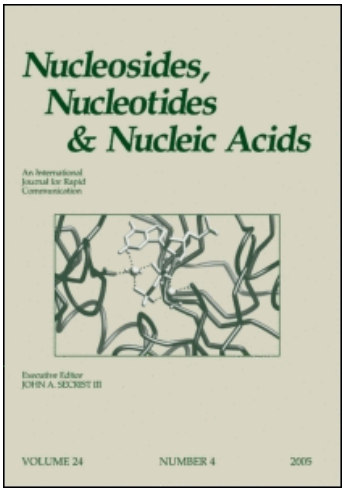

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Synthesis of Oligodeoxyribonucleotide Analogues by Use of Deoxyribonucleoside-3'-yl O-bis(1,1,1,3,3,3-Hexafluoro-2-Propyl) Phosphites as New Key Intermediates

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SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDE ANALOGUES BY USE OF DEOXYRIBONUCLEOSIDE-3'-YL O-BIS(1,1,1,3,3,3-HEXAFLUORO-2-PROPYL) PHOSPHITES AS NEW KEY INTERMEDIATES

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ABSTRACT

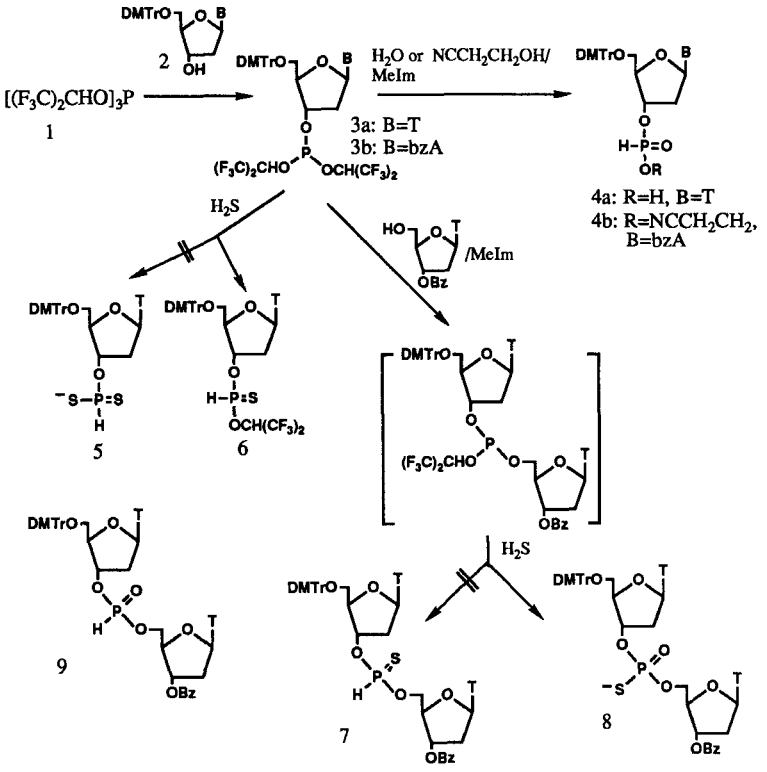
The deoxyribonucleoside-3'-yl O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite units (**3**) could be converted into the O-nucleosidyl phosphonate, O-2-cyanoethyl O-nucleosidyl phosphonate, and O-1,1,1,3,3,3-hexafluoro-2-propyl O-nucleosidyl phosphonothioate. Compound **3a** was activated by methylimidazole to give the dithymidylate derivatives (**8**). The appropriately protected nucleosidyl phosphonates (**3**) were applied to the synthesis of oligodeoxyribonucleotides used as antisense oligonucleotides.

INTRODUCTION

The O-nucleosidyl phosphonates have been frequently used for oligonucleotide synthesis.[1-3] They are useful intermediates for the preparation of several phosphate esters and their analogues. The internucleotidic phosphate can be converted into the phosphate[1-3], phosphoramidate[4], alkylphosphonate[5], phosphorothioate[4,6], and phosphorodithioate[7-11]. These analogues have found application as inhibitors of translation of RNA into protein and as potential anti-viral agents.[12-19]

Recently, we have reported[20,21] a simple method for the synthesis of deoxyribonucleoside-3'-yl phosphonates by use of bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphonate. This reaction proceeds via an N-phosphonylpyridine intermediate. Based upon the above facts, a new building block, deoxyribonucleoside-3'-yl O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphites (**3**)[22]

Dedicated to Professor Dr. Tohru Ueda



Scheme 1

were tested and applied successfully to the synthesis of medium size oligodeoxyribonucleotides on a solid support.[23-25]

In this paper, we wish to report an efficient approach to the synthesis of several kinds of phosphonate units and internucleotidic phosphate analogues starting from appropriately protected deoxyribonucleoside-3'-yl O-bis(1,1,1,1,3,3,3-hexafluoro-2-propyl) phosphites (**3**) as new key intermediates.

RESULTS AND DISCUSSION

First, we examined the preparation of 5'-O-dimethoxytritylthymidine-3'-yl phosphonate (**4a**) and O-2-cyanoethyl 5'-O-dimethoxytrityl-N^6-benzoyldeoxyadenosine-3'-yl phosphonate (**4b**) by use of the corresponding deoxyribonucleoside-3'-yl O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite units (**3**). The phosphitylating agent, tris(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (**1**) (1.1 mol equiv.) was treated with nucleosides (**2**) (1.0 mol equiv.) in

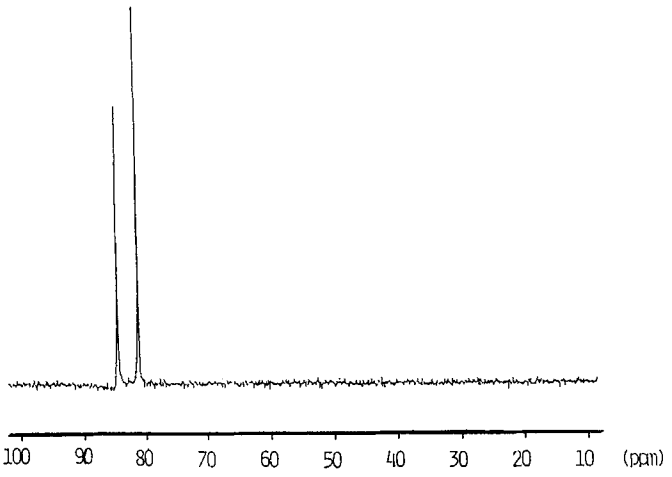


Fig. 1. ^{31}P-NMR spectrum of 5'-O-dimethoxytritylthymidine-3'-yl O-1,1,1,3,3,3-hexafluoro-2-propyl phosphonothioate (**6**).

CH_2Cl_2 at room temperature for 10 min, followed by treatment with H_2O or 2-cyanoethanol in the presence of N-methylimidazole (MeIm) as a catalyst. After the usual work-up and chromatography, **4a**[1] (91%) and **4b**[26] (96%) were obtained, respectively. ^{31}P-NMR showed that the contamination by the 3'-3' linked product and the decyanoethylated product from **4b** was not detected. These phosphonate units were employed as key intermediates for the synthesis of oligodeoxyribonucleotides and their analogues.

Next, we examined the preparation of oligonucleotides having phosphorodithioate linkages by the use of **3a**. **3a** was treated with dry a solution of H_2S saturated in THF. After 30 min, the reaction was monitored by ^{31}P-NMR. The spectrum of the reaction mixture showed that the signal of **3a** completely disappeared and new signals were observed at 81.18 and 84.57 ppm. The chemical shift suggested that **3a** was converted not into the desired 5[8] (53.64 and 53.02 ppm) but into 5'-O-dimethoxytritylthymidine-3'-yl O-1,1,1,3,3,3-hexafluoro-2-propyl phosphonothioate (**6**) (Fig. 1). The result clearly indicates that the nucleoside-3'-phosphonothioate (**6**) was isolated in 89% yield after purification by silica gel chromatography. Further, we examined the synthesis of dinucleoside (3'-5') phosphonothioate diester (**7**)[8,9] by use of **3a**. The phosphite unit (**3a**) (1.0 mole equiv.) was treated with 3'-O-benzoylthymidine (1.2 mole equiv.) in the presence of MeIm in dry CH_3CN at room temperature. After 10 min, the mixture was treated with a solution of dry H_2S saturated in THF for 30 min. After the usual work-up

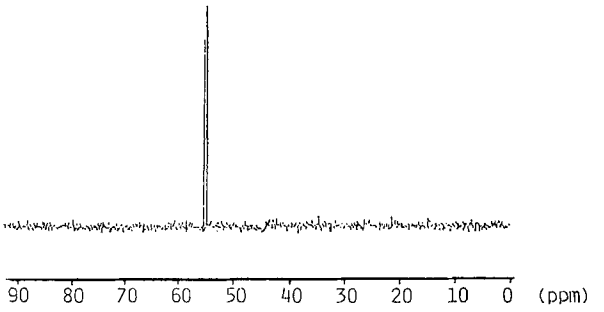
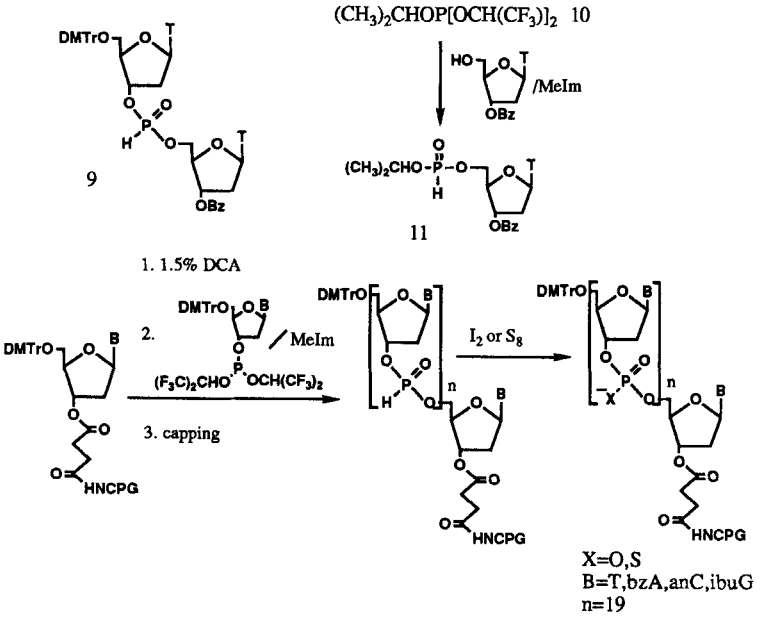


Fig. 2. ^{31}P-NMR spectrum of dinucleoside phosphorothioate (**8**).

involving extraction with CH_2Cl_2, coevaporation, and column chromatography, a colorless oily substance was obtained. To our surprise, ^{31}P-NMR analysis of the purified compound suggested the presence of only one sulfur (Fig. 2). Based upon this data we concluded that the structure of the compound should be assigned as the dinucleoside (3'-5') phosphorothioate diester (**8**).

It has been shown by Andrus et al.[27] that a capping reaction for an unreacted 5'-hydroxyl group on a solid support is required for the chemical synthesis of oligodeoxyribonucleotides by the H-phosphonate approach. We first tested the utility of the agent used with the phosphoramidite approach for a capping (Ac_2O/DMAP) of unreacted 5'-hydroxyl group.[28] However, the coupling reaction did not proceed smoothly and the product contained some impurities which could not be separated by HPLC. We have tried the capping reaction by use of bis(1,1,1,3,3,3-hexafluoro-2-propyl) 2-propyl phosphite (HFPP) (**10**) which could be easily prepared in 82% yield by treatment of 2-propyl phosphorodichloridite with 1,1,1,3,3,3-hexafluoro-2-propanol in the presence of triethylamine. The reactivity of **10** was first examined by the reaction of 3'-O-benzoylthymidine (1.2 mole equiv.) with **10** (1.0 mole equiv.) in the presence of MeIm in CH_3CN at room temperature. After 10 min, the reaction mixture was treated with 0.1 M MeIm in THF:H_2O (98:2, v/v) and was monitored by ^{31}P-NMR. The spectrum of the reaction mixture showed that a signal of the capping agent **10** completely disappeared and new signals were observed at 8.07 and 7.22 ppm. The chemical shift suggested that **10** was readily converted into the corresponding H-phosphonate diester (**11**).

The new phosphite approach including the new capping reagent described here was demonstrated in the synthesis of an oligodeoxyriboonucleotide and its phosphorothioate analogues. The antisense oligodeoxyribonucleotide, 5'-



Scheme 2

dCACCCAATTCTGAAAATGGA-3' (ODNs-tat), the complementary sequence to the HTLV-III mRNA splice acceptor site[29,30] was synthesized on an automated synthesizer by the new phosphite approach including the new capping reagent. The fully protected DNA on solid support was treated with conc. ammonia at 55°C for 6 h. The 5'-tritylated oligomer was separated by reversed phase C-18 silica gel and detritylated with 80% AcOH. The deblocked oligomer was further purified by reversed phase C-18 HPLC (Fig. 3). The main peak was found to be homogeneous by TSKgel DEAE 2SW and gel electrophoresis. The composition of four nucleosides was determined by reversed phase C-18 HPLC after hydrolysis of the oligomer with snake venom phosphodiesterase and alkaline phosphatase.

For the phosphorothioate, the oxidation step was replaced by treatment with 5% sulfur in CS_2/pyridine/triethylamine (45:45:10) for up to 2 h, depending on chain length. After the usual deprotection, isolation of the desired oligomer, 5'-dCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsAsTsGsGsA-3' (S-ODNs-tat) was performed by reversed phase C-18 HPLC (Fig. 3b). The main peak was found to be homogeneous by TSKgel DEAE 2SW and by gel electrophoresis. In this case, a small amount of oligomer attached to CPG was taken before the final treat-ment with sulfur and oxidized with 0.1 M I_2 solution to

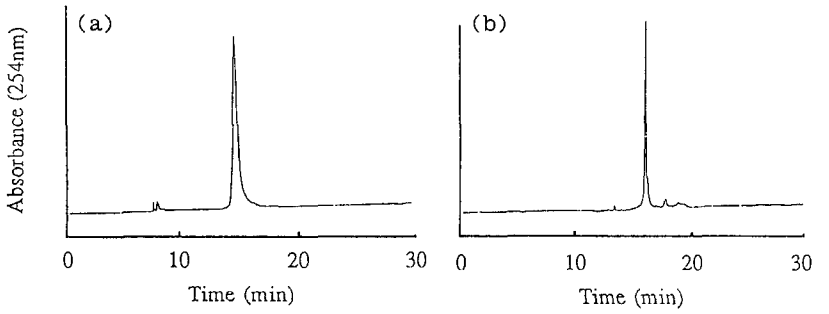


Fig. 3. Reversed phase HPLC of dCACCCAATTCTGAAAATGGA (a) and dCsAsCsCsCsAs-AsTsTsCsTsGsAsAsAsAsTsGsGsA (b). Elution was performed with a linear gradient of acetonitrile (5-50%) in 0.1 M triethylammonium acetate (pH 7.0) during 30 min.

phosphodiesters. The product was used for determination of base composition by enzymatic degradation to nucleosides followed by HPLC.

The S-ODNs-tat and ODNs-tat synthesized here were tested for anti-HIV activity. The S-ODNs-tat possessed slightly higher anti-HIV activity than S-dC_{28}[18] itself. These results will be reported.[31]

EXPERIMENTAL SECTION

General materials and methods.

^1H and ^{31}P-NMR spectra were recorded on a Bruker AMX 400 spectrometer with TMS and 80%H_3PO_4 as internal standards. Ultraviolet spectra were recorded on a Shimadzu UV-160 spectrometer. Thin layer chromatography (TLC) was carried out on Merck Kieselgel 60F_{254} plates which were developed in system A (CH_2Cl_2-MeOH, 9:1, v/v), system B (CH_2Cl_2-MeOH, 95:5, v/v). Reversed phase TLC was carried out on Merck silanized silica gel;[RP-8F 60F_{254}]plates with a mixture of acetone and 0.02 M triethylammonium acetate (TEAA) (6:4, v/v) as the eluting agent. Column chromatography was carried out on silica gel (BW-300; Fuji Davison Co.Ltd.) and alkylated silica gel (C-18, Waters Associates Inc.).

THF was continuously refluxed from sodium/benzophenone and distilled prior to use. CH_2Cl_2 was distilled from P_2O_5 and stored over activated 4-A° molecular sieves. CH_3CN was distilled twice from P_2O_5 and from CaH_2 and then stored over activated 4-A° molecular sieves. Pyridine was distilled twice from p-toluenesulfonyl chloride and from CaH_2 and then stored over activated 4-A° molecular sieves. DMF, N-methylimidazole, and lutidine were freshly distilled from CaH_2. Dicyclohexylcarbodiimide (DCC) and CS_2 were redistilled before use. 1,1,1,3,3,3-Hexafluoro-2-propanol was purchased from Sentral Glass Co. Ltd. and distilled before use. Long-chain alkylamino controlled pore glass (LCAA-CPG) was purchased from Electro Nucleonics Inc. Snake venom phosphodiesterase and alkaline phosphatase were purchased from Böehringer Mannheim.

The chain elongation steps were carried out in an Applied Biosystems Model 381A DNA synthesizer using CPG column containing 0.2 μmol of partially-protected dT and dA.

20% Polyacrylamide/7 M urea gel electrophoresis was run at 400V. Reversed phase HPLC was performed on a Tosoh PPCM system using a TSKgel oligo-DNA RP for analysis and Inertsil ODS for purification with a linear gradient of CH_3CN in 0.1 M triethylammonium acetate (pH 7.0). For anion exchange HPLC, the TSKgel DEAE-2SW, DEAE-NPR, and DEAE 5PW columns were used with a linear gradient of ammonium formate in 20% CH_3CN.

The dT-CPG (39 μmol/g) and dA-CPG (28 μmol/g) were prepared as described previously.[32] Tris(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (**1**) and deoxyribonucleoside-3'-yl O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite units (**3**) were prepared as described previously.[24,25]

Preparation of 5'-O-dimethoxytritylthymidine-3'-ly phosphonate (4a).

After coevaporation with dry pyridine, 5'-O-dimethoxytritylthymidine (**2a**) (540 mg, 1.0 mmol) was dissolved in THF (10 ml) and a catalytic amount of MeIm and tris(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (**1**) (0.33 ml, 1.1 mmol) was added. The reaction was complete in 10 min, and a mixture of 1 M triethylammonium bicarbonate (TEAB) (pH 7.4) and triethylamine (50:1, v/v) was added to the reaction mixture. After 5 min, the product was extracted with CH_2Cl_2 (2 X 15 ml), washed with 1 M TEAB (pH 7.4) and dried (Na_2SO_4). The CH_2Cl_2 layer was concentrated and the residue was applied to a silica gel column and eluted with a stepwise gradient of MeOH (0-3%) in CH_2Cl_2 containing triethylamine (2%). The appropriate fractions were pooled, washed with 1 M TEAB (pH 7.4) and dried (Na_2SO_4). The CH_2Cl_2 layer was concentrated *in vacuo* to give the corresponding H-phosphonate unit **4a** (648 mg, 91%). ^{31}P-NMR ($CDCl_3$, 85%H_3PO_4) δ 2.83 ppm.

Preparation of O-2-cyanoethyl 5'-O-dimethoxytrityl-N^6-benzoyldeoxyadenosine-3'-yl phosphonothioate (4a).

After coevaparation with dry pyridine, 5'-O-dimethoxytrityl-N^6-benzoyl-deoxyadenosine (**2b**) (658 mg, 1.0 mmol) was dissolved in THF (10 ml) and a catalytic amount of MeIm and tris(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (**1**) (0.33 ml, 1.1 mmol) was added. The reaction was complete in 10 min, and a mixture of 2-cyanoethanol (0.21 ml, 3 mmol) and MeIm (0.3 ml, 3.6 mmol) was added to the reaction mixture. After 20 min, the product was extracted with CH_2Cl_2 (2 X 20 ml), washed with 0.1 M TEAB (pH 7.4) and dried (Na_2SO_4). The CH_2Cl_2 layer was evaporated and the residue was applied to a silica gel column and eluted with a stepwise gradient of MeOH (0-3%) in CH_2Cl_2. The appropriate fractions were collected and concentrated *in vacuo* to give the corresponding **4b** (762 mg, 96%). ^{31}P-NMR (C_5D_5N, 85%H_3PO_4) δ 7.92 ppm.

Preparation of 5'-O-dimethoxytritylthymidine-3'-yl O-1,1,1,3,3,3-hexafluoro-2-propyl phosphonothioate (6).

5'-O-Dimethoxytritylthymidine-3'-yl O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (**3a**) (521 mg, 1.0 mmol) was treated with dry H_2S saturated THF (10 ml) for 30 min. Evaporation of solvent afforded a gum which was redissolved in ethyl acetate and washed with 1 M TEAB (pH 7.4). The ethyl acetate layer was dried (Na_2SO_4), concentrated to a gum, dissolved in CH_2Cl_2 and fractionated using silica gel column chromatography (0-5% MeOH in CH_2Cl_2:triethylamine 99.5:0.5, v/v). The product was isolated in 89% (999 mg) yield. ^{31}P-NMR ($CDCl_3$, 85%H_3PO_4) δ 81.18, 84.57 ppm.

Synthesis of 5'-O-dimethoxytritylthymidine-3'-yl, 3'-O-benzoylthymidin-5'-yl phosphorothioate (8).

3'-O-Benzoylthymidine (415 mg, 1.2 mmol) was dried by coevaporation with dry THF (10ml) and dissolved in dry CH_3CN (5 ml). The resulting solution was mixed with **3a** (521 mg, 1.0 mmol) and MeIm (0.16 ml, 2.0 mmol) and the reaction mixture was stirred for 10 min. To the solution was added dry H_2S saturated THF (10 ml) and the mixture was stirred for 30 min. Evaporation of the solvent afforded a gum which was redissolved in ethyl acetate and washed with 1 M TEAB (pH 7.4). The product was dried over Na_2SO_4, concentrated to a gum, dissolved in CH_2Cl_2 and fractionated using silica gel column chromatography (0-10% MeOH in CH_2Cl_2:triethylamine 99.5:0.5, v/v). The product was isolated in 85% yield. ^{31}P-NMR ($CDCl_3$, 85%H_3PO_4) δ 56.89, 57.04 ppm.

Synthesis of capping agent (10).

To a mixture of 2-propyl phosphorodichloridite (20.1 g, 125 mmol) and triethylamine (48.8 ml, 350 mmol) in dry ether (150 ml) under cooling at -20°C was 1,1,1,3,3,3-hexafluoro-2-propanol (52.8 ml, 500 mmol) in dry ether (50 ml). The mixture was allowed to warm up to room temperature, and was stirred for an additional 12 h. Petroleum ether (100 ml) was then added. Precipitates were kept overnight at 4°C and were filtered. The filtrate was concentrated, and the residue was distilled under reduce pressure. The main fraction (43.3g, 82%) was collected and obtained as a colorless liquid: b.p. 48°C/18mmHg ^{31}P-NMR ($CDCl_3$, 85% H_3PO_4) δ139.9 ppm.

Synthesis of oligodeoxyribonucleotides

The LCAA-CPG support loaded with first nucleoside (0.2 μmol) was packed in a small ABI column of an Applied Biosystems 381A DNA Synthesizer. The reaction cycle of chain elongation was carried out by a control programmed series of reagent and solvent washes based on a program of the DNA synthesis with the following modifications:

1) coupling: 0.25 M phosphite unit (**3**) and 0.5 M methylimidazole in dry CH_3CN in delivered in 4 alternating bursts of 4 sec (MeIm) followed by 10 sec (phosphite + MeIm) with wait time 5 min.

2) unblocking: 3% trichloroacetic acid in CH_2Cl_2 delivered in 5 X 10 sec bursts with intermediate 1 sec reverse flushes.

3) hydrolysis: 0.1 M MeIm in 2% aqueous THF solution delivered in two 10 sec bursts with total intermediate wait time of 120 sec.

4) capping: 0.5 M HFPP and 1.5 M MeIm in CH_3CN in delivered one two 10 sec bursts with total intermediate wait time 5 min.

Deprotection and isolation of oligodeoxyribonucleotides

After oxidation, the column was washed with CH_3CN and ether. Further the column was treated with concentrated ammonia for 1 h at room temperature. The solution was eluted from column and heated in a sealed vial at 55°C for 5-8 h. The solution was concentrated and the residue was dissolved in water. The solution was passed through a membrane filter (EKICRODISC 13, Gelman Sciences Japan).[33] The deprotected oligonucleotide was analyzed and purified by the anion exchange HPLC or reversed phase HPLC. The appropriate fractions were collected and lyophilized from sterile water. The purity and chain length were analyzed by anion exchange HPLC and PAEG.

Enzymatic digestions

The oligonucleotide (0.5 A_{260} units) was dissolved in 0.01 M TRIS/HCl buffer (pH 8.8) (500 µl) and digested with snake venom phosphodiesterase (5 µg) at 37°C for 2 h. The mixture was further incubated with alkaline phosphatase (5 µg) at 37°C for 1 h. Degradation products were analyzed by the reversed phase HPLC using a TSKgel oligo-DNA RP with a nonlinear gradient of CH_3CN (5% during 60 min) in 0.1 M TEAA (pH 7.0).

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