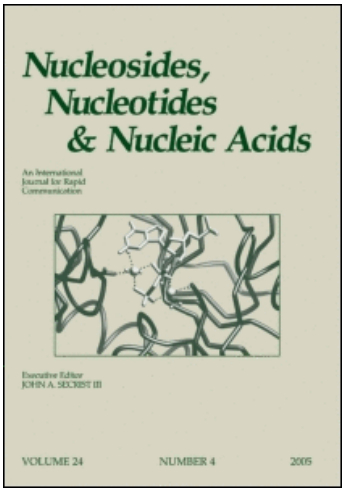

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

A Novel Thymidine Phosphoramidite Synthon for Incorporation of Internucleoside Phosphate Linkers During Automated Oligodeoxynucleotide Synthesis

David Tabatadze[a]; Paul Zamecnik[a]; Ivan Yanachkov[b]; George Wright[b]; Katherine Pierson[a]; Surong Zhang[c]; Alexei Bogdanov Jr.[c]; Valeri Metelev[cd]
[a] Cancer Center, Massachusetts General Hospital, Charlestown, Massachusettes, USA [b] GL Synthesis, Inc., Worcester, Massachusetts, USA [c] Department of Radiology, University of Massachusetts Medical School, Worcester, Massachusetts, USA [d] Department of Chemistry, Moscow State University, Moscow, Russian Federation

To cite this Article Tabatadze, David , Zamecnik, Paul , Yanachkov, Ivan , Wright, George , Pierson, Katherine , Zhang, Surong , Bogdanov Jr., Alexei and Metelev, Valeri(2008) 'A Novel Thymidine Phosphoramidite Synthon for Incorporation of Internucleoside Phosphate Linkers During Automated Oligodeoxynucleotide Synthesis', Nucleosides, Nucleotides and Nucleic Acids, 27: 2, 157 — 172
To link to this Article: DOI: 10.1080/15257770701795938
URL: http://dx.doi.org/10.1080/15257770701795938

Nucleosides, Nucleotides, and Nucleic Acids, 27:157–172, 2008

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770701795938

Taylor & Francis
Taylor & Francis Group

A NOVEL THYMIDINE PHOSPHORAMIDITE SYNTHON FOR INCORPORATION OF INTERNUCLEOSIDE PHOSPHATE LINKERS DURING AUTOMATED OLIGODEOXYNUCLEOTIDE SYNTHESIS

David Tabatadze,[1] Paul Zamecnik,[1] Ivan Yanachkov,[2] George Wright,[2] Katherine Pierson,[1] Surong Zhang,[3] Alexei Bogdanov, Jr.,[3] and Valeri Metelev[3,4]

[1]*Cancer Center, Massachusetts General Hospital, Charlestown, Massachusettes, USA*
[2]*GL Synthesis, Inc., Worcester, Massachusetts, USA*
[3]*Department of Radiology, University of Massachusetts Medical School, Worcester, Massachusetts, USA*
[4]*Department of Chemistry, Moscow State University, Moscow, Russian Federation*

□ *A novel thymidine phosphoramidite synthon was synthesized and successfully used for incorporation of primary amino groups, attached through a triethylene glycol linker to the internucleoside phosphates, at desired locations during automated oligodeoxynucleotide synthesis. The synthesized amino-linker bearing oligonucleotides are stable under deprotection conditions and exhibit Watson-Crick base-pairing properties. Covalent labeling of oligonucleotides with carbocyanine near-infrared fluorochromes resulted in 2.5 times higher labeling yields when compared with oligonucleotides containing base-attached aminolinkers. We anticipate that the developed synthetic approach will be useful for nucleotide sequence-specific attachment of single or multiple ligands or reporter molecules.*

Keywords Thymidine phosphoramidite synthon; sequence-specific attachment; single ligand; multiple ligands; reporter molecules

INTRODUCTION

The use of synthetic phosphodiester oligonucleotides as therapeutic agents faces two major challenges: 1) rapid degradation of the compounds

Received 9 April 2007; accepted 2 August 2007.

This work was supported in part by grants from the G. Harold and Leila V. Mathers Foundation and National Institutes of Health Grant 5 R01 AI060872–02. The work of S. Z., V. M., and A. B. was supported in part by National Institutes of Health Exploratory Grant R21 CA116144. The authors thank Mrs. Karen Pierson for technical assistance.

Address correspondence to Paul Zamecnik, Cancer Center, Massachusetts General Hospital, 149 Thirteenth St, Charlestown, MA 02129. E-mail: paul_zamecnik@hms.harvard.edu

in vivo and 2) low uptake and inefficient transport through plasma membranes. To overcome these problems, various approaches have been tested in recent decades. One successful strategy has been synthesis of chemically modified oligonucleotides, particularly chemical modification of the backbones of oligonucleotides, insofar as this approach provides preservation of Watson-Crick hybridization properties of compounds. Synthetic oligonucleotides with phosphorothioate,[1] methylphosphonate,[2] boranophosphate,[3] benzylphosphonate,[4] modifications, locked nucleic acid,[5,6] peptide nucleic acid,[7] morpholino derivatives,[8–9] and oligonucleotides with modified terminal groups[10–13] have been widely tested as novel therapeutic agents[14–16] and diagnostic tools.[17]

For some applications, native or modified synthetic oligonucleotides have to be linked to nonnucleotide molecules or surfaces. The nonnucleotide molecules could be fluorescent dyes or quenchers, metal chelators, ligands for various proteins, enzymes, receptors, transporters, or other biologically active molecules, hydrophobic residues, or even other oligonucleotides. The resultant conjugates may be useful as hybridization probes in DNA sequencing and microarray technology, as diagnostic and therapeutic agents, electron and fluorescent microscopy probes, and have roles in crystallography, affinity chromatography, and in cell biology research.[18–22] Because oligonucleotides, with the exception of phosphorothioates, lack appropriately reactive functional groups for conjugate synthesis, introduction of such groups is necessary. One of the most convenient and widely used groups for conjugation purposes is the aliphatic amino group. It can be coupled selectively and under mild conditions with ligands (reporters) bearing carboxylic groups or their activated derivatives, sulfonyl chlorides, isocyanates and isothiocyanates, aldehydes and alkylating residues, or other electrophilic functionalities. The amino group is usually attached to the oligonucleotide by a linker (spacer). The nature and the length of the linkers are important for the synthesis and the function of the conjugates. Short linkers can create spatial restrictions, which lower the reactivity of the amino group and interfere with the function of the ligand and the oligonucleotide. Hydrophobic linkers, even when of sufficient length, tend to collapse in aqueous environments, assuming globular conformations and again creating spatial crowding. The site of the spacer attachment is another important factor determining the properties of the conjugates. Traditionally, the spacer is attached at the 5′-end of the oligonucleotide upon the completion of the automated synthesis.[23–26] The attachment to the 3′-end on nonstandard supports also has been explored.[27] The drawbacks of both methods are that they do not allow position-specific modifications. If multiple reporters are to be attached, they cluster at the end of the oligonucleotide with no convenient strategy to vary their relative positions. When the reporter has to be placed within a certain sequence, or when

multiple reporters are to be placed within a desired distance, the intrastrand modification is necessary. The standard approach is to attach the linker to C-5 of the pyrimidine bases or C-8 of the purines. Deoxyribonucleoside phosphoramidites of this type, with trifluoroacetyl-protected amino groups, are commercially available. It is obvious that the design of the spacer and the place of its attachment in those synthons are driven more by consideration of synthetic convenience than by functionality. In the case of purines, an aliphatic spacer is attached to C-8 through an amino group, and in the case of pyrimidines, a carbamoylvinyl group introduced at C-5 is in conjugation with the base. Both alternatives could potentially result in interfering with normal base pairing. The attachment of the linker to the internucleoside phosphates enables sequence-specific and multi-site labeling, minimally perturbing the Watson-Crick base pairing and, importantly, providing the stabilization of the oligonucleotides toward nucleases.[28,29] Two major types of internucleoside modifications have been described: 1) using a spacer linked to the sulphur of phosphorothioates: 2) phosphoramidate nitrogen-linked spacers.[24] The first type of linker is prepared by alkylation of phosphorothioates, and the second by oxidation of H-phosphonates in the presence of 1,ω-diamines. The drawbacks of these strategies include incompatibility with the commonly used phosphoramidite-based automated oligonucleotide synthesis, and the possibility that they may require additional post-synthetic modifications.

The attachment of a linker to the oxygen of the internucleoside phosphates has been explored by using a sequence-specific attachment of 2-aminoethyl groups.[30] However, the short two-carbon atom spacer is not optimal for further attachment of reporters or ligands to the amino group. Increasing the length of the spacer results in its destabilization, because of the intramolecular attack at the terminal amino group of carbon atom adjacent to the phosphate through a favorable five or six atom cyclic transition state, resulting in the spacer's scission. In fact, the 4-trifluoroacetamidobutyl group has been developed as an alternative to the cyanoethyl group for phosphate protection in oligonucleotide synthesis.[31] Therefore, we hypothesized that by increasing the length of the spacer to 9 atoms an unfavorable 10 atom cyclic transition state would be created.[32] We decided to test ω-aminoalkyl derivatives that are sufficiently stable under alkaline deblocking conditions for position- specific attachment of ligands or reporter groups, to the internucleoside phosphates.

In this paper we report the synthesis of a thymidine phosphoramidite monomer, which was used for incorporating aliphatic primary amino groups through a triethylene glycol spacer to the internucleoside phosphate at desired locations. Various fluorescent or quenching reporter groups were covalently attached to the amino groups of the synthesized oligonucleotides in high yields.

MATERIALS AND METHODS

2-(2-(2-Aminoethoxy)ethoxy)ethanol, 3. A mixture of 2-(2-(2-chloroethoxy)ethoxy)ethanol, **1** (50.0 g, 0.297 mol) and sodium azide (19.28 g, 0.297 mol) in 450 ml N,N-dimethylformamide was stirred overnight at 90°C. The mixture was cooled, diluted with 500 ml tetrahydrofuran, and, after stirring for 1 hour, it was filtered. The solid salts were washed with tetrahydrofuran. The combined filtrate and washings, which contain the azide **2**, were stirred and treated with triphenylphosphine (83.76 g, 0.319 mol), added in two portions, and stirred continuously for 48 hours while protected from contact with air. Water (8.1 ml) was added, and stirring was continued for another 48 hours. The mixture was evaporated on a membrane pump vacuum, and then on an oil pump vacuum, at a bath temperature of 45°C. The residue was stirred with 850 ml of water and filtered. The solid was washed with water, and the combined filtrate and washings were concentrated on a membrane pump vacuum at a bath temperature of 70°C. The resulting yellowish oil was distilled under vacuum (0.2 mm Hg) to give **3** as a colorless oil: 39.3 g, 88% yield. ^{1}H NMR ($CDCl_3$): δ 3.54–3.72 (m, 8H, C*H*$_2$-O), 3.51 (t, 2H, C*H*$_2$-OH), 2.82 (t, 2H, C*H*$_2$-NH_2), 2.30 (bs, 3H, NH_2+OH); ^{13}C NMR (Me_2SO-d_6): δ 73.82, 73.24, 70.63, 70.49, 61.06, 42.14; ESI+ MS: $[M+H]^+$ 150.3, calculated for $C_5H_{16}NO_3$ 150.2.

Trifluoro-*N*-(2-(2-(2-hydroxyethoxy)ethoxy)ethyl)acetamide, 4. Methyl trifluoroacetate (3.20 g, 2.52 ml, 25 mmol) was added drop wise through a septum with stirring and gentle cooling to **3** (2.98 g, 20 mmol), and the mixture was sealed and left at room temperature for 24 hours. The volatiles were evaporated under membrane pump vacuum and bath temperature of 65°C to give **4** as a slightly yellowish oil which crystallized when stored in the refrigerator: 4.83 g, 98% yield. ^{1}H NMR (Me_2SO-d_6): δ 9.41 (t, 1H, NH), 4.57 (bs, 1H, OH), 3.55–3.28 (m, 12H, CH_2); ^{13}C NMR (Me_2SO-d_6): δ 156.60 (q, J 36.1 Hz, CO), 115.96 (q, J 116 Hz, CF_3), 73.15, 70.50, 70.46, 68.67, 61.08, 39.99.

5′-*O*-DMTr-thymidine-3′-*O*-(2-(2-(2-trifluoroacetamidoethoxy)ethoxy)ethyl)-*N*,*N*-diisopropylphosphoramidite, 6. Bis-(diisopropylamino)chlorophosphine (1.334 g, 5 mmol) was weighed in an argon-filled glove bag and loaded in a flame-dried, then cooled under argon vial equipped with a stirring bar. The vial was sealed with a Teflon-lined septum, and 20 ml of dry benzene were added through the septum under argon, followed by dry diisopropylamine (0.607 g, 0.848 ml, 6 mmol). The clear solution was cooled to 10°C, and a solution of **4** (1.23 g, 5 mmol) in 2.6 ml of dry benzene was added drop wise with vigorous stirring and cooling during ca. 3 minutes. The mixture was stirred under argon for 1 hour at room temperature. At that time a ^{31}P NMR spectrum of the reaction mixture showed complete consumption of bis-(diisopropylamino)chlorophosphine.

The reaction mixture was filtered under positive pressure of argon through a glass fiber filter, and the solid was washed with dry benzene. The combined filtrate and washings were evaporated under membrane vacuum to yield **5** as oil. The oil was dissolved in 10 ml of dry N,N-dimethylformamide, and the solution was added through a septum to 5′-*O*-DMTr-thymidine (3.00 g, 5.5 mmol) in an argon-filled, flame dried flask, followed by 3 ml of a 0.45 M tetrazole solution in dry acetonitrile. This mixture was stirred for 1 hour under argon at room temperature. Triethylamine (0.5 ml) was added, and the reaction mixture was evaporated under vacuum. The resulting oil was dissolved in 50 ml dichloromethane and loaded onto a silica gel column (30×5 cm), equilibrated with hexane:ethyl acetate:triethylamine, 66:33:2, and eluted with a gradient of hexane:ethyl acetate:triethylamine, 66:33:2, to ethyl acetate:triethylamine, 100:2 (4 l), at a flow rate of 50 ml/min. The fractions containing **6** were pooled and evaporated to give 2.40 g of solid. This material was 95% pure by HPLC and contained 1.7 mol% of bis-(DMTr-T)-N,N-diisopropylphosphoroamidite. Because this impurity can cause branching during oligonucleotide synthesis, the product was repurified on a silica gel column which was eluted first with an isocratic mixture of hexane:ethyl acetate:triethylamine, 59:39:2, (2 l), and then with a gradient of hexane:ethyl acetate:triethylamine, 59:39:2, -hexane:ethyl acetate:triethylamine, 39:59:2, (4 l). The fractions containing pure **6** were pooled and evaporated under vacuum. The residue was dissolved in dry benzene and lyophilized to give 1.84 g (40% yield) of **6** as a colorless powder. HPLC: two diastereomers in ratio 2:1; purity, 96% and no bis-(DMTdT)-phosphoramidite contamination. ^{1}H NMR (C_6D_6): δ 10.3 (bs, 1H, NH-1), 7.8–7.5 (m, 4H, H-6+Ar-H+NH), 7.48–7.37 (m, 4H, Ar-H), 7.25–7.15 (m, 2H, Ar-H), 7.13–7.04 (m, 1H, Ar-H), 6.83–6.75 (m, 4H, Ar-H), 6.65–6.53 (m, 1H, H-1′), 4.86–4-76 (m, 1H, H-4′), 4.33 and 4.18 (2 m in ratio 2:1, 1H, H-3′), 3.83–3.20 (m, 16H, CH_2O, C*H*$_2$NH, H-5′5″, NC*H*Me_2), 3.37 (s, 6H, CH_3O), 2.64–2.26 (m, 2H, H-2′2″), 1.56 (s, 3H, 5-Me), 1.18–1.02 (12H, CH*Me*$_2$); ^{13}C NMR* (C_6D_6): δ 164.82^{b}, 164.75^{a}; 159.73^{a}, 159.71^{b}; 157.64^{b} (q; $^{2}J_{C\text{-}F}$ = 36.1 Hz), 157.60^{a} (q; $^{2}J_{C\text{-}F}$ = 36.1 Hz); 151.52^{b}, 151.48^{a}; 145.65^{a}, 145.55^{b}; 136.37; 136.32^{b}, 136.00^{a}; 131.01; 129.07; 128.92; 128.63; 127.72; 117.13^{b} (q; $^{1}J_{C\text{-}F}$ = 288.0 Hz), 117.18^{a} (q; $^{1}J_{C\text{-}F}$ = 288.0 Hz); 114.04; 111.57; 110.22; 87.67; 86.33^{a} (d; $^{3}J_{C\text{-}P}$ = 4.0 Hz), 86.01^{b} (d; $^{3}J_{C\text{-}P}$ = 6.3 Hz); 85.72; 74.00^{a} (d; $^{2}J_{C\text{-}P}$ = 15.5 Hz), 73.89^{b} (d; $^{2}J_{C\text{-}P}$ = 17.2 Hz); 71.86^{b} (d; $^{3}J_{C\text{-}P}$ = 6.9 Hz), 71.79^{a} (d; $^{3}J_{C\text{-}P}$ = 7.4 Hz); 71.11^{b}, 71.05^{a}; 70.76^{b}, 70.71^{a}; 64.08^{a}, 63.87^{b}; 63.46^{b} (d; $^{2}J_{C\text{-}P}$ = 16.1 Hz), 63.33^{a} (d; $^{2}J_{C\text{-}P}$ = 16.6 Hz); 55.25; 43.76 ($^{2}J_{C\text{-}P}$ = 12.6 Hz); 40.90^{b} (d; $^{3}J_{C\text{-}P}$ = 3.4

*Most of the ^{13}C resonances are split due to the presence of two diastereomers in ratio 2:1. In those cases the signal associated with the more abundant diastereomer is indicated with the superscript "a," and the signal ue to the less abundant diastereomer—with the superscript "b."

Hz), 40.60[a] (d; $^{3}J_{C\text{-}P}$ = 4.6 Hz); 40.35[b], 40.27[a]; 25.06; 24.97; 12.25; ^{31}P NMR (^{1}H dec., C_6D_6): δ 149.69, 149.51 (s, in ratio 1:2); ^{19}F NMR (^{1}H dec., C_6D_6): δ −75.86, −75.92 (s, in ratio 1:2); ESI+ MS: $[M+Et_3NH]^+$ 1020.23, calculated for $C_{51}H_{74}F_3N_5O_{11}P$ 1020.51; ESI-MS: $[M\text{-}H]^-$ 917.50, calculated for $C_{45}H_{57}F_3N_4O_{11}P$ 917.37.

Synthesis of oligonucleotides was performed using phenoxyacetyl protected dA, 4-isopropyl-phenoxyacetyl protected dG, acetyl protected dC and dT phosphoramidites (all from Glen Research, Sterling VA) on a 394 DNA/RNA synthesizer (Applied Biosystems) by using a standard protocol for phosphoramidite synthesis. Synthesis was performed on 1 μM dT-CPG columns (Glen Research). Coupling duration for the incorporation of the novel dT phosphoramidite monomer **6** at the targeted location of the oligonucleotide sequence was increased up to 3 minutes. A 50 mM solution of monomer in anhydrous acetonitrile was used. Coupling efficiency according to the DMTr monitor reading was 99%. Deprotection of oligonucleotides was carried out for 5 hours at room temperature using concentrated ammonium hydroxide. Four oligonucleotides containing either one (**I-III**) or two (**IV**) internucleoside aminolinkers were synthesized (Table 1).

INITIAL PURIFICATION OF OLIGONUCLEOTIDES

The ion-pair reverse-phase HPLC method was used for the isolation and purification of modified oligonucleotides on a C18 column (Microsorb-MV 100-5, 250 × 4.6 mm, Varian, USA). For the linear eluting gradient the following buffers were used: A, 2% acetonitrile in 0.1 M triethylammonium acetate (TEAA), pH 7 (Glen Research); B, 50% acetonitrile in 0.1 M TEAA, pH 7. The column was eluted at 1 ml/min at room temperature. To isolate trityl-on oligonucleotides the following gradient was used: 90% A-10% B for 3 minutes, then to 100% B in 22 minutes. Detritylation was performed by treating with 80% acetic acid for 30 minutes followed by ethanol/sodium acetate precipitation. The purity of obtained oligonucleotides was determined by reverse phase HPLC using a gradient of 90% A-10% B for 3 minutes, then to 50% A-50% B in 47 minutes.

Conjugation of Oligonucleotides Bearing Aminolinkers with Dabcyl and QSY21 NHS Esters

15 nmol of oligonucleotide bearing an aminolinker were dissolved in 40 μl of 0.1 M $NaHCO_3$. Three 10 μl portions of a saturated solution of dabcyl NHS ester (AnaSpec, USA) in N,N-dimethylformamide were added to the oligonucleotide solution at 20-minute intervals, and the inhomogeneous mixture was left overnight at room temperature in the dark. Aqueous 2 M $LiClO_4$ (0.1 ml) and 0.7 ml of acetone were added, the

TABLE 1 Synthesized oligonucleotides bearing one or two amino reporter groups at various positions and the corresponding dye-conjugated products

No.	Oligonucleotides[a]	Molecular mass[b]	
		Calculated	Observed
I	CGGAAAGT*CCCTCATAGCT	5904.0	5904.2
II	TGGAAAGCTTTT*TACAGTT	5964.1	5964.4
III	TGGAAAGCTTTCTAT*AGTT	5964.1	5965.2
IV	TGGAAAGCTTTT*TAT*AGTT	6110.3	6111.6
V	CGGAAAG^TCCCTCATAGCT	NA	
VI	CGGAAAGT$^{Cy3.5}$CCCTCATAGCT	NA	
VII	CGGAAAGT^{QSY21}CCCTCATAGCT	NA	
VIII	CGGAAAGT^{800CW}CCCTCATAGCT	6888.9	6888.0
IX	CGGAAAGT^{Cy7}CCCTCATAGCT	NA	
X_1	CGGAAAGT^{dabcyl}CCCTCATAGCT (fraction 1)	6155.8	6155.6
X_2	CGGAAAGT^{dabcyl}CCCTCATAGCT (fraction 2)	6155.8	6155.4
XI	TGGAAAGCTTTT^{800CW}TACAGTT	6949.0	6950.0
XII	TGGAAAGCTTTT^{dabcyl}TACAGTT	6215.3	6216.0
XIII	TGGAAAGCTTTT^{Cy7}TACAGTT	NA	
XIV	TGGAAAGCTTTCTAT^{dabcyl}AGTT	6215.3	6216.4
XV	TGGAAAGCTTTCTAT^{800CW}AGTT	6949.0	6950.0
XVI	TGGAAAGCTTTT^{dabcyl}TAT^{dabcyl}AGTT	6612.8	6613.2
XVII	CGGAAAG^{800CW}TCCCTCATAGCT	6870.9	6870.8
XVIII	AGCTATGAG^{800CW}GGACTTTCCG	6942.9	6942.0

[a]T* is T-O-P (=O)-$OCH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$; $T^{Cy3.5}$ is T-O-P(=O)-$OCH_2CH_2OCH_2CH_2OCH_2CH_2$NH-Cy3.5; T^{QSY21} is T-O-P(=O)-$OCH_2CH_2OCH_2CH_2OCH_2CH_2$NH-QSY21; T^{800CW} T-O-P(=O)-$OCH_2CH_2OCH_2CH_2OCH_2CH_2$NH-800CW; T^{dabcyl}- is T-O-P(=O)-$OCH_2CH_2OCH_2CH_2OCH_2CH_2$NH-dabcyl; T^{Cy7} T-O-P(=O)-$OCH_2CH_2OCH_2CH_2OCH_2CH_2$NH-Cy7; G^ is -NH-$(CH_2)_6$-$NH_2$ at the 8 position of guanine; and G^{800CW} is –NH-$(CH_2)_6$-NH-800CW at the 8 position of guanine.

[b]By MS.

NA, not analyzed.

mixture was vortexed, and precipitates were collected after centrifugation at 10000 rpm for 7 minutes. The oligonucleotides were then re-precipitated as above followed by Micro Bio-Spin 6 column (Bio-Rad Laboratories) purification according to the manufacturer's recommendations. After spin-chromatography, oligonucleotides were purified by reverse phase HPLC using the conditions described above. Purified oligonucleotides **X**, **XII**, **XIV** and **XVI** (Table 1) were concentrated in a centrifugal vacuum concentrator (SpeedVac, Savant) to 50–100 μl, and were precipitated by standard ethanol/sodium acetate treatment.

Conjugation of Oligonucleotides Bearing an Aminolinker with Cy3.5, Cy7, or 800CW NHS Esters

10 nmol of aminolinker-bearing oligonucleotide were dissolved in 30 μl of 0.1 M $NaHCO_3$. Two 7.5 μl portions of dye solutions (0.5 mg in 30 μl dimethylsulfoxide; Cy3.5, Cy7 NHS esters were from Amersham-GE

Healthcare (USA), and heptamethine cyanine IRDye 800CW NHS ester was from Li-COR (USA) were added over a 1 hour interval, and the homogeneous reaction mixture was left overnight at room temperature in the dark. The oligonucleotides were precipitated in a mixture of 0.1 ml water, 16 μl 3M sodium acetate (pH 5.5), and 0.7 ml ethanol. The mixture was left at −80°C for 3 hours, and centrifuged at 10000 rpm for 8.5 minutes. The labeled oligonucleotides **VI**, **VIII**, **IX**, **XI**, **XIII**, **XV**, **XVII**, and **XVIII** (Table 1) were purified by spin-chromatography, then by reverse phase HPLC, and finally by ethanol/sodium acetate precipitation, as described above.

Spectral measurements were performed using Cary 50 Bio UV-visible spectrophotometer. Melting temperatures were determined with a step-wise Peltier temperature ramping bath in 2-degree mode in a temperature-controlled cuvette holder. Electrospray mass spectrometry (MS) was performed with a LTQ Linear Ion trap mass spectrometer (Thermo, Inc) with use of a Nanomate (Advion, Inc.) robot. Spectra were obtained in the negative ion mode with 1.8 kV spray voltage and 0.8 PSI back pressure (UMMS Proteomic and Mass Spectrometry Core Facility, University of Massachusetts Medical School, Worcester, MA, USA). Sample solutions were adjusted to 75% methanol before MS analysis. The full mass spectra were acquired for 30 sec from m/z 400–2000 using 2 ms scans and 10 ms maximum injection time.

RESULTS AND DISCUSSION

Synthesis of Synthon 6

In order to introduce one or more amino groups attached by an appropriate linker to the internucleoside phosphate group in a position specific manner, using a standard phosphoramidite chemistry with no post-synthetic modifications, we designed a phosphoramidite synthon—5′-*O*-DMTr-thymidine-3′-*O*-(2-(2-(2-trifluoroacetamidoethoxy)ethoxy)ethyl)-N,N-diisopropylphosphoramidite, (**6**, see Figure 1). The 9 atom, triethylene glycol linker was expected to have minimal tendency to undergo cyclization by a nucleophilic attack of the terminal amino group on the carbon atom adjacent to the phosphate group. In addition, the polyethylene glycol spacers are hydrophilic in nature, conformationally flexible in both organic and aqueous environments, and are biologically inert.[33,34] The trifluoroacetyl protection of the amino group was chosen due to its stability under the automated oligonucleotide synthesis conditions, and easy removal during the basic deprotection of the oligonucleotide.

The synthesis of amidite **6** is outlined in Figure 1. First, commercially available 2-(2-(2-chloroethoxy)ethoxy)ethanol, **1**, was converted, via the azide **2**, to 2-(2-(2-aminoethoxy)ethoxy)ethanol, **3**, in a one pot synthesis in 88% yield. The amino group of **3** was protected as the trifluoroacetamide by

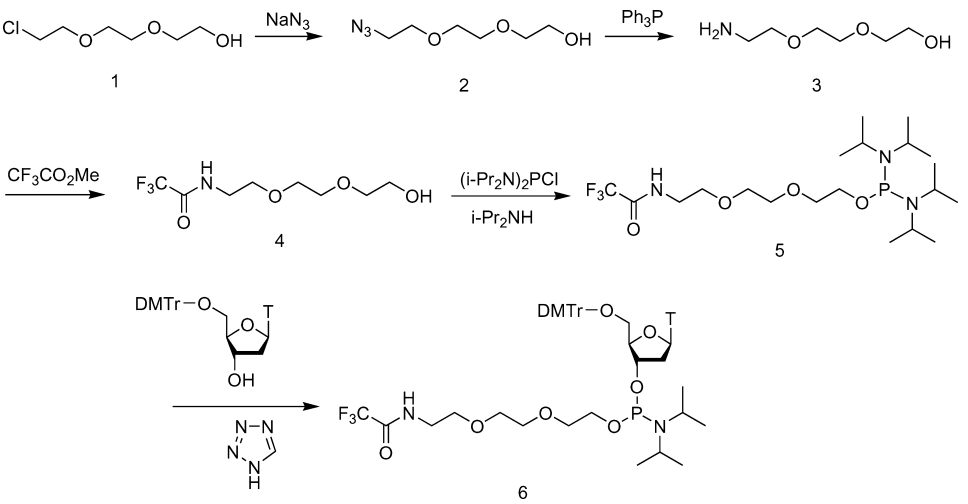

FIGURE 1 A scheme of synthon 6 synthesis.

reaction with excess of methyl trifluoroacetate to give **4** in 98% yield. Next, the diamidite **5** was prepared by reaction of **4** with bis(diisopropylamino)-chlorophosphine and diisopropylamine, and, without isolation, was converted to the targeted amidite **6** by reaction with a slight excess of 5′-*O*-DMTr-dT in the presence of tetrazole. Synthon **6** was isolated in 52% yield (from **4**) and 95% purity by silica gel chromatography. This material was repurified to remove a small amount (1.7 mol%) of $(DMTr\text{-}T)_2PN(i\text{-}Pr)_2$, which could cause branching during oligonucleotide synthesis, to give, after lyophilization from benzene, **6** in 40% final yield.

Stability and Coupling Efficiency of Synthon 6

After storing synthon **6** for six months at −20°C, no apparent decrease in coupling efficiency was detected. Usually the compound was dissolved in anhydrous acetonitrile under argon and was kept on the synthesizer for two weeks at room temperature. The coupling yield of the monomer for the 3 minutes coupling duration was 98–99% after 7–8 days, and for the same coupling conditions coupling yield decreased to 90% after additional 10–12 days. Coupling yields were assessed using a DMTr detector reading on the synthesizer. Based on this finding we conclude that dry monomer stored at −20°C preserves its intactness over six months and is stable in dry acetonitrile for at least 1 week.

OLIGONUCLEOTIDE SYNTHESIS

Initially we attempted to synthesize an oligonucleotide by combining standard phosphoramidite monomers (benzoyl protected dA and dC, and

iso-butyryl protected dG) with our novel thymidine phosphoramidite synthon, **6**. The average coupling yield for **6** was 96–97% under standard conditions. When coupling time was increased up to 3 minutes, the coupling efficiency increased to 99%. The HPLC analysis after standard deprotection showed that major portion of the final product was cleaved into several small fragments (data not shown). Since phosphotriesters are less stable in basic environment than phosphodiesters[35,36] we decided to use "ultramild" phosphoramidite monomers (phenoxyacetyl protected dA, 4-isopropyl-phenoxyacetyl protected dG and acetyl protected dC from Glen Research) instead of standard ones. The use of "ultramild" monomers enabled deprotection under milder conditions (5 hours of ammonium hydroxide treatment at room temperature instead of 14–16 hours at 55°C), which resulted in a great improvement of the yield of the final product. We demonstrated the wide applicability of internucleotide amino linkers by synthesizing four 19-mer oligonucleotides containing one or two amino linker groups at different positions (see Table 1). Also, for comparison purposes, few 19-mer oligonucleotides (as example **V**) were synthesized using a commercial G-phosphoramidite monomer with an amino group attached through an aminohexyl linker to C-8 of the base. Ion-paired reverse phase HPLC on a C18 column was used for the post-deprotection purification of the modified oligonucleotides. Their purity and identity were determined by analytical reverse phase HPLC and ESI mass spectrometry (see Table 1).

Watson-Crick base paring properties of the modified oligonucleotides were evaluated by comparison of melting temperatures of a duplex formed between the amino-functionalized oligonucleotide CGG AAA GT*C CCT CAT AGC T (**I**) and its complementary unmodified oligonucleotide 3′ GCC TTT CAG GGA GTA TCG A 5′ (duplex 1) and a control, unmodified duplex with the same sequence (duplex 2). The melting temperatures were almost equal, i.e. 65 and 66°C for duplexes 1 and 2, respectively. Therefore, the presence of aminolinker phosphotriester group in the middle of the oligonucleotide sequence did not appear to interfere with Watson-Crick hybridization properties of the oligonucleotides.

Conjugation of the Amino-Modified Oligonucleotides with Fluorescent Dyes

Amino-functionalized oligonucleotides were conjugated with **three** different red and far-red fluorochromes (Cy3.5, Cy7, and 800CW) as well as with two quenchers (dabcyl and QSY21). The conjugates were purified by ion-pair reverse phase HPLC, and characterized by HPLC analysis, mass spectrometry and UV/VIS spectroscopy (for details, see Materials and Methods and Table 1). The results of mass spectrometric analysis of the products correlated well with their calculated molecular masses

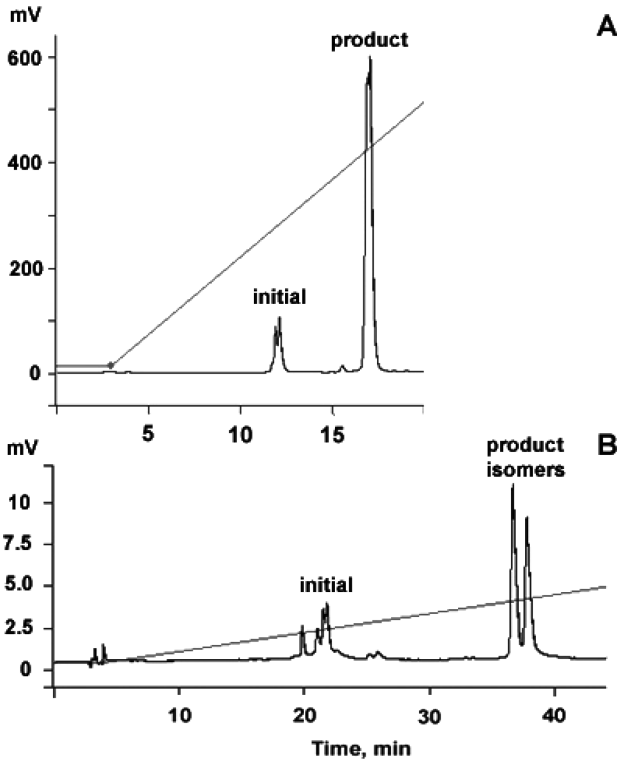

FIGURE 2 Reverse phase HPLC analysis of reaction of amino-linker modified oligonucleotide **I** with dabcyl NHS ester leading to CGGAAAGT^{dabcyl}CCCTCATAGCT (**X**). Panel A, steep gradient. Panel B, shallow gradient. In the case of the shallow gradient the product is separated into two fractions, $\mathbf{X_1}$ and $\mathbf{X_2}$, due to the presence of two diasteriomers, identical by mass spectrometry (Table 1).

(Table 1). HPLC analysis showed that covalent attachment of dyes to the aminolinker phosphotriester groups afforded high yields of target products (80–90%). For example, Figures 2A and 2B show HPLC profiles of CGGAAAGT^{dabcyl}CCCTCATAGCT (**X**) under conditions of steep and shallow gradients of acetonitrile, respectively. In the case of the shallow gradient the product **X** showed separation into two peaks, probably due to two different diastereomeric forms of the product as a result of creation of additional chiral center at the triester phosphorus atom. Those two peaks ($\mathbf{X_1}$ and $\mathbf{X_2}$) were collected separately, and their mass spectra were compared. The mass-to-charge values for both isomers were equal and in good agreement with the calculated masses of expected product (Table 1).

High yields of dye conjugation to the oligonucleotides at the internucleotide phosphotriester were observed for all compounds synthesized. The major factors explaining the high yields presumably are the steric accessibility and hydrophilic nature of the amino linker moiety. Covalent attachment of a dye to an aminolinker group at the internucleotide triester

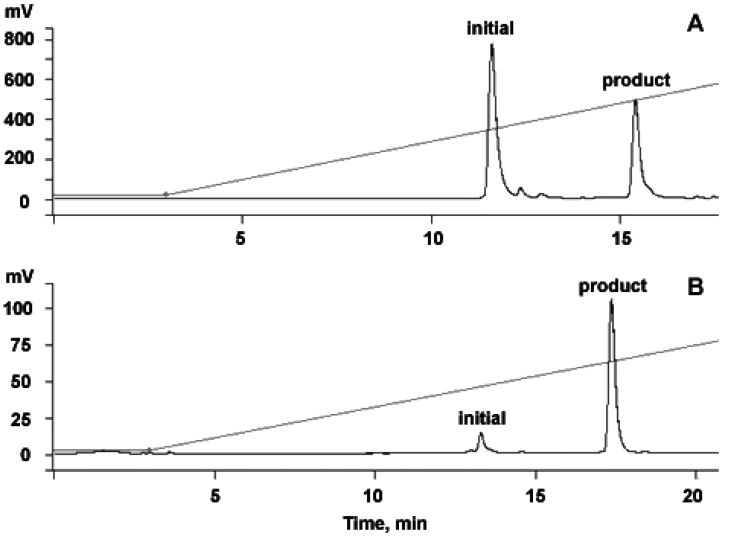

FIGURE 3 Reverse phase HPLC analysis of reaction mixtures of coupling of oligonucleotides with heptamethine cyanine IRDye 800CW. Panel A, CGGAAAGˆTCCCTCATAGCT (**V**). Panel B, CGGAAAGT*CCCTCATAGCT (**I**).

provided significantly higher yield than the attachment via an aminolinker of the heterocyclic base. Figure 3 shows the HPLC analysis of the reaction mixtures resulting from labeling of CGGAAAGˆTCCCTCATAGCT (**V**) where the amino group is attached via hexylamino linker to C-8 of G, and CGGAAAGT*CCCTCATAGCT (**I**) where the amino group is attached to the phosphotriester, with IRDye 800CW NHS ester. Both reactions were performed at the same time and under identical conditions. Yield of **VIII**

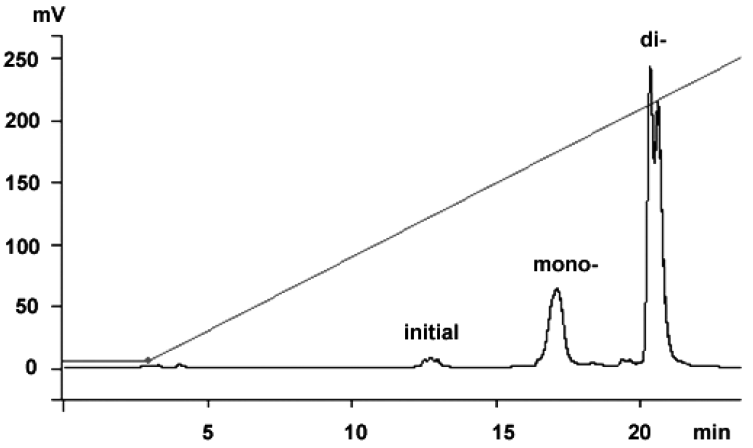

FIGURE 4 Reverse phase HPLC analysis of the reaction mixture of coupling of TGGAAAGCTTTT*TAT*AGTT (**IV**) with dabcyl NHS ester. The presence of residual **IV**, the mono-adduct and the target product (TGGAAAGCTTTT^{dabcyl}TAT^{dabcyl}AGTT, **XVI**) are shown.

where dye is attached to the aminolinker at the phosphotriester (panel A, ~90%) was approximately 2.5 times higher than the yield of **XVII** where linking was performed via the aminolinker on the nucleotide base (panel B, ~35%). The same trend was observed in other experiments with NHS ester of heptamethine cyanine IRDye 800CW dye (the yield of **XVIII** was about 35%, however, thecompounds **XI** and **XV** were obtained with the 85% yield) as well as with dabcyl NHS ester and the corresponding oligonucleotides (data not shown).

A di-amino-functionalized oligonucleotide was labeled successfully to give the doubly labeled oligonucleotide in 50% yield. Figure 4 shows

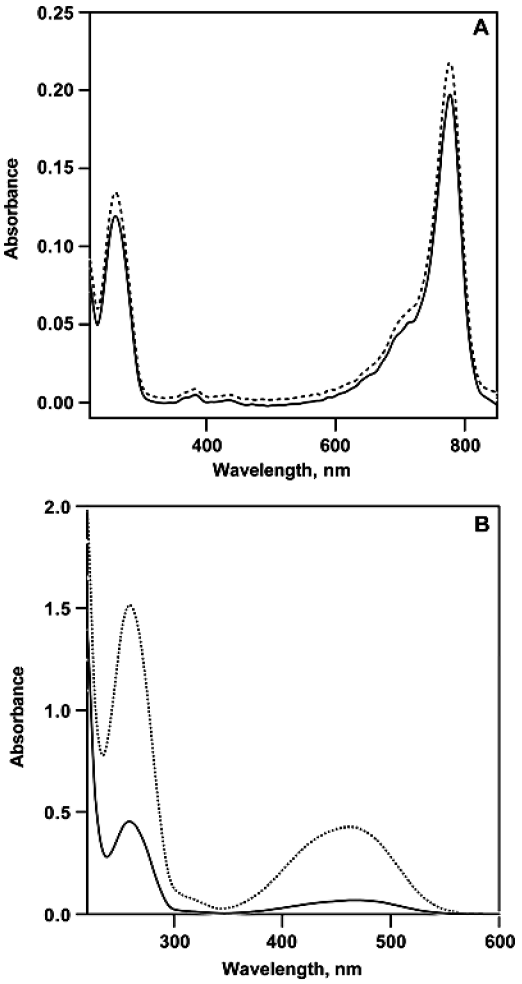

FIGURE 5 UV/VIS spectra of dye-labeled oligonucleotides. Panel A, CGGAAAGT^{800CW}CCCTCATAGCT (**VIII**, dotted line) and TGGAAAGCTTTT^{800CW}TACAGTT (**XI**, solid line). Panel B, TGGAAAGCTTTCTAT^{dabcyl}AGTT (**XIV**, solid line) and TGGAAAGCTTTT^{dabcyl}TAT^{dabcyl}AGTT (**XVI**, dotted line).

HPLC analysis of the reaction mixture of TGGAAAGCTTTT*TAT*AGTT (**IV**) and dabcyl NHS ester (for coupling conditions see Materials and Methods). The chromatogram shows three major peaks that correspond to the starting material, the mono-adduct, and the target product TGGAAAGCTTTT^{dabcyl}TAT^{dabcyl}AGTT (**XVI**), respectively. Further evidence for labeling of oligonucleotides was found by comparison of UV/VIS spectra of a mono and di-labelled oligonucleotides. In Figure 5A are shown the UV/VIS spectra of CGGAAAGT^{800CW}CCCTCATAGCT (**VIII**) and TGGAAAGCTTTT^{800CW}TACAGTT (**XI**), in which the absorbance maximum at 780 nm indicates the presence of covalently attached 800CW dye. In Figure 5B are the UV/VIS spectra of dabcyl-labelled TGGAAAGCTTTCTAT^{dabcyl}AGTT (**XIV**) and TGGAAAGCTTTT^{dabcyl}TAT^{dabcyl}AGTT (**XVI**), both of which show absorbance maxima at 470 nm, indicating the presence of dabcyl groups. As expected, the double-labeled oligonucleotide **XVI** has a twice higher 470/260 nm absorbance ratio than oligonucleotide **XIV** (Figure 5B).

We performed a limited stability study of one of the novel oligonucleotide conjugates, **VIII**, which contained 800CW dye at the eighth nucleoside from the 5′-end (see Table 1). When dissolved in deionized water and stored at −20°C for approximately four months, HPLC analysis showed no signs of degradation (Figure 6).

In conclusion, we have synthesized and successfully used a novel phosphoramidite synthon that enables facile incorporation of primary amino groups attached via a triethylene glycol linker to the internucleoside phosphates during the automatic oligonucleotide synthesis. The novel aminolinker bearing oligonucleotides have preserved Watson-Crick base-pairing properties, are stable under deprotection and conjugation conditions, and are superior in their labeling yields to oligonucleotides containing

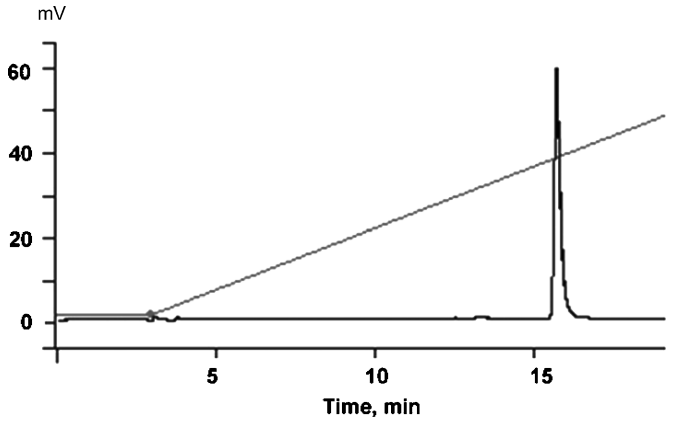

FIGURE 6 Reverse phase HPLC analysis of CGGAAAGT^{800CW}CCCTCATAGCT (**VIII**) dissolved in deionized water and stored at −20°C for approximately 4 months.

base-attached aminolinkers. The novel synthon and ligand-conjugation method can be used for high yield, sequence-specific attachment of single or multiple ligands or reporter molecules to synthetic oligonucleotides.

REFERENCES

1. Padmapriya, A.A.; Tang, J.Y.; Agrawal, S. Large-scale synthesis, purification, and analysis of oligodeoxynucleotide phosphorothioates. *Antisense Res. Dev.* **1994**, 4, 185–199.
2. Reddy, M.P.; Farooqui, F.; Hanna, N.B. Elimination of transamination side product by the use of dC^{Ac} methylphosphonamidite in the synthesis of oligonucleoside methylphosphonates. *Tetrahedron Lett* **1996**, 37, 8691–8694.
3. Shaw, B.R.; Madison, J.; Sood, A.; Spielvogel, B.F. Oligonucleoside boranophosphate (borane phosphonate). *Methods Mol. Biol.* **1993**, 20, 225–243.
4. Samstag, W.; Eisenhardt, S.; Offensperger, W.B.; Engels, J.W. Synthesis and properties of new antisense oligodeoxynucleotides containing benzylphosphonate linkages. *Antisense Nucleic Acids Drug Dev.* **1996**, 6, 153–156.
5. Kurreck, J.; Wyszko, E.; Gillen, C.; Erdmann, V. Design of antisense oligonucleotides stabilized by locked nucleic acids. *Nucleic Acids Res.* **2002**, 30, 1911–1918.
6. Crinelli, R.; Bianchi, M.; Gentilini, L.; Magnani, M. Design and characterization of decoy oligonucleotides containing locked nucleic acids. *Nucleic Acids Res.* **2002**, 30, 2435–2443.
7. Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S.; Driver, D.; Berg, R.; Kim, S.; Norden, B.; Nielsen, P. PNA hybridizes to complementary oligonucleotides obeying the watson-crick hydrogen-bonding rules. *Nature* **1993**, 365, 566–568. (article)
8. Stirchak, E.P.; Summerton, J.E.; Weller, D.D. Uncharged stereoregular nucleic acid analogs: 2. Morpholino nucleoside oligomers with carbamate internucleoside linkages. *Nucleic Acids Res.* **1989**, 17, 6129–6141.
9. Sinha, N.D.; Biernat, J.; McManus, J.; Koster, H. Polymer support oligonucleotide synthesis XVIII: Use of beta-cyanoethyl-n,n-dialkylamino-/n-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. *Nucleic Acids Res.* **1984**, 12, 4539–4557.
10. Shchepinov, M.S.; Udalova, I.A.; Bridgman, A.J.; Southern, E.M. Oligonucleotide dendrimers: Synthesis and use as polylabelled DNA probes. *Nucleic Acids Res.* **1997**, 25, 4447–4454.
11. Shchepinov, M.S.; Mir, K.U.; Elder, J.K.; Frank-Kamenetskii, M.D.; Southern, E.M. Oligonucleotide dendrimers: Stable nanostructure. *Nucleic Acids Res.* **1999**, 27, 3035–3041.
12. Horn, T.; Chang, C.A.; Urdea, M.S. Chemical synthesis and characterization of branched oligodeoxyribonucleotides (bDNA) for use as signal amplifiers in nucleic acid quantification assays. *Nucleic Acids Res.* **1997**, 25, 4842–4849.
13. Guzaev, A.; Salo, H.; Azhayev, A.; Lonnberg, H. New approach for chemical phosphorylation of oligonucleotides at the 5′-terminus. *Tetrahedron* **1995**, 51, 9375–9384.
14. Monteith, D.K.; Levin, A.A. Synthetic Oligonucleotides: The development of antisense therapeutics. *Toxicol. Pathol.* **1999**, 27, 8–13.
15. Ma, D.D.; Rede, T.; Naqvi, N.A.; Cook, P.D. Synthetic oligonucleotides as therapeutics: the coming of age. *Biotechnol. Annu. Rev.* **2000**, 5, 155–196.
16. Wilson, J.A.; Richardson, C.D. Future promise of siRNA and other nucleic acid based therapeutics for the treatment of chronic HCV. *Infect. Disord. Drug Targets* **2006**, 6, 43–56.
17. Landegren, U.; Kaiser, R.; Caskey, C.T.; Hood, L. DNA diagnostics—Molecular techniques and automation. *Science* **1988**, 242, 229–237.
18. Goodchild, J. Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconj. Chem.* **1990**, 1, 165–187.
19. Da Ros, T.; Spalluto, G.; Prato, M. Oligonucleotides and oligonucleotide conjugates: A new approach for cancer treatment. *Current Med. Chem.* **2005**, 12, 71–88.
20. Boutorine, A.S.; Grimm, G.N.; Héléne, C. Methods of attaching unprotected oligonucleotides to DNA-binding, fluorescent, or reactive ligands for synthesis of antisense or gene-directed agents and probes. *Mol. Biol.* **2000**, 34, 804–813.

21. Urban, E.; Noe, R.C. Structural modifications of antisense oligonucleotides. *Il Farmaco* **2003**, 58, 243–258.
22. Silverman, A.P.; Kool, E.T. Detecting RNA and DNA with templated chemical reactions. *Chem. Rev.* **2006**, 106, 3775–3789.
23. Tang, J.Y.; Agrawal, S. Incorporation of multiple reporter groups on synthetic oligonucleotides. *Nucleic Acids Res.* **1990**, 18, 6461.
24. Agrawal, S.; Zamecnik, P.C. Site specific functionalization of oligonucleotides for attaching two different reporter groups. *Nucleic Acids Res.* **1990**, 18, 5419–5423.
25. Agrawal, S.; Christodoulou, Ch.; Gait, M.J. Efficient methods for attaching non-radioactive labels to the 5′ ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res.* **1986**, 14, 6227–6245.
26. Kachalova, A.V.; Stetsenko, D.A.; Romanova, E.A.; Tashlitsky, V.N.; Gait, M.J.; Oretskaya, T.S. A new and efficient method for synthesis of 5-conjugates of oligonucleotides through amide-bond formation on solid phase. *Helv. Chim. Acta* **2002**, 85, 2409–2417.
27. Markiewicz, W.T.; Gröger, G.; Rösch, R.; Zebrowska, A.; Markiewicz, M.; Klotz, M.; Hinz, M.; Godzina, P.; Seliger, H. A new method of synthesis of fluorescently labelled oligonucleotides and their application in DNA sequencing. *Nucl. Acids Res.* **1997**, 25, 3672–3680.
28. Wenninger, D.; Hinz, M.; Hahner, S.; Hillenkamp, F.; Seliger, H. Enzymatic and hybridization properties of oligonucleotide analogues containing novel phosphotriester internucleotide linkage. *Nucleos. Nucleot.* **1998**, 17, 2117–2125.
29. Awad, A.M.; Sobkowski, M.; Seliger, H. Enzymatic and hybridization properties of oligonucleotide analogs containing novel phosphoramidate internucleotide linkages. *Nucleosides, Nucleotides and Nucl. Acids* **2004**, 23, 777–787.
30. Seliger, H.; Krist, B.; Berner, S. Specific intrachain introduction of reporter groups into oligonucleotides as substituents at internucleotidic linkages. *Nucleos. Nucleot.* **1991**, 10, 303–306.
31. Wilk, A.; Grajkowski, A.; Phillips, L.R.; Beaucage, S.L. The 4-[N-methyl-N-(2,2,2-trifluoroacetyl)amino]butyl group as an Alternative to the 2-cyanoethyl group for phosphate protection in the synthesis of oligodeoxyribonucleotides. *J. Org. Chem.* **1999**, 64, 7515–7522.
32. Winnik, M. Cyclization and the conformation of hydrocarbon chains. *Chem. Rev.* **1981**, 81, 491–524.
33. Greenwald, R.B.; Conover, Ch.D.; Choe, Y.H. Poly(ethylene glycol) conjugated drugs and prodrugs: A comprehensive review. *Critical Rev. Ther. Drug Carrier Systems* **2000**, 17, 101–161.
34. Zalipsky, S. Functionalized Poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjugate Chem.* **1995**, 6, 150–165.
35. Caruthers, M.H.; Barone, A.D.; Beaucage, S.L.; Dodds, D.R.; Fisher, E.F.; McBride, L.J.; Matteucci, M.; Stabinsky, Z.; Tang, J.Y. Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. *Methods Enzymol.* **1987**, 154, 287–313.
36. Bannon, P.; Verly, W. Alkylation of phosphates and stability of phosphate triesters in DNA. *Eur. J. Biochem.* **1972**, 31, 103–111.