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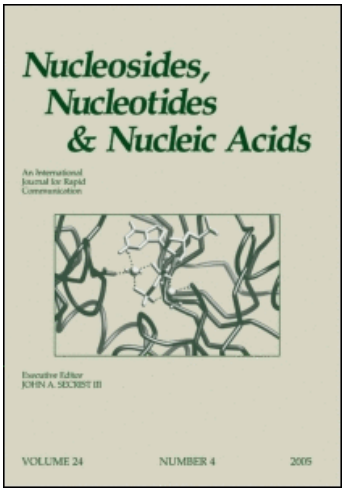

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Syntheses and Properties of Dansylated Deoxycytidine 3',5'-Bisphosphate Derivatives Useful for 3'-Labeling of Oligoribonucleotides

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NUCLEOSIDES & NUCLEOTIDES, 11(10), 1713-1730 (1992)

SYNTHESES AND PROPERTIES OF DANSYLATED DEOXYCYTIDINE 3',5'-BISPHOSPHATE DERIVATIVES USEFUL FOR 3'-LABELING OF OLIGORIBONUCLEOTIDES

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Abstract: For non-RI labeling of RNAs with fluorescence markers, deoxycytidine 3',5'-bisphosphate derivatives (**1** and **2**) were synthesized as dansyl donors which could be linked to the 3'-terminus of RNAs by T4 RNA ligase catalized joining reactions. Ligations of GpApC with these dansyl donors in the presence of T4 RNA ligase were studied.

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INTRODUCTION

A number of methods for non-RI labeling of DNAs have recently been developed.[1] These techniques facilitated rapid sequencing and detection of DNA fragments.[2] Compared with non-RI labeling of DNAs, there have been reported only few papers concerning non-RI labeling of RNAs.[3-6] Much attention has been paid to the recent new aspects on the structure and function of RNAs, since several important discoveries such as self-splicing[7] and ribozymes[8] have been reported in the molecular biochemistry of RNAs. For the monitoring of such reactions, non-RI labeling of RNA substrates would be convenient and powerful tools in laboratory work since special facilities for RI materials were unnecessary.

The dansyl (DNS) group has been frequently utilized as a fluoresence source for labeling of a large number of natural products since its presence could be readily detected directly and visibly with reasonable sensitivity by a UV detector. To our knowledge, however, only two papers have been reported about labeling of RNAs by the use of DNS-containing reagents[3,4] Yang reported that the DNS group could be attached to the 5'-terminal phosphate of tRNA *via* a diphosphate linkage by the use of 6-(*N*-dansylamino)hexyl phosphoromorpholidate.[3] This labeling method required an impractical non-aqueous reaction. On the other hand, Gumport reported the 3'-ligation of tRNAs with adenosine 6-(*N*-dansylamino)hexyl diphosphate as a donor of the DNS group.[4] However, we observed that DNS donors of this type were inefficiently linked to

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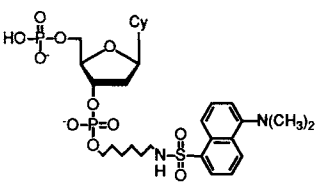
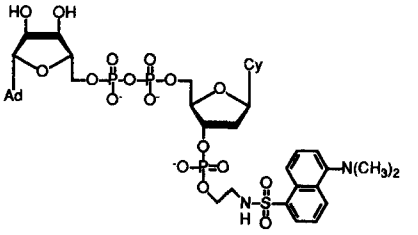


1: pdCp-HA-DNS



2: $A^{5'}$ppdCp-EA-DNS

the 3'-terminus of tRNAs by RNA ligase.[9] Our preliminary results led us to synthesize more effective DNS donors useful for developments of chemistry and biochemistry of RNAs.

In this paper, we report the synthesis of two DNS-containing deoxycytidine 3',5'-bisphosphate derivatives (**1** and **2**).

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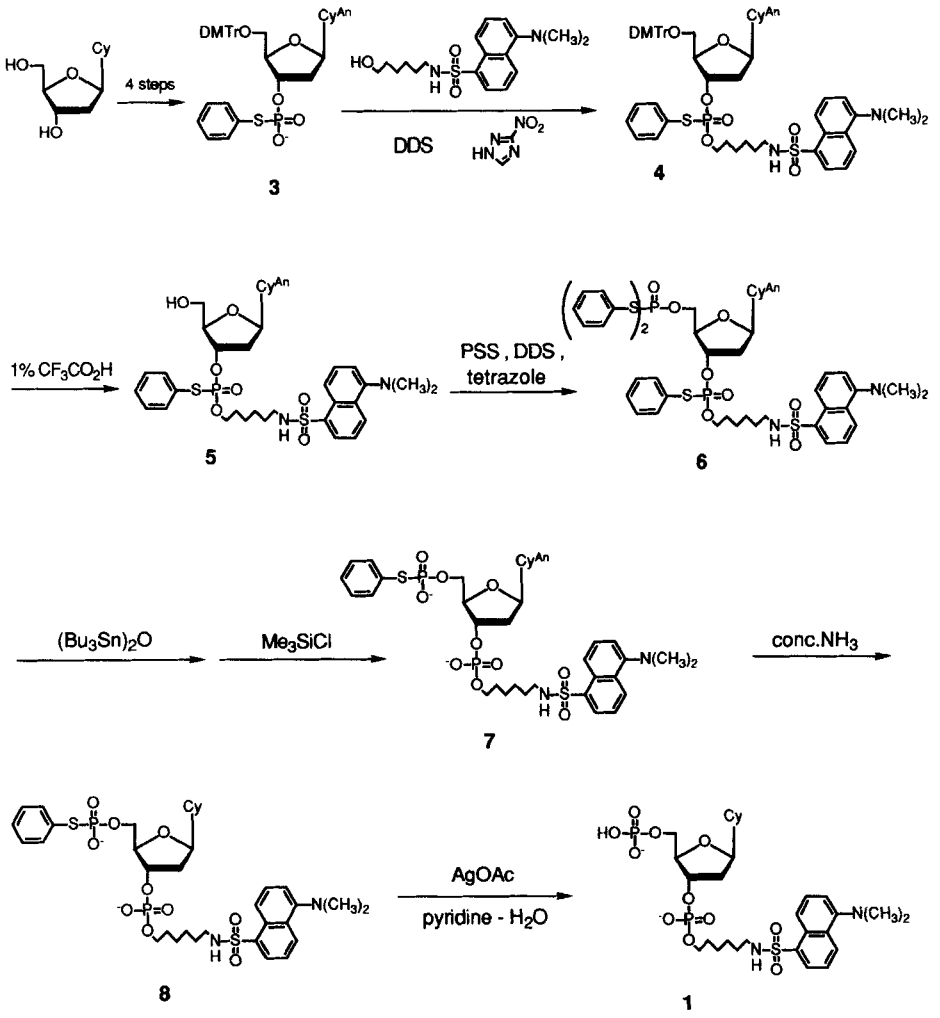
Results and Discussion

Synthesis of pdCp-HA-DNS

In T4 RNA ligase catalyzed joining reactions between two RNA fragments, cytidine is superior to the other common ribonucleosides of adenosine, guanosine, and uridine as 5'-terminal ribonucleosides of 5'-phosphorylated donors as far as the efficiency of ligation is concerned.[10] It is also known that T4 RNA ligase can catalyze the joining between 3'-terminal free oligoribonucleotides and 3',5'-bisphosphorylated deoxyribonucleosides.[11-14] In this case, 3',5'-bisphosphorylated deoxycytidine is the best as a donor among the four common 3',5'-bisphosphorylated deoxyribonucleotides. In consideration of these facts as well as the convenience of synthetic strategy, we designed 3',5'-bisphosphorylated deoxycytidine derivative (**1**) containing the DNS group at the 3'-phosphate residue.

In this experiment, we have chosen a hexamethylene group as the linker between the 3'-phosphate group and the DNS group. At the beggining of this study, we considered that the use of the relatively long spacer would resulted in avoidance of the quenching due to the intramolecular interaction between the cytosine moiety and the DNS group, since fluorophore-containing nucleic acids such as $tRNA^{Phe}$-formycin exhibited significant quenching.[6]

The outline of the synthesis of **1** is shown in Scheme 1. Deoxycytidine 3'-phosphorothioate derivative (**3**) was synthesized by the known procedure[15] from deoxycytidine via a 4-step reaction. Compound **3** was condensed with 6-(*N*-dansylamino)hexanol in the presence of isodurenedisulfonyl dichloride (DDS)[14] and 1,2,4-1*H*-triazole (NT)[16] to give the 3'-*O*,*O*,*S*-triester derivative (**4**) in 93% yield.



Scheme 1

Treatment of **4** with 1% trifluoroacetic acid in CH_2Cl_2 gave the 5'-hydroxyl derivative (**5**) in 93% yield. Phosphorylation of **5** with cyclohexylammonium *S,S*-diphenyl phosphorodithioate (PSS)[17,18] in the presence of DDS and tetrazole[19] afforded the 3',5'-bisphosphorylated deoxycytidine derivative (**6**) in 88% yield. Treatment of **6** with bis(tributyltin) oxide[20] in pyridine followed by addition of chlorotrimethylsilane resulted in quantitative formation of the dissociated species (**7**) which was further deblocked with ammonia to give the 5'-phosphorothioate derivative (**8**) quantitatively. The final

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dephenylthioation of **8** with silver acetate[18] gave the desired labeling compound **1**. This compound could be easily purified by reverse-phase column chromatography using a C_{18} column (Waters) because of the sufficient lipophilicity of the DNS group. This is one of useful properties of DNS-containing nucleotide materials. The use of iodine in place of silver acetate for hydrolysis of the P-S bond of **8** resulted in a complex mixture of at least 7 products. Control experiments using 6-(*N*-dansylamino)hexanol suggested that the DNS group was readily decomposed even by this mild oxidizing reagent. The silver-ion catalyzed hydrolysis of **8** gave a clean result, showing the sole product of **1** on TLC. Compound **1** was characterized by 500 MHz ^{1}H NMR. The UV spectrum (FIG. 1A) of **1** was similar to that (FIG. 1B) of a 1:1 mixture

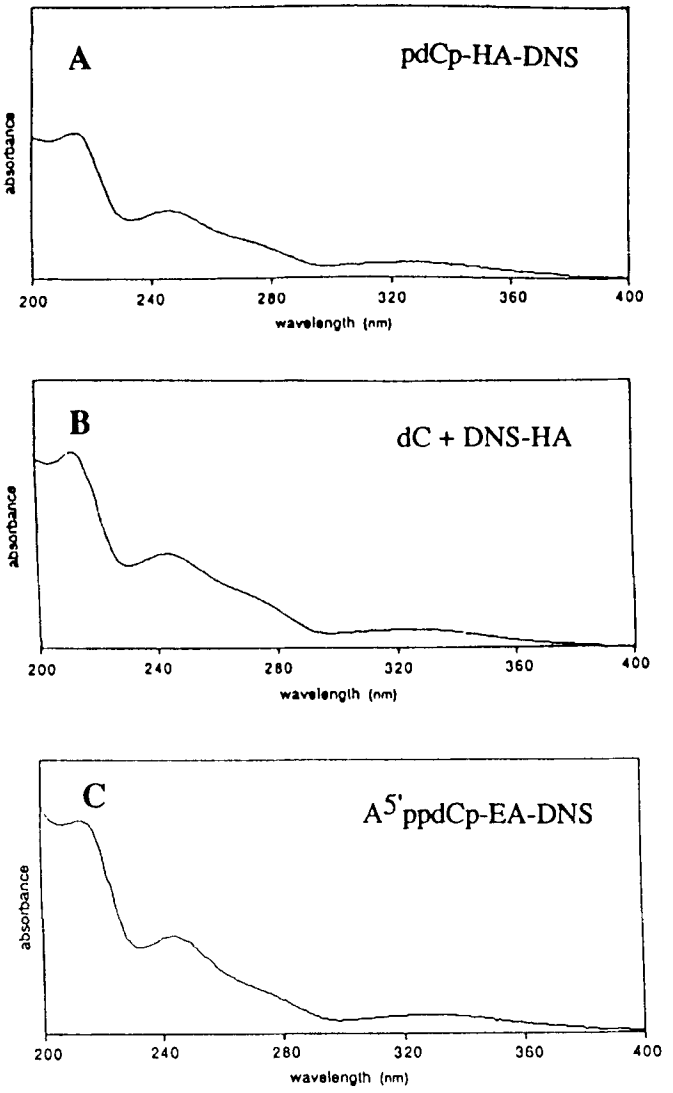

FIG. 1. The UV spectra of DNS derivatives

of deoxycytidine and 6-(*N*-dansylamino)hexanol. The fluorescence spectrum of **1** is shown in FIG. 2A. The reverse-phase HPLC analyses of this material by UV and fluorescence detectors are shown in FIG. 3. In the HPLC profile (FIG. 3A) monitored by UV, the peak of **1** appeared at a retention time of ca. 30 min, suggesting the great contribution of the DNS group to the lipophilicity of the molecule as discussed before.

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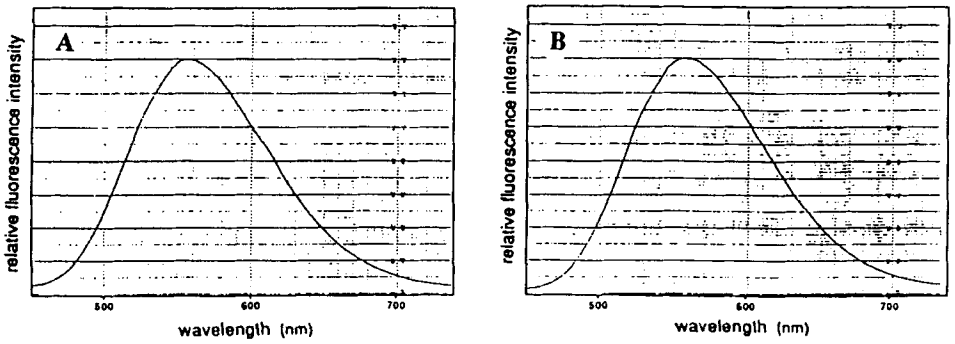

FIG. 2. The fluorescence spectra of **1** (A) and **2** (B).

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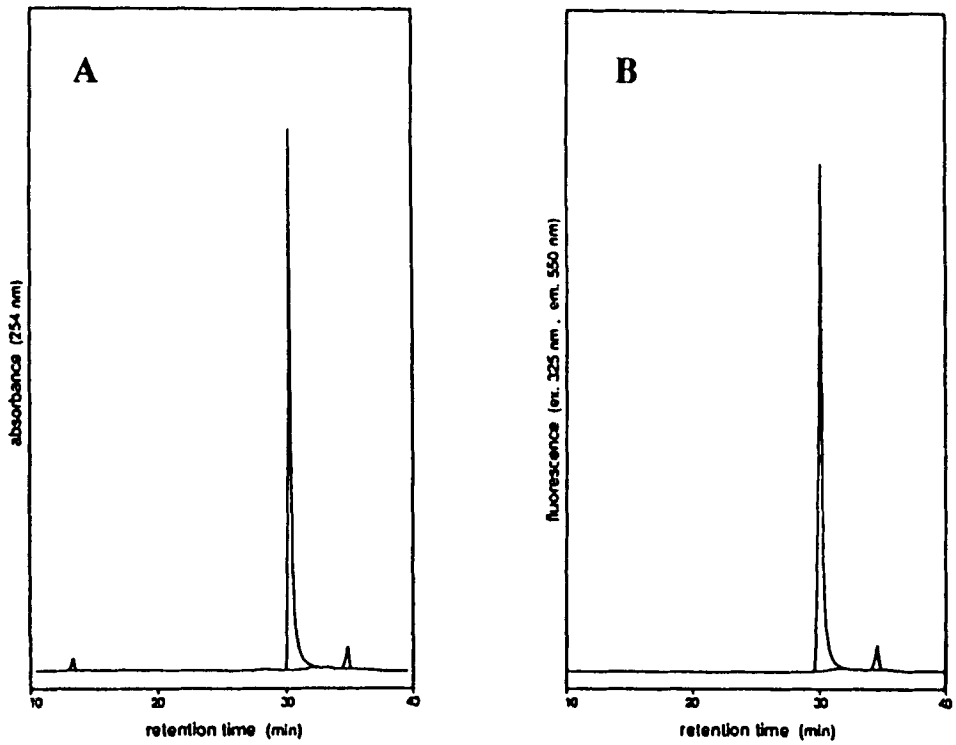
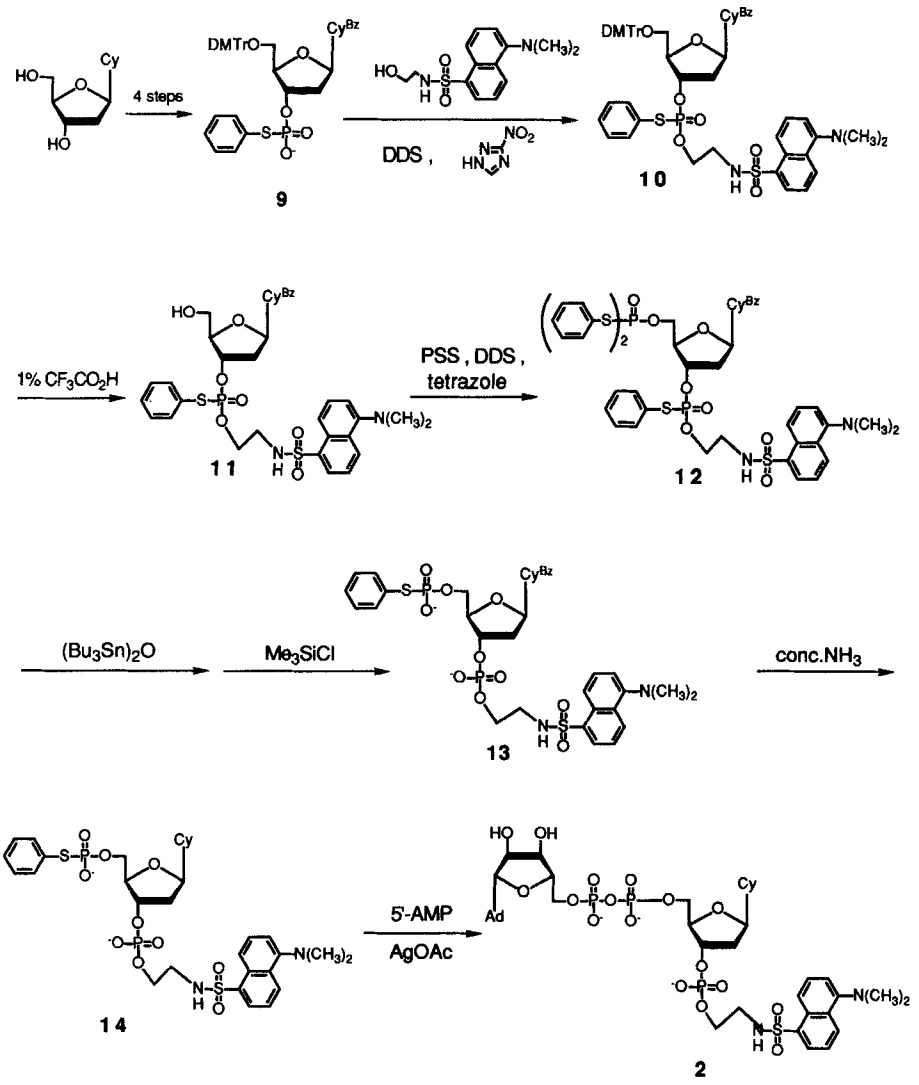


FIG. 3. The reverse-phase HPLC profile of **1** (A: UV, B: fluorescence)



Scheme 2

Synthesis of $A^{5'}$ppdCp-HA-DNS

5'-Adenylated oligoribonucleotides or deoxyribonucleotides are known to be better substrates in T4 RNA ligase catalyzed joining.[21-23] This enables us to avoid the use of ATP in the joining reaction. Therefore, we also synthesized compound **2** as shown in Scheme 2. In this experiment, the linker between the 3'-phosphate group and the DNS group was changed from the hexamethylene group to the ethylene one to test if the quenching occurred when the shoter spacer was used.

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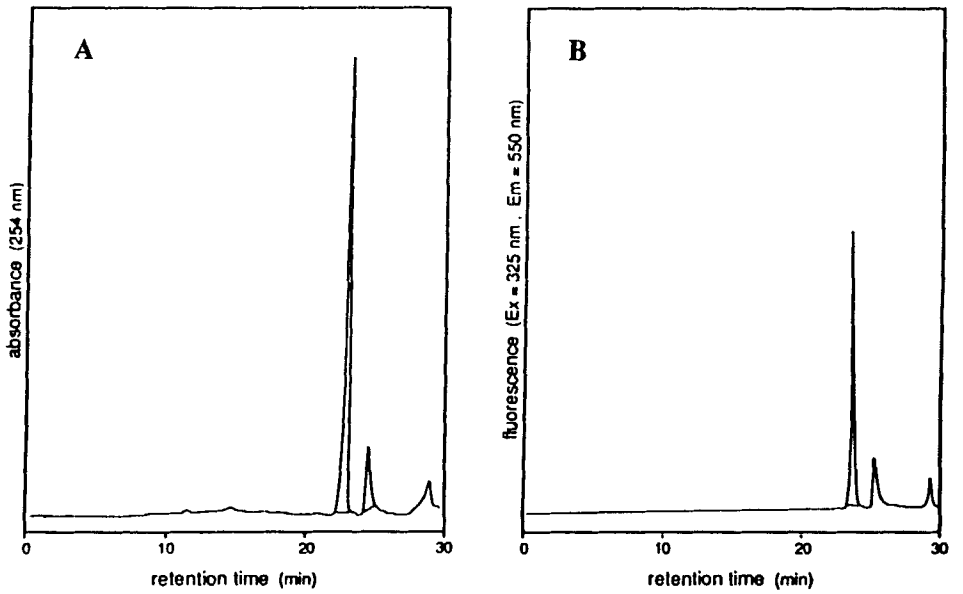

FIG. 4. The reverse-phase HPLC profile of crude **2** (A: UV, B: fluorescence)

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The key intermediate (**13**) was synthesized *via* a 6-step reaction in a manner similar to that described in the case of **1**.Compound **13** was deacylated by treatment with ammonia to give compound **14**. Condensation of **14** with 5'-AMP was carried out in the presence of silver acetate in DMF-pyridine. Reverse-phase column chromatography gave compound **2** as a crude material in 28% yield. The HPLC analysis of this sample suggested that it contained a byproduct. The byproduct was probably a pyrophosphate derivative, $[p(dC)p\text{-}EA\text{-}DNA]_2$, since the relative ratio of the peak intensity of the byproduct in the fluorescence and UV spectra was higher than that of the corresponding peaks of **2**. Such symmetric pyrophosphate products were often observed in this type of unsymmtric pyrophosphate bond formation. Further purification of **2** by repeated reverse-phase column chromatography has failed although TLC showed a sufficient difference in their Rf values. Several papers concerning the mechanism of T4 RNA ligase catalyzed joining reactions suggested that T4 RNA ligase recognized strongly the adenylic acid residue of adenylated ribonucleotides (A^5'ppNu) so that A^5'ppNu were considerably better substrates compared with diribonucleoside pyrophosphate derivatives represented by Nu^5'ppNu' in which Nu was not adenosine but another ribonucleoside.[10-14] On the basis of these reasons, it seemed to us that the symmetric pyrophosphate mentioned above did not interfere with the ligation and remained intact. Therefore, we used this crude material

for labeling of GpApC. During the synthesis of **2**, DNS-containing synthetic intermediates **10-14** as well as **2** could be easily detected as fluorescence spots on TLC by UV iradiation. The sensitivity was almost similar to that of compounds **4-8** as well as **1**. Therefore, it was concluded that both the ethylene and hexamethylene groups could be used as linkers without significant quenching of the DNS group for this kind of studies.

3'-Labeling of GpApC with pdCp-HA-DNS and A$^{5'}$ppdCp-HA-DNS

The 3'-Labeling of an oligoribonucleotide, GpApC, with DNS-containing deoxycytidine 3',5'-bisphosphate derivatives **1** and **2** was studied. Ligations under the standard conditions were monitored at the reaction times of 1 h, 4 h, 8 h, and 12 h by reverse-phase HPLC as shown in FIG. 5. These results are summarized in Table 1. As seen from Table 1, the efficiency of ligation after 1 h in the case of **2** is better than that in the case of **1** but both reactions of GpApC with **1** and **2** could reach to completion when the mixtures were incubated for 8 h and 12 h, respectively. In the case of the ligation of GpApC with **2**, it was confirmed that the pyrophosphate $[p(dC)p\text{-}EA\text{-}DNA]_2$ (retention time: ca. 24 min) remained intact during the ligation as seen in FIG. 5B. The ligated products were detected easily as materials having both UV and fluorescence absorbance by UV and fluorescence detectors attached to HPLC, since the ratio of UV to fluorescence intensities in the ligated materials always increased compared with that in the starting DNS donor molecule **1** or **2**. Enzyme analysis of these products was performed by the use of nuclease P1. For instance, enzyme degradation of GpApCp-dCp-HA-DNS, which was separated by reverse-phase HPLC, with nuclease P1 gave an expected mixture of G, pA, pC, pdC and p-HA-DNS as shown in FIG. 6.

In order to examine the fluorescence sensitivity of the DNS group linked to the 3'-termini of oligoribonucleotides at the 3'-terminal position, the reaction of GpApC with p-dCp-EA-DNS was analyzed after incuvation for 55 h by polyacrylamide gel electrophoresis as shown in FIG. 7. Direct detection of the ligation product with eyes was possible at the level of 10 pmol although FIG. 7 did not give a clear spot after taking a picture which showed that the limitation of detection was 100 pmol. This order in sensitivity would be still sufficient for studies such as mechanistic elucidation of ribozymes using electrophoresis. In conclusion, it should be noted that the DNS-containing cytidylate derivatives **1** and **2** would be used as 3'-labeling reagents of oligoribonucleotides for biological studies. The lipophilc property of the DNS group would be helpful for isolation of 3'-labeled materials by the use of reverse-phase HPLC

EXPERIMENTAL

^{1}H NMR spectra were recorded at 60 MHz on a Hitachi 24B spectrometer and at 500 M Hz on a JEOL-GX500 spectrometer with Me_4Si or DSS as the external reference. UV

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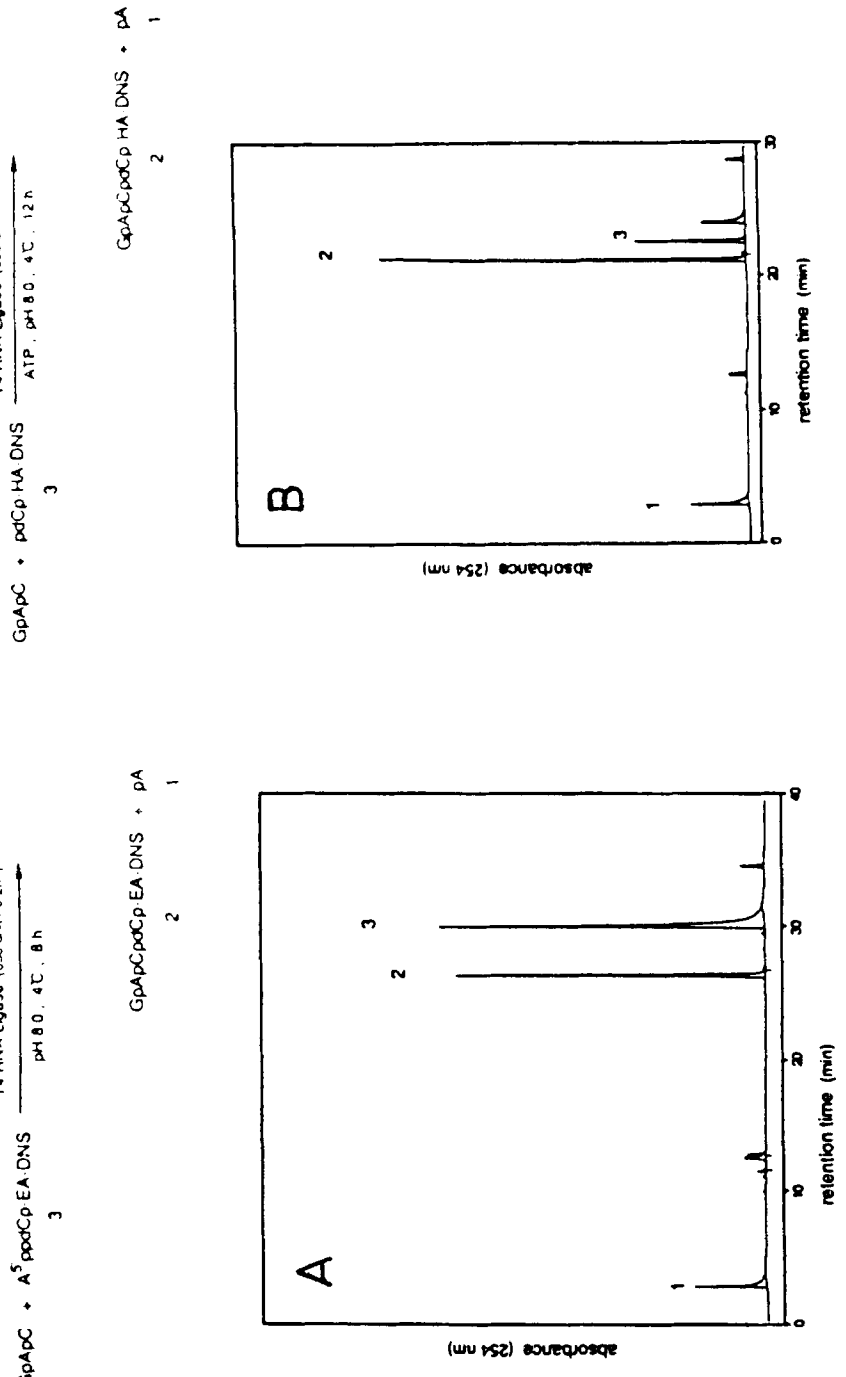

FIG. 5. The reverse-phase HPLC of the mixture obtained after ligations of GpApC with **1** (A) and **2** (B)

TABLE 1. Time course of 3'-labeling reaction of GpApC with **1** and **2**

dansyl derivative	T4 RNA ligase (unit/0.2 ml)	time	yield of product (%)
pdCp-HA-DNS (**1**)	65	1 h	49
pdCp-HA-DNS (**1**)	65	4 h	90
pdCp-HA-DNS (**1**)	65	8 h	99
pdCp-HA-DNS (**1**)	65	12 h	100
$A^{5'}$ppdCp-EA-DNS (**2**)	65	1 h	70
$A^{5'}$ppdCp-EA-DNS (**2**)	65	4 h	97
$A^{5'}$ppdCp-EA-DNS (**2**)	65	8 h	100

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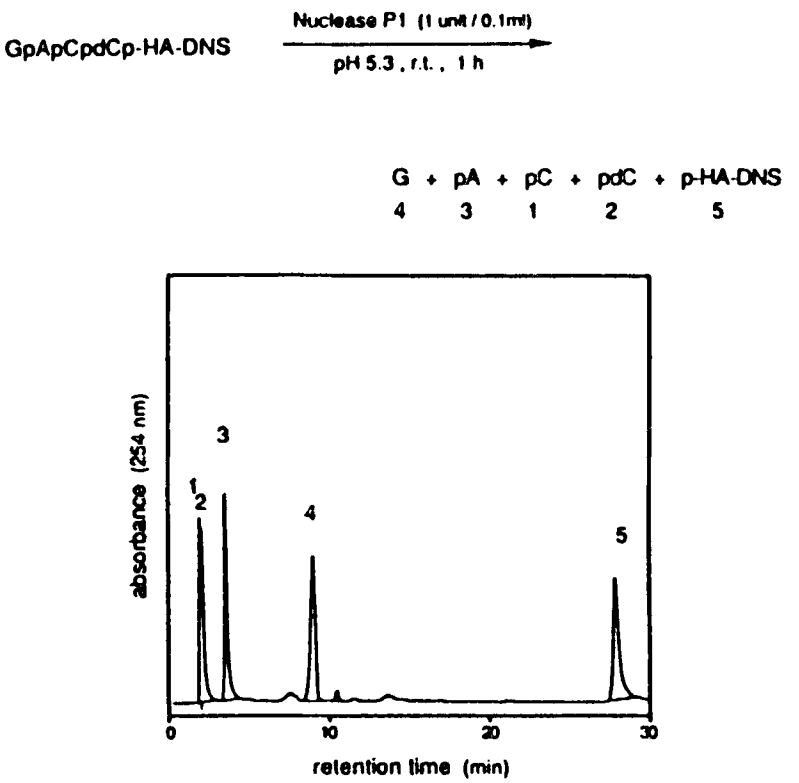

FIG. 6. The enzyme assay of GpApCp-dCp-HA-DNS with nuclease P1

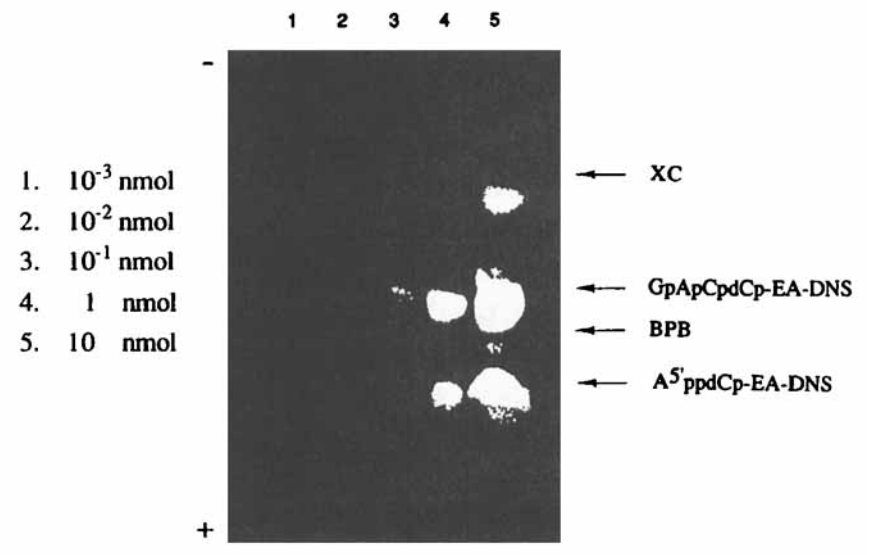

FIG. 7. Polyacrylamide gel electrophresis of the mixture obtained after GpApC was incubated with **2** in the presence of T4 RNA ligase.

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spectra were obtained on a Hitachi U2000 spectrophotometer. The UV spectra of the two compounds (pdCp-HA-DNS and A5'ppdCp-EA-DNS) and the 1:1 mixture (dC + DNS-HA) shown in FIG.1 were measured by dissolving them in water at appropriate concentrations. The fluorescence spectra of **1** and **2** were obtained on a Nihon Bunkou Fluorescence spectrophotometer by dissolving them in water at appropriate concentrations. Paper chromatography was performed by use of a descending technique with Whatman 3MM papers and Toyo Roshi 51 papers using the following solvent systems: 2-propanol-conc. aqueous ammonia-water, 7:1:2, v/v/v. Column chromatography was performed with silica gel C-200 purchased from Wako Co., Ltd., and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. TLC was performed on precoated TLC plates of silica gel 60 F-254 (Merck). Reverse-phase HPLC was performed on a Waters LC Module 1 apparatus using a μBondasphere C_{18} column at 50 °C with a linear gradient starting from 0.1 M NH_4OAc, pH 7.0 and applying CH_3CN at a flow rate of 1.0 mL/min for 30 min (System A). The HPLC profiles depicted in FIGS. 3, 4, and 6 were obtained under the conditions of System A. were obtained under the conditions of System A. Uridine was purchased from Yamasa Co., Ltd. Pyridine was distilled two times from *p*-toluenesulfonyl chloride and from calcium hydride and then stored over molecular sieves 3A. T4 RNA ligase was purchased from Takara

Shuzou Co. Ltd. and nuclease P1 was purchased from Yamasa Shouyu Co. Ltd. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

***6-(N*-Dansylamino)hexanol.** A mixture of dansyl chloride (1.35 g, 5 mmol) and 6-amino-1-hexanol (1.17 g, 10 mmol) was dissolved in dry pyridine (20 ml) and heated at 70 °C for 30 min. Water was added and the solution was adjusted to pH 3~4. Extraction was performed 3 times with CH_2Cl_2. The organic phase was collected, dried over Na_2SO_4, and filtered. The filtrate was evaporated and the residue was chromatographed on a column of silica gel with CH_2Cl_2-MeOH to give the title compound[24] (1.70 g, 97%) as oil: ^1H NMR (60 MHz, $CDCl_3$) δ 0.80-2.07 (10H, m, CH_2), 2.87 (6H, s, $N(CH_3)_2$), 3.45 (2H, t, J = 5.8Hz, CH_2NH), 5.03 (1H, m, $NHSO_2$), 6.90-7.61 (3H, m, ArH), 8.00-8.65 (3H, m, ArH) . Anal. Calcd. for $C_{18}H_{26}N_2O_3S \cdot 1/4H_2O$: C, 60.91; H, 7.53; N, 7.89. Found: C, 61.12; H, 7.67; N, 7.80.

5'-*O*-(4,4'-Dimethoxytrityl)-4-*N*-anisoyldeoxycytidine(3')-(*S*-Phenyl)thiophospho-6-(*N*-dansylamino)hexanol (4). A mixture of the O,S-diester (3) (0.937 g, 1 mmol) , 6-(*N*-dansylamino)hexanol (0.526 g, 1.5 mmol), and 3-nitro-1*H*-1,2,4-triazole (0.228 g, 2 mmol) was rendered anhydrous by repeated coevaporations with dry pyridine and finally dissolved in dry pyridine (10 ml). To the mixture was added DDS (0.994 g, 3 mmol), and the resulting solution was stirred at room temperature for 30 min. The solvent was removed under reduced pressure and the residue was partitioned between CH_2Cl_2-sat. $NaHCO_3$. The organic layer was collected and the aqueous phase was extracted with $CHCl_3$. The organic extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (20 g) with CH_2Cl_2-MeOH to give **4** (1.09 g, 93%): ^1H NMR (60 MHz, $CDCl_3$) δ 0.65-1.77 (8H, m, CH_2), 2.00-2.52 (1H, m, 2'-H), 2.83 (6H, s, $N(CH_3)_2$), 3.36 (2H, m, 5'-H), 3.63-4.37 (5H, m, sugar-H), 3.72 and 3.79 (9H, s each, CH_3O) , 4.71-5.40 (1H, m, 3'-H), 5.40-6.29 (2H, m, 5-H and 1'-H), 6.80-8.59 (30H, m, ArH) . Anal. Calcd. for $C_{61}H_{66}N_5O_{11}PS_2 \cdot H_2O$: C, 63.25; H, 5.91; N, 6.05; S, 5.54. Found: C, 63.09, H, 5.80; N, 6.36; S, 5.40.

[4-*N*-Anisoyldeoxycytidine(3')]-(*S*-Phenyl)thiophospho-[6-(*N*-dansylamino)hexanol] (5). A solution of **4** (0.584 g, 1.5 mmol) in CH_2Cl_2 (22 ml) was treated with trifluoroacetic acid (0.22 ml) at 0 °C for 90 min. The mixture was diluted with CH_2Cl_2 and the CH_2Cl_2 solution was washed 3 times with sat. $NaHCO_3$.

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The aqueous layer was combined and reextracted with $CHCl_3$. The organic extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Chromatography on a column of silica gel (12 g) gave **5** (0.402 g, 93%): ^1H NMR (60 MHz, $CDCl_3$) δ 0.93-1.71 (8H, m, CH_2), 2.00-3.20 (10H, m, contains $N(CH_3)_2$ at 2.82), 3.57-4.27 (5H, m, sugar-H), 3.77 (3H, s, CH_3O), 4.87-5.37 (1H, m, 3'-H), 5.83-6.27 (2H, m, 5-H and 1'-H), 6.69-8.43 (16H, m, ArH).

[5'-*O*-Bis(phenylthio)phosphoryl-4-*N*-anisoyldeoxycytidine(3')]-(S-phenyl)thiophospho-[6-(*N*-dansylamino)hexanol] (6). A mixture of **5** (0.401 g, 0.464 mmol), PSS (0.266 g, 0.696 mmol), and 1*H*-tetrazole was rendered anhydrous by repeated coevaporations with dry pyridine, and finally dissolved in dry pyridine (17 ml). DDS (0.461 g, 1.39 mmol) was added to the mixture. After being stirred at room temperature for 30 min, the solution was evaporated and the residue was dissolved in CH_2Cl_2. The CH_2Cl_2 solution was washed 3 times with sat. $NaHCO_3$. The aqueous layers were combined and reextracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed to give **6** (0.46 g, 88%): ^1H NMR (60 MHz, $CDCl_3$) δ 0.83-2.27 (10H, m, CH_2, 2'-H), 2.50-3.22 (2H, m, sugar-H and CH_2), 2.83 (6H, s, $N(CH_3)_2$), 3.27-4.67 (5H, m, sugar-H and CH_2), 3.80 (3H, s, CH_3O), 4.77-5.35 (1H, m, 3'-H), 5.75-6.36 (2H, m, 5-H and 1'-H), 6.66-8.55 (26H, m, ArH) . Anal. Calcd. for $C_{52}H_{57}N_5O_{11}P_2S_4 \cdot H_2O$: C, 55.75; H, 5.31; N, 6.25; S, 11.45. Found: C, 55.72; H, 5.06; N, 6.29; S, 11.44.

Diammonium Salt of [5'-*O*-[Phenylthio(hydroxy)phosphoryl]deoxycytidine(3')]-phospho-[6-(*N*-dansylamino)hexanol] (8). Compound **6** (0.226 g, 0.2 mmol) was dissolved in pyridine (6 ml) and bis(tributyltin) oxide (5.0 ml, 10 mmol) was added. After being stirred at room temperature for 2 h, the mixture was treated with chlorotrimethylsilane (1.2 ml, 20 mmol) at room temperature for 5 min. The solvent was removed under reduced pressure and the residue was dissolved in pyridine-water (4:1, v/v, 20 ml). The solution was washed 3 times with hexane (20 ml). The aqueous layer was concentrated under reduced pressure. The residue was dissolved in pyridine (26 ml) and conc. ammonia (52 ml) was added. After being kept at room temperature for 24 h, the mixture was evaporated under reduced pressure. The residue was dissolved in water and applied to a column of C-18 silica gel (Waters, 12 g). Elution was performed by stepwise addition of 0~50% methanol-containing water to give **8** (0.17 g, quant.). This material was used in the next experiment.

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Trisodium salt of [5'-*O*-(dihydroxyphosphoryl)deoxycytidine(3')]-phospho-[6-(*N*-dansylamino)hexanol] (1). Compound **8** (50 mg, 59.1 mmol) was dissolved in pyridine-water (2:1, v/v, 2 ml) and silver acetate (0.395 g, 2.36 mmol) was added. After being stirred vigorously at room temperature for 1 h, the mixture was diluted with pyridine-water (2:1, v/v, 8 ml) and bubbled by hydrogen sulfide gas with cooling until a clear supernatant solution had been obtained. The xcess hydrogen sulfide gas was removed by evaporation for 5 min and the resulting precipitate was centrifuged. The supernatant was decanted and evaporated under reduced pressure. The residue was chromatographed on a column of C-18 silica gel (Waters) with 0~50% methanol-containing water to give fractions containing the pyridinium salt of **1**. The fractions were collected, and combined, and evaporated under reduced pressure, and dissolved in water. The solution was passed through a column of Dowex 50 W-X8, 100-200 mesh, Na form). The column was eluted with water. The eluate was lyophilized to give trisodium salt of **1** (89%): ^{1}H NMR (500 MHz, D_2O) δ 0.84-0.91 (4H, m, γ-CH_2), 1.10-1.19 (4H, m, β-CH_2), 2.32 (1H, ddd, J = 5.8, 8.5, 14.1 Hz, 2'-H), 2.44 (1H, ddd, J = 0.6, 5.8, 14.1Hz, 2'-H), 2.86 (6H, s, $N(CH_3)_2$), 2.92 (2H, m, 5'-H), 3.64 (2H, m, CH_2O), 3.93 (2H, m, CH_2N), 4.33 (1H, m, 4'-H), 4.81 (1H, m, 3'-H), 6.14 (1H, d, J = 7.3Hz, 5-H), 6.37 (1H, dd, J = 5.8, 8.5Hz, 1'-H), 7.40 (1H, d, J = 7.6Hz, ArH), 7.66 (1H, t), 7.68 (1H, t), 8.09 (1H, d, J = 7.6Hz, ArH), 8.22 (1H, d, J = 7.3Hz, 6-H), 8.29 (1H, d, J = 8.5Hz, ArH), 8.48 (1H, d, J = 8.5Hz, ArH): UV(H_2O) λmax 245 nm, 329 nm, λmin 232 nm, 297 nm.

2-(*N*-Dansylamino)ethanol. Dansyl chloride (0.50 g, 1.85 mmol) was added to a 33% aqueous solution (15 ml, 74 mmol) of ethanolamine. The mixture was heated at 80 °C for 20 min and cooled to room temperature. The solution was diluted with water and adjusted to pH 3~4 by addition of 1 M HCl. Extraction was performed 3 times with CH_2Cl_2-BuOH (5:1, v/v) and the organic extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Chromatography on a column of silica gel (10 g) with CH_2Cl_2-MeOH gave the title compound (0.532 g, 98%) : ^{1}H NMR (60 MHz, $CDCl_3$) δ 2.37 (1H, m), 2.82 (6H, s, $N(CH_3)_2$), 3.00 (2H, t, J = 4.8Hz, CH_2N), 3.52 (2H, t, J = 4.8Hz, CH_2O), 5.42 (1H, m), 6.91-7.52 (3H, m, ArH), 8.00-8.43 (3H,m, ArH)

[5'-*O*-(4,4'-Dimethoxytrityl)-4-*N*-benzoyldeoxycytidine(3')]-(*S*-phenyl)thiophospho-[6-(*N*-dansylamino)ethanol] (10). A mixture of the *O,S*-diester **9** (0.907 g, 1 mmol), 2-(*N*-dansylamino)ethanol (0.442 g, 1.5 mmol), and 3-nitro-1*H*-1,2,4-triazole (0.232 g, 2 mmol) was rendered anhydrous by repeated

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coevaporations with dry pyridine and finally dissolved in dry pyridine (10 ml). DDS (0.994 g, 3 mmol) was added and the mixture was stirred at room temperature for 25 min. The same workup as described in the case of the synthesis of **4** followed by chromatography gave **10** (0.916 g, 85%): ^{1}H NMR (60 MHz, $CDCl_3$) δ 1.56-4.31 (12H, m, sugar H and CH_2), 2,79 (6H, s, $N(CH_3)_2$), 3.68 (3H, s, CH_3O), 4.88-5.35 (1H, m, 3'-H), 6.61-8.54 (17H, m, ArH and 1'-H).

[4-*N*-Benzoyldeoxycytidine(3')]-(S-phenyl)thiophospho-[2-(*N*-dansylamino)ethanol (11). A solution of **10** (0.916 g, 0.847 mmol) in CH_2Cl_2 (30 ml) was treated with trifluoroacetic acid (0.3 ml) at 0 °C for 30 min. The mixture was diluted with CH_2Cl_2 and the CH_2Cl_2 solution was washed 3 times with sat. $NaHCO_3$. The aqueous layers were combined and reextracted with $CHCl_3$. The organic extracts were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Chromatography on a column of silica gel (20 g) gave **11** (0.531 g, 80%): ^{1}H-NMR (60 MHz, $CDCl_3$) δ 2.77 (6H, s, $N(CH_3)_2$), 3.13 (2H, m, 5'-H), 3.76 (2H, s, CH_2O), 4.06 (2H, s, CH_2N), 4.85-5.52 (1H, m, 3'-H), 6.62-8.53 (17H, m, 1'-H and ArH) .

[5'-*O*-Bis(phenylthio)phosphoryl-4-*N*-benzoyldeoxycytidine(3')]-(*S*-phenyl)thiophospho-[2-(*N*-dansylamino)ethanol] (12). A mixture of **11** (0.156 g, 0.2 mmol), PSS (0.115 g, 0.3 mmol), and 1*H*-tetrazole (0.056 g, 0.8 mmol) was rendered anhydrous by repeated coevaporations with dry pyridine, and finally dissolved in dry pyridine (8 ml). DDS (0.199 g, 0.6 mmol) was added to the mixture and the mixture was stirred at room temperature for 30 min. The same workup described in the case of 6 followed by chromatography gave **12** (0.149 g, 72%): ^{1}H NMR (60 MHz, $CDCl_3$) δ 1.56-2.19 (2H, m, 2'-H), 2.27-3.37 (2H, m, 2'-H), 2.81 (6H, s, $N(CH_3)_2$), 3.77-4.55 (5H, m, CH_2O, CH_2N and 4'-H), 4.64-5.30 (1H, m, 3'-H), 5.78-6.34 (1H, m, 5-H), 6.40-6.80 (1H, m, 1'-H), 6.96-8.55 (27H, m, ArH) . Anal. Calcd. for $C_{48}H_{47}N_5O_{10}P_2S_4$: C, 55.22; H, 4.54; N, 6.71; S,12.28. Found: C, 55.14; H, 4.54; N, 6.83; S, 12.08.

Diammonium Salt of [5'-*O*-[Phenylthio(hydroxy)phosphoryl]deoxycytidine(3')]-phospho-[2-(*N*-dansylamino)ethanol] (14). Compound **12** (0.627 g, 0.6 mmol) was dissolved in pyridine (18 ml) and bis(tributyltin) oxide (15.3 ml, 30 mmol) was added. After being stirred at room temperature for 3 h, the mixture was treated with chlorotrimethylsilane (7.6 ml, 60 mmol) at room temperature for 5 min. Then conc. ammonia (5 ml) was added and stirring was continued for 5 min. The solvent was removed under reduced pressure and the residue was dissolved in pyridine-water (2:1, v/v,

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20 ml). The solution was washed 3 times with hexane (20 ml). The aqueous layer was concentrated under reduced pressure. The residue was dissolved in pyridine (60 ml) and conc. ammonia (120 ml) was added. After being kept at room temperature for 2 days, the mixture was evaporated under reduced pressure. The residue was dissolved in water and applied to a column of C-18 silica gel (Waters, 36 g). Elution was performed by stepwise addition of 0~50% methanol-containing water to give **14** (1830 A_{322}, 70%): ^{1}H-NMR (500 MHz, D_2O) δ 1.81-1.84 (1H, m, 2'-H), 2.10-2.14 (1H, m, 2'-H), 2.85 (6H, s, $N(CH_3)_2$), 3.15 (2H, m, 5'-H), 3.73 (2H, m, CH_2O), 3.91 (1H, m, 4'-H), 3.99-4.04 (2H, m, CH_2N), 4.49 (1H, m, 3'-H), 5.85 (1H, d, J = 7.6Hz, 5-H), 6.10 (1H, m, 1'-H), 7.17 (3H, m, ArH), 7.37-7.43 (3H, m, ArH), 7.61-7.68 (3H, m, ArH), 8.24 (1H, d, J = 7.6Hz, 6-H), 8.32 (1H, d, J = 8.8Hz, ArH), 8.40 (1H, d, J = 8.2Hz, ArH): UV(H_2O) λmax 243 nm, 322 nm, λmin 235 nm, 307 nm.

Trisodium Salt of Adenosine(5')-diphospho-(5')deoxycytidine(3')-phospho-[2-(*N*-dansylamino)ethanol] (2). The diammonium salt of **14** (450 A_{322} unit, 0.1 mmol) and disodium salt of 5'-AMP were converted to the pyridinium salts by using ion exchange resin (Dowex 50 W-X2, 100-200 mesh, pyridinium form). They were mixed with trioctylamine (0.874 ml, 4 mmol) and the mixture was rendered anhydrous by repeated coevaporations with dry pyridine. The dried mixture was dissolved in dry pyridine (6 ml) and silver acetate (0.50 g, 3 mmol) was added. After being stirred at room temperature for 1 h, the mixture was bubbled with hydrogen sulfide gas at 0 °C until a clear supernatant solution had been obtained. The resulting silver sulfide was removed by centrifuge and the supernatant was collected. The clear solution was evaporated and chromatographed on a reverse-phase C-18 column (Waters, 16 g) with a stepwise gradient of 0~20% methanol in 0.1 M ammonium acetate to give fractions containing **2**. The fractions were combined and lyophilized several times from its aqueous solution to remove ammonium acetate. The lyophilized sample was dissolved in water and passed through Dowex 50W-X8, 100-200 mesh, Na form) to give trisodium salt of **2** (145 A_{322}, 28%, 85% pure): UV(H_2O) λmax 250 nm, 325 nm, λmin 233.5 nm, 305 nm. The fluorescence spectrum of this material is shown in FIG. 2.

Ligation of GpApC with DNS-Containing pCp Derivatives. In an eppendorf tube, GpApC (0.05 μmol) and an appropriate labeling reagent (0.1 μmol) were dissolved in 20 μl of 0.5 M Tris - HCl (pH 8.0) containing 0.1 M $MgCl_2$ and 20 μl of 0.1 M DTT. In the case of ligation using **1**, 0.3 μl of 10 mM ATP was added. The mixture was diluted with sterile water to a volume of 200μl. Then T4 RNA ligase (65 unit, 1.3 μl) was added and the resulting mixture was incubated at 4 °C. At an appropriate time, part of

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the mixture was taken , heated at 100 °C for 30 sec, and analyzed or purified by reverse-phase HPLC.

Enzyme Assay of GpApCp-dCp-HA-DNS. A freeze dried sample of GpApCp-dCp-HA-DNS (10 ml, 1 nmol) was dissolved in 39 µl of 20 mM AcOH-AcONa buffer (pH 5.3) containing 0.1 mM $ZnCl_2$. Nuclease P1 (1 unit, 1 µl) was added and the mixture was incubated at room temperature for 60 min. The mixture was heated at 100 °C for 30 sec and analyzed by reverse phase HPLC.

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