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Synthesis and Properties of Deoxyoligonucleotides Containing Putrescinylthymine (Nucleosides and Nucleotides. LXXVI)[1)]

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Putrescinylthymidine was prepared by the reduction of the Schiff base formed from a 2′-deoxy-5-formyluridine derivative and *N*-phthaloylputrescine, followed by deprotection. The following deoxyoligonucleotides containing putrescinylthymine (T^P) were synthesized; dodecathymidylic acids containing two to four T^P residues, self-complementary decanucleotides (AAGAATTCTT) and dodecanucleotides (AGATAGCTATCT) in which T residues were partly replaced by T^P, and related oligomers. Oligonucleotides containing T^P were resistant to nuclease S1 digestion and were poor substrates to venom phosphodiesterase. The thermal stability (T_m) of the duplex structure of oligomers containing T^P was not enhanced in spite of the expected electrostatic binding between the putrescinyl and phosphoryl residues, and was rather sequence-dependent.

Keywords——deoxyoligonucleotide; putrescinylthymine; putrescinylthymidine; melting temperature (T_m); duplex stability; nuclease S1; venom phosphodiesterase

It is well known that bacteriophage deoxyribonucleic acids (DNAs) contain various modified pyrimidine bases such as 5-hydroxymethylcytosine,[2)] 5-methylcytosine,[3)] 5-hydroxymethyluracil,[4)] 5-(4,5-dihydroxypentyl)uracil,[5)] α-glutaminylthymine,[6)] and putrescinylthymine (5-(4-aminobutylamino)methyluracil, T^P, the base moiety of **1**).[7)] Among them, the hypermodified bases[5-7)] have attracted much interest in relation to their functions in these DNAs. For example, phage ΦW-14 DNA contains up to 50% T^P in place of thymine.[7,8)] This modification results in a higher melting temperature (T_m, 99.3 °C) than expected from the GC content (90.3 °C), and lower buoyant density (1.666) than that expected (1.716).[7,9)] The enhanced thermal stability of this DNA has been explained in terms of the reduction of the anionic electrostatic repulsion between phosphoryl moieties by the presence of the cationic putrescinyl groups.[9)] A recent report suggested a function of this basic moiety in the efficient packaging of the DNA in the virus particle.[10)]

Since the T^P moieties in the DNA are not modified any further, unlike other modified bases such as 5-hydroxymethylcytosine, a study of oligonucleotides containing T^P should be useful for direct estimation of the function of this base moiety in the viral DNA. This paper describes the synthesis of putrescinylthymidine and deoxyoligonucleotides of defined sequence containing T^P in place of thymine, and the properties of these modified oligomers. A preliminary account of a part of this work has appeared.[11)]

Synthesis of Putrescinylthymidine

Initial attempts at obtaining putrescinylthymidine (**1**) by substitution of 5-acetoxymethyl-3′,5′-di-*O*-benzoyl-2′-deoxyuridine or 3′,5′-di-*O*-acetyl-5-bromomethyl-2′-deoxyuridine with N^1-phthaloylputrescine resulted in a low yield of **1**. We have recently found that the 5-aminomethyluridines can be obtained by the Schiff base formation of 5-formyluridine with amines followed by reduction with borohydride.[12)] This route seemed to

be useful for the present purpose. 3′,5′-Di-*O*-acetyl-5-formyl-2′-deoxyuridine (**2**), prepared by oxidation of the thymidine with potassium peroxysulfate,[13] was treated with *N*-phthaloylputrescine (**3**) in dioxane at room temperature to give the Schiff base **4**, which was further treated with sodium borohydride at low temperature. After chromatographic purification of the product, 3′,5′-di-*O*-acetyl-*N*-phthaloylputrescinylthymidine (**5**) was obtained in 30% overall yield. Ammonolysis of **5** afforded putrescinylthymidine (**1**), isolated as the mono-oxalate. The structure of **1** was confirmed by the nuclear magnetic resonance

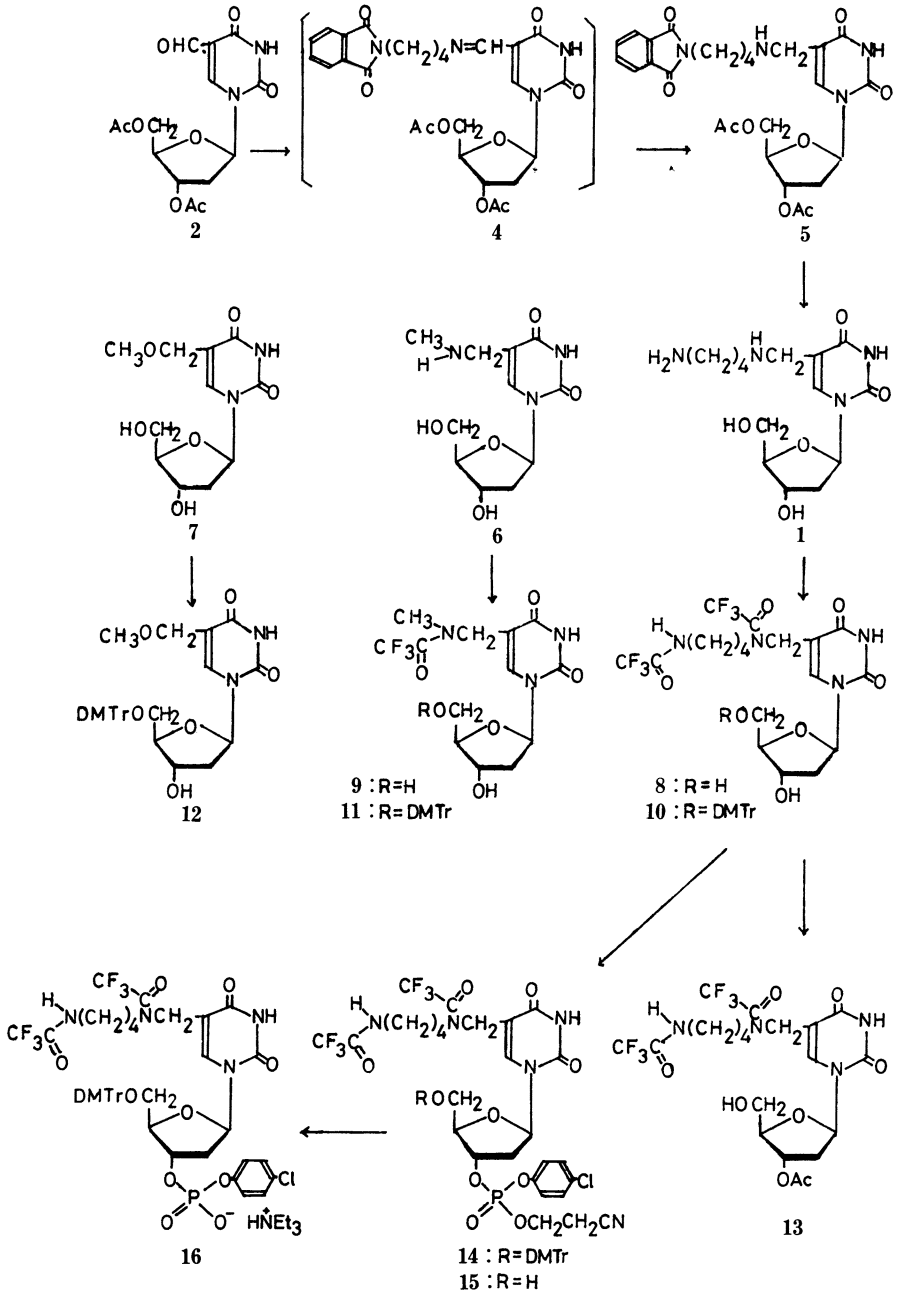

Chart 1

(NMR) spectrum and the result of elemental analysis. As reference compounds, 2′-deoxy-5-(methylaminomethyl)uridine (**6**) and 2′-deoxy-5-(methoxymethyl)uridine (**7**) were prepared from 3′,5′-di-*O*-acetyl-5-bromomethyl-2′-deoxyuridine by methylammonolysis or methanolysis, respectively.

Synthesis of Deoxyoligonucleotides Containing Putrescinylthymine (T^P), 5-(Methylaminomethyl)uracil (T^N), and 5-(Methoxymethyl)uracil (T^O)

Prior to oligonucleotide synthesis, the side-chain amino groups of **1** and **6** were protected with a trifluoroacetyl function by treatment with ethyl trifluoroacetate in the presence of triethylamine[14] in methanol to give *N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine (T^{PF}, **8**) and *N*-trifluoroacetyl-5-methylaminomethyl-2′-deoxyuridine (T^{NF}, **9**), respectively. These compounds and **7** were then converted to the 5′-dimethoxytrityl derivatives (**10**, **11**, **12**) by the usual procedure. Compound **10** was further converted to the 3′-*O*-acetyl derivative (**13**), 3′-phosphotriesters (**14** and **15**) and 3′-phosphodiester (**16**) by the usual procedures.[15,16] Compounds **11** and **12** were also converted to the properly protected nucleotides, and these monomer units were used in oligonucleotide synthesis. Dinucleotide monophosphate, T^PT, was prepared by condensation of **16** with 3′-*O*-acetylthymidine by the use of triisopropylbenzenesulfonyl chloride (TPSCl) in the presence of *N*-methylimidazole,[17] followed by deprotection. TT^P was likewise prepared from **13** and the appropriate nucleotide component.

The oligonucleotides TTCTT, TT^PCTT, $TTCT^PT$, AAGAATTCTT, $AAGAATT^PCTT$, $AAGAATTCT^PT$, and $AAGAATT^PCT^PT$ were prepared by the liquid phase phosphotriester method.[17] The results at the stage of the final condensation steps are summarized in Table I. The deprotection was performed by successive treatment with *N*,*N*,*N*′,*N*′-tetramethylguanidinium *p*-nitrobenzaldoximate (TMGNBO),[18] conc. NH_4OH, and 80% AcOH. The oligomers were purified by diethylaminoethyl (DEAE)-cellulose column chromatography or reversed phase column chromatography,[19] and high performance liquid chromatography (HPLC). The dodecanucleotide AGATAGCTATCT and analogs containing T^P in place of T at positions 4, 8, and 10 were prepared by the solid-phase phosphotriester method using aminomethylpolystyrene as the support.[20] Dodecathymidylate (T_{12}) and analogs containing T^P at positions 3 and 9 ($T^P_{3,9}$), 3, 7, and 11 ($T^P_{3,7,11}$), and 2, 5, 8, 11 ($T^P_{2,5,8,11}$) were also prepared. Similarly, $T^P_{3,9}$ and $T^O_{3,9}$ were prepared. The results are summarized in Table II with the yield of each condensation step. The oligomers prepared by this method were purified by a combination of gel-filtration and HPLC to a purity of >99%.

Digestion of Oligonucleotides by Nucleolytic Enzymes

It has been reported that ΦW-14 DNA is resistant to deoxyribonuclease (DNase) I or venom phosphodiesterase (VPDase) digestion[21] and T^P-nucleoside was detectable after nuclease S1 digestion of heat-denatured DNA followed by VPDase and alkaline phosphatase (APase) action.[22] Therefore, T_{12} and analogs containing T^P, T^N or T^O were treated with nuclease S1, VPDase, and nuclease P_1 to test their susceptibility. Nuclease S1 completely cleaved T_{12} at 50 °C overnight, giving 5′-TMP (pdT) and thymidine (dT) in 11 : 1 ratio. Under similar conditions $T^P_{3,9}$, $T^N_{3,9}$, and $T^O_{3,9}$ showed several peaks other than those of pdT and dT on HPLC, suggesting resistance due to modification of the methyl function of thymine residue.

VPDase (with APase) cleaved $T^O_{3,9}$ completely to give dT and dT^O in the expected ratio. $T^N_{3,9}$ showed one peak in addition to those of dT and dT^N. In the case of $T^P_{3,9}$, dT^P was not detected, and instead, two peaks probably due to incomplete hydrolysis were detected. One of them was confirmed to be TT^P by co-chromatography with an authentic sample, and the other peak was assumed to be TTT^P or $TTTT^P$, since its retention time was not identical with that of authentic T^PT. In fact, VPDase digestion of T^PT gave dT and dT^P, while TT^P was completely resistant.

TABLE I. Synthesis of Penta- and Decadeoxyribonucleotides Containing T^P

3′-Diester component mg (mmol)	5′-Hydroxy component mg (mmol)	*N*-Methyl-imidazole mg (mmol)	TPSCl mg (mmol)	Reaction time h	Oligomer mg yield (%)	Deprotected oligomer (T_m)
DMTrTTp 250 (0.2)	$C^{an}TT_{ac}$ 222 (0.18)	98 (1.2)	182 (0.6)	2	$DMTrTTC^{an}TT_{ac}$ 230 (75)	TTCTT
$DMTrTT^{PE}p$ 90 (0.06)	$C^{an}TT_{ac}$ 62 (0.05)	33 (0.4)	50 (0.16)	3	$DMTrTT^{PF}C^{an}TT_{ac}$ 90 (65)	TT^PCTT
$DMTrTT^{PF}p$ 105 (0.07)	$C^{an}T^{PF}T_{ac}$ 62 (0.05)	40 (0.48)	55 (0.18)	1.5	$DMTrTT^{PF}C^{an}T^{PF}T_{ac}$ 114 (65)	TT^PCT^PT
$DMTrTTC^{an}p$ 70 (0.04)	$T^{PF}T_{ac}$ 30 (0.03)	16 (0.26)	36 (0.12)	1.5	$DMTrTTC^{an}T^{PF}T_{ac}$ 77 (78)	$TTCT^PT$
$DMTrA^{bz}A^{bz}G^{ib}p$ 128 (0.07)	$A^{bz}A^{bz}p$(CE) 68 (0.06)	33 (0.4)	61 (0.2)	1	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$(CE) 120 (70)	
$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$ 30 (0.01)	$TTC^{an}TT_{ac}$ 18 (0.008)	9 (0.1)	16 (0.05)	2	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}TTC^{an}TT_{ac}$ 33 (56)	AAGAATTCTT (22 °C)
$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$ 36 (0.012)	$TT^{PF}C^{an}TT_{ac}$ 24 (0.01)	11 (0.13)	20 (0.06)	4	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}TTC^{an}TT_{ac}$ 39 (77)	$AAGAATT^PCTT$ (16 °C)
$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$ 40 (0.015)	$TTC^{an}T^{PF}T_{ac}$ 37 (0.015)	8 (0.1)	15 (0.05)	1	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}TTC^{an}T^{PF}T_{ac}$ 42 (68)	$AAGAATTCT^PT$ (27 °C)
$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$ 40 (0.015)	$TT^{PF}C^{an}T^{PF}T_{ac}$ 32 (0.011)	11 (0.13)	20 (0.06)	3	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}TT^{PF}C^{an}T^{PF}T_{ac}$ 35 (50)	$AAGAATT^PCT^PT$ (15 °C)

Abbreviations: The internucleotidic phospho groups are omitted. DMTr, 5′-*O*-dimethoxytrityl; an, N^4-anisoyl; ac, 3′-*O*-acetyl; bz, N^6-benzoyl; p, *p*-chlorophenylphosphoryl; (CE), 2-cyanoethyl; ib, N^2-isobutyryl; P, 5-putrescinyl; PF, *N*,*N*′-ditrifluoroacetylputrescinyl.

TABLE II. Oligodeoxyribonucleotides Prepared by the Solid-Phase Phosphotriester Method, and Their Melting Temperatures (T_m)[a]

AGA-TA-GC-TA-TC-T 68 84 60 86 77[c]	46 °C	TTTT-TTTT-TTT-T 61 55 94	29 °C[b]	T_{12}
AGA-T^PA-GC-TA-TC-T 69 66 77 79 69	37 °C	TT-T^PTT-TTT-T^PTT-T 87 84 82 87	23.5 °C	$T^P_{3,9}$
AGA-TA-GC-T^PA-TC-T 65 78 81 55 70	40 °C	TT-T^PTT-TT^P-TTT-T^P-T 85 83 71 75 74	18.5 °C	$T^P_{3,7,11}$
AGA-TA-GC-TA-T^PC-T 70 85 81 78 52	37 °C	TT^P-TTT^P-TTT^P-TTT^P-T 79 74 64 78	13.5 °C	$T^P_{2,5,8,11}$
		TT-T^NTT-TTT-T^NTT-T 78 69 82 100	20 °C	$T^N_{3,9}$
		TT-T^OTT-TTT-T^OTT-T 80 86 76 92	25 °C	$T^O_{3,9}$

a) T_m's were measured in 0.1 M NaCl–0.01 M sodium cacodylate at pH 7.2. *b*) T_m's of the duplex with poly(dA). *c*) Yields (%) of the condensation steps as measured in terms of the amount of released dimethoxytrityl groups.

However, as has been reported,[22] nuclease S1 treatment followed by VPDase (with APase) treatment resulted in complete hydrolysis of the modified oligonucleotides to the nucleoside components. Thus, treatment of AGATAGCT^{P}ATCT, for example, with the combination of the nucleases gave dC, dT^P, dG, dT and dA in the expected ratio. Other oligonucleotides gave similar results. Nuclease P_1 also cleaved the modified nucleotides but the rates were very low (data not shown). Taking account of the synthetic routes, these results indicate that the oligomers synthesized have the expected sequences. The usual two-dimensional fingerprinting for sequence analysis of natural oligonucleotides[23] was not attempted because of the expected incomplete (non-random) hydrolysis by nuclease P_1 and VPDase.

Melting Temperature Profiles of Oligodeoxynucleotides Containing T^P

Dodecathymidylates containing T^P, T^N, or T^O were annealed with poly(dA) and their melting temperatures (T_m) were measured (Table II). The natural poly(dA): T_{12} showed the highest T_m (29 °C) among them. The heteroduplex with $T^O_{3,9}$ showed a slight decrease of the T_m (25 °C) whereas that of $T^N_{3,9}$ showed a still lower T_m (20 °C). This may be because that the NH proton of the 5-CH_2NHCH_3 function of T^N can form a hydrogen bond to the 4-C=O group, thus reducing the hydrogen-bond-forming ability of that carbonyl with the adenine moiety, resulting in decreased duplex stability. The duplex with $T^P_{3,9}$ also showed a lower T_m (23.5 °C) as compared to the unmodified duplex but higher than that of poly(dA): $T^N_{3,9}$. Thus, the primary amino group at the putrescinyl moiety should have some stabilizing effect.

In general, polyamines and diamines bind to DNA and stabilize it[24] and this stability has been attributed to their binding to the minor groove of the DNA helix by ion-pair formation with the phosphoryl anions of the DNA chain.[25] The unusually high T_m of ΦW-14 DNA has also been explained in terms of a similar effect of the putrescinyl moiety.[9] Thus, the T_m of the poly(dA) : oligo(dT) duplex should be elevated by increasing the number of T^P substitutions in place of T. However, the present results show that this is not the case, and as the number of substitutions increased from 2 to 4 the T_m became even lower (poly(dA) : $T^P_{3,9}$, 23.5 °C; poly(dA) : $T^P_{3,7,11}$, 18.5 °C; poly(dA) : $T^P_{2,5,8,11}$, 13.5 °C). Therefore the reported stabilizing effect of the putrescinyl moiety may not be due to ionic interaction with the phosphate anion; instead, interactions with other moieties of DNA such as with base moieties should be taken into account.

Therefore, the T_m's of some self-complementary oligomers containing T^P were compared

with those of the unmodified oligomers (Tables I and II). These oligomers are in the usual B-type duplexes as revealed by their circular dichroism (CD) spectra (data not shown). In the case of AGATAGCTATCT, the substitution of T^P for T resulted in lowering of T_m's compared to that of the natural dodecamer (T_m 46 °C). However, there seems a certain sequence dependency, and the dodecamer having a C-residue on the 5′-side of T^P showed the highest T_m (40 °C) among the substituted decamers. In the case of the decamer AAGAATTCTT (Table I), the substituted decamer having T^P on the 3′-side of C showed a higher T_m (27 °C) than the natural decamer (22 °C) while the sequence isomer having T^P on the 5′-side of C showed a lower T_m (16 °C). This again shows that there must be some sequence specificity involved in the thermal stability of DNA resulting from the introduction of T^P residues. In oligomers where two T^P residues are located closer together, the T_m's tend to decrease, as shown by AAGAATT^{P}CT^{P}T (<5 °C).

We cannot yet draw any definite conclusion about the role of the putrescinyl residues of T^P in the thermal stability of DNA of ΦW-14. However, it may be concluded that the presence of the putrescinyl residue does not necessarily stabilize the DNA by electrostatic interaction with the phosphoryl moieties, as do polyamines or diamines. The putrescinyl moiety in T^P is located in the major groove of the DNA helix, whereas diamines are bound in the minor groove of DNAs.[25] Although the possibility of ionic interaction of the putrescinyl moiety with the phosphoryl group is not fully excluded, interactions with the base moieties nearby in the duplexes may be involved in the thermal stability of ΦW-14 DNA.

Experimental

General Methods——The ultraviolet (UV) spectra and T_m's were measured with a Shimadzu UV-240 spectrophotometer equipped with a temperature controller and thermometer. CD spectra were measured on a JEOL J-40 or J-500 spectropolarimeter. NMR spectra were recorded on a JEOL FX-100 or 200 FT spectrometer with tetramethylsilane as an internal standard. All exchangeable protons were confirmed by addition of D_2O. Mass spectra (MS) were measured on a JEOL D-300 spectrometer. Melting points were determined on a Yamato MP-21 micromelting point apparatus and are uncorrected. Thin layer chromatography (TLC) and reversed phase TLC were performed on Merck Kieselgel $60F_{254}$ plates and Merck Kieselgel $60F_{254}$ silanisiert, respectively. Silica gel used for column chromatography was Merck Kieselgel 60 (70—230 mesh). The columns used for HPLC were Merck Lichrosorb RP-18 or Whatman Chromedia LPR-2 on a Hitachi 638-30 liquid chromatograph. VPDase and nuclease S1 were purchased from Boehringer Mannheim Co., *E. coli* APase from Worthington Biochemical Inc., and nuclease P_1 from Yamasa Shoyu Co. Poly(dA) was purchased from Sigma Chemical Co. The deoxyribonucleosides for oligonucleotide synthesis were purchased from Yamasa Shoyu Co.

Monophthaloylputrescine (3) Hydrochloride——4-Phthalimidobutyronitrile[26] (10.0 g, 46.7 mmol) was hydrogenated over PtO_2 (200 mg) in EtOH (500 ml) containing concentrated HCl (10 ml) for 1 week. The catalyst was filtered off, the filtrate was concentrated, and the residue was crystallized from EtOH to give **3** as the hydrochloride (8.8 g, 74%), mp 189—191 °C. ^{1}H-NMR (DMSO-d_6) δ: 8.0 (3H, br s, NH_3^+), 7.89 (4H, s, Pht), 3.57 (2H, t, PhtNCH$_2$), 2.67 (2H, t, N-CH$_2$), 1.58 (4H, m, CH$_2$CH$_2$). *Anal.* Calcd for $C_{12}H_{15}ClN_2O_2$: C, 56.58; H, 5.94; Cl, 13.92; N, 11.00. Found: C, 56.38; H, 5.91; Cl, 14.42; N, 10.95.

3′,5′-Di-*O*-acetyl-2′-deoxy-5-(phthalimidobutylaminomethyl)uridine (5)——a) A mixture of 3′,5′-di-*O*-acetyl-2′-deoxy-5-formyluridine[13a,27] (**2**, 1.64 g, 4.8 mmol), monophthaloylputrescine hydrochloride (**3**, 1.85 g, 7.2 mmol) and triethylamine (2.4 g, 24 mmol) in dioxane (10 ml) was stirred for 2 h. $NaBH_4$ (370 mg, 10 mmol) was added to the solution in an ice-bath. After 30 min, the mixture was concentrated to a half of the initial volume, and 80% AcOH was added to neutralize the solution. The solvent was removed *in vacuo* and the residue was extracted with $CHCl_3$. The organic layer was concentrated and the concentrate was chromatographed on silica gel (1.5 × 40 cm). The eluate with $CHCl_3$–MeOH (10 : 1) was concentrated to leave **5** (1.8 g, 62%) as a foam. ^{1}H-NMR ($CDCl_3$) δ: 7.81, 7.71 (2H each, m, Pht), 6.31 (1H, dd, H-1′), 5.22 (1H, br d, H-3′), 5.0—4.2 (2H, br, NH), 4.34 (2H, dd, H-5′), 4.26 (1H, m, H-4′), 3.70 (2H, t, PhtNCH$_2$), 3.52 (2H, q, 5-CH$_2$, J_{gem}=14.2 Hz), 2.67 (2H, t, NCH$_2$), 2.46, 2.33 (1H each, dd, H-2′), 2.12, 2.11 (3H each, s, Ac). 1.9—1.5 (4H, m, CH$_2$CH$_2$).

b) 3′,5′-Di-*o*-acetylthymidine (1.0 g, 3.07 mmol) was brominated with Br_2 (0.18 ml, 3.3 mmol) in CCl_4 (200 ml) under irradiation by the reported procedure.[27] The product was dissolved in acetonitrile (40 ml) and treated with monophthaloylputrescine hydrochloride (5.0 g, 19.6 mmol) and K_2CO_3 (4.14 g, 30 mmol) in acetonitrile (70 ml) under cooling in an ice bath for 1 h. The precipitate was filtered off, the filtrate was concentrated, and the residue was chromatographed on silica gel (1.5 × 40 cm) to give **5** (860 mg, 52%). The physical constants were similar to those of

the product obtained in a).

Putrescinylthymidine Mono-oxalate (1)——Compound **5** (860 mg, 1.6 mmol) was dissolved in 40% aqueous $MeNH_2$ (15 ml) and the mixture was kept at room temperature for 3 d. The solvent was removed *in vacuo* and the residue was adsorbed on Amberlite IRC 50 resin (H^+ form, 100 ml). The resin was washed with H_2O (120 ml) and the product was eluted with 0.3 M NH_4OH. The eluate was concentrated to leave an oily residue (500 mg). This was taken up in a small volume of MeOH, and the solution was neutralized with oxalic acid. The precipitate was collected, then crystallized from MeOH, mp 188—190.5 °C (dec.). UV $\lambda_{max}^{H_2O}$ nm (ε): 264 (8800), λ_{max}^{pH1}: 264 (9600), λ_{max}^{pH12}: 264 (5300). ^{1}H-NMR (D_2O) δ: 8.15 (1H, s, H-6), 6.27 (1H, t, H-1′, J=6.4 Hz), 4.44 (1H, m, H-3′), 4.07 (1H, m, H-4′), 4.02 (2H, s, 5-CH_2), 3.85 (1H, m, H-5′), 3.08 (4H, m, NCH_2), 2.41 (2H, m, H-2′), 1.78 (4H, m, CH_2CH_2). *Anal.* Calcd for $C_{16}H_{26}N_4O_9 \cdot 2/3H_2O$: C, 44.52; H, 6.38; N, 12.98. Found: C, 44.77; H, 6.22; N, 12.95.

2′-Deoxy-5-(*N*-methyl-*N*-trifluoroacetylaminomethyl)uridine (9)——A solution of 3′,5′-di-*O*-acetylthymidine (2.0 g, 6.1 mmol) in CCl_4 (400 ml) was treated with Br_2 (0.4 ml, 7 mmol) under irradiation.[27] After 1 h, the solvent was removed *in vacuo* and the residue (5-bromomethyl derivative) was dissolved in CH_3CN (30 ml). A saturated solution of $MeNH_2$ in acetonitrile (30 ml) was added dropwise to the solution in an ice-bath, and the whole was kept for 15 min. The solvent was evaporated off and the residue was taken up in 1.5 M NH_4OH (100 ml). After 1 h at room temperature, the solvent was removed and the residue was absorbed on Dowex 50W × 8 resin (H^+, form, 3 × 30 cm). The column was washed with H_2O, then eluted with 0.5 M NH_4OH. The eluate was concentrated, the residue (1.2 g) was dried by codistillation with pyridine, and the residue was dissolved in MeOH. Ethyl trifluoroacetate (4.3 g, 30 mmol) and triethylamine (3.0 g, 30 mmol) were added and the solution was kept overnight at room temperature. The solvent was evaporated off and the residue was applied to a column of silica gel (1.5 × 50 cm). The eluate with $CHCl_3$–MeOH (15 : 1) was concentrated to leave **9** as a foam (1.3 g, 58%). An aliquot was crystallized from MeOH, mp 159—163 °C. High-resolution MS *m/z*: 367.0992. Calcd ($C_{13}H_{16}F_3N_3O_6$): 367.0999. MS *m/z*: 367 (M^+), 251 (B+H), 117 ($M^+ - B$). ^{1}H-NMR (D_2O) δ: 7.94, 7.90 (1H, s each, H-6), 6.30 (1H, t, H-1′), 4.50 (1H, m, H-3′), 4.37 (2H, s, 5-CH_2), 4.07 (1H, m, H-4′), 3.82 (2H, m, H-5′), 3.23, 3.21 (3H, s each, NCH_3), 2.39 (2H, m, H-2′).

2′-Deoxy-5-(methylaminomethyl)uridine Acetate (6)——A solution of **9** (110 mg, 0.3 mmol) in concentrated NH_4OH (3 ml) was kept at room temperature for 30 min. The solvent was removed *in vacuo* and the residue was dissolved in 10% AcOH (1 ml). The solvent was removed and the residue was crystallized from aqueous MeOH to give **6** (60 mg, 72.3%), mp 113—115 °C. UV $\lambda_{max}^{H_2O}$ nm (ε): 265 (10800); λ_{max}^{pH1}: 265 (9600); λ_{max}^{pH12}: 264 (6400). ^{1}H-NMR (D_2O) δ: 8.14 (1H, s, H-6), 6.28 (1H, t, H-1′), 4.48 (1H, m, H-3′), 4.08 (1H, m, H-4′), 4.00 (2H, s, 5-CH_2), 3.83 (2H, dd, H-5′), 2.73 (3H, s, NCH_3), 2.41 (2H, m, H-2′), 1.92 (3H, s, acetate). *Anal.* Calcd for $C_{13}H_{21}N_3O_7 \cdot 5/4H_2O$: C, 44.12; H, 6.69; N, 11.87. Found: C, 44.35; H, 6.46; N, 11.58.

2′-Deoxy-5′-dimethoxytrityl-5-(methoxymethyl)uridine (12)——3′,5′-Di-*O*-acetylthymidine (3.0 g, 9.2 mmol) was monobrominated by the procedure[27] described above and the product was dissolved in 5 M NaOMe in MeOH (20 ml). After 30 min, the solution was neutralized by addition of AcOH. The solvent was removed *in vacuo*, the residue was taken up in MeOH (20 ml), the insoluble material was filtered off, and the filtrate was concentrated to leave a syrup of crude 2′-deoxy-5-(methoxymethyl)uridine (**7**, 2.1 g). Compound **7** (1.0 g, 3.67 mmol) was dissolved in pyridine (2 ml). Dimethoxytrityl chloride (1.24 g, 3.67 mmol) was added to the pyridine solution, and after 1 h at room temperature MeOH (1 ml) was added. The solvent was removed, the residue was taken up in $CHCl_3$, and the organic layer was washed with aqueous $NaHCO_3$ solution and dried over Na_2SO_4. The solvent was removed and the residue was chromatographed (silica gel, 1.5 × 50 cm). The eluate with benzene–AcOEt (1 : 1) was concentrated to leave **12** (39% from the thymidine) as a foam. ^{1}H-NMR ($CDCl_3$) δ: 8.54 (1H, br s, NH), 7.77 (1H, s, H-6), 7.47—7.23, 6.88—6.79 (9H and 4H, m and d, phenyl protons), 6.36 (1H, t, H-1′), 4.51 (1H, m, H-3′), 4.04 (1H, m, H-4′), 3.9—3.75, 3.80 (2H and 6H, m and s, 5-CH_2 and CH_3O–Ph), 3.40 (2H, t, H-5′), 3.06 (3H, s, CH_3O), 2.4—2.1 (2H, m, H-2′).

2′-Deoxy-5-(methoxymethyl)uridine (7)——Pure **7** was obtained by deprotection of **12**. Compound **12** (200 mg, 0.35 mmol) was dissolved in 80% AcOH (3 ml). After 30 min, the solvent was removed and the residue was dried by co-distillation with toluene. The residue was partitioned between H_2O and $CHCl_3$. The aqueous layer was concentrated and the residue was crystallized from AcOEt to give **7** (80 mg, 85%), mp 116—117 °C (lit. 120—125 °C[28]). UV $\lambda_{max}^{H_2O}$ nm (ε): 264 (11500); λ_{max}^{pH1}: 264 (10500, lit., 10700); λ_{max}^{pH12}: 264 (9500). MS *m/z*: 272 (M^+), 156 (B+H), 117 ($M^+ - B$). ^{1}H-NMR (D_2O) δ: 7.98 (1H, s, H-6), 6.29 (1H, t, H-1′), 4.46 (1H, m, H-3′), 4.25 (2H, s, 5-CH_2), 4.04 (1H, m, H-4′), 3.80 (2H, dd, H-5′), 3.37 (3H, s, CH_3O), 2.41 (2H, m, H-2′). *Anal.* requires for $C_{11}H_{16}N_2O_6$: C, 48.52; H, 5.92; N, 10.29. Found: C, 48.28; H, 5.85; N, 10.11.

***N*,*N*′-Bis(trifluoroacetyl)putrescinylthymidine (8)**——Compound **1** (1.1 g, 2.56 mmol), ethyl trifluoroacetate (3.55 g, 25 mmol) and triethylamine (1.25 g, 12.5 mmol) were dissolved in MeOH (30 ml). After 2 d at room temperature, the solvent was removed and the residue was chromatographed (silica gel, 1.5 × 50 cm). The eluate with $CHCl_3$–MeOH (30 : 1) was concentrated and the residue was crystallized from MeOH to give **8** (800 mg, 62.1%), mp 168—170 °C. MS *m/z*: 520 (M^+), 423 ($M^+ - 97$), 404 (B+H), 117 ($M^+ - B$). ^{1}H-NMR (DMSO-d_6) δ: 11.50, 11.46 (1H, br s each, N^{3}H), 9.44 (1H, br, NH), 7.84, 7.83 (1H, s each, H-6), 6.13 (1H, m, H-1′), 5.22, 4.99 (1H each, m, HO-5′, 3′), 4.24 (1H, br s, H-3′), 4.18 (2H, s, 5-CH_2), 3.78 (1H, m, H-4′), 3.4—3.2, 3.16 (4H and 2H, m each, H-5′, N–CH_2 and NCH_2), 2.1 (2H, m, H-2′), 1.8—1.4 (4H, m, CH_2CH_2). *Anal.* Calcd for $C_{18}H_{22}F_6N_4O_7$: C, 41.54; H, 4.26; N, 10.77. Found: C, 41.43; H, 4.28; N, 10.81.

5′-*O*-Dimethoxytrityl-*N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine (10)——Compound **8** (670 mg, 1.3 mmol) was dimethoxytritylated by the usual procedure to give **10** (550 mg, 67%) after chromatographic separation. ^{1}H-NMR ($CDCl_3$) δ: 9.8—9.6 (2H, br, NH), 7.93 (1H, s, H-6), 7.45—7.15, 6.83 (9H and 4H, m and d, Ph), 6.24 (1H, t, H-1′), 4.5 (1H, m, H-3′), 3.95 (1H, m, H-4′), 3.87 (2H, s, 5-CH_2), 3.80, 3.78 (3H each, s, CH_3O), 3.5—3.2 (6H, m, H-5′ and NCH_2), 2.40, 2.27 (1H each, m, H-2′), 1.70 (4H, br s, CH_2CH_2). A small amount (6.8%) of by-product (3′,5′-bis(dimethoxytrityl)derivative) was also obtained as a foam.

3′-*O*-Acetyl-*N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine (13)——Compound **10** (250 mg, 0.3 mmol) in pyridine (3 ml) was treated with Ac_2O (120 mg, 1.2 mmol) at room temperature overnight. The solvent was removed and the residue was partitioned between $CHCl_3$ and $NaHCO_3$–H_2O. The organic layer was dried, the solvent was removed *in vacuo*, and the residue was dessolved in 5 ml of 2% benzensulfonic acid (in $CHCl_3$–MeOH, 7 : 3). After 10 min at room temperature, the solution was diluted with $CHCl_3$, and washed with $NaHCO_3$–H_2O. The dried organic layer was chromatographed (silica gel, 0.9 × 50 cm), the eluate with $CHCl_3$–MeOH (30 : 1) was concentrated, and the residue was crystallized from EtOH to give **13** (100 mg, 60%), mp 141—142 °C. ^{1}H-NMR ($CDCl_3$) δ: 10.16 (1H, s, NH), 8.42 (1H, s, H-6), 7.5 (1H, br s, NH), 6.23 (1H, t, H-1′), 5.39 (1H, m, H-3′), 4.36—4.16 (3H, m, H-4′, CH_2-5), 3.92 (2H, m, H-5′), 3.56—3.37 (4H, m, CH_2–N), 2.46 (2H, m, H-2′), 1.79—1.66 (4H, m, CH_2CH_2). *Anal.* Calcd for $C_{20}H_{24}F_6N_4O_8$: C, 42.71; H, 4.30; N, 9.96. Found: C, 42.29; H, 4.30; N, 10.07.

2′-Deoxy-5′-*O*-dimethoxytrityl-5-(*N*-methyl-*N*-trifluoroacetylaminomethyl)uridine (11)——Compound **9** (330 mg, 0.9 mmol) was dried by co-distillation with pyridine and the residue was dissolved in pyridine (1 ml). Dimethoxytrityl chloride (340 mg, 1 mmol) was added and the solution was kept at room temperature for 1 h. A few drops of MeOH were added, then the solvent was removed *in vacuo*, and the residue was partitioned between $CHCl_3$ and H_2O. The organic layer was concentrated and the residue was applied to a silica gel column (1.2 × 40 cm). The eluate with $CHCl_3$–MeOH (30 : 1) was concentrated to leave **11** (456 mg, 76%) as a foam. ^{1}H-NMR ($CDCl_3$) δ: 8.76 (1H, br s, NH), 7.82 (1H, s, H-6), 7.57—7.13 and 6.90—6.78 (9H and 4H, m each, Ph), 6.26 (1H, t, H-1′), 4.45 (1H, m, H-3′), 4.05—3.90 (3H, m, H-4′, CH_2-5), 3.78 (6H, s, OCH_3), 3.42 (2H, d, H-5′), 3.24, 3.22 (3H, s each, CH_3–N), 2.23 (2H, m, H-2′), 2.15 (1H, br s, HO-3′).

5′-*O*-Dimethoxytrityl-*N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine 3′-(4-Chlorophenyl 2-cyanoethyl)phosphate (14)——Compound **10** (520 mg, 0.63 mmol) was dried by co-distillation with pyridine and the residue was dissolved in pyridine (2 ml). *p*-Chlorophenyl phosphoroditriazolide (0.1 M in tetrahydrofuran, 8 ml)[15] was added to the solution. After 30 min, *N*-methylimidazole (308 mg, 3.75 mmol) and 2-cyanoethanol (438 mg, 6.25 mmol) were added and the solution was concentrated to a half of its original volume, then kept for 1.5 h at room temperature. The reaction was quenched by addition of a small volume of H_2O under cooling in an ice bath, and the solution was extracted with $CHCl_3$. The organic layer was washed with $NaHCO_3$–H_2O, then dried over Na_2SO_4, and the solvent was removed. The residue was applied to a silica gel column (10 × 50 cm). The eluate with $CHCl_3$–MeOH (20 : 1) was concentrated to leave **14** (590 mg, 88%). ^{1}H-NMR ($CDCl_3$) δ: 9.0 (1H, br s, N^3H), 8.64 (1H, br s, NH), 7.96 (1H, s, H-6), 7.69—7.03 and 6.81 (9H and 4H, m and d, Ph), 6.23 (1H, t, H-1′), 5.1 (1H, m, H-3′), 4.32—4.18 (4H, m, CH_2-5, CH_2CH_2CN), 3.85 (1H, br s, H-4′), 3.77 (6H, s, CH_3O), 3.7—3.2 (6H, m, H-5′ and CH_2N), 2.70—2.58 (2H, m, CH_2CN), 2.42 (2H, m, H-2′), 1.61 (4H, br s, CH_2CH_2).

***N*,*N*′-Bis(trifluoroacetyl)putrescinylthymidine 3′-(4-Chlorophenyl 2-cyanoethyl)phosphate (15)**——Compound **14** (prepared from 0.5 mmol of **10**) was dissolved in a 2% solution of benzenesulfonic acid ($CHCl_3$–MeOH, 7 : 3, 5 ml). After 20 min, the solution was diluted with $CHCl_3$ and washed with 5% $NaHCO_3$ and H_2O, then dried over Na_2SO_4. The solvent was removed and the residue was chromatographed (silica gel, 1.5 × 50 cm). The eluate with $CHCl_3$–MeOH (20 : 1) was concentrated to leave **15** (290 mg, 59.4% from **10**). This was used immediately for the next step.

Triethylammonium 5′-*O*-Dimethoxytrityl-*N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine 3′-(4-Chlorophenyl)-phosphate (16)——a) Compound **10** (400 mg, 0.48 mmol) in dioxane (1 ml) was treated with *p*-chlorophenyl-phosphoroditriazolide (0.1 M in dioxane, 3.5 ml) at room temperature for 1.5 h. Aqueous pyridine (90%, 1 ml) was added and the mixture was concentrated to a volume of *ca.* 2 ml. This was extracted with $CHCl_3$ and the organic layer was washed with 0.1 M triethylammonium bicarbonate. Pyridine was added to the solution, the solvent was removed *in vacuo* and the residue was dried by co-distillation with pyridine several times to leave **16** (480 mg, 91%). This was used for the next step.

b) Compound **14** (590 mg, 0.55 mmol) in pyridine (3 ml) was treated with triethylamine (1.0 g, 10 mmol) at room temperature for 4 h. The solvent was removed *in vacuo* and the residue was dried by co-distillation with pyridine to leave **16**, which was stored at −20 °C until use.

Synthesis of TT^p and T^{p}T——A solution of the triethylammonium salt of 5′-*O*-dimethoxytritylthymidine 3′-(*p*-chlorophenyl)phosphate (96 mg, 0.12 mmol) and **13** (56 mg, 0.10 mmol) in pyridine (1 ml) was treated with TPSCl (156 mg, 0.5 mmol) and *N*-methylimidazole (82 mg, 1 mmol) at room temperature for 1 h. The reaction was quenched by addition of H_2O (0.1 ml) in an ice bath, and $CHCl_3$ was added. The organic layer was washed with 5% $NaHCO_3$ and H_2O, then dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue was applied to a column of silica gel (0.9 × 50 cm). The eluate with $CHCl_3$–MeOH (30 : 1) was concentrated to leave the protected dimer (130 mg, 93%) as a foam. The foam was taken up in conc. NH_4OH (15 ml) and pyridine (5 ml), and the mixture was kept at room temperature for 12 h, then at 55 °C for 4 h. The solvent was removed *in vacuo* and the residue was dissolved in 80%

AcOH (5 ml). After 1 h at toom temperature, the solvent was removed *in vacuo*, and the residue was co-distilled with toluene to remove a trace of AcOH. The residue was dissolved in 0.1 M triethylammonium bicarbonate and the solution was washed with EtOAc several times, then concentrated. The residue was applied to a column of Amberlite IRC 50 (H^+) resin (1 × 40 cm) and the column was washed with H_2O, then eluted with 0.3 M NH_4OH. The eluate ($A_{260} > 1$) was concentrated and the residue was chromatographed on DEAE-cellulose. The eluate with 0.1 M triethylammonium bicarbonate was concentrated to leave TT^P ($TOD_{260} = 1100$). ^{1}H-NMR (D_2O, the number 1 in parentheses indicates the 5′-nucleoside protons and 2, the 3′-nucleoside protons) δ: 8.00 (1H, s, H-6(2)), 7.65 (1H, s, H-6(1)). TT^P was not hydrolyzed by VPDase and APase (37 °C, 3 d) as checked by HPLC. T^PT was prepared by a similar method. The diester (125 mg, 0.12 mmol) from **14** and 3′-*O*-acetylthymidine (39 mg, 0.1 mmol) were condensed to give the protected dimer (140 mg, quant.), which was deprotected to leave T^PT ($TOD_{260} = 1030$). Digestion with VPDase and APase (37 °C, 3 d) gave dT and dT^P ($T/T^P = 1.05$). ^{1}H-NMR (D_2O): δ: 7.85 (1H, s, H-6(2)), 7.69 (1H, s, H-6(1)), 6,24, 6.23 (1H each, t, H-1′), 4.67 (1H, m, H-3′(2)), 4.59—4.00 (4H, m, H-4′(1, 2), H-5′(1)), 3.83—3.69 (5H, m, CH_2-5, H-3′(1), H-5′(2)), 3.01, 2.83 (2H each, m, CH_2N), 2.6—2.4, 2.35—2.1 (1H and 3H, m, H-2′(1, 2)), 1.90 (3H, s, CH_3(2)), 1.70 (4H, m, CH_2CH_2).

Synthesis of Oligomers by the Liquid-Phase Phosphotriester Method——The following oligomers were prepared by liquid-phase oligonucleotide-block condensation *via* phosphotriester method[16,17] starting from **15** or **16** for introduction of T^P: TTCTT, TT^PCTT, TT^PCT^PT, AAGAATTCTT, $AAGAATTT^PCTT$, $AAGAATTCT^PT$, and $AAGAATT^PCT^PT$.

Synthesis of Dodecanucleotides by the Solid-Phase Phosphotriester Method——The following dodecanucleotides were prepared by the solid phase phosphotriester method developed by Itakura *et al.*[20] on aminomethylpolystyrene as the polymer support: AGATAGCTATCT, $AGAT^PAGCTATCT$, $AGATAGCT^PATCT$, $AGATAGCTAT^PCT$, TTTTTTTTTTTT, $TT^PTTT^PTTT^PT$, $TTT^PTTTT^PTTTT^PT$, $TTT^PTTTTTT^PTTT$, $TTT^NTTTTTT^NTTT$, and $TTT^OTTTTTT^OTTT$.

Annealing of Poly(dA) : Oligo(dT)——Poly(dA) (0.86 OD_{265}/ml) and oligo(dT) containing T^P, T^N or T^O (0.89 OD_{265}/ml) were annealed in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.2) by the reported procedure.[29]

Digestion of Oligothymidylates by Nuclease S1——Oligothymidylate or an analog containing T^P, T^N, and T^O (0.5—1.0 OD unit) was incubated in a buffer (50 mM $AcONH_4$, 50 mM NaCl, 5 mM $ZnSO_4$, pH 5.0, 60 μl) with nuclease S1 (5×10^5 unit/ml, 10 μl) at 50 °C overnight. The hydrolyzate was analyzed by HPLC. In the case of T_{12}, the molar ratio of 5′-TMP and thymidine was 1.0 : 10.8.

Digestion of Oligothymidylates by VPDase——The dodecathymidylate containing T^P at positions 3 and 9, T^N at positions 3 and 9, or T^O at positions 3 and 9 (*ca.* 2 OD units) was incubated with VPDase (1 mg/ml, 10 μl) and bacterial alkaline phosphatase (360 units/ml, 5 μl) in a buffer (100 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 8.9) at 37 °C for 15 h. The hydrolyzate was analyzed by HPLC.

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