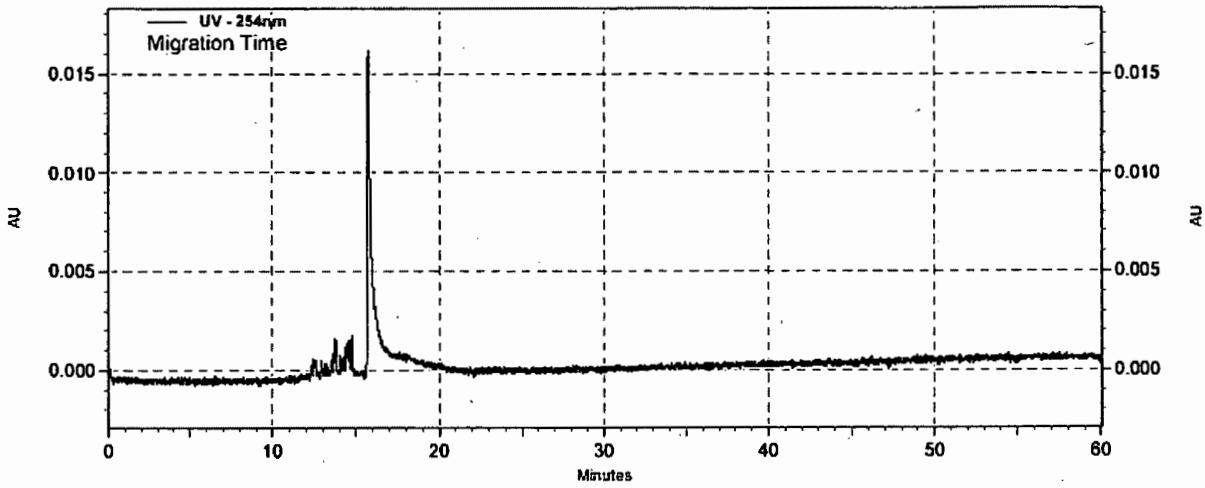


Figure 11.

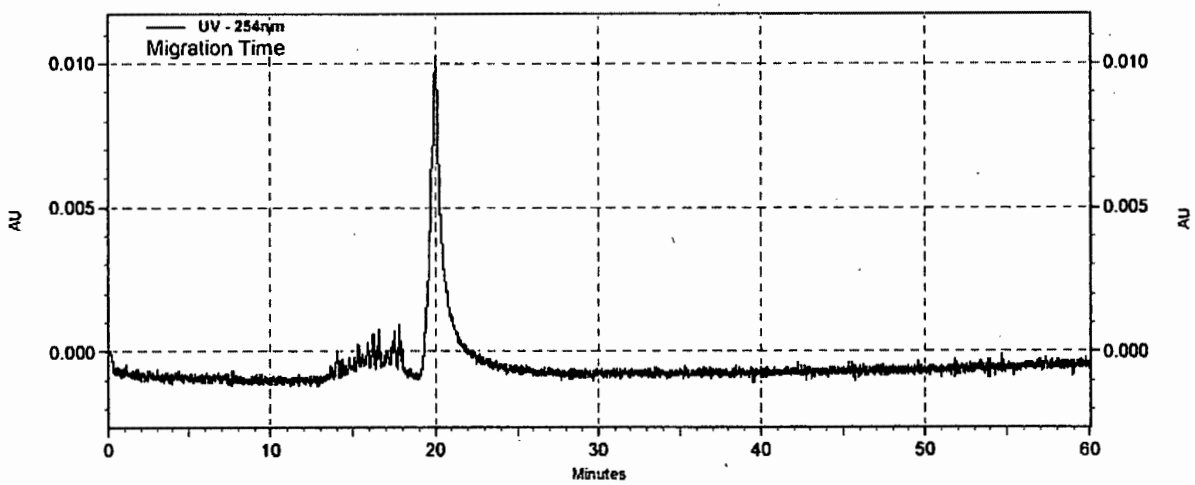
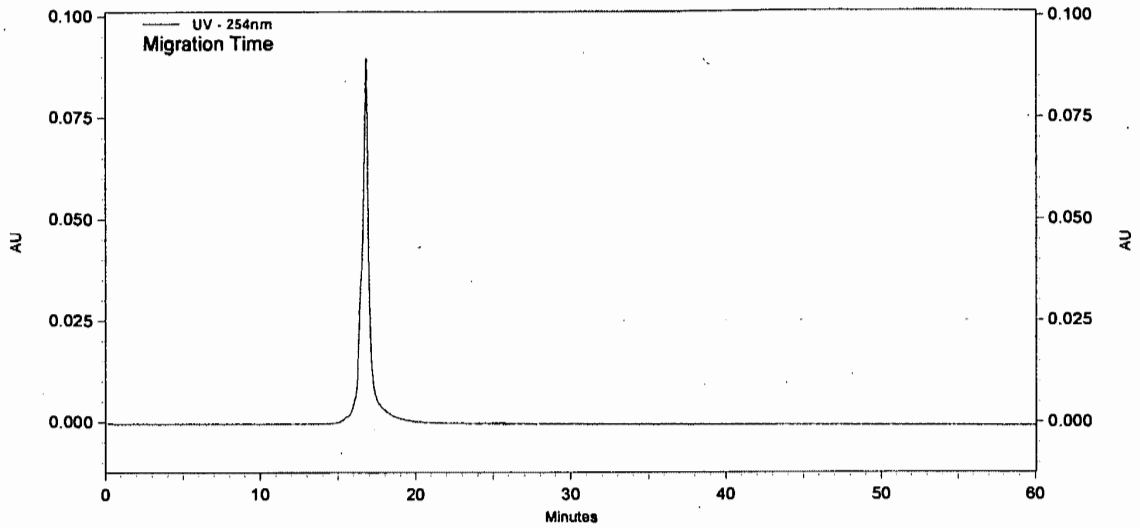


Figure 12.



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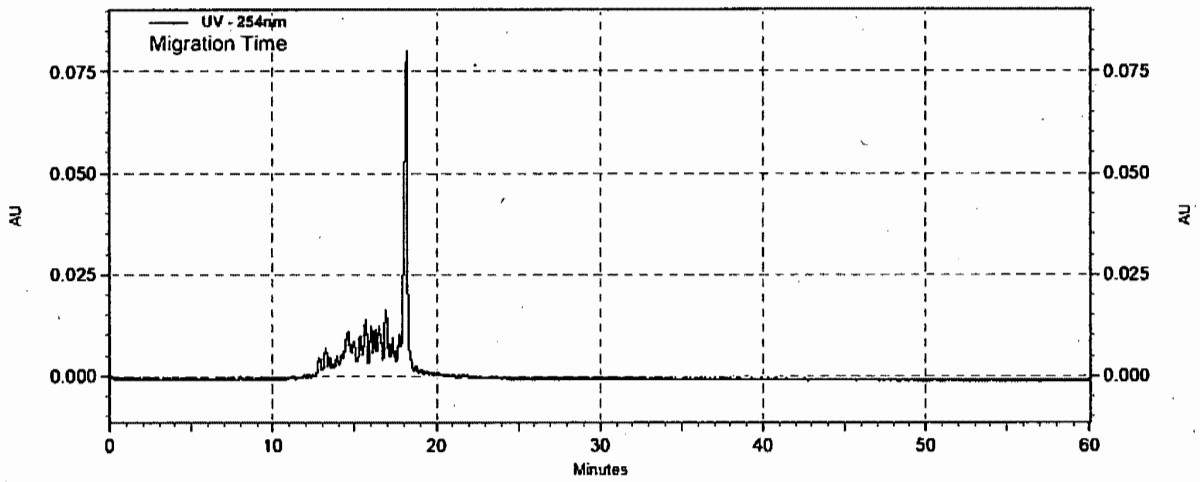

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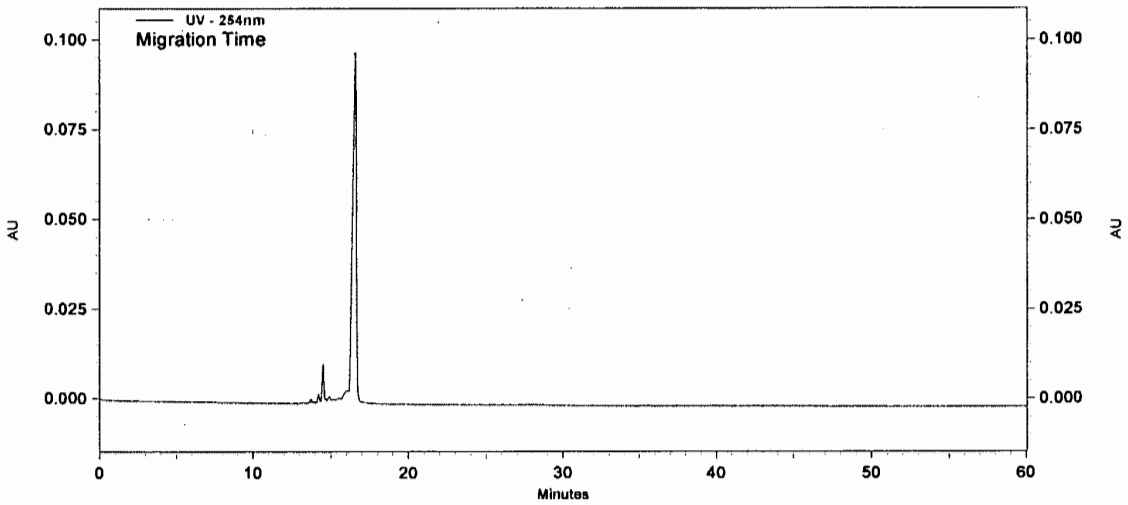

Figure 15.

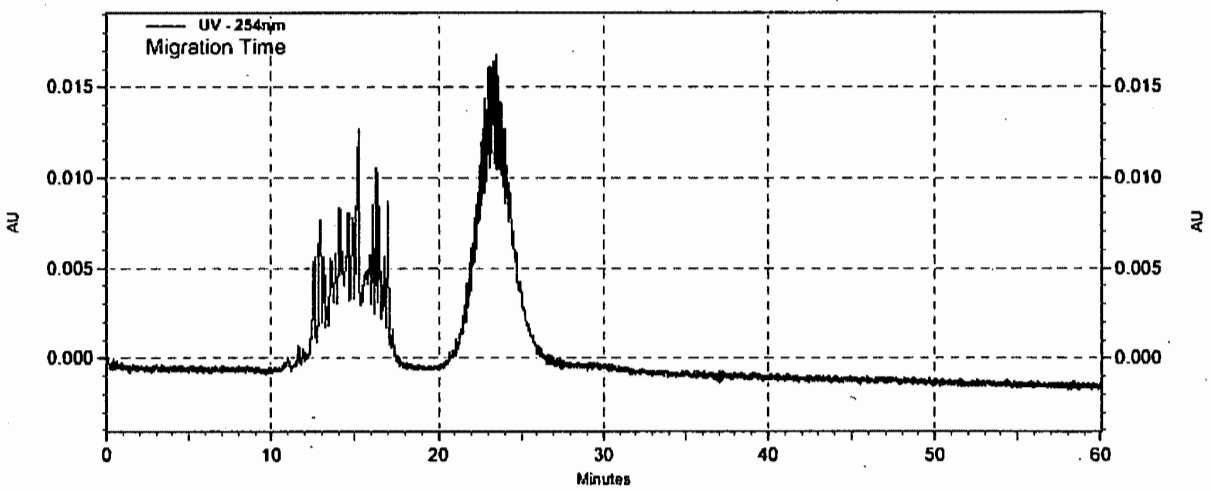

Figure 16.

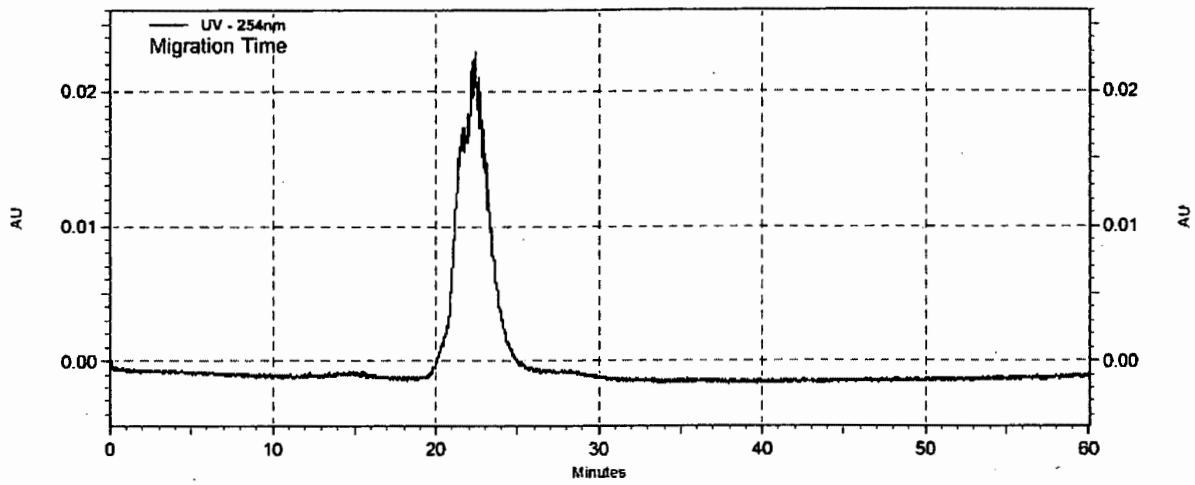

Figure 17.

Figure 18.

PATENT APPLICATION SERIAL NO._____

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FEE RECORD SHEET

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