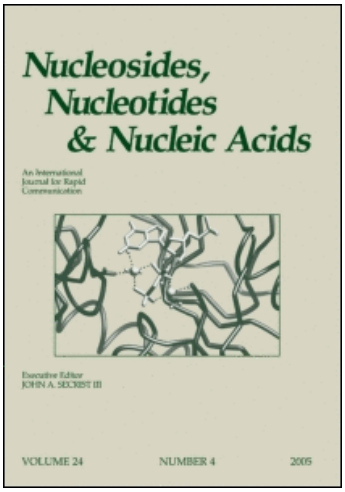

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FACILE POST-SYNTHETIC DERIVATIZATION OF OLIGODEOXYNUCLEOTIDE CONTAINING 5-METHOXYCARBONYLMETHYL-2'-DEOXYURIDINE

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ABSTRACT: 5-Methoxycarbonylmetyl-2'-deoxyuridine residue was incorporated into oligoDNAs containing either an exclusive thymidine residue (dT) or all four natural deoxynucleoside residues (dA, dG, dC, dT) *via* a phosphoramidite method. The treatment of the fully protected oligomer bound to controlled pore glass (CPG), with a variety of polyamine resulted in the release of the oligomer from CPG and the incorporation of the polyamine at the 5-position of the uracil component, simultaneously and in good yields.

Modification of oligonucleotides with various functional molecules such as an intercalating agent, a metal chelating agent, a fluorescent dye, etc., has been attracting wide interest in terms of applying such modified oligonucleotides to biological and biophysical studies.[1] One of the most popular methods for the modification of DNA is the incorporation of a primary amine containing moiety to the DNA strand as the linker molecule and further reaction of the terminal amino group with a reactive form of the functional molecule. For example, Orgel et al. demonstrated the conjugation of hexamethylene-diamine with 5'-phosphate of oligoDNA and further derivation of the terminal primary amine with the *N*-hydroxysuccinimidyl ester of biotin, in their pioneer works.[2,3] Derivatizable amine-containing molecules can also be incorporated to the 5'-terminus of DNA by the use of a specially prepared phosphoramidite reagent bearing a protected primary amine residue.[4]

This paper is dedicated to the late Professor Tsujiaki Hata.

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More recently, Matsuda et al. demonstrated an elegant method of oligoDNA modification utilizing a novel nucleoside residue.[5] They reported that the treatment of oligopyrimidylates containing 5-methoxycarbonyl-2'-deoxyuridine or 5-trifluoroethoxy-carbonyl-2'-deoxycytidine residue with ethylenediamine or hexamethylenediamine resulted in the corresponding oligomers carrying the amino-linkers at the 5-position of the pyrimidine nucleoside derivatives.

The method is very attractive since one can site-specifically introduce desirable amino-linkers at the 5-position of the pyrimidine base in DNA, which would not interfere with the formation of a duplex with their complementary oligonucleotide, after the assembly of DNA. Also in this method, time-consuming protection procedures generally required for reactive primary amine moieties on the nucleobases prior to the assembly of DNA can be excluded.

Previously we have reported a short communication describing a facile synthesis of a novel nucleoside, 5-methoxycarbonylmetyl-2'-deoxyuridine, from arabinoamino-oxazoline and the introduction of a variety of a primary amine containing molecules to the 5-position of the nucleoside by an ester-amide exchange reaction.[6a] The amine-modified uridine components with an amine protecting group were incorporated to oligoDNA in place of thymidine residue by use of phosphoramidite chemistry.[6b] In the course of our study of the preparation of derivatizable DNA molecules, we have found that 5-methoxycarbonylmetyl-2'-deoxyurine can also be used as a suitable key component for the post-synthetic modification of oligoDNA with a polyamine molecule. The method is also applicable to the DNA containing all four natural nucleosides (dA, dG, dC, dT). Here, we wish to describe the preparation of oligoDNA containing 5-methoxycarbonylmetyl-2'-deoxyurine residue and the site-specific derivatization of the oligoDNA with various polyamine molecules by a post-synthetic manner.

Preparation of CPG-bound oligoDNA containing 5-Methoxycarbonyl-methyl-2'-deoxyuridine residue.

5-Methoxycarbonylmethyl-2'-deoxyuridine (**2**) was prepared by simply treating 3',5'-di-*O*-acetyl-5-methoxycarbonylmethyl-2'-deoxyuridine (**1**)[6] with triethylamine (TEA) in the presence of *N,N*-dimethylaminopyridine (DMAP) in methanol. Under these conditions, no side reaction such as the formation of 2'-deoxyuridine 5-carboxylate was observed and the yield of the product was satisfactory (> 80 %). The nucleoside was protected at its 5'-position with a dimethoxytrityl group (DMTr) and converted to the corresponding 3'-phosphoramidite derivative **4** by the standard phosphitylation procedure.[7] The compound **4** was incorporated into two types of oligoDNAs (**5'-DMTr-ODN-1-CPG** and **5'-DMTr-ODN-2-CPG**, where CPG stands for a

controlled pore glass support) using an automated DNA synthesizer (ABI 381-A) in 1 μmol scale synthesis. During the assembly of the oligomers, however, the reaction period of 30 sec. usually applied for unmodified phosphoramidite reagents was found to be inadequate for **4**. To achieve a high rate of coupling, therefore, the coupling period of 360 sec. was applied for **4** using 0.1 M CH_3CN solution. The average coupling yield of **4** estimated by trityl color assay[8] was approximately 95% (Scheme 1).

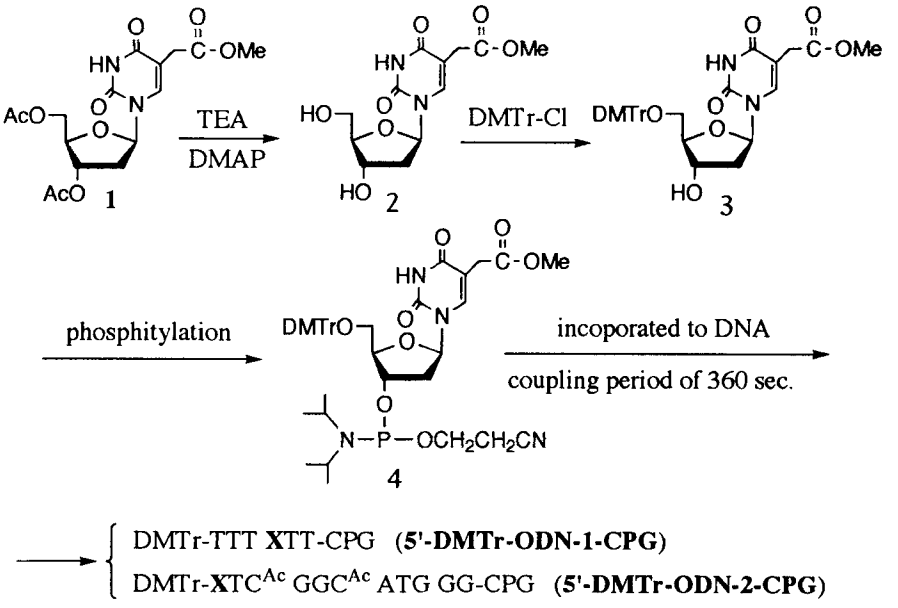
As was reported previously, the treatment of N^4-benzoyl deoxycytidine (dC^{bz}), a commonly used component in oligoDNA synthesis, with an amine derivative produces a significant amount of the N^4-transaminated product.[9] We have used, therefore, N^4-acetyl deoxycytidine (dC^{Ac}) phosphoramidite[10] instead of the dC^{bz} phosphoramidite for the preparation of **5'-DMTr-ODN-2-CPG** containing all four natural nucleoside residues to avoid such undesirable side reaction (Scheme 1). The coupling yield of dC^{Ac} phosphoramidite unit was satisfactory (> 96 %).

Site-specific derivatization of oligoDNA containing 5-Methoxycarbonylmethyl-2'-deoxyuridine residue with polyamine.

At first, **5'-DMTr-ODN-1-CPG**, containing an exclusive thymidine residue along with a single 5-modified 2'-deoxyuridine residue, was used to study the feasibility of the current method. Thus, **5'-DMTr-ODN-1-CPG** (38.1 mg) was divided into four equal portions (9 mg) and each portion was treated with excess of a polyamine solution in ethanol (50 %) at room temperature for 12h. The solutions were concentrated with N_2 evaporator followed by dilution with triethylammonium acetate buffer (pH 7.0), gel filtration with Sephadex G-25, and HPLC purification using a C-18 column. In each sample, the main fraction containing the DMTr bearing oligomer was isolated and subjected to detritylation followed by ethanol precipitation and gel filtration. The obtained oligomers were further subjected to enzyme digestion using snake venom phosphodiesterase, alkaline phosphatase and nuclease P1 to confirm the incorporation of the polyamine functions to the modified uridine residue (Scheme 2).

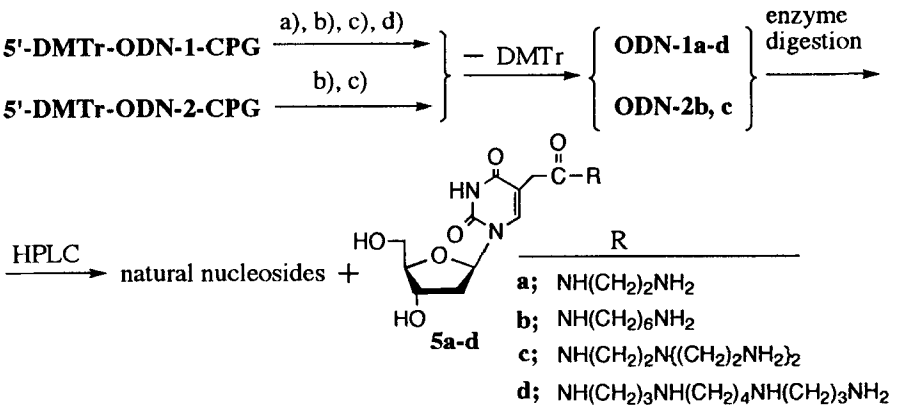
In HPLC analysis, all oligomers gave two peaks corresponding to thymidine and the polyamine-derived 2'-deoxyuridine residue (**5a-d**) after the enzyme digestion. The typical example is shown in FIG. 1-a. The structures of the latter compounds were identified by the co-injection of the independently prepared authentic samples.[6] The nucleoside composition calculated from each HPLC analysis is listed in TABLE 1.

Each nucleoside composition agrees well with the desired ratio of dT:dM = 5:1. The results of the enzyme digestion clearly demonstrate that the treatment of **5'-DMTr-ODN-1-CPG** affects the release of the oligomer from CPG and the incorporation of the polyamine molecule at the 5-position of the uridine residue, simultaneously and in a site-



Oligomers are fully protected form. **X**, C^{Ac}, and CPG correspond to 5-methoxycarbonylmethy deoxyuridine residue, N^4-acetyl deoxycytidine residue, and the controlled pore glass support, respectively.

SCHEME 1



5'-DMTr-ODN 1 and **2** were treated with a) ethylenediamine, b) hexamethylenediamine, c) tris(2-aminoethyl) amine, and d) spermine in ethanol (50%) at room temperature, respectively. After removal of the DMTr group, oligomers were subjected to enzyme digestion to give natural nucleosides and C-5 polyamine-derived deoxyuridine analogs (**5a-d**).

SCHEME 2

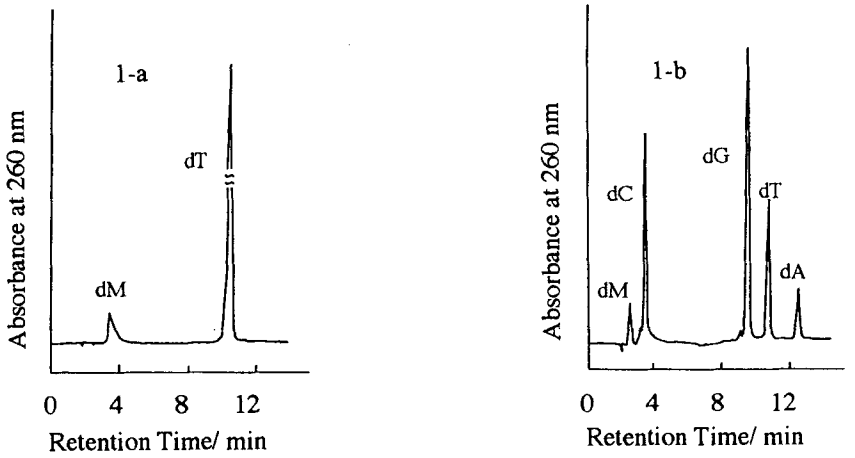


FIG. 1. HPLC analysis of the enzyme digested oligomers performed by reversed phase HPLC on Wakosil 5C-18 column (4 mmϕ x 250 mm) using 0.1 M TEAA (pH 7.0) with a linear gradient of 2.1-30.1% acetonitrile. dM corresponds to a C-5 polyamine-derived 2'-deoxyuridine nucleoside. a); **ODN-1d**, b); **ODN-2c**

TABLE 1. The yields and the results of nucleoside composition analysis of ODN-1s. (d-TTT MTT)

Oligomer	C-5 substituted group	Isolated yield (OD)	Nucleoside composition (dT : dM)
ODN-1a	ethylenediamine	4.3	5.3 : 1.1[a)]
ODN-1b	hexamethylenediamine	2.8	4.9 : 0.9[b)]
ODN-1c	tris(2-aminoethy)amine	3.2	5.2 : 1.0[c)]
ODN-1d	spermine	2.8	5.0 : 1.1[d)]

The nucleoside composition analysis of the oligomers was carried out by hydrolysis of the ODNs uing snake venom phosphodiesterase, nuclease P_1, and alkaline phosphatase. dM corresponds to C-5 polyamine-derived 2'-deoxyuridine nucleoside (**5a-d**). a); **5-a**, b); **5-b**, c); **5-c**, d); **5-d**.

specific manner. Also, the reaction seems to be clean since no detectable unknown peaks were observed in the HPLC analysis.

We further extended the study to apply the current method to an oligomer containing all four natural deoxynucleoside residues (dA, dG, dC, dT). Thus, **5'-DMTr-ODN-2-CPG** (34.5 mg) was divided into two equal portions (16 mg) and each portion was treated with either tris(2-aminoethyl)amine or hexamethylenediamine in ethanol (50 %). It should be noted that, in the latter case, additional conc. NH_4OH

treatment was necessary to achieve complete removal of the base-labile protecting group. After work-up in the same manner as above, the isolated, fully deprotected oligomer was subjected to enzyme digestion. FIG. 1-b shows the typical example of the enzyme digestion experiment analyzed by HPLC. As is clearly shown in FIG. 1-b, only four distinctive peaks corresponding to the four natural nucleosides along with the polyamine derived 5-modified deoxyuridine were generated by the enzyme digestion. No detectable unknown peaks were observed. The nucleoside composition estimated by each HPLC analysis is listed in TABLE 2.

The results of nucleoside composition analysis are almost parallel to the desired sequence in which the ratio of dA: dG: dC: dT: dM = 1:5:2:2:1 for both ODN-2b and -2c. These results indicate that the treatment of the CPG-bound fully protected oligoDNA containing a protected form of four natural nucleosides, although an unusual acetyl group was used as the *N*-4 protection group of the deoxycytidine, and 5-methoxycarbonylmetyl-2'-deoxyurine with a polyamine derivative is both effective to release the oligomer from the support as well as to incorporate the polyamine molecule into the uridine residue.

Furthermore, the use of dC^{Ac} as a deoxycytidine component in oligoDNA is proved to be effective to suppress the undesirable amine exchange side reaction at the *N*-4 position during the treatment of the oligomer with amine derivatives, as was expected. Also, it should be noted that the current deoxyuridine derivative has an extra methylene bridge at the C-5 position compared to the previously reported 5-methoxycarbonyl analog.[5-a] In the polyamine-modified form, therefore, the possible formation of an intramolecular hydrogen bond between the O-6 of uracil base and the hydrogen atom of the amide bond mentioned in a previous report[5-b] would be avoided.

In conclusion, we have successfully demonstrated that the current method will provide a convenient route to the modification of oligoDNA with various polyamine-type reagents in a post-synthetic manner. The method is also proved to be applicable for the oligomer containing all four natural bases.

The obtained oligomers will allow us to make further modification with other functional molecules having the capability to react with a primary amine moiety attached on the oligomer. The study of such additional modification of polyamine-derived oligoDNA prepared by the current method is progressing in our group and will be reported elsewhere.

EXPERIMENTAL

5-Methoxycarbonylmethyl-2'-deoxyuridine (2). A mixture of 3',5'-di-*O*-acetyl-5-methoxy-carbonylmethyl-2'-deoxyuridine (**1**) (326 mg, 0.848 mmol),[6] dimethylaminopyridine (10 mg) and triethylamine (1 ml) in methanol (5 ml) was stirred

TABLE 2. The yields and the results of nucleoside composition analysis of ODN-2s. (d-MTC GGC ATG GG)

Oligomer	C-5 substituted group	Isolated yield (OD)	Nucleoside composition (dA : dG : dC : dT : dM)
ODN-2b	hexamethylenediamine	19.3	0.9 : 5.2 : 2.2 : 2.1 : 0.9[a)]
ODN-2c	tris(2-aminoethy)amine	21.5	0.8 : 4.8 : 2.3 : 1.9 : 0.8[b)]

The nucleoside composition analysis of the oligomers was carried out by hydrolysis of ODNs using snake venom phosphodiesterase, nuclease P_1, and alkaline phosphatase. dM corresponds to C-5 modified 2'-deoxyuridine nucleoside (**5-b, 5-c**). a); Compound **5-b** as dM, b); Compound **5-c** as dM.

for 14 h. at room temperature. The mixture was evaporated to dryness and the residue was purified by silica-gel column chromatography (15% of methanol in C_2H_2). 5-Methoxycarbonylmethyl-2'-deoxyuridine (**2**) was isolated as a white powder (213 mg, 81.3%). An analytical sample was recrystallized from H_2O. mp 159-160 °C. *Anal* Calcd for $C_{12}H_{16}N_2O_7 \cdot 1/2H_2O$: C, 46.60; H, 5.53; N, 9.06. Found: C, 46.32; H, 5.18; N, 9.19. ^{1}H-NMR (D_2O) δ: 7.81 (1H, s, H-6), 6.25 (1H, t, H-1'), 4.67-4.39 (1H, m, H-3'), 4.04-3.99 (1H, m, H-4'), 3.86-3.67 (5H, m, H-5' and OCH_3), 3.41 (2H, s, C5-CH_2-), 2.39-2.32 (2H, m, H-2').

5'-*O*-(4,4'-Dimethoxytrityl)-5-Methoxycarbonylmethyl-2'-deoxyuridine (3). To a mixture of **2** (275 mg, 0.916 mmol) and dimethylaminopyridine (7 mg) in pyridine (5 ml) was added a solution of dimethoxytrityl chloride (466 mg, 1.38 mmol) in pyridine (5 ml) followed by triethylamine (500 μl) and the mixture was stirred at room temperature for 14 h. The reaction mixture was poured into cold sat. $NaHCO_3$ (100 ml) and extracted with CH_2Cl_2 (3 x 50 ml). The organic layer was collected, dried over $MgSO_4$, and evaporated. The obtained gum was further co-evaporated with toluene and the obtained residue was purified by silica-gel column chromatography using 10% methanol in CH_2Cl_2 containing 0.1% triethyamine as a eluent. The appropriate fractions were collected and evaporated. The residue was dissolved in CH_2Cl_2 (5 ml) and the solution was dropwised to a stirred hexane (250 ml) under cooling to precipitate the desired product. Yield. 479 mg (86.8%). mp 101-102 °C. *Anal* Calcd for $C_{33}H_{34}N_2O_9$: C, 65.77; H, 5.69; N, 4.65. Found: C, 65.27; H, 5.87; N, 4.91. ^{1}H-NMR ($CDCl_3$) δ: 7.77 (1H, s, H-6), 7.39-6.80 (13H, m, Ar), 6.45 (1H, t, H-1'), 4.66-4.60 (1H, m, H-3'), 4.07-4.04 (1H, m, H-4'), 3.80 (3H, s, CH_3O-Ar), 3.60 (3H, s, OCH_3), 3.56-3.35 (2H, m, H-5'), 2.69-2.37 (4H, m, C5-CH_2- and H-2').

3'-*O*-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5'-*O*-(4,4'-dimethoxytrityl)-5-methoxycarbonylmethyl-2'-deoxyuridine (4). Diisopropylamine (140 μl, 1.00 mmol) was added dropwise to a solution of dichloro-(2-cyanoethoxy)phosphine (63μl, 0.50 mmol) in dry CH_2Cl_2 (5 ml) under argon atmosphere at 0 °C. The mixture was stirred at room temperature for 30 min. A portion (4 ml) of the solution was added dropwise to a stirred solution of **3** (200 mg, 0.332 mmol) in CH_2Cl_2 (5 ml) containing *N*-ethyl-*N,N*-diisopropylamine (140 μl, 0.80 mmol) under argon atmosphere at room temperature. After stirring for 30 min. dry methanol (20 μl) was added to this mixture and the mixture was pored into cold CH_2Cl_2 (30 ml, pre-washed with sat. $NaHCO_3$). After washing the organic layer with sat. $NaHCO_3$ (30 ml), the separated organic layer was dried over $MgSO_4$ and evaporated to dryness. The obtained residue was purified by silica-gel column column chromatography using ethyl acetate-methanol-triethylamine (16/1/2, v/v/v) as an eluent. The appropriate fractions were collected and evaporated. The residue was dissolved in CH_2Cl_2 (3 ml) and the solution was added dropwise to stirred hexane (250 ml) under cooling to precipitate the desired product. Yield. 187 mg (70.2%). mp 72.4-73.8 °C. *Anal* Calcd for $C_{42}H_{51}N_4O_{10}P_1$: C, 62.82; H, 6.41; N, 6.98. Found: C, 62.64; H, 6.22; N, 6.81. ^{31}P-NMR ($CDCl_3$) δ: 145.28 and 145.69 ppm.

5-[*N*-(3-aminopropyl)-4-aminobutyl]-3-aminopropyl]carbamoylmethyl-2'-deoxyuridine (5d). The compound was prepared from **1** as described before.[6b] In brief, a mixture of **1** (206 mg, 0.536 mmol), spermine (842 μl, 5.36 mmol), and 4-dimethylaminopyridine (0.01 g, 0.082 mmol) in methanol (2 ml) was stirred at 50 °C for 24 h. After the removal of the volatile material, the residue was dissolved in a small amount of methanol and added dropwise to ether to precipitate 5-[*N*-(3-aminopropyl)-4-aminobutyl]-3-aminopropyl]carbamoylmethyl-2'-deoxyuridine (5d) as an oily residue. Yield. 154 mg (61.1%). MS Found: *m/z* 471.3. Calcd for $C_{21}H_{39}N_6O_6$: MH^+, 471.29. ^{1}H-NMR (D_2O) δ: 7.76 (1H, s, H-6), 6.31 (1H, t, H-1'), 4.50-4.43 (1H, m, H-3'), 4.07-4.01 (1H, m, H-4'), 3.87-3.73 (2H, m, H-5'), 3.35-3.27 (4H, m, C5-CH_2- and -$NHCH_2$-), 3.05-2.85 (8H, m, -$NHCH_2$- x4), 2.43-2.32 (2H, m, H-2'), 2.03-1.64 (10H, -CH_2- x5).

Assembly of OligoDNA containing 5-Methoxycarbonyl-methyl-2'-deoxyuridine residue. OligoDNAs were prepared by normal phosphoramidite procedure using automated DNA synthesizer (ABI 380-A) in 1 μmol scale. The coupling period for **4** was, however, extended to 360 sec. from 30 sec. used for other dA, dG, dC, dT phosphoramidite reagents. The coupling yield of **4** and dC^{Ac} phosphoramidite estimated by normal trityl colour assay using perchrolic acid solution[8] were 94.8% and 96.2%. respectively.

Derivatization of oligoDNA containing 5-Methoxycarbonyl-methyl-2'-deoxyuridine residue with polyamine. A part of **5'-DMTr-ODN-1-CPG** (38.1 mg), an oligomer connected to CPG-support, was divided into four equal portions (9.0 mg each) and each portion was treated with 50 % solution of ethylenediamine, hexamethylenediamine, tris(2-aminoethy)amine, or spermine in ethanol (1 ml) at room temperature for 12 h. After the removal of volatile material by N_2 evaporator, each sample was diluted with 0.1 M triethyammonium acetate (TEAA, pH 7.0) to adjust the volume approximately 1 ml and subjected to gel filtration using Sephadex G-25 column. The appropriate fractions were collected and dried by evaporation. The obtained residue was dissolved in 0.1 M TEAA (pH 7.0) and purified by reversed phase HPLC on Wakosil 5C-18 column (10 mmϕ x 250 mm) using 0.1 M TEAA (pH 7.0) with a linear gradient of 20-40% acetonitrile. The isolated DMTr-bearing oligomer was treated with 10% acetic acid (10 min) followed by ethanol precipitation and Sephadex G-25 gel filtration in the usual manner.

The derivatization of **5'-DMTr-ODN-2-CPG** was performed in essentially the same manner as above. **5'-DMTr-ODN-1-CPG** (34.5 mg) was divided into two equal portions (16.0 mg) and subjected to the derivatization with either tris(2-aminoethyl)amine or hexamethylenediamine and subsequent purification. In the latter case, however, the oligomer was further treated with conc. NH_4OH (2 ml) at 55 °C for 12 h in a sealed vessel before the removal of volatile material by N_2 evaporator.

Enzyme digestion of the polyamine-derived oligoDNA. The polyamine-derived oligomers (ca. 0.5 $OD_{260\,nm}$) were treated overnight with snake venom phosphodiesterase (0.5 units) and alkaline phosphatase (1 units) in 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$ at 37 °C. Subsequently, nuclease P1 (ca. 5 units) and 0.1 M sodium acetate (pH. 5.3) were added to this mixture. The whole mixture was incubated at 37 °C overnight. The reaction mixture was analyzed by reversed phase HPLC on Wakosil 5C-18 column (4 mmϕ x 250 mm) using 0.1 M TEAA (pH 7.0) with a linear gradient of 2.1-30.1% acetonitrile and the flow rate of 1 ml/min.

Identification of the polyamine-derived nucleosides **5a-d** was done by the co-injection of the authentic samples prepared by the reaction of **1** with polyamine.[6b] The composition ratios of the nucleosides were calculated from areas of the peaks in the HPLC chart. The extinction coefficients at 260 nm of **5a-d** used for the calculation were as follows; **5a**, 8000[6b]; **5b**, 9300[6b]; **5c**, 7900[6b]; **5d**, 8200. The values were calculated from absorption spectra of the nucleosides and are not exact values, although they are sufficient for the calculation of the nucleoside composition analysis.

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