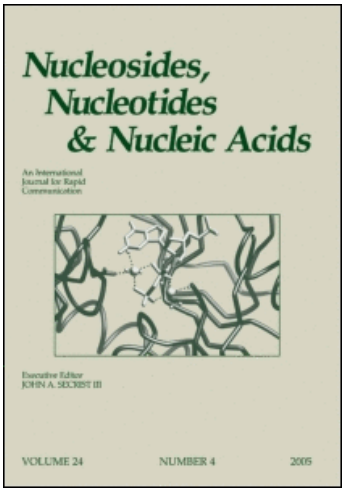

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Synthesis and Nuclease Stability of Dinucleotides Containing an *Anti*-conformationally Constrained Acyclic Thymidine

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SYNTHESIS AND NUCLEASE STABILITY OF DINUCLEOTIDES CONTAINING AN *ANTI* CONFORMATIONALLY CONSTRAINED ACYCLIC THYMIDINE

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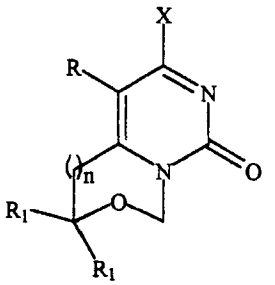
ABSTRACT: Novel heterodimers containing an *anti* conformationally constrained acyclic thymidine were prepared and the nuclease resistance of the modified dinucleotides were studied.

INTRODUCTION

In recent years modified oligonucleotides have become an area of increased activity due to their potential use as antiviral and antitumoral agents. [1] These oligonucleotides are modified either in the base, the sugar or the phosphate moiety. All these modifications are intended to fulfill certain requirements such as stability against enzymatic breakdown, high solubility in biological fluids, penetration into cells and sufficient binding to their complementary sequence. Sugar-modified oligonucleotides, [2] including α-oligonucleotides, 2'-O-methyloligoribonucleotides, acyclic oligonucleotides[3-5] and locked sugar pucker oligonucleotides[6-8] have been prepared to address these issues. Among these sugar-modified oligonucleotides, acyclic oligonucleotides have gained little attention probably because they showed a large decrease in melting temperature and thus a large decrease in duplex stability. [4] The decrease in duplex stability may be attributed to the loss of entropy as a cyclic sugar has been replaced by a flexible acyclic sugar. One way to overcome the problem due to the loss of entropy is to prepare conformationally constrained analogues of acyclic nucleosides as the building units for incorporation into



X = OH, NH_2
R = H, CH_3, Cl, Br, I
R_1 = CH_2OH, H
n = 1, 2

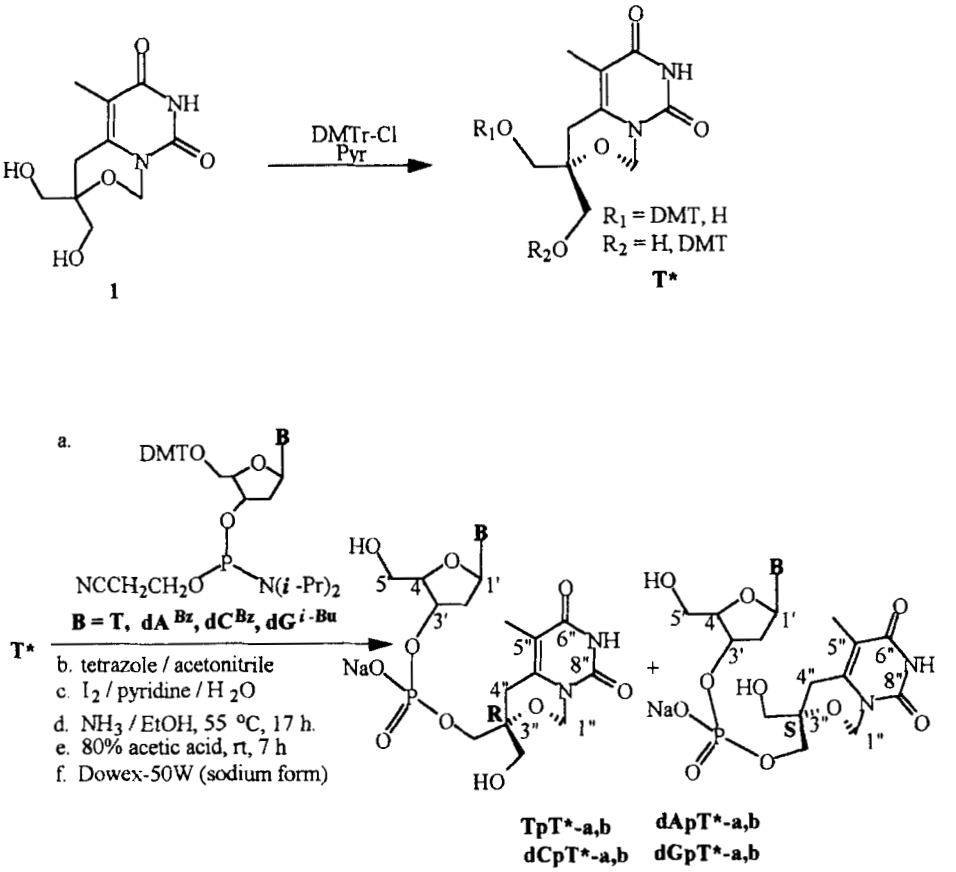
FIGURE 1. The chemical structure of *anti*-conformationally constrained pyrimidine acyclic nucleosides.

oligonucleotides. Recently, we have synthesized a series of *anti*-conformationally constrained pyrimidine acyclic nucleosides (FIGURE 1).[9-15] Some of them showed slight activity against HIV[10] or breast cancer cell line.[13] We think these conformationally constrained pyrimidine acyclic nucleosides can be used as building units for oligonucleotide synthesis. These *anti*-conformationally constrained pyrimidine acyclic oligonucleotides may reduce the loss of entropy of the unconstrained, acyclic nucleosides, and possibly enhance the binding feature to their target sequence. As a first step of this program of oligonucleotide research, we report here on the synthesis and nuclease stability of dinucleotides containing an *anti* conformationally constrained acyclic thymidine.

RESULTS AND DISCUSSION

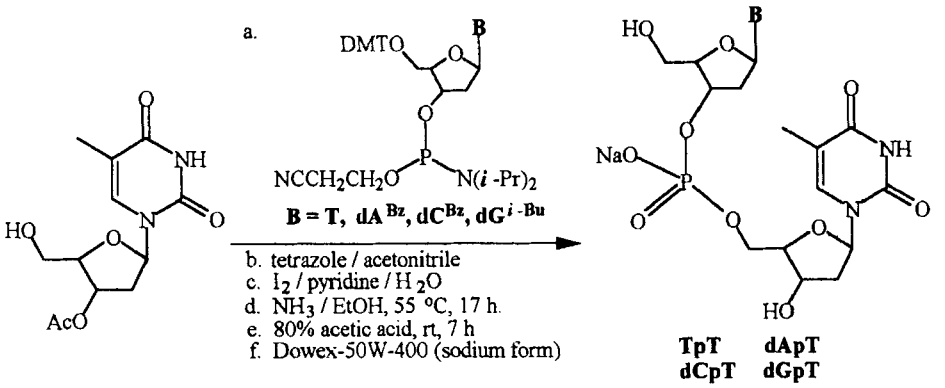
Chemical

The synthesis of the dinucleotides containing an *anti* conformationally constrained acyclic thymidine involved the preparation of **1** following the methodology previously described.[10] Mono-tritylation of the two primary hydroxyl groups on **1** with 1 equivalent of 4,4'-dimethoxytrityl chloride in the presence of pyridine and triethylamine afforded mono-tritylated **T*** as a racemic (R and S) mixture. Without isolation of the mixture, coupling of **T*** with the corresponding nucleoside phosphoramidites (**T**, $\mathbf{dA^{Bz}}$,



SCHEME 1

dC^{Bz}, and $dG^{i\text{-}Bu}$) in the presence of tetrazole, followed by oxidation with iodine gave the fully protected dinucleotides in high yield. Deprotection of the cyanoethyl and trityl protecting groups of these dinucleotides with aqueous ammonia and 80% acetic acid respectively afforded the corresponding dinucleotides. The resulting compounds were passed through a column of Dowex 50W-400 resin to give the sodium salt of the dinucleotides (**TpT***, **dAPT***, **dCPT***, and **dGPT***) each as a mixture of two diastereomers (**a** and **b**), one diastereomer containing the phosphodiester linkage between C3' of natural nucleosides and C3"R of constrained pyrimidine acyclic nuceoside (*i.e.* C3'-C3"R) and another having the linkage between C3' and C3"S (*i.e.* C3'-C3"S)(SCHEME 1). An attempt to separate each diastereomer (C3'-C 3"R and C 3'-C 3"S) from the



SCHEME 2

mixture of each dinucleotide by reverse phase column chromatography (RP-18, water / methanol = 95 : 5) failed. Each diastereomeric mixture of the four modified dinucleotides were characterized by ^{1}H and ^{31}P nmr, Mass, and elementary analysis. ^{31}P nmr spectra of the diastereomeric mixture of the four modified dinucleotides showed two peaks corresponding to internucleotide phosphate signal. This was considered as evidence that the two diastereomers (C 3'-C 3"R and C 3'-C 3"S) were present in the mixture. In order to assess the enzymatic stability of the four modified dinucleotides, three nucleases, nuclease S1, Bovine Spleen Phosphodiesterase (BS PDE), and Snake Venom Phosphodiesterase (SV PDE) were used. Four natural dinucleotides (**TpT**, **dAPT**, **dCPT**, and **dGPT**) were also synthesized (SCHEME 2) and used in the studies of enzymatic stability as a control group. The enzymatic degradation of the dinucleotides was followed by reverse phase high performance liquid chromatography (HPLC). Interestingly, each diastereomeric mixture of the four modified dinucleotides could be separated by reverse phase HPLC using 10% methanol in 0.1 M triethylammonium acetate, pH = 7 as the mobile phase. Therefore the four diastereomers with short retention time were designated **a** (**TpT*-a**, **dAPT*-a**, **dCPT*-a**, and **dGPT*-a**) and the other four diastereomers having longer retention time than **a** were designated **b** (**TpT*-b**, **dAPT*-b**, **dCPT*-b**, and **dGPT*-b**). The hydrolysis of each diastereomeric mixture of the four modified dinucleotides by three nucleases was assessed by measuring of each diastereomeric signal with an integrator and the results were tabulated in TABLE 1. It

TABLE 1. Enzymatic stability of the modified dinucleotides toward nuclease S1, bovine spleen phosphodiesterase (BS PDE) and snake venom phosphodiesterase (SV PDE).

	$t_{1/2}$(min)		
	Nuclease S1	BS PDE	SV PDE
dApT	6.2	3	8.7
dApT*-a	47.8	320	S
dApT*-b	111.8	S	S
dGpT	3.96	3.3	4.6
dGpT*-a	25.5	520	S
dGpT*-b	64.2	S	S
dCpT	2.75	6.4	13
dCpT*-a	34.6	S	S
dCpT*-b	71.7	S	S
TpT	2.25	3.4	8.5
TpT*-a	24.75	533	S
TpT*-b	60.3	S	S

p: phosphate; dA: 2'-deoxyadenosine; dG: 2'-deoxyguanosine; dC: 2'-deoxycytidine
T: thymidine; T*: *anti* conformationally constrained acyclic thymidine
S: completely stable (> 24 hr) under the reaction condition.

was found that the modified dinucleotides were much more slowly cleaved than the natural dinucleotides by nuclease S1and BS PDE. However, they were highly resistant to degradation by SV PDE. Nuclease S1 degradation stability followed the order **dApT*** > **dCpT*** > **dGpT*** > **TpT***, while for BS PDE the stability order was **dCpT-a*** > **TpT*-a** > **dGpT*-a** > **dATP*-a**. It was of interest that nuclease S1 and BS PDE digested each diastereomer **b** of the modified dinucleotides slower than the corresponding diastereomer **a**. It may be concluded that diastereomers **a** were better substrate for nuclease S1 and BS PDE than diastereomers **b**. This information helped us to postulate structures for diastereomers **a** and **b**. For diastereomers **a** to be a better substrate for nuclease S1 and BS PDE, the C3'-C3"R linkage must be similar to the C3'-C5' linkage found in natural polynucleotides. Diastereomers **b**, on the other hand, must contain the alternative C3'-C3"S configuration. However, the exactly structural assignment of diastereomers **a** and **b** needs to be verified conclusively by other means.

In conclusion, in this paper a synthesis of the modified dinucleotides containing an *anti* conformationally constrained acyclic thymidine is reported. The modified

dinucleotides possess excellent enzymatic stability toward nuclease S1, bovine spleen phosphodiesterase and snake venom phosphodiesterase. Further experiments, incorporating the modified dinucleotides by automatic procedures into oligonucleotide sequences are currently in progress.

EXPERIMENTAL SECTION

General Methods. Melting points were taken on a BUCHI 530 apparatus and are uncorrected. The silica gel used for chromatography was silica gel 60 70-230 mesh (E. Merck, Darmstadt, Germany), TLC was performed on prescored DC-Alufolien Kieselgel $60F_{254}$ (E. Merck, Darmstadt, Germany). Reverse phase-TLC was performed on prescored DC-Fertigplatten Rp-$18F_{254}$ (E. Merck, Darmstadt, Germany). Compounds were visualized by exposing the plates to UV light (254 nm). Evaporations were carried out at < 50 °C using a rotary evaporator upon reduced pressure (water aspirator). Solvent ratios are reported as v/v. ^{1}H and ^{13}C nmr spectra were obtained using Varian 300 nmr spectrometer. ^{31}P nmr spectra were recorded on a Bruker AMX-400. Where necessary, deuterium exchange experiments were used to obtain proton shift assignments. Analytical samples were dried under reduced pressure at 78 °C in the presence of P_2O_5 for at least 12 h unless otherwise specified. Elemental analyses were obtained from Perkin-Elmer 2400 Elemental Analyzer.

(±) 3-Hydroxymethyl-3-dimethoxytrityloxymethyl-5-methyl-(1*H*,3*H*,4*H*,7*H*)-pyrimido[1,6-*c*][1,3]oxazin-6,8-dione (T*). A mixture of compound **1** (1.1 g, 4.5 mmol), 4,4'-dimethoxyltrityl chloride (1.7 g, 4.6 mmol), dry pyridine (30 mL) and triethylamine (2 mL) was stirred under anhydrous condition at room temperature for 15 h. The reaction was quenched with methanol (2 mL) and the solvent was removed under reduced pressure. Dissolve the residue in chloroform (50 mL), wash the solution with 1 M aqueous sodium bicarbonate (2 x 50 mL), dry it over sodium sulfate, filter it, and evaporate solvent under reduced pressure. The residue was chromatographed on silica gel and eluted with ethyl acetate / dichloromethane (4:1 v/v) containing 0.5% triethylamine. Evaporation of solvent from the product fractions yielded pure compound **T***, 1.96 g (81%). Mp 126-128 °C; R_f = 0.3 (AcOEt / CH_2Cl_2 = 4:1 + 0.5% triethylamine); ^{1}H nmr

($CDCl_3$) δ 8.56 (s, 1H, NH), 7.32-7.16 (m, 8H, aryl), 6.78 (m, 5H, aryl), 5.40-5.29 (d, J = 8.0 Hz, 2H, H_2-1), 3.72 (s, 6H, OCH_3), 3.55 (s, 2H, CH_2O), 3.13 (d, J = 12.0 Hz, 2H, CH_2O), 2.93-2.78 (d, J = 16.0 Hz, 2H, H_2-4), 1.80 (s, 3H, CH_3); Ms (*m/z*): 546 MH^+, Anal. Calcd. for $C_{31}H_{32}O_7N_2$ (544.60): C, 68.37%; H, 5.92%; N, 5.14%. Found: C, 68.44%; H, 6.22%; N, 4.91%.

Thymidylyl-(3', 3")-[3-hydroxymethyl-5-methyl-(1*H*,3*H*,4*H*,7*H*)-pyrimido[1,6-*c*][1,3]oxazin-6,8-dione]phosphate, sodium salt (TpT*-a and TpT*-b)

T* (710 mg, 1.3 mmol) was dried by coevaporation twice with pyridine. A mixture of previously dried **T***, thymidine phosphoramidite (2 g, 2.6 mmol) and tetrazole (400 mg, 5.7 mmol) in anhydrous acetonitrile (15 mL) was stirred for 30 min at room temperature under nitrogen atmosphere. Then a solution of iodine (0.5 g) in dichloromethane / pyridine / water (1:3:1) was added dropwise until no decoloration was observed, and the mixture was further stirred for 30 min. The mixture was diluted with dichloromethane (200 mL) and washed twice with 200 mL of brine (the first time containing a small amount of sodium sulfite to reduce excess iodine). The organic phase was dried, evaporated to dryness and purified by column chromatography to yield 1.45 g of the intermediate as a white powder. This intermediate was dissolved in ethanol (3 mL) and treated with concentrated ammonia (2 mL). After stirring for 30 min at room temperature, 10 mL of concentrated ammonia was added. The mixture was kept for 8 h at 55 °C, evaporated and coevaporated with dry pyridine and with dry tetrahydrofuran. The residue was treated with 80% aqueous acetic acid (20 mL) for 7 h at room temperature. The reaction mixture was washed with dichloromethane twice. The aqueous phase was evaporated and coevaporated twice with water to dryness. The residue was passed through a DOWEX 50W-X4 column to convert the dinucleotide as the sodium salt form. This dinucleotide was further purified by reverse phase column chromatography (RP-18, water / methanol = 95 : 5) to yield 215 mg (29%) of **TpT*-a,b** as a white powder. Mp 241-245 °C; R_f = 0.55 (RP-18, water / methanol = 95 : 5); ^{31}P nmr (D_2O): δ -0.43, -0.49; ^1H nmr (D_2O) δ 7.48 (s, 1H, H-6), 6.10 (t, J = 8.0 Hz, 1H, H-1'), 5.26 (d, J = 12.0 Hz, 2H, H_2-1"), 4.61 (m, 1H, H-3'), 4.01 (q, J = 4.0 Hz, 1H, H-4'), 3.74-3.59 (m, 4H , H_2-5', CH_2OP-3"), 3.51 (s, 2H, CH_2O-3"), 2.95-2.87 (m, 2H , H_2-4"), 2.32-2.19 (t, J = 4.0 Hz, 2H, H_2-2'), 1.75 (s, 3H, CH_3-5"), 1.72 (s, 3H, CH_3-5); Ms (*m/z*): 569 MH^+; Anal. Calcd. for

$C_{20}H_{25}O_{12}N_4PNa\bullet 4H_2O$ (640.47): C, 37.50%; H, 5.34%; N, 8.65%. Found: C, 37.42 %; H, 5.29 %; N, 8.71 %.

2'-Deoxyadenosylyl-(3',3")-[3-hydroxymethyl-5-methyl-(1*H*,3*H*,-4*H*,7*H*)-pyrimido[1,6-*c*][1,3]oxazin-6,8-dione]phosphate, sodium salt (dApT*-a and dApT*-b) **dApT*** was prepared in a similar manner to the preparation of **TpT***. Yield 24%; Mp 235-238 °C; R_f = 0.30 (RP-C18, water / methanol = 95 : 5); ^{31}P nmr (D_2O): δ -0.32, -0.38; ^1H nmr (D_2O) δ 8.12 (s, 1H, H-2), 8.01 (s, 1H, H-8), 6.26 (t, J = 4.0 Hz, 1H , H-1'), 5.21 (d, J = 12.0 Hz, 2H , H_2-1"), 4.75 (m, 1H, H-3'), 4.18 (m, 1H, H-4'), 3.78 (s, 2H , CH_2OP-3"), 3.68 (d, J = 4.0 Hz, 2H , H_2-5'), 3.52 (s, 2H, CH_2O-3"), 2.83-2.30 (m, 2H , H_2-4"), 2.48.-2.52 (m, 2H , H_2-2'), 1.74 (s, 3H, CH_3); Ms (*m/z*): 578 MH^+ ; Anal. Calcd. for $C_{20}H_{25}O_{10}N_7PNa\bullet 6H_2O$ (685.51): C, 35.10%; H, 5.43%; N, 14.30%. Found: C, 35.33%; H, 5.13%; N, 14.23%.

2'-Deoxycytidylyl-(3',3")-[3-hydroxymethyl-5-methyl-(1*H*,3*H*,4*H*,7*H*)-pyrimido[1,6-*c*][1,3]oxazin-6,8-dione]phosphate, sodium salt (dCpT*-a and dCpT*-b) **dCpT*** was prepared in a similar manner to **TpT***. Yield 27%; Mp 248-250 °C; R_f = 0.50 (RP-C18, water / methanol = 95 : 5); ^{31}P nmr (D_2O): δ -0.38, -0.44; ^1H nmr (D_2O) δ 7.68 (d, 1H, H-6), 6.12 (t, J = 8.0 Hz, 1H , H-1'), 5.89 (d, J = 8.0 Hz, 1H, H-5), 5.26 (m, 2H, H_2- 1"), 4.65 (m, 1H, H- 3'), 4.06 (m, 1H, H-4'), 3.75 (s, 2H, CH_2OP -3"), 3.66 (d, 2H , H-5'), 3.51 (s, 2H, CH_2O -3"), 2.96-2.90 (d, J = 4.0 Hz, 2H, H_2-4"), 2.39-2.16 (t, J = 4.0 Hz, 2H , H_2 -2'), 1.78 (s, 3H, CH_3); Ms (*m/z*): 554 M^+; Anal. Calcd. for $C_{19}H_{24}O_{11}N_5PNa\bullet 4H_2O$ (624.45): C, 36.54%; H, 5.17%; N, 11.20%. Found: C, 36.59%; H, 5.19%; N, 11.16%.

2'-Deoxyguanosylyl-(3',3")-[3-hydroxymethyl-5-methyl-(1*H*,3*H*,4*H*,7*H*)-pyrimido[1,6-*c*][1,3]oxazin-6,8-dione]phosphate, sodium salt (dGpT*-a and dGpT*-b) **dGpT*** was prepared in a similar manner to **TpT***. Yield 29%; Mp 251-254 °C; R_f = 0.53 (RP-C18, water / methanol = 95 : 5); ^{31}P nmr (D_2O): δ -0.26, -0.38; ^1H nmr (D_2O) δ 7.80 (s, 1H, H-8), 6.10 (t, J = 8.0 Hz, 1H , H-1'), 5.26 (d, J = 12.0 Hz, 2H, H-1"), 4.75 (m, 1H, H-3'), 4.11 (m, 1H, H-4'), 3.77(s, 2H, CH_2OP-3"), 3.65 (d, J = 8.0 Hz, 2H , H_2-5'), 3.52 (s, 2H, CH_2O-3"), 282-2.86 (m, 2H , H_2-4"), 2.46-2.66 (m, 2H ,H_2-2'), 1.73 (s, 3H, CH_3); Ms (*m/z*): 594 MH^+; Anal. Calcd. for $C_{20}H_{25}O_{11}N_5PNa\bullet 5H_2O$ (683.49): C, 35.16%; H, 5.16%; N, 14.35%. Found: C, 35.55%; H, 5.04%; N,14.39%.

Thymidylyl-(3',5')-thymidine phosphate, sodium salt (TpT) **TpT** was prepared in a similar manner to **TpT***. Yield 29.8%; Mp 235-239 °C; R_f = 0.61 (RP-C18, water / methanol = 95 : 5); ^{31}P nmr (D_2O): δ -0.85; ^1H nmr ($CDCl_3$) δ 7.53, 7.50 (s, 1H each, H-6, H-6a), 6.17, 6.02 (t, J = 8.0 Hz, 1H each, H-1', H-1a'), 4.65, 4.44 (m, 1H each, H-3', H-3a'), 4.03-3.90 (m, 4H, H-4', H-4a', H_2-5a'), 3.65 (m, 2H, H_2-5'), 2.41-2.15 (m, 4H, H_2-2', H_2-2a'), 1.73-1.71 (s, 6H , CH_3); Ms (*m/z*): 569 MH^+; Anal. Calcd. for $C_{20}H_{26}O_{12}N_4PNa\bullet 5H_2O$ (657.47): C, 36.48%; H, 5.31%; N, 8.51%. Found: C, 36.39%; H, 4.95%; N,8.50%.

2'-Deoxyadenosylyl-(3',5')- thymidine phosphate, sodium salt (dApT) **dApT** was prepared in a similar manner to **TpT***. Yield 33%; Mp 223-228 °C; R_f = 0.47 (RP-C18, water / methanol = 95 : 5); ^{31}P nmr (D_2O): δ -0.96; ^1H nmr ($CDCl_3$) δ 8.06 (s, 1H, H-2), 7.91 (s, 1H, H-8), 7.31 (s, 1H, H-6'), 6.16 (t, J = 4.0 Hz, 2H, H_2-1'), 6.00 (t, J = 8.0 Hz, 2H , H_2-1a'), 4.70 (m, 1H, H-3'), 4.39 (t, J = 4.0 Hz, 1H, H-3a'), 4.15 (m, 1H, H-4'), 4.04 (m, 1H, H-4a'), 3.93 (d, J = 8.0 Hz 2H, H_2-5a'), 3.65 (m, 2H, H_2-5'), 2.64 (d, J = 4.0 Hz , 2H, H_2-2'), 2.11 (t, J = 4.0 Hz, 2H, H_2-2a'), 1.47 (s, 3H, CH_3); Ms (*m/z*): 578 MH^+ ; Anal. Calcd. for $C_{20}H_{25}O_{10}N_7PNa\bullet 5H_2O$ (667.50): C, 35.98%; H, 5.28%; N, 14.68%. Found: C, 35.79%; H, 4.96%; N,14.66%.

2'-Deoxyguanosylyl-(3',5')-thymidine phosphate, sodium salt (dGpT) **dGpT** was prepared in a similar manner to **TpT***. Yield 34%; Mp 229-232 °C; R_f = 0.68 (RP-C18, water / methanol = 95 : 5); ^{31}P nmr (D_2O): δ -0.94; ^1H nmr ($CDCl_3$) δ 7.75 (s, 1H, H-8), 7.39 (s, 1H, H-6a), 6.02-6.11 (m, 2H, H-1', H-1a'), 4.72 (m, 1H, H-3'), 4.40 (m, 1H, H-3a'), 3.92-4.13 (m, 4H, H-4', H-4a', H_2-5a'), 3.66 (m, 2H, H-5'), 2.14-2.61 (m, 4H, H_2-2', H_2-2a'), 1.54 (s, 3H, CH_3); Ms (*m/z*): 594 MH^+; Anal. Calcd. for $C_{20}H_{25}O_{11}N_7PNa\bullet 5H_2O$ (682.48): C, 35.15%; H, 5.02%; N, 14.36%. Found C, 34.76%; H, 4.63%; N,14.22%.

2'-Deoxycytidylyl-(3',5')-thymidine phosphate, sodium salt (dCpT) **dCpT** was prepared in a similar manner to **TpT***. Yield 33%; Mp 231-235 °C; R_f = 0.60 (RP-C18, water / methanol = 95 : 5); ^{31}P nmr (D_2O): δ -0.83 ; ^1H nmr ($CDCl_3$) δ 7.67 (d, J = 8.0 Hz, 1H, H-6), 7.54 (s, 1H, H-6a), 6.02-6.16 (m, 2H, H-1', H -1a'), 5.82 (d, J = 8.0 Hz, 1H, H-5), 4.65 (m, 1H, H-3'), 4.43 (m, 1H, H-3a'), 3.68-4.04 (m, 4H, H-4' , H-4a', H_2-5a'), 3.63 (m, 2H, H_2-5'), 2.16-2.42 (m, 4H, H_2-2' , H_2-2a'), 1.72 (s, 3H, CH_3); Ms

(*m/z*): 554 MH^+; Anal. Calcd. for $C_{19}H_{24}O_{11}N_5PNa \bullet 6H_2O$ (660.48): C, 34.55%; H, 5.49%; N, 10.60%. Found: C, 34.91%; H, 5.23%; N,10.78%.

Enzymatic stability of the dimers

Reverse phase HPLC on an analytical Cosmosil C-18 column (5 μm, 250 x 4.6 mm i.d.) was performed with a Model 110B Beckman pump. Detection was carried out with a Soma-S7702 UV-VIS detector set at 263 nm. A mobile phase of 10% methanol in 0.1 M triethylammonium acetate (pH = 7) pumped at a flow rate of 1.0 ml/min was used. The hydrolysis of the dimers by several nucleases was judged by integration of the dimer signal with a Shimadzu LC-R6A integrator. Results are indicated in Table 1.

Nuclease S1

A solution of the dimer (1 $OD^{263\ nm}$) in 270 μL of deionized water was mixed with the following buffer (30 μL): 0.3 M sodium acetate (pH 4.8), 0.5 M sodium chloride, 0.01 M zinc chloride. Nuclease S1 (2 μL = 2000 U) was then added. The mixture was incubated at 37 °C and aliquots (10 μL) were removed at given times and mixed with the following blocking buffer (390 mL): 10 mM sodium phosphate (pH 6.8). This mixture was heated at 95 °C for 5 min and then cooled again. 50 μL volumes were injected on HPLC.

Bovine spleen phosphodiesterase

A solution of the dimer (1 $OD^{263\ nm}$) in 200 μL of deionized water was mixed with 100 μL of a 3 mM sodium citrate (pH 6.0). 8 μL (0.1 U) of bovine spleen phosphodiesterase [a solution of the enzyme in 50 mM sodium citrate (pH 6.0), 50% glycerol (v/v)] was then added. The rest was carried out as described for nuclease S1.

Snake venom phosphodiesterase

A solution of the dimer (1 $OD^{263\ nm}$) in 200 μL of deionized water was mixed with the following buffer (100 μL): 0.3 M Tris.HCl (pH 8.8), 0.3 M sodium chloride, 0.040 M magnesium chloride. 10 μL (0.1 U) of snake venom phosphodiesterase [a solution of the enzyme in 5 mM Tris.HCl (pH 7.5), 50% glycerol (v/v)] was then added. The rest was carried out as described for nuclease S1.

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