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PREPARATION OF A NEW PHOSPHORYLATING AGENT: S-(N-MONOMETHOXYTRITYLAMINOETHYL)-O-(o-CHLOROPHENYL)PHOSPHOROTHIOATE AND ITS APPLICATION IN OLIGONUCLEOTIDE SYNTHESIS

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Abstract - A new phosphorylating agent, S-(N-monomethoxytritylaminoethyl)-O-(o-chlorophenyl)phosphorothioate (3), was prepared. It was used for phosphorylation with a 5'-hydroxyl group of an oligonucleotide. After base labile protecting groups were removed, the partially deprotected oligonucleotide was separated and converted to the oligonucleotide with an aminoethyl or a phosphoryl group at the 5'-end by treatment with 80% acetic acid or iodine-water, respectively. By using 14 as a linker between a 3'-hydroxyl group and a resin, an oligonucleotide with a phosphate at the 3'-end was prepared.

The syntheses of ppT, pppT, A5'pp5'T and A5'ppp5'T were also performed by treatment of 5'-O-(N-monomethoxytritylaminoethylthiophosphoryl)thymidine with tri-n-octylammonium salt of phosphoric acid, pyrophosphoric acid, pA and ppA, respectively.

INTRODUCTION

Because the synthetic methods for oligodeoxyribonucleotide are well established, the demand for the synthesis of modified oligonucleotide is increasing. As the modification of 5'-hydroxyl group, oligonucleotides containing an aminoalkyl[1-8], a mercapto[8-11], an aldehyde[12] or a phosphoryl group[10,13-16] at the 5'-end were prepared. The oligonucleotide with an aliphatic amino or mercapto group at the 5'-end, where the amino or mercapto group was attached to a fluorescent compound, is used as a primer for DNA sequence determination[17,18]. When the amino group of the oligonucleotide is linked to biotin[19-21] or ethylenediaminetetraacetic acid (EDTA)[1], it is also used as a hybridization probe for a certain DNA or in DNA strand cleavage studies. The oligonucleotide with a phosphoryl group at the 5'-end is the substrate for DNA ligase reaction[14,16]. It is directly used for the synthesis[10] of a protein gene. Therefore, the oligonucleotide with an aminoalkyl or a phosphoryl group at the 5'-end is useful in biological studies.

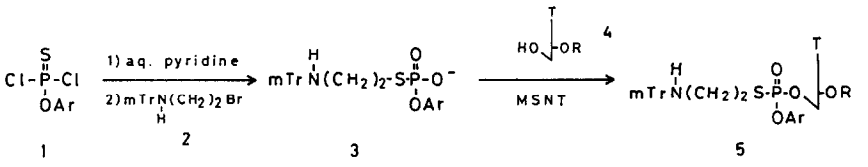
Here we try to prepare two compounds from a common intermediate. In order to prepare the intermediate, N-monomethoxytritylaminoethylthio group is employed for the terminal phosphoryl protection of an oligonucleotide. In this system, the 5'-terminal amino and phosphoryl groups are generated by treatment with an acid[5,16] and iodine[22], respectively. We report the preparation of the phosphorylating agent, S-(N-monomethoxytritylaminoethyl)-O-(o-chlorophenyl)phosphoro-

thioate, and its applications to the synthesis of the 3'-phosphorylated oligonucleotide and nucleoside 5'-di- and tri-phosphate.

RESULTS AND DISCUSSION

Synthesis of phosphorylating agent (3)

In our previous paper[23], 3'-O-levulinylthymidine-5'-o-chlorophenyl-phosphoranilidate was successively treated with NaH, CS_2 and N-monomethoxytritylaminoethylbromide (2) to prepare 5a, a common intermediate for the synthesis of oligonucleotide with an aminoalkyl or a phosphoryl group at the 5'-end, according to the reported procedure. However, this procedure needs lots of manipulations and the yield of 5a was relatively low (32%). We tried to develop an another method. An o-chlorophenylphosphorodichloridothioate (1) was prepared by the reaction of o-chlorophenylphosphorodichloridite with $PSCl_3$ according to the Tolkmith's procedure[24]. After hydrolysis of 1 with aqueous pyridine and the subsequent treatment of 2, a phosphorylating agent (3) was obtained with 46% yield. A mixture of 3'-O-benzoylthymidine (4b) and 3 (2 eq.) was treated with 1-mesitylene-sulfonyl-3-nitrotriazole (MSNT) (3 eq.) in pyridine for 1 hr, and compound 5b was obtained in 75% yield. When thymidine bound to polystyrene (4c) was treated with 3 (6 eq.) and MSNT for 1 hr, the phosphorylation yield of 94 % was obtained using the trityl cation assay procedure.



Ar = o-chlorophenyl
mTr = monomethoxytrityl

R = a, $-C(O)(CH_2)_2C(O)CH_3$
b, $-C(O)C_6H_5$
c, $-C(O)(CH_2)_2C(O)NHCH_2$~Ⓟ

Chart 1

In order to show the usefulness of this phosphorylating agent in the synthesis of oligonucleotides, a hexadeoxyribonucleotide (TCTCTC) was prepared on a polystyrene support by succesive condensation of properly protected CT, CT and T to the C-resin using the standard phosphotriester method[25]. Then a mixture of 3 (6 eq.) and the hexamer resin (6) was treated with MSNT for 1 hr. The resin was treated with 0.5M N^1,N^1,N^3,N^3-tetramethyl-guanidinium syn-pyridine-2-aldoxi-

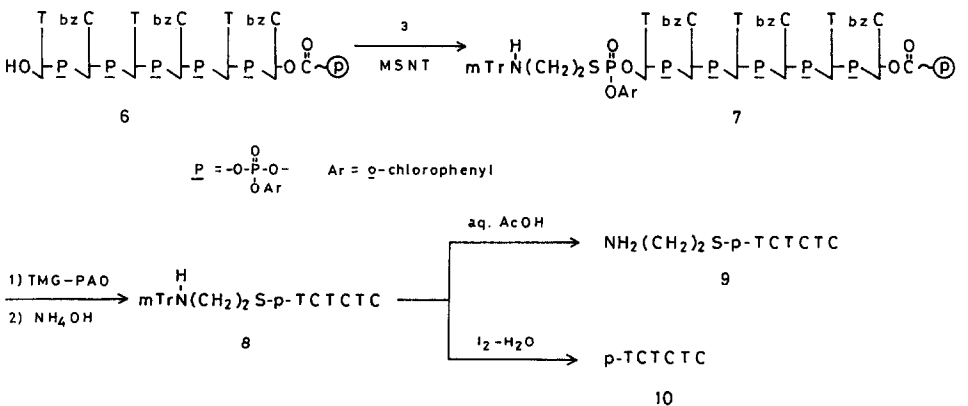


Chart 2

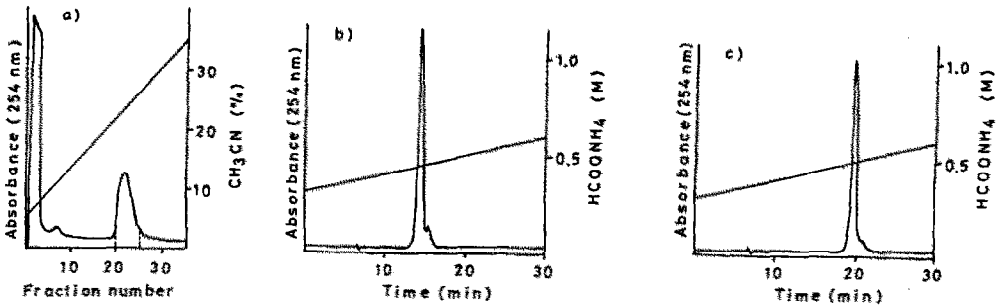


Fig 1. a) Separation of mTrNH$(CH_2)_2$SpTpCpTpCpTpC (8) by reversed-phase column chromatography. Analysis of b) $NH_2(CH_2)_2$SpTpCpTpCpTpC (9) and c) pTpCpTpCpTpC (10) by anion exchange HPLC.

mate (TMG-PAO) for 15 hr, followed by NH_4OH at 55°C for 6 hr to remove benzoyl and o-chlorophenyl groups and to release the nucleotidic compounds from the resin. Compound 8 was separated on a C-18 silica gel column using a linear gradient of CH_3CN in 50 mM triethylammonium acetate (TEAA) (pH 7) (Fig. 1). The partially deblocked hexamer (8) was easily separated from the truncated oligonucleotide and non-nucleotidic materials. Then 8 was subjected to two different deblocking procedures. When 8 was treated with 80% acetic acid for 1 hr, 5'-aminoethylated hexamer (9) was obtained in 82% yield. On the other hand, when 8 was treated with iodine-water for 4 hr, 5'-phosphorylated hexamer (10) was obtained in 73% yield. The structures of 9 and 10 were confirmed by enzymatic digestion. Compound 9 was completely hydrolyzed to $NH_2CH_2CH_2$SpT, pdC and pT in a ratio of 1:3:2 by nuclease P_1 digestion. On the other hand, 10 was hydrolyzed to pT and pdC in equal amounts.

Synthesis of nucleoside-resin linked via phosphoryl linkage

An aminomethylated polystyrene (11) was first treated with succinic anhydride for 20 hr. The resin was no longer ninhydrin positive. Then it was activated by pentachlorophenol and dicyclohexylcarbodiimide (DCC) for 20 hr. The resin (13) was treated with phosphorylating agent (14), which was prepared by acid treatment of 3, for 1 hr. Unreacted pentachlorophenyl activated carbonyl

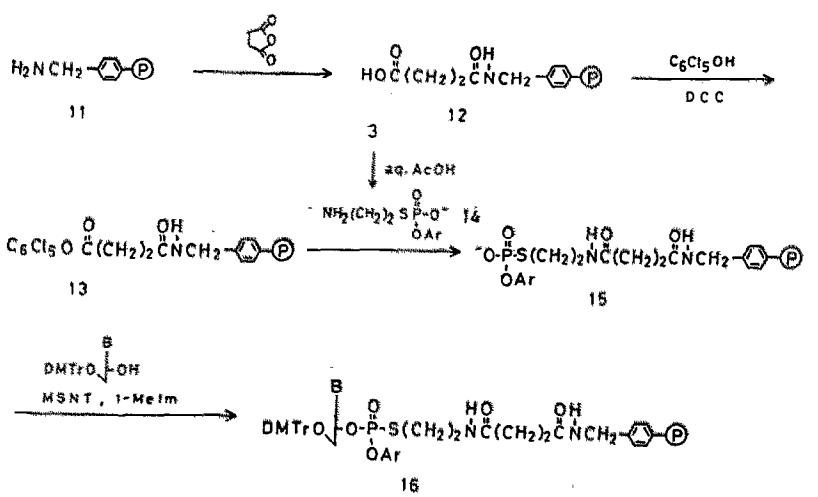


Chart 3

group was capped by addition of morpholine. Then the phosphorylated resin (15) and 3'-hydroxyl group of 5'-O-dimethoxytritylthymidine or 5'-O-dimethoxytrityl-N-benzoyldeoxycytidine was treated with MSNT and 1-methylimidazole to prepare the nucleoside resin (16). The amount of the nucleoside loaded to the resin was 55-65 μmol per gram. Unreated phosphoryl group of 15 was capped by treatment of MeOH with MSNT and 1-methylimidazole.

In order to prepare 3'-phosphorylated oligonucleotide, T-resin (16, B=T) was first treated with 3 % trichloroacetic acid to generate 5'-hydroxyl group. It was treated with 5'-dimethoxytrityl N-benzoyldeoxycytidine 3'-o-chlorophenylphosphate and MSNT for 20 min at 40°C to make the dimer resin (17). Compound 17 was treated with PAO-TMG for 12 hr at room temperature and NH_4OH for 5 hr at 55°C. By this treatment, about 10 % of nucleotide was released from the resin. Even if the NH_4OH treatment was prolonged to 24 hr, the amounts of the cleaved products did not increase. Most of the nucleotides was still bound to the resin. When the resin was treated with I_2-H_2O for 5 hr, the resin was no longer trityl positive. All the products which were in the supernatant, were applied to a reversed phase column. The dimer which still possessed dimethoxytrityl group was isolated. It was treated with 80 % aqueous acetic acid to obtain d-CpTp. The dimer was identified by enzymatic digestion. By treatment of d-CpTp with bacterial alkaline phosphatase, it was converted to d-CpT which was hydrolyzed to dC and pT by venom phosphodiesterase in equal amounts.

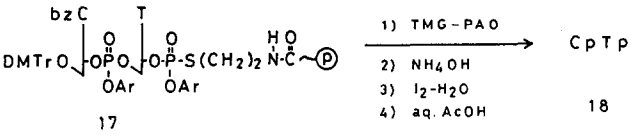


Chart 4

By the same procedure, the hexadeoxyribonucleotide resin with a nucleotide sequence, TCTCTC, was prepared. After treatment with PAO-TMG and NH_4OH as usual, the resin was treated with I_2-H_2O. The hexamer containing a dimethoxytrityl group was isolated by reversed phase column chromatography as shown in Fig. 2. Then the trityl group was removed by 80 % acetic acid and the unprotected hexamer was analyzed by ion-exchange HPLC. As shown in Fig. 2b, d-TCTCTCp was obtained as a main peak. The identification of the hexamer was performed by enzymatic

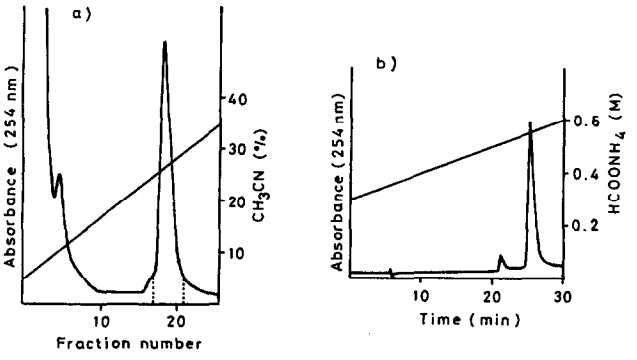


Fig 2. a) Separation of DMTrTpCpTpCpTpCp by reversed-phase column chromatography. b) Analysis of TpCpTpCpTpCp by anion exchange HPLC.

digestion. The hexamer was not hydrolyzed by venom phosphodiesterase indicating the presence of 3'-phosphate. However after treatment of the hexamer with bacterial alkaline phosphatase to give d-TCTCTC, it was hydrolyzed with venom phosphodiesterase to afford T, pT and pdC in a ratio of 1:2:3.

Synthesis of nucleoside polyphosphates

Finally, in order to show another application of the N-monomethoxytrityl-aminoethylthio protecting group, thymidine 5'-di- and triphosphates, A5'pp5'T and A5'ppp5'T were synthesized (Chart 5). Compound 5b was treated with NH_4OH at 55°C for 12 hr to synthesize 19, which was isolated using a C-18 silica gel column. Compound 19 was treated with tri-n-octylammonium salt of phosphoric acid (10 eq.) or pyrophosphoric acid (10 eq.) in pyridine in the presence of iodine (20 eq.) for 10-15 hr. From the reaction with phosphoric acid, thymidine 5'-diphosphate (20) was obtained with 38% yield after purification with a DEAE sephadex A-25 column. From the reaction with pyrophosphoric acid, thymidine 5'-triphosphate (21) was obtained with 21% yield as well as 20 (25%). Compound 19 was next treated with adenosine 5'-phosphate or adenosine 5'-diphosphate in the presence of iodine for 20-25 hr. After isolation by reversed phase C-18 HPLC or anion-exchange HPLC, 22 and 23 were obtained in 75% and 50% yields, respectively (Fig. 3).

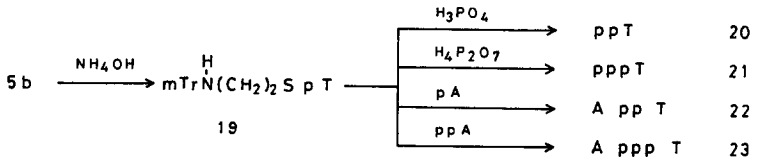


Chart 5

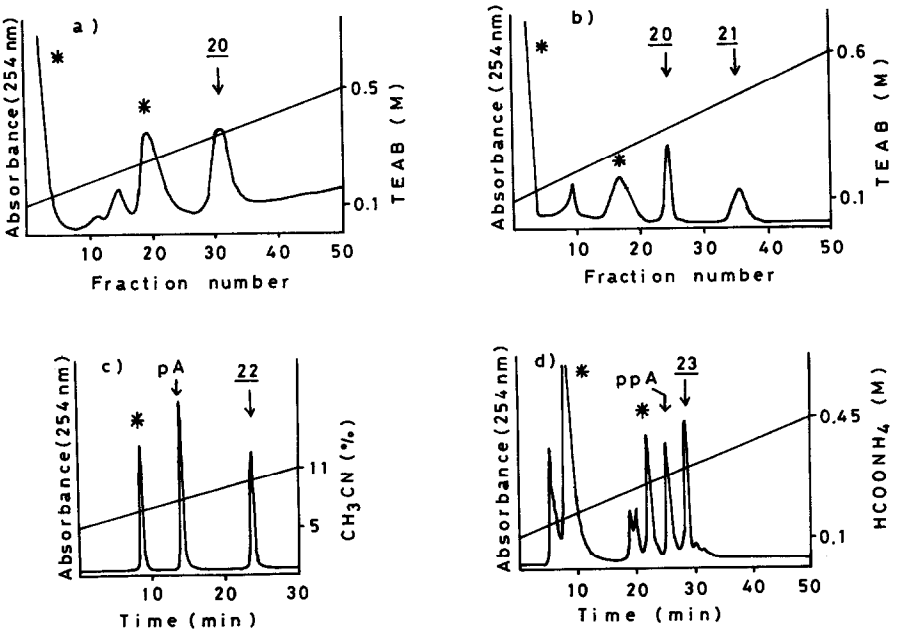


Fig 3. Analysis of ppT (20), pppT (21),AppT (22) and ApppT (23) by a,b) Sephadex A-25 column chromatography, c) reversed-phase C-18 HPLC and d) anion exchange HPLC. *, non-nucleotidic compound.

In conclusion, by using the phosphorylating agent (3) at the last coupling step in oligonucleotide synthesis, 5'-aminoalkylated and phosphorylated hexanucleotides were obtained from a common intermediate by different deprotection procedures. Although the present method is based on the phosphotriester method, it can be applied to the phosphite or H-phosphonate method if one prepares 3'-phosphoramide or H-phosphonate derivatives from compound 5. The compound 3 was useful for the preparation of nucleoside resin (16), with which the 3'-phosphorylated oligonucleotides were synthesized by the 3' → 5' chain elongation. Using a combination of 16 and 3, the 3',5'-diphosphorylated oligonucleotide[26] can be prepared. The compound 3 was also used for the synthesis of nucleoside 5'-di- or triphosphates and dinucleoside 5'-polyphosphates. Accordingly, it is also possible to prepare the adenylated donor intermediate[27] in the T_4 RNA ligase reaction or the cap structure[28] of eukaryotic messenger RNA.

EXPERIMENTAL

Thin-layer chromatography (TLC) was performed on a plate of Kieselgel $60F_{254}$ (Merck). For column chromatography, Kieselgel 60 (Merck) was used. Reversed-phase chromatography was performed on an alkylated silica gel (C18, 55-105 μ, Waters) column. High pressure liquid chromatography (HPLC) was performed on an Altex 322 Mp chromatography system. The reversed-phase HPLC was composed of nucleosil (C18, 5μ, Macherey-Nagel) packed under 500 Kg/cm^2 in a stainless steel column (φ0.6 × 20 cm). For anion exchange HPLC, DEAE 2SW was purchased from Toyo Soda Co.

Aminomethylated polystyrene co-polymer with 1 % divinylbenzene was purchased from Peptide Institute, Inc. Japan. Snake venom phosphodiesterase and nuclease P1 were purchased from Boehringer Mannheim and Yamasa Shoyu Co. The conditions for enzymatic digestion were described previously[16,23].

UV spectra were measured on a Hitachi model 200-10 spectrophotometer. ^{31}P NMR spectra was recorded with a JEOL GX500 spectrometer operating at 202.42 MHz using trimethyl phosphate as an external standard.

Preparation of phosphorylating agent (3)

To a solution of O-(o-chlorophenyl)phosphorodichloridothioate (180 μl, 1 mmol) in pyridine (2 ml), H_2O (72 μl, 4 mmol) was added with stirring at 0°C. After 10 min at room temperature, diisopropylamine (0.35 ml, 2 mmol) and N-monomethoxytritylaminoethylbromide, prepared from monomethoxytritylation of 2-aminoethylbromide (4 mmol) followed by purification on a column of silica gel, were added to the mixture. The mixture was stirred at room temperature for 12 hr, then diluted with CH_2Cl_2 (20 ml) and washed with 0.1 M triethylammonium bicarbonate (TEAB, pH 7.5) (15 ml × 3). The organic layer was evaporated to oil, which was then dissolved in CH_2Cl_2 (5 ml) and added into n-hexane (100 ml) with stirring. The whole reaction mixture was kept overnight at -20°C and the supernatant was removed. The resultant oil was applied onto a column of silica gel (φ3 × 4 cm). Elution was performed with increasing MeOH concentration in CH_2Cl_2 containing NEt_3 (1 %). The fractions containing the desired product (TLC, CH_2Cl_2:MeOH = 10:1, Rf 0.32) were collected and evaporated to give a white solid as triethylammonium salt. Yield was 292 mg (46 %). ^{31}P NMR ($CDCl_3$) : 11.9 ppm.

Preparation of 5b

3'-O-Benzoylthymidine (4b) (34.6 mg, 0.1 mmol) was mixed with 3 (128 mg, 0.2 mmol) and co-evaporated with pyridine (5 ml) to remove a moisture. Then the

mixture was treated with MSNT (89 mg, 0.3 mmol) in pyridine (5 ml) for 1 hr. The completion of reaction was confirmed by TLC (CH_2Cl_2:MeOH=10:1, Rf 0.37 → 0.68). The mixture was diluted with CH_2Cl_2 (20 ml) and washed with 0.1 M TEAB (10 ml × 3). The organic layer was evaporated to an oil which was separated on the column of silica gel (φ3 × 3 cm). Elution was performed with a stepwise gradient of MeOH in CH_2Cl_2 containing NEt_3 (1 %). The fractions containing the desired product were pooled and evaporated. The residue was taken up in CH_2Cl_2 (3 ml) and dropped into n-hexane (50 ml) with stirring. The white precipitates were collected and dried under vacuum. Yield was 65 mg (0.075 mmol, 75 %).

Preparation of d-mTrNH$(CH_2)_2$SpTpCpTpCpTpC (8)

By the standard phosphotriester method[24], C-resin (5 μmol) was treated succesively with 3 eq. of protected CT, CT and T to prepare the hexamer-resin (6). A mixture of 6 and 3 (20 mg, 30 μmol) in pyridine (0.5 ml) was once evaporated. To the residue in pyridine (0.5 ml), MSNT (20 mg, 67 μmol) was added and the mixture was allowed to stand for 1 hr. The phosphorylated hexamer-resin (7) was then treated with 0.5 M PAO-TMG in dioxane-pyridine-H_2O (5:3:1, 1 ml) for 15 hr and evaporated. The residue was heated in a mixture of NH_4OH-pyridine (2 ml-0.5 ml) at 55° for 6 hr. The resin was filtered off and the solution was evaporated to a small volume, which was applied on a C_{18} column (φ0.7 × 8 cm). The products were eluted with a linear gradient of CH_3CN (5 → 35 %) in 50 mM TEAA (total 100 ml). Fractions between the dotted lines in Fig. 1a were pooled and 58 A_{260} units of 8 (1.21 μmol, 24 %) were obtained.

Preparation of d-$NH_2(CH_2)_2$SpTpCpTpCpTpC (9)

Compound 8 (20 A_{260} unit) was treated with 80 % aqueous AcOH (2 ml) at room temperature for 1 hr. The mixture was diluted with H_2O (2 ml) and washed with ether (7 ml × 2). The aqueous layer was separated by anion exchange HPLC which was performed with a linear gradient of $HCOONH_4$ (0.3 → 0.6 M, 30 min) in 20 % CH_3CN at a flow rate of 0.5 ml/min. Yield was 16.3 A_{260} units.

Compound 9 ($2A_{260}$ units) was hydrolyzed with nuclease P_1. The mixture was analyzed by paper electrophoresis (pH 7.5 and 3.5) to separate $NH_2(CH_2)_2$SpT (0.28 A_{267} at pH 7.5), pT (0.57 A_{267} at pH 7.5) and pdC (1.06 A_{280} at pH 2). The ratio found was 1.00:2.07:2.93.

Preparation of d-pTpCpTpCpTpC (10)

Compound 8 (15 A_{260} units) was dissolved in pyridine-H_2O (3:1 v/v, 0.5 ml) containing 75 mM I_2 and kept at room temperature for 4 hr. The mixture was diluted with H_2O (2 ml), washed with ether (7 ml × 3) and evaporated. The residue was applied onto a column of Sephadex G-25 (φ1.2 × 19 cm) and eluted with 20 mM TEAB to remove the non-nucleotidic compounds. Fractions eluted at the void volume were collected and 10 was isolated by anion exchange HPLC. The elution condition was the same as that for the isolation of 9. Yields was 11 A_{260} units.

Compound 10 (1 A_{260} unit) was hydrolyzed with nuclease P_1. The mixture was analyzed by paper electrophoresis (pH 3.5) to separate pdC (0.53 A_{280} at pH 2) and pT (0.42 A_{267} at pH 7.5). The ratio found was 1.00:1.05.

Preparation of nucleoside resin (16)

Succinic anhydride (1 g, 10 mmol) was added to aminomethylpolystyrene (11) (1 g) (200 μmol of NH_2 group) which was suspended in pyridine (10 ml). The mixture was shaken for 20 hr. The resin was collected and successively washed

with pyridine (10 ml × 2), CH_2Cl_2 (10 ml × 2), MeOH (10 ml × 2), CH_2Cl_2 (10 ml × 2) and ether (10 ml × 2) before drying. The succinylated resin showed a negative result in the ninhydrin test.

To the above succinylated resin (12) suspended in pyridine (10 ml), pentachlorophenol (1.33 g, 5 mmol) and DCC (1.4 g, 6.8 mmol) was added. The mixture was shaken for 12 hr. The pentachlorophenyl activated resin (13) was collected and successively washed with pyridine (10 ml × 2), CH_2Cl_2 (10 ml × 2), MeOH (10 ml × 2) and CH_2Cl_2 (10 ml × 2) and dried.

Compound 3 (641 mg, 1 mmol) was treated with 80 % AcOH (5 ml) for 30 min to give 14. The mixture was evaporated to oil, which was dissolved in pyridine (6 ml) and NEt_3 (0.5 ml). The above solution (0.9 ml) was added to 13 (120 mg) and the mixture was shaken for 1 hr. The resin 13 was collected and washed with pyridine (1 ml × 2). The reaction with 14 was repeated again. Then the resin was treated with 10 % morpholine in pyridine (1 ml) for 10 min. The resin was collected, washed successively with pyridine (1 ml × 3) and CH_2Cl_2 (1 ml × 3) and dried.

The above resin (60 mg) was mixed with 5',N-protected nucleoside (0.1 mmol) in pyridine and evaporated to remove any moisture. It was treated with MSNT (60 mg, 200 μmol) and 1-methylimidazole (32 μl, 400 μmol) in pyridine (1 ml) for 1 hr at 35°C. Then the resin was washed once with pyridine (1 ml) and treated with MeOH (10 μl), MSNT (60 mg, 200 μmol), 1-methylimidazole (32 μl, 400 μ mol) in pyridine (1 ml) for 30 min at 35°C. The resin was collected, washed successively with pyridine (1 ml × 3) and CH_2Cl_2 (1 ml × 3) and dried. The polystyrene was loaded with the nucleoside to the extent of 55-65 μmol per gram.

Preparation of d-CpTp and d-TpCpTpCpTpCp

Using the nucleotide resin (16, B=T or bzC), protected T or CT, CT and T^{29} were successively condensed by the standard phosphotriester method[25]. The dimer or hexamer resin thus obtained was treated with PAO-TMG and NH_4OH as described in the synthesis of 8. The resin was washed with pyridine (1 ml × 3), then treated with 75 mM I_2 in pyridine-H_2O (3:1) (1 ml) for 4 hr. The resin was filtered off and washed with 50 % aqueous pyridine (4 ml). The aqueous layers were combined and evaporated. The residue was applied on a C_{18} column (φ0.7 × 8 cm) and separated with a linear gradient of CH_3CN (5 → 35 %) in 50 mM TEAA (total 100 ml). Fractions around 20 % CH_3CN concentration were pooled and evaporated. The residue was treated with 80 % AcOH for 40 min. The mixture was washed with ether (6 ml × 2) and the aqueous layer was evaporated. An aliquot of d-CpTp sample was analyzed by reversed phase HPLC for identification. d-TpCpTpCpTpCp was isolated by anion exchange HPLC (Fig. 2b) and 17 A_{260} units (0.35 μmol) were obtained from 16 (B=C) (1 μmol).

The hexamer (d-TpCpTpCpTpCp) (3 A_{260} units) was treated with a bacterial alkaline phosphatase then with snake venom phosphodiesterase. The mixture was analyzed by paper electrophoresis (pH 3.5) to separate T(0.38 A_{267} at pH 7.5), pdC (1.54 A_{280} at pH 2) and pT (0.83 A_{267} at pH 7.5). The ratio found was 1.00:2.08:2.92.

Preparation of $mTrNH(CH_2)_2SpT$ (19)

Compound 5b (8.7 mg, 10 μmol) was heated in a mixture of pyridine (1 ml) and NH_4OH (1.5 ml) at 55°C for 12 hr. The mixture was evaporated to a small volume and separated by a reversed phase C-18 silica gel column chromatography (φ0.7 × 6 cm). Elution was performed with a linear gradient of CH_3CN (10 → 50 %) in 50 mM

TEAA (total, 100 ml). The fractions containing 19 were collected and evaporated. Yield was 70 A_{260} units.

Preparation of ppT (20)

To a solution of 19 (40 A_{260} unit) in pyridine-H_2O (4:1 v/v, 1 ml), tri-n-octylamine (40 µl, 160 µmol) and phosphoric acid (3.9 mg, 40 µmol) were added. The mixture was evaporated. To remove traces of moisture, the residue was dissolved in pyridine (1 ml) and evaporated again. This process was repeated five times. To the resultant oil, I_2 (20 mg, 80 µmol) dissolved in pyridine (1 ml) was added. The mixture was stirred for 10-15 hr, then diluted with H_2O (1 ml) and washed with ether (7 ml × 3). The aqueous layer was applied onto a column of DEAE-Sephadex A-25 (φ1.7 × 8 cm), which was eluted with a linear gradient of TEAB (0.1 → 0.5 M) (total 300 ml). The fractions containing 5'-TDP, which was eluted at 0.35 M TEAB concentration, were collected. Yields was 15.1 A_{260} units (38 %). The structure was confirmed by comparing with the authentic sample.

Preparation of pppT (21)

Compound 19 (40 A_{260} units) was treated with pyrophosphoric acid (4.7 mg, 40 µmol) in pyridine (1 ml) in the presence of tri-n-octylamine (40 µl, 160 µmol) as well as I_2 (20 mg, 80 µmol) as described above. The mixture, after washing with ether, was separated on a column of DEAE-Sephadex A-25 (φ1.7 × 8 cm) which was eluted with a linear gradient of TEAB (0.1 → 0.6 M, total 300 ml). 5'-TDP and 5'-TTP were eluted at 0.35 M and 0.45 M TEAB concentration, respectively. Yields were 9.9 A_{260} units (25 %) for 5'-TDP and 8.2 A_{260} units (21 %) for 5'-TTP. The structure were confirmed by comparing with the authentic samples.

Preparation of A5'pp5'T (22)

Tri-n-octylammonium salt of 19 (40 A_{260} units) and 5'-AMP (2.8 mg, 8 µmol) were treated with I_2 (20 mg, 80 µmol) in pyridine (1 ml) for 20-25 hr as above. The mixture was separated by reversed phase HPLC using linear gradient of CH_3CN (5 → 11 %) in 0.1 M TEAA. A5'pp5'T was eluted at 10 % CH_3CN concentration and obtained 60 A_{260} units (75 %). ^{31}P NMR (H_2O-D_2O, 4:1): -13.8 ppm.

Compound 22 (A_{260} units) was hydrolyzed with snake venome phosphodiesterase. The mixture was analyzed by paper electrophoresis (pH 3.5) to prepare pA (1.38 A_{259} at pH 7.5) and pT (0.98 A_{267} at pH 7.5). The ratio found was 1.00:1.07.

Preparation of A5'ppp5'T (23)

Tri-n-octylammonium salt of 19 (40 A_{260} units) and 5'-ADP (3.4 mg, 8 µmol) were treated with I_2 (20 mg, 80 µmol) in pyridine (1 ml) for 20-25 hr as described above. The mixture was separated by anion exchange HPLC using linear gradient of $HCOONH_4$ (0.1 → 0.45 M) in 20 % CH_3CN. A5'ppp5'T was eluted at 0.3 M $HCOONH_4$ and obtained 40 A_{260} units (50 %). ^{31}P NMR (H_2O-D_2O, 4:1): -14.1 ppm (2P), -25.7 ppm (1P).

Compound 23 (A_{260} units) was hydrolyzed with snake venome phosphodiesterase. The mixture was analyzed by paper electrophoresis (pH 3.5) to prepare pA (0.855 A_{259} at pH 7.5) and pT (0.600 A_{267} at pH 7.5). The ratio found was 1.00:1.05.

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