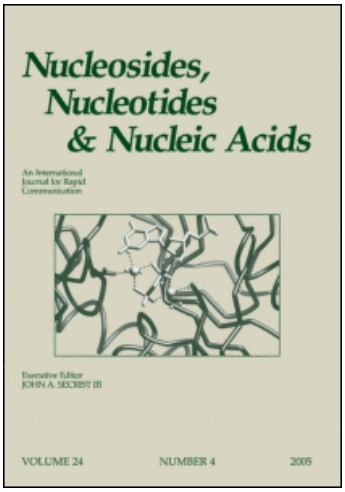

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DINUCLEOSIDE PHOSPHATES CONTAINING ARABINOSE OR DEOXYXYLOSE. HYDROLYSIS BY EXONUCLEASES AND STACKING PROPERTIES

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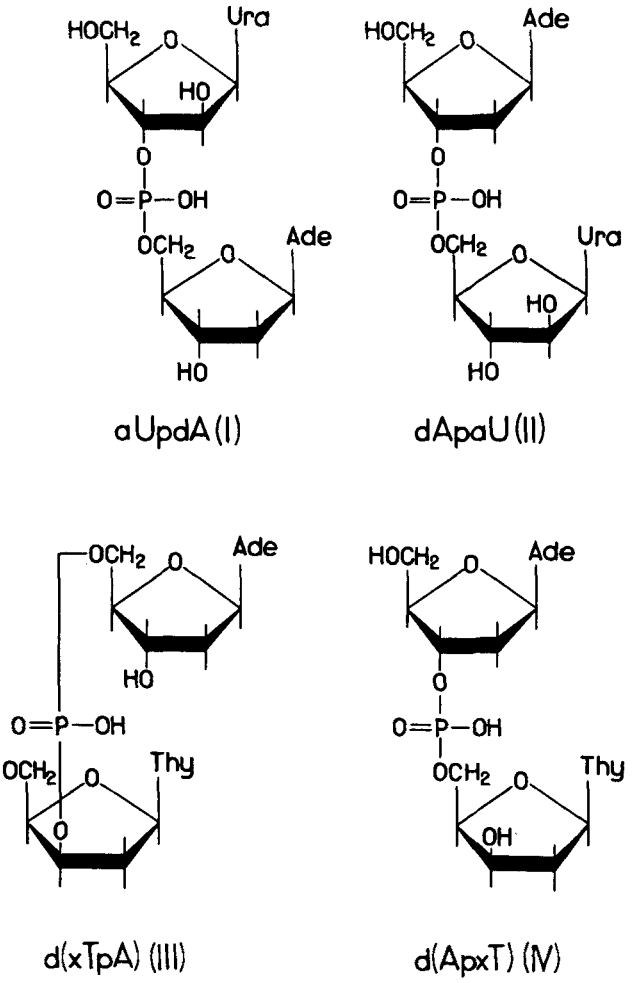
ABSTRACT. Two pairs of isomeric dinucleoside monophosphates containing either 1-(β-D-arabinofuranosyl)uracil (arabino-U, aU) or 1-(β-D-2-deoxy-*threo*-pentofuranosyl) thymine (2′-deoxyxylo-T, dxT) were synthesized. The kinetics of the hydrolysis of these dimers by venom and spleen phosphodiesterases (PDE) were studied. In addition, circular dichroism was used to study their stacking behaviour. A correlation was found to exist between the enzymatic cleavage rate and the conformation of the dimers. It was found that the configuration change at C-2′ or C-3′ in the 5′-terminal nucleoside residue, especially the inversion of 3′-hydroxyl involved into the internucleotide 3′-5′-phosphodiester linkage formation, was critical.

INTRODUCTION

Significant biological activity is an important property of the arabino- and xylonucleosides. The molecular basis of their influence on biosynthesis and biotransformation of the nucleic acids *in vivo* are being studied extensively [1]. For example, such nucleosides were shown to be incorporated into some DNAs; the arabino-A and arabino-C nucleosides were found to inhibit the primary steps of replication [2]. Ohtsuka et al. [3] were first to demonstrate a decrease in the *EcoRI* restriction endonuclease hydrolysis of the synthe-



SCHEME

tic oligonucleotides containing an *EcoRI* recognition site with a single change of dA for arabino-A. Up to date there is no detailed examination of the influence of single arabino- and xylonucleosides on the intrinsic mechanisms of nucleic acid-protein interaction and the nucleic acid metabolism enzymes. The data concerning the stability of the oligonucleotides with modified sugar moieties to nucleases currently

gain additional importance due to the search for the nuclease-insensitive oligonucleotide probes.

Dinucleoside monophosphates are the simplest models of the nucleic acids containing the nucleosides with altered configuration at C-2'- or C-3'-atoms. That is why we synthesized four dinucleoside monophosphates (compounds (I)-- (IV) SCHEME) containing arabino-U or 2'-deoxyxylo-T and studied their hydrolysis by snake venom and spleen phosphodiesterases . d(TpA) (V) was used as control.

RESULTS AND DISCUSSION

aU and dxT were obtained from the natural counterparts by use of anhydro nucleoside derivatives. CD, UV and ^{1}H NMR spectral data confirming the structure of these compounds have been reported previously [4,5]. In the course of the synthesis of MeOTraU it was found that the intermediate MeOTr-2',3'-uridine thiocarbonate (VI) formation took no more than 24 hours in contrast to 5 days reported earlier [6]. The purification of (VI) using the silica column chromatography increases the yield of the final product. The structure and purity of the key syntons containing anomalous sugars - MeOTr-O^2,2'-anhydroU, MeOTrdxT and MeOTraU was confirmed by UV and ^{1}H NMR spectroscopy data (see Experimental).

Dinucleoside monophosphates (I)-(IV) were synthesized by the phosphotriester approach, protected aU and dxT being the HO-components. To synthesize aUpdA (I) we used 5'-MeOTr-O^2,2'-anhydrouridine (VII), as a nucleoside component. The anhydro cycle-containing dinucleoside monophos-

phate formed in the course of the coupling reaction of compound (VII) with N^6-benzoyl-3'-levulinoyl-2'-deoxyadenosine -5'-(p-chlorophenyl)phosphate [(ClPh)pdbzA(Lev)] was subsequently treated with ammonium hydroxide in pyridine. This treatment resulted in both the removal of the alkali labile blocking groups and rupturing the O^2,2'-anhydro linkage. The latter reaction could be monitored spectrophotometrically [7]. The O^2,2'-anhydro cycle is stable in the condensation reactions and it can selectively protect the 2'-hydroxy function in the course of the synthesis of the arabinose-containing oligonucleotides.

Dinucleoside phosphates (I)-(V) were completely deblocked and isolated by HPLC.

The hydrolysis of the obtained dinucleoside phosphates (I)-(IV) by snake venom and spleen PDE was studied in detail. In the control experiments the hydrolysis of the unmodified dinucleoside phosphate d(TpA) (V) has been performed.

In order to further examine the basis for the substrate specificity of the compounds (I)-(IV) we carried out a kinetic analysis.The hydrolysis products were analysed using by HPLC. Retention times of nucleosides and nucleoside-5 '(or 3 '-) phosphates were identical to those of reference samples.FIG.1 shows the chromatography separation profile of the spleen PDE hydrolysis and describes the conditions of the analysis.

To characterize the substrate specificity of the dimers (I)-(V) the values of K_m and V_{max} were determined (see TABLE). The Michaelis-Menten equation was applied because

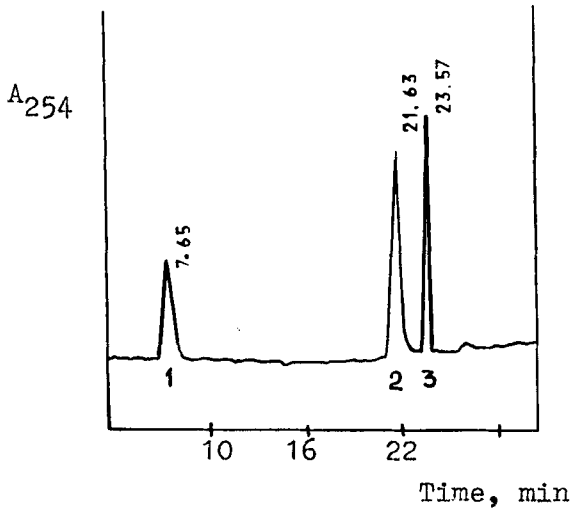


FIG. 1. HPLC analysis of the reaction mixture containing products of aUpdA hydrolysis with spleen PDE (30 min incubation). 1 - pdA; 2 - aU; 3 - aUpdA. The analysis was performed on the reversed phase column (Lichrosorb RP18) in 50 mM tris-acetate buffer, pH 7.0, using ethanol gradient elution (0-25%).

$[S_o) >> E$ in all the experiments. The graphics were plotted according to the least squares method using the "Replay" facility of the Spectra-Physics SP4200 integration system.

FIG. 2 exemplified the data for the snake venom PDE hydrolysis. It is clear from the TABLE that the dinucleoside phosphates (I)-(V) can be hydrolyzed by the snake venom as well as by the spleen PDE.As shown in TABLE and FIG.2, K_m values for the compounds (I)-(IV) tested are similar to that of d(TpA). This fact led us to suggest that the 3'-hydroxyl-

TABLE. UV spectroscopy data and kinetic parameters of hydrolysis for dinucleoside monophosphates by phosphodiesterases

Compound	UV *		Snake Venom PDE		Spleen PDE	
	(nm) λ_{max}	(nm) λ_{min}	K_m, mM	$V_{max} \cdot 10^2$, $\frac{mM}{min}$	K_m, mM	$V_{max} \cdot 10^2$, $\frac{mM}{min}$
aUpdA (I)	260	231	0.35±0.02	2.7±0.15	0.33	1.0±0.15
dApaU (II)	260	233	1.0±0.05	2.5±0.15	0.10	0.6±0.032
d(xTpA) (III)	261	231	0.3±0.02	0.3±0.02	**	**
d(ApxT) (IV)	262	232	0.91±0.05	2.0±0.1	0.12	0.53±0.03
d(TpA) (V)	260	231	0.31±0.02	3.7±0.2	0.52	12±0.51

* The spectra were measured in 0.02 M phosphate buffer, pH 7.3

** Under standard experimental conditions the hydrolysis does not occur.

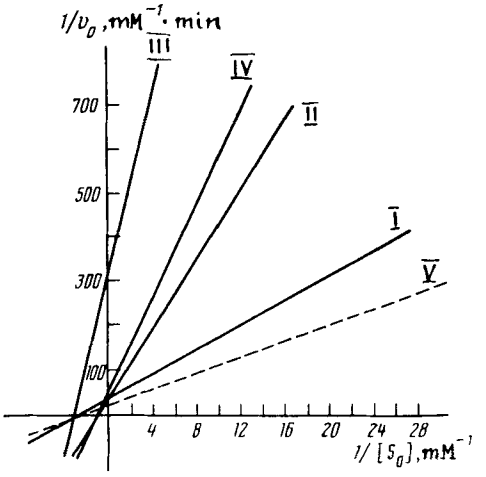


FIG. 2. Kinetic analysis. The reaction mixture (50 μl) contained snake venom PDE (5 a.u./ml) and one of the following substrates: I - aUpdA; II - dApaU; III -d(xTpA); IV - d(ApxT); V - d(TpA). The substrates concentrations ranged from 0.05 to 0.8 M.

or 2'-hydroxyl inversion was not critical for binding of the substrates to the enzyme active site. Nevertheless the hydrolysis rates of the modified substrates differed significantly. V_{max} values for the hydrolysis of the modified dimers by both snake venom and spleen PDE were considerably lower as compared with that of d(TpA) (V). It seems reasonable that the change of the configurations hinders the formation of the productive complex, thus interfering with the hydrolysis.

The most appreciable decrease takes place in case of d(xTpA) (III). So, when exposed to snake venom PDE the V_{max}

value for (III) is diminished tenfold and spleen PDE is incapable of hydrolyzing this substrate, even after 10 hours (standard conditions). A 10-fold increase of the spleen PDE concentration resulted in hydrolysis of 30% of d(xTpA) in 8 hours.

We suggest that the inversion of the hydroxy function at C3′ atom of the 5′-terminal nucleoside residue involved in the 3′-5′-internucleotide bond formation alters the geometry of the phosphodiester fragment, such that the interactions with the active site are suboptimal.The stability of the 2′-5′-bound arabinonucleosides to spleen PDE hydrolysis [8] is consistent with this view.

The dimer conformation may be an important factor which affects the rate of enzymatic cleavage. In the present paper we have studied the intramolecular stacking properties of dimers (I)-(IV) to characterize their conformational stability. It is known that the intensity change of CD effect on the temperature gives information about the base stacking in dinucleotides [9].

FIG. 3 shows the CD spectra of isomeric dimers (I) and (II) containing aU and dpA at 6 and 80°C; the dependences of the main band intensity on the temperature are presented on the inserts.

It is seen that the striking differences exist in the termal behaviour between these two compounds. The temperature dependence of $\Delta\varepsilon_{270}$ for dApaU is obvious and doesn't differ greatly from that for dAprU used as control. Notice that the base stacking tendency of mixed dimers, containing

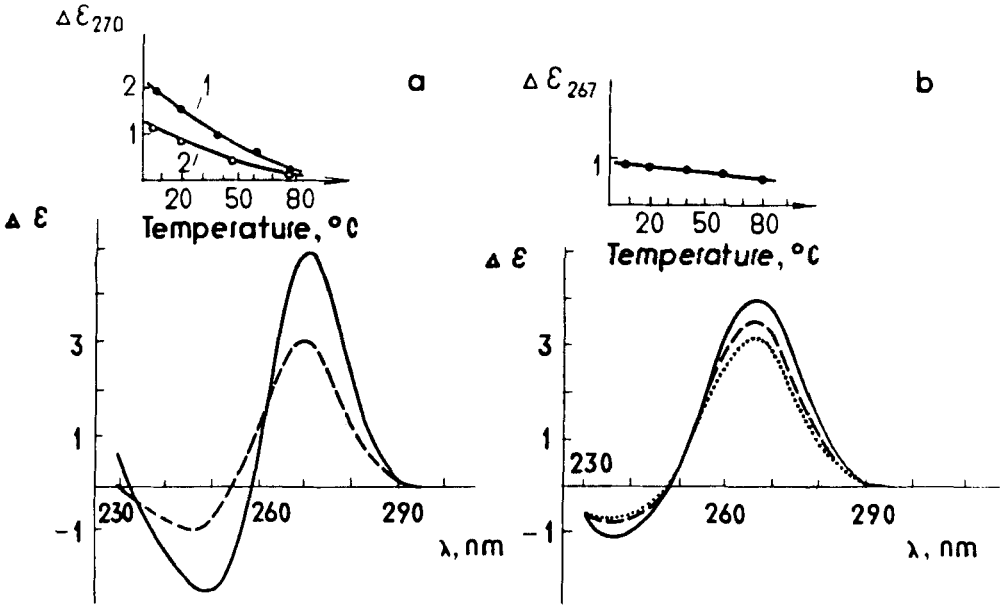


FIG. 3. CD spectra of dApaU (a) and aUpdA (b) at different temperatures: —— 6°C, --- 80°C, ... the contribution of the monomers aU and pdA. On the inserts: temperature dependence of CD of dApaU (a, curve 1), aUpdA (b), and dAprU (a, curve 2). The intensity of CD band is taken at the maximum of the positive longer wavelength band and is corrected for the contribution of monomers constituant. All spectra were measured in 0.02 M phosphate buffer, pH 7.3.

deoxyribose in the 5'-terminal residue, is much lower then that of ribonucleotide analogs [10]. The important feature of dimer (II) is the change of its CD spectrum with temperature, in particular, the shift in position of the cross-over point. These data led to the conclusion that a consi-

derable conformational change of dimer (II) occurs in this temperature range. On the other hand, the CD spectrum of aUpdA (I) is similar to that of the corresponding aU and dpA monomers and the temperature dependence of $\Delta\varepsilon_{267}$ has a very small slope (FIG. 3b).This means that the dimer (I) shows a very little stacking tendency.

The same difference in behaviour was observed in two pairs of dinucleoside phosphate isomers - ApaC and aCpA and also UpaC and aCpU [10]. These results indicate that in the case of pyrimidine-purine or pyrimidine-pyrimidine dinucleoside phosphates the substitution of 5′-terminal arabinose (aNp-) for ribose or deoxyribose causes unstacking; the presence of arabinose in 3′-terminal residue (-paN) does not impede base-base interactions. However, it is important to note that in arabinose-containing purine-purine dimer - aApaA - base stacking does occur [9].

The intensity and the shape of CD spectrum of d(xTpA) (III) at low temperature are not comparable with that of d(ApxT) (IV) and also with the spectrum representing the monomer contribution (FIG.4). The negative band at 275 nm is a characteristic feature of low temperature CD spectrum of d(xTpA), demonstrating that the sign of the Cotton effect is inverted. Increase of temperature induces a change in the total profile of the curve: namely, the shift of the wavelength of maximum and the disappearance of negative band at 275 nm (FIG. 4b). It appears that a left-handed conformation predominates in the stacked state of this dimer [11]. It should be noted that UV spectrum of

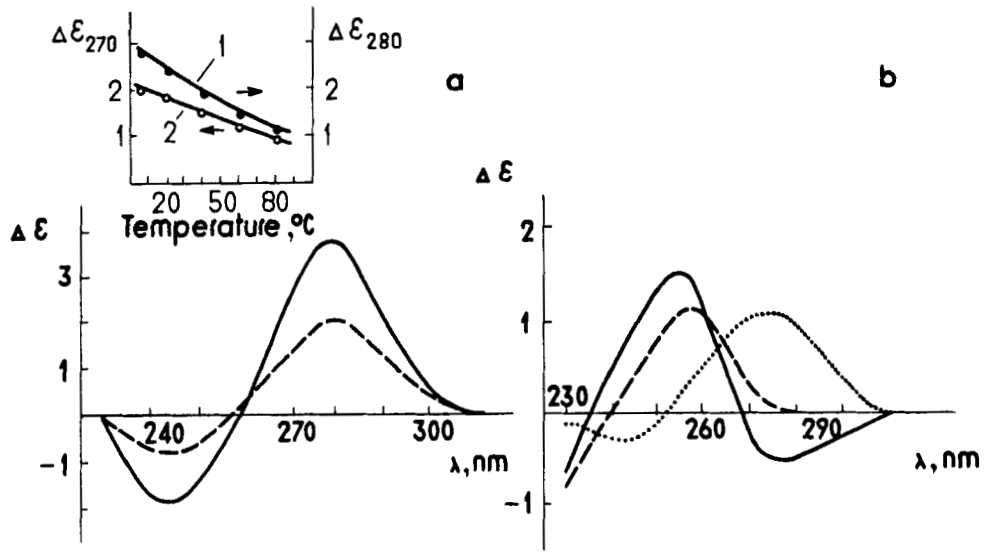


FIG. 4. CD spectra of d(ApxT) (a) and d(xTpA) (b) at different temperatures: —— 6°C, --- 80°C, ... the contribution of the monomers dxT and dpA. On the insert: temperature dependence of CD of d(ApxT) (curve 1) and d(TpA) (curve 2). Conditions as in FIG. 3.

(III) is almost identical with that of unmodified d(TpA) (see TABLE).

The CD spectrum of d(ApxT), containing deoxyxylose in 3'-terminal nucleoside residue, is characteristic of right-handed stacked conformation. As seen from FIG.4a, the intensity of the positive Cotton band of d(ApxT) and d(ApT) appreciably depends on the temperature. Notice that for these compounds the λ_{max} of positive and negative bands are not temperature dependent.

Summarizing briefly, the configuration change at C3′ or C2′ atoms in 3′-terminal nucleoside residue of dinucleoside monophosphate does not impede conformational stability and geometry of the molecule, whereas such modifications in 5′-terminal pentose change dramatically the dimer conformation. Thus, the bases are predominantly unstacked in aUpdA and there is the left-handed direction of the helical turn in d(xTpA).

EXPERIMENTAL

Used in the work were: nucleosides, monomethoxytrityl chloride, 2,4,6-triisopropylbenzenesulphonyl chloride (TPS), imidazole, thiocarbonyldiimidazole (Im_2CS), N-methylimidazole (CH_3Im), dimethylaminopyridine (DMAP) (Merck, FRG). (ClPh)pdbzA(Lev) (Novosibirsk, USSR), snake venom and spleen PDE (Sigma, FRG).

^1H NMR spectra were obtained on VXR-400 (Varian, USA) in $CDCl_3$ using $CHCl_3$ as an internal standard. UV spectra were recorded on 150-20 Spectrophotometer (Hitachi, Japan). CD spectra were recorded with a Roussel-Jouan III dicgrograph. Dependences of CD on temperature were studied in thermostatic quartz cuvettes (Hellma, FRG) with path length 10 mm. Temperature was monitored with a copper-constantant thermocouple. The concentrations were determined from the absorption spectra. Molar extinction coefficients of aU and dxT were taken equal to that of corresponding unmodified nucleosides. The circular dichroism values are expressed per nucleotide residue.

Thin layer chromatography (TLC) was performed on Kieselgel 60 F_{254} (Merck) in system chloroform-methanol (95:5, v/v). Column chromatography was carried out on Silica gel L 40/100 (Czechoslovakia) using 280 nm UV detection. HPLC was carried out on a SP 8100 (Spectra Physics, USA) liquid chromatograph equipped with a gradient mixing system, UV absorption detector (254 nm) and a photometer output recorder. The separation was achieved using an ethanol concentration gradient (0-10%) in tris-HCl buffer (pH 7.0) as an eluent, 1ml/min, 40°C.

MeOTr-2',3'-thiocarbonyluridine (VI). To a solution of MeOTrU (1.5 g, 3 mmol) in dry tetrahydrofuran was added Im_2CS (0.61 g, 3.3 mmol). The reaction mixture was stirred for 24 h at room temperature and then concentrated *in vacuo*. The residue was dissolved in chloroform (50 ml) and washed with water (3x10 ml). The organic layer was dried with Na_2SO_4, concentrated to a small volume and chromatographed on a silica gel column (20x5 cm). Elution was performed with a chloroform-ethanol gradient (0-4% ethanol). The appropriate fractions, as judged by TLC, were concentrated under reduced pressure to afford pure (VI) as white solid. Yield 1.30 g (2.4 mmol, 80%).

MeOTr-O^2,2'-anhydrouridine(VII) was prepared from the compound (VI) by refluxing with Im for 30 min as described in [6]. UV (EtOH) λ_{max} 230 nm, λ_{min} 222 nm, shoulder at 249-253 nm. H^1NMR ($CDCL_3$) δ 6.09 (d, 1, H-1', $J_{1',2'}$= 5.8Hz), 5.21 (dd, 1,H-2', $J_{2',3'}$= 1.4 Hz), 4.52 (m, 1, H-3'), 4.31 (dt, 1, H-4', $J_{4',3'}$= 4.4 Hz, $J_{4',5'}$= 6.0 Hz), 3.07 (d, 2, H-5'), 5.96 (d, 1, H-5, $J_{5,6}$= 7.5 Hz).

MeOTraU was prepared from the compound (VII) by treatment with NaOH in 50% ethanol as in [7].The rupture of the anhydro linkage was checked by TLC. UV (EtOH) λ_{max} 230, 264 nm, λ_{min} 227, 249 nm. H^1NMR ($CDCl_3$) δ 6.08 (d, 1, H-1', $J_{1',2'}$= 5.1 Hz), 4.40 (dd, 1, H-2', $J_{2',3'}$= 5.2 Hz), 4.30 (dd, 1, H-3', $J_{3',4'}$=5.6 Hz), 3.94 (m, 1, H-4', $J_{4',5'a}$=3.9 Hz, $J_{4',5'b}$= 3.3 Hz), 3.48 and 3.51 (AB, 2, H-5'a and H-5'b, J_{AB} = 11.0 Hz), 5.35 (d, 1, H-5, $J_{5,6}$= 8.2 Hz).

MeOTrdxT was prepared by a three-step procedure via the anhydrothymidine nucleoside according to the method of Miller and Fox [12]. Each step of synthesis was analyzed by TLC. UV (EtOH) λ_{max} 267 nm, λ_{min} 250 nm). H^1NMR ($CDCl_3$) δ 6.19 (dd, 1, H-1', $J_{1',2'a}$= 2.5 Hz, $J_{1',2'b}$= 8.2 Hz), 2.14 (dd, 1, H-2'a, $J_{2'a,2'b}$= 14.4 Hz), 2.58 (m, 1, H-2'b, $J_{2'b,3'}$= 5.3 Hz), 4.46 (m, 1, H-3'), 4.02 (m, 1, H-4', $J_{4',3'}$= 3.1 Hz), 3.49 (dd, 1, H-5'a, $J_{5'a,4'}$= 5.6 Hz, $J_{5'a,5'b}$= 10.2 Hz), 3.62 (dd, 1, H-5'b, $J_{5'b,4'}$= 5,1 Hz), 3.12 (d, 1, OH-3', J = 2.3 Hz).

Dinucleoside phosphates (I), (III) and (V). The 1 mmol 3'-OH-component (compound (VII), MeOTrdxT or MeOTrdT) and the 1.2 mmol P-component (ClPh)pdbzA(Lev) were dried by evaporation with anhydrous pyridine (at least four times). Then a solution TPS (3 mmol) and MeIm (6 mmol) in 3 ml of dry pyridine was added. After 20 min to 1 hr the reaction mixtures were analyzed by TLC. The reactions were terminated by adding 2 ml ice-cold water and kept at room temperature for 15 min. Then reaction mixtures were concentrated to dryness. The residues were dissolved in chloroform (50 ml),

washed with 0.1 M triethylammonium bicarbonate (2x15 ml) and water (2x10 ml). The organic layer was dried (Na_2SO_4), concentrated to a small volume and chromatographed on the silica gel column (1x10 cm), using a linear gradient of ethanol in chloroform (0-10%) as an eluent. The fractions, containing the required products, were pooled and evaporated. Yields 80-87%.

The fully protected dimers were completely deblocked by treatment of 80% acetic acid (2 h, 20°C) then ammonium hydroxide in pyridine (20 h, 50°C).

Dinucleoside phosphates (II) and (IV). Previously MeOTraU and MeOTrdxT were benzoylated with an excess of benzoyl anhydride in pyridine as described in [13], followed by detritylated with 80% acetic acid (2 hr,20°C). $aU(Bz)_2$ or dxT(Bz) were condensed with MeOTrdbzAp(ClPh) using the standard phosphotriester protocol. Termination and work up of the reaction mixtures were as described above for dimers (I), (III) and (V).

Snake venom phosphodiesterase-catalyzed hydrolysis of compounds (I)-(IV). One A_{260}(0.04 μmol) of dinucleoside phosphate dissolved in 100 μl buffer (0.04 M ammonium bicarbonate, pH 8.5) was treated with 5 μl snake venom PDE. Previous experiments showed that this amount of the enzyme was sufficient to completely hydrolyze 0.04 μmol of d(TpA) at 37°C in 1 hr. The mixture was incubated at 37°C. Aliquots of 5 μl were withdrawn every 10 min. The reaction was terminated with bentonit. The aliquots were diluted by 10-fold with water and analyzed by HPLC.

Spleen phosphodiesterase-catalyzed hydrolysis of compounds (I)-(IV) was carried out in 0.65 M citric buffer, pH 6.2 for 2 hr. The procedure, termination and workup of the reaction mixtures were as described above.

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