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Synthesis of Adenylyl-(3′→5′)-guanosine and some Analogues as Probes to Explore the Molecular Mechanism of Stimulation of Influenza Virus RNA Polymerase

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Influenza virus mRNA synthesis is primed by a capped oligonucloetide which is cleaved off from a cellular mRNA by a viral protein. The dinucloetide A3′p5′G can be used as a primer for the viral RNA polymerase mediated RNA synthesis in a cell-free system.

Analogues of A3′p5′G have therefore been synthesized using the phosphotriester approach, and their priming ability for the influenza virus mRNA synthesis has been determined. An absence of the 2′-hydroxyl function in the guanosine residue in the dinucleotide, as in A3′p5′dG, drastically decreased its priming ability. Similarly, an alteration of the 3′→5′ phosphate linkage to a 2′→5′ phosphodiester linkage affected the priming ability quite severely. However a dinucleotide, with the 2′-hydroxyl function omitted in the adenosine moiety, as in dA3′p5′G, could still stimulate the mRNA synthesis. None of the modified dinucleotides inhibited A3′p5′G or globin mRNA primed influenza mRNA synthesis.

The molecular mechanism of the influenza mRNA synthesis has recently been analyzed in great detail.[1-3] The virion contains an RNA polymerase which seems to be responsible for the synthesis of the capped and polyadenylated mRNA. In the infected cell, a capped oligoribonucleotide containing 12–14 nucleotides is cleaved off from a cellular mRNA and it is used at the 5′-end of the influenza mRNA. Since this priming can take place also in a cell-free assay using purified viruses,[4] the nuclease activity found in the virions seems to be responsible for the cleavage of the cap containg primer from cellular mRNA.[5] In this cell-free assay a dinucleotide, A3′p5′G (*12*), can also serve as a primer of mRNA synthesis for the influenza virion polymerase.[6,7] It is therefore possible that modified dinucleotides, such as the analogues of A3′p5′G, may function as inhibitors of influenza RNA polymerase activity. We have previously[8] reported results showing the importance of methylation of the cap structure for its function as a primer, and that *12* and the cap m^{7}GpppAm interact with different sites on the polymerase complex.

The purpose of this work was to explore the effect of structural modifications of A3′p5′G, through the preparation of a few analogues, in order to understand the molecular mechanism that controls the initiation of influenza virus mRNA synthesis or its inhibition.

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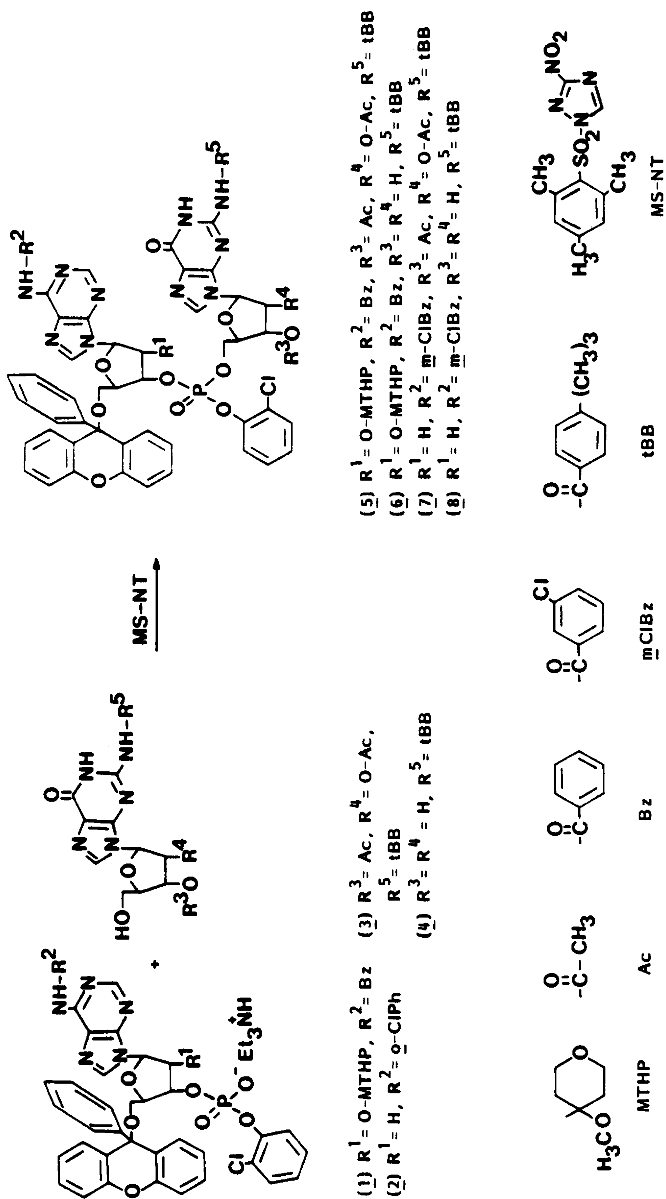
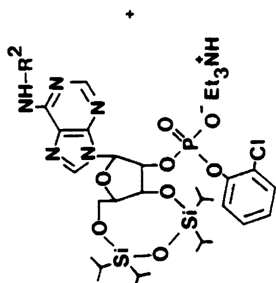
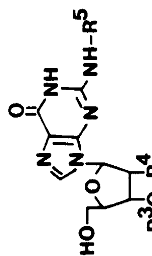

MS-NT
(1) R1 = O-MTHP, R2 = Bz
(2) R1 = H, R2 = o-ClPh
(3) R3 = Ac, R4 = O-Ac, R5 = tBB
(4) R3 = R4 = H, R5 = tBB
(5) R1 = O-MTHP, R2 = Bz, R3 = Ac, R4 = O-Ac, R5 = tBB
(6) R1 = O-MTHP, R2 = Bz, R3 = R4 = H, R5 = tBB
(7) R1 = H, R2 = m-ClBz, R3 = Ac, R4 = O-Ac, R5 = tBB
(8) R1 = H, R2 = m-ClBz, R3 = R4 = H, R5 = tBB
MTHP
Ac
Bz
mClBz
tBB
MS-NT



(9) R^2 = Bz

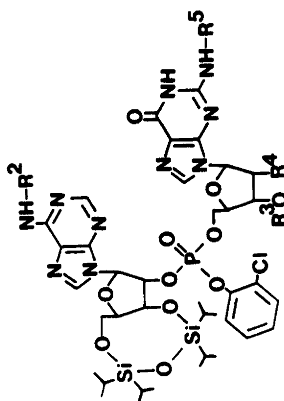
+



(3) R^3 = Ac, R^4 = O-Ac, R^5 = tBB

(4) R^3 = R^4 = H, R^5 = tBB

MS-NT →



(10) R^2 = Bz, R^3 = Ac, R^4 = O-Ac, R^5 = tBB

(11) R^2 = Bz, R^3 = R^4 = H, R^5 = tBB

Table 1. Effect of dinucleotides, (*12–17*), (2 o.d. units at A_{260}) on the activity of influenza RNA Polymerase A and B in primed and unprimed reaction (see Experimental).[a]

Dinucleotides	Influenza RNA polymerase activity in absence of primers (% inhibition; – indicates stimulation)		Influenza RNA polymerase activity in presence of primers (% inhibition; – indicates stimulation)			
			A3'p5'G		Globin mRNA	
	A	B	A	B	A	B
A3'p5'G (*12*)	−4267	−13426	2	–	−59	–
A3'p5'dG (*13*)	−753	−304	17	14	10	19
dA3'p5'G (*14*)	−3545	−10454	6	0	−39	−1
dA3'p5'dG (*15*)	−388	−198	4	7	0	10
A2'p5'G (*16*)	−2948	−7914	25	3	−54	−6
A2'p5'dG (*17*)	−439	−73	1	19	−19	27

[a] A=influenza A. B=influenza B.

CHEMISTRY

Fully protected dinucleoside monophosphates, *5* to *8* and *10* and *11*, have been prepared using the methods that have been developed in the phosphotriester approach.[9]

Preparation of 2',5'-bis-protected ribonucleoside block: (1). Preparation of an optimally protected ribonucleoside is a prerequisite to the preparation of the 3'-phosphodiester block (*1*). The general route of preparation of such a block has been described previously.[10] The selective introduction of the 3'-phosphodiester function has been carried out by the reaction of 2-chlorophenylphosphorobis-(1,2,4-triazolide)[11] followed by a hydrolysis step to obtain *1* in an almost quantitative yield.

Table 2. HPLC quantitation of dinucleotides (*12–17*) after they were fully digested with *Crotalus adamanteus* snake venom phosphodiesterase.

Compound		Fragments obtained after digestion	R_t(min)	Ratios obs.	Ratios calc.
A3'p5'G	(*12*)	G	6.03	1.13	1
		A	9.03	1.00	1
A3'p5'dG	(*13*)	dG	6.46	1.15	1
		A	8.86	1.00	1
dA3'p5'G	(*14*)	G	5.81	0.94	1
		dA	9.64	1.00	1
dA3'p5'dG	(*15*)	dG	6.02	1.10	1
		dA	9.31	1.00	1
A2'p5'G	(*16*)	G	5.52	1.05	1
		A	8.36	1.00	1
A2'p5'dG	(*17*)	dG	6.22	1.15	1
		A	8.53	1.00	1

Preparation of 5'-protected-2'-deoxyribonucleoside block: (2). The preparation of a block such as *2* has also been reported in the literature.[12]

Preparation of 3',5'-bis-protected-2'-O-(2-chlorophenyl)phosphate (9). Preparation of such derivatives is based upon the synthesis of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)nucleosides which may be conveniently prepared in a high yield using a literature procedure.[13]

General methods of condensation reactions. Condensation of 5'-protected phosphodiester block (*1*, *2* or *9*) to a 5'-hydroxyl block[10,12] (*3* or *4*) has been carried out in dry pyridine solution by employing a slight excess of the former compound in the presence of a 3–5 fold excess of 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MS–NT).[15] The yields of fully protected dinucleotides (as powders) varied from 75 to 85 %.

The fully protected dinucleotides, thus obtained, have been subsequently deprotected using 4-nitrobenzaldoximate ions,[15] followed by a treatment of aqueous ammonia and then either a brief treatment of fluoride ions[14] or 80 % acetic acid[10] has been employed (experimental section). The deprotected dimers, *12* to *17*, were subsequently purified by DEAE Sephadex A25 column chromatography using triethylammonium bicarbonate buffer (pH 7.5, linear gradient: 0.001 to 0.13 M) as shown in Fig. 1. The compounds that eluted under these main peaks were collected. Thus compounds *12* to *17* were obtained in 90, 89, 92, 88, 79 and 85 % yields respectively. These dimers, *12* to *17*, were found to be pure as checked by reverse phase HPLC; elution profiles are shown in Fig. 1. These fully deprotected dinucleotides and their intermediates have been characterized spectroscopically (experimental). Finally, the fully deprotected dinucleotides have also been characterized by enzymatic and alkaline digestions. The monomeric components that were produced in these digests were quantified by analytical HPLC procedures,[16] as shown in Tables 2, 3 and 4 respectively, to substantiate the structure of the target dinucleotides.

We have subsequently studied the relative rates of hydrolyses of fully deprotected dinucleotides with *Crotalus Adamanteus* snake venom phosphodiesterase, calf spleen phosphodiesterase and 0.1 M sodium hydroxide, as shown in Table 4.

Table 3. HPLC quantitation of dinucleotides (*12*–*17*) after they were fully digested with calf spleen phosphodiesterase.

Compound		Fragments obtained after digestion	R_t(min)	Ratios	
				obs.	calc.
A3'p5'G	(*12*)	G	5.25	1.02	1
		3'-AMP	20.60	1.00	1
A3'p5'dG	(*13*)	dG	6.11	1.11	1
		3'-AMP	16.86	1.00	1
dA3'p5'G	(*14*)	G	5.56	0.91	1
		3'-AMP	15.86	1.00	1
dA3'p5'dG	(*15*)	dG	6.25	1.02	1
		3'-AMP	13.14	1.00	1
A2'p5'G	(*16*)	no cleavage	13.58	–	–
A2'p5'dG	(*17*)	no cleavage	14.23	–	–

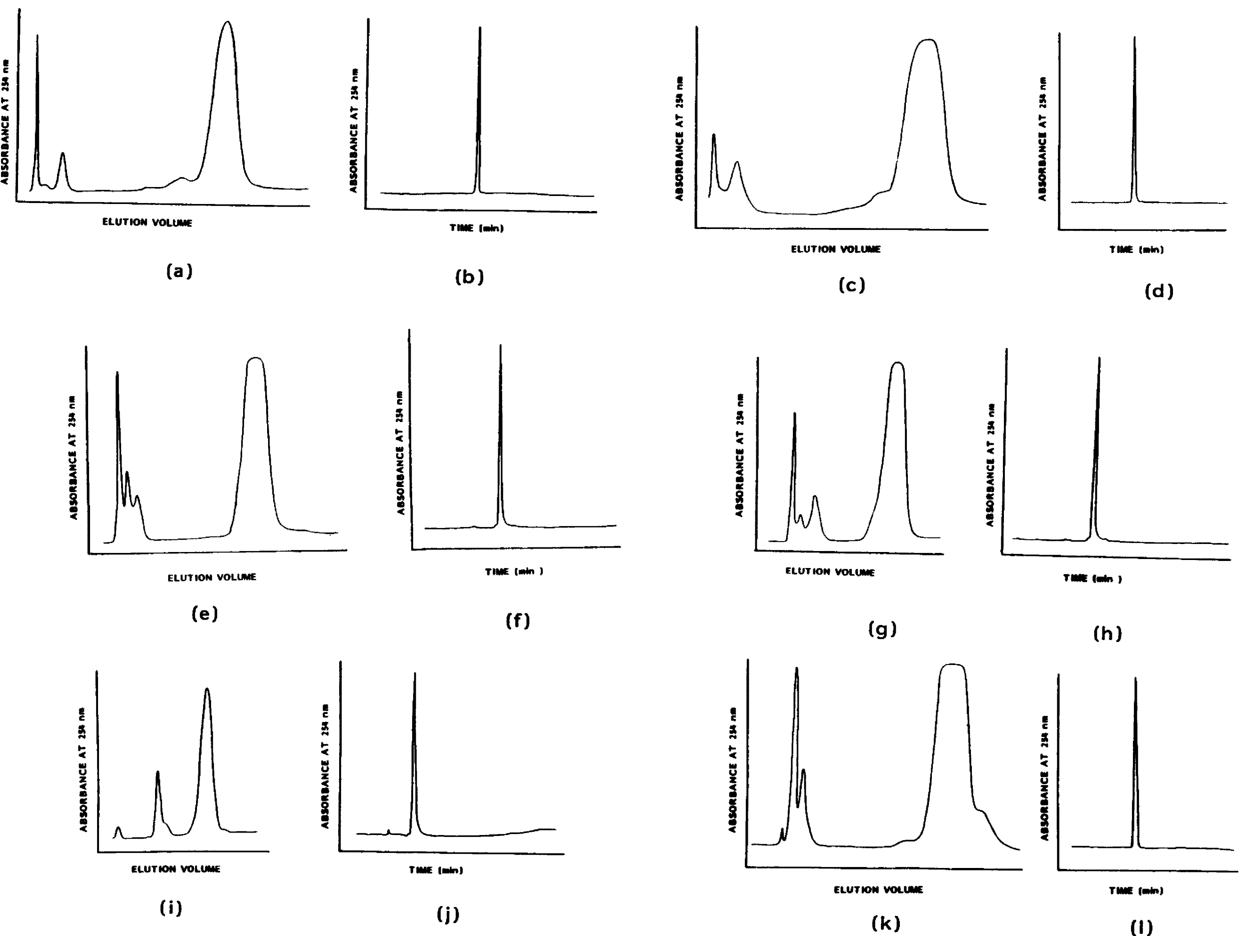


Fig. 1. (a), (c), (e), (g), (i) and (k) are DEAE Sephadex elution profiles of fully deprotected dimers *12* to *17* respectively using triethylammonium bicarbonate buffer (pH 7.5, linear gradient: 0.001 M to 0.13 M; 300 ml each) (b), (d), (f), (h), (j) and (l) are reverse phase HPLC elution profiles of the compounds which eluted under the main peaks in DEAE Sephadex elution profiles shown in (a), (c), (e), (g), (i) and (k) respectively (Spherisorb ODS 10μ column, 0.0005 M tetrapentylammonium phosphate (TPeAmP) buffer, pH 7.0, in 0–20 % acetonitrile in a linear gradient mode for 30 min in the mobile phase). R_t of compounds in elution profiles (b), (d), (f), (h), (j) and (1) are 14.66, 14.72, 12.98, 13.27, 13.58 and 12.39 min respectively.

A comparison of half-lives of hydrolyses of dinucleotides with snake venom phosphodiesterase suggests that the dimers with the 2′-deoxyribose unit at the 5′-end, as in *14* and *15*, are three-fold more stable than the corresponding dimers with the ribose unit at the 5′-end: *12* and *13* respectively. It also emerges from a comparison of *12* and *13* with *16* and *17* that this enzyme does not discriminate between dimers containing either a 3′→5′ or a 2′→5′ phosphodiester linkage. Similarly, a comparison of half-lives of hydrolyses of dinucleotides with calf spleen phosphodiesterase indicates that the nature of the pentafuranose moiety at the 5′-end of the dimer seems to control the relative rate of hydrolyses; whereas, the dinucleotides contaning a 2′→5′ phosphodiester linkage is not at all a substrate of this enzyme. A perusal of the data on alkaline hydrolyses of these dinucleotides suggest that dimers with a 3′→5′ phosphodiester linkage and a vicinal 2′-hydroxyl function, as in *12* and *13*, are more resistant to hydrolyses than the corresponding dimers with the 2′→5′ phosphodiester linkage and a vicinal 3′-hydroxyl function. The dinucleotides without the vicinal hydroxyl function were completely stable to alkaline hydrolyses as expected.

Effects of modified dinucleotides, (13) to (17), on Influenza RNA polymerase activity. In order to explore the essential structural feature(s) that is/are important for the priming ability of A3′p5′G, we have prepared its five structural analogues *13* to *17*.

It is clear from Table 1 that substitution of the 2′-hydroxyl function by a hydrogen in the guanosine residue, as in *13*, decreases the stimulation of mRNA synthesis in the absence of primers. Such a lack of stimulation was observed for an isomeric substrate *17*; showing that the 2′-hydroxyl group in the guanosine moiety is important for the stimulation of RNA polymerase activity. Similarly, the activity was diminished with the 2′→5′ analogue *16*, which is an isostere of *12*. Such a difference may be attributed to the overall change of stacking-unstacking equilibrium between two base residues in the dinucleotides due to the change of the phosphate backbone structure. It should be noted that *14*, in which the 2′-hydroxyl function in the adenosine moiety has been substituted by a hydrogen, could still stimulate the influenza RNA synthesis. Compound *15* was very similar to *13* and *17*. None of these modified dinucleotides could inhibit influenza RNA polymerase activity.

Table 4. Alkaline hydrolysis of dinucletides (*12*–*17*) with 0.1 M sodium hydroxide and HPLC quantitation of the reaction mixture.

Compound		Fragments obtained after hydrolysis	R_t(min)	Ratios	
				obs.	calc.
A3′p5′G	(*12*)	G	5.74	1.13	1
		2′(3′)-AMP	21.31, 22.95	1.00	1
A3′p5′dG	(*13*)	dG	6.56	1.03	1
		2′(3′)-AMP	17.24, 19.97	1.00	1
dA3′p5′G	(*14*)	no cleavage	15.06	–	–
dA3′p5′dG	(*15*)	no cleavage	15.81	–	–
A2′p5′G	(*16*)	G	4.82	1.12	1
		2′(3′)-AMP	18.37, 21.38	1.00	1
A2′p5′dG	(*17*)	dG	5.66	0.99	1
		2′(3′)-AMP	21.05, 23.00	1.00	1

DISCUSSION

None of these modified dinucleotides showed any inhibition of the influenza RNA polymerase activity at concentrations shown in Table 1. Recently, it has been shown that the dinucleotides G3′p5′m^{5}C and A3′p5′V (V=Virazole) could inhibit the influenza RNA polymerase activity to some extent.[17,18] Our observations along with these published reports clearly suggest that any modification of the sugar residue of guanosine part of A3′p5′G *12* affects the stimulating ability of an analogue in influenza RNA synthesis. However, the behaviour of G3′p5′m^{5}C and A3′p5′V show that it could be possible to find potential inhibitors among modified dinucleotides. It is still left to be shown if any of the modified dinucleotides can get incorporated into the viral RNA.

EXPERIMENTAL

Virus. Influenza A Victoria 3/75 X -47 and Influenza B Hong Kong 8/73, grown in eggs were purchased from Orion OY, Helsinki. Virus core structures were prepared as described by Plotch et al.[5]

Polymerase assay and purification of viral cores. The cell-free influenza RNA polymerase assay was carried out in the following way: a 50 µl reaction mixture containing viral cores, ^{3}H-GTP (40 µM, 10.5 Ci/mmol), ATP (1 mM), CTP (0.1 mM), UTP (0.1 mM), tris-hydrochloride (80 mM, pH 7.9), sodium chloride (100 mM), magnesium chloride (6 mM), DTT (1 mM) and 8 % glycerol (v/v) was incubated for 20 min at 30 °C in the presence of dinucleotides: *12* to *17*. A3′p5′G (*12*) and globin mRNA were used as primers in concentrations of 100 µM and 1 µg/50 µl respectively. Synthesis of viral RNA was determined as TCA precipitable radioactivity.[11]

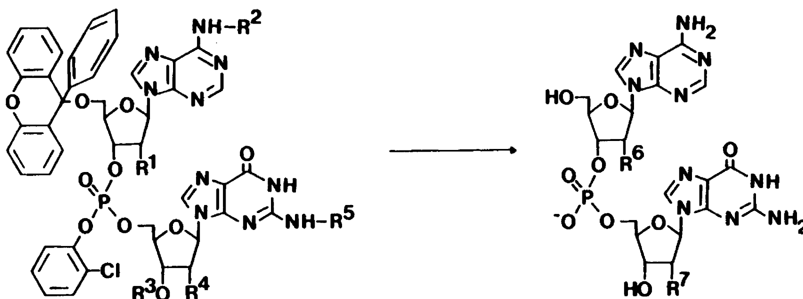
Analytical methods and chemistry. ^{1}H NMR spectra were measured at 60 MHz with a Perkin-Elmer R 600 and at 90 MHz with a Jeol FX 90Q spectrometer using tetramethylsilane as an internal standard in δ scale. ^{31}P NMR spectra were recorded at 36 MHz in the same solvent mixture as for ^{1}H NMR using 85 % phosphoric acid as an external standard in δ scale. UV absorption spectra were recorded with a Cary 2200 spectrophotometer. Reactions were monitored by using Merck pre-coated silica gel 60 F_{254} plates using the following solvent systems:

(A) 10 % methanol–chloroform (v/v)

(B) 1 M ammonium acetate–ethanol (1.5:8.5, v/v).

High performance liquid chromatography (HPLC) was performed on a spherisorb ODS 10 µ column[16] with the help of LDC equipments, model III pumps, UV III monitor and a gradient master; Merck Kieselgel G was used for short column chromatography.[19] Dried solvents were prepared using literature procedures.[10] The key reagents: 1,1,3,3-tetraisopropyl-1,3-dichloro-1,3-disiloxane,[13] 9-chloro-9-phenylxanthene (Px–Cl)[20] 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MS–NT),[15] 2-chlorophenylphosphorobis-(1,2,4-triazolide)[11] were prepared using literature procedures.

2′-O-(Methoxytetrahydropyranyl)-5′-O-(9-phenylxanthen-9-yl)-6-N-benzoyladenylyl-(3′→5′)-2′,3′-di-O-acetyl-2-N-t-butylbenzoylguanosine (5). To a dry pyridine solution (10 ml/mmol) of triethylammonium salt of 2′-*O*-(methoxytetrahydropyranyl)-3′-*O*-(2-chlorophenylphosphate)-5′-*O*-(9-phenylxanthen-9-yl)-6-*N*-benzoyladenosine (*1*) (207 mg, 0.20 mmol) and 2′,3′-di-*O*-acetyl-2-*N*-t-butylbenzoylguanosine (*3*) (96 mg, 0.17 mmol) was added 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MS–NT) (150 mg, 0.51 mmol) and the reaction mixture was stirred for 40 min at 20 °C. The reaction was quenched by the addition of a saturated aqueous sodium bicarbonate solution (0.1 ml). The stirring was continued for another 10 min and the reaction mixture was poured into a separating funnel containing saturated aqueous sodium bicarbonate solution (100 ml). The resultant mixture was extracted with chloroform (3×50 ml). The chloroform extracts were pooled and concentrated to give a syrup which was co-evaporated with toluene (2×30 ml). The residue was then fractionated by short column (2 cm×2 cm) chromatography on silica gel using the following eluents: pure dichloromethane, 2 % ethanol–chloroform and 4 %



(5) R^1= O-MTHP, R^2= Bz, R^3= Ac, R^4= O-Ac, R^5= tBB
(6) R^1= O-MTHP, R^2= Bz, R^3= R^4= H, R^5= tBB
(7) R^1= H, R^2= m-ClBz, R^3= Ac, R^4= O-Ac, R^5= tBB
(8) R^1= H, R^2= m-ClBz, R^3= R^4= H R^5 = tBB

(12) R^6= R^7= OH
(13) R^6= OH, R^7= H
(14) R^6= H, R^7= OH
(15) R^6= R^7= H

ethanol–chloroform mixture. Appropriate fractions were collected and concentrated *in vacuo* to give the desired compound 5 as a glass which was precipitated from light petroleum (30–50 °C) to give a white powder. Yield 180 mg (76 %). R_f=0.52 (solvent A).

^{1}H NMR ($CDCl_3$) 8.71 (s, 1H) H-8 of 9-adeninyl- group; 8.33 (s, 1H) H-2 of 9-adeninyl-group; 8.04–6.90 (m, 27H) aromatic protons; 6.3 (d, 9.6 Hz, 1H) H-1′ of adenosine moiety; 6.2 (d, 4.8 Hz, 1H) H-1′ of guanosine moiety; 6.04–5.60 (m, 3H) H-2′ and -3′ of guanosine and -3′ of adenosine moiety; 5.14 (*m*, 1H) H-2′ of adenosine; 4.60 (m, 3H) H-5′ and -4′ of guanosine and -4′ of adenosine moiety; 3.40 (m, 6H) H-5′ of adenosine moiety and the MTHP group; 2.60 (s, 3H) methoxyl of MTHP; 2.19 (s, 3H) acetyl-; 2.01 (s, 3H) acetyl-; 1.64 (m, 4H) MTHP; 1.17 (s, 9H) tBB. ^{31}P NMR ($CDCl_3$): −8.84.

*2′-O-(Methoxytetrahydropyranyl)-5′-O-(9-phenylxanthen-9-yl)-6-*N*-benzoyl adenylyl)-(3′→5′)-2-*N-t*-butylbenzoyl-2′-deoxyguanosine (6).* Compound 6 was prepared using essentially a reaction condition as described for 5 with the following exceptions:

(*1*) (248.3 mg, 0.24 mmol), (*4*) (86 mg, 0.20 mmol) and MS–NT (296, 1.0 mmol) in dry pyridine (20 ml) for 20 min. A standard work-up and purification over a short column of silica gel using 4.5 % ethanol–chloroform mixture gave the title compound. Yield 167 mg (66 %), R_f=0.46 (solvent A).

^{1}H NMR ($CDCl_3$): 8.65 (s, 1 H) H-8 of 9-adeninyl-; 8.37 (s, 1H) H-2 of 9-adeninyl-; 8.00–7.00 (m, 27H) aromatic protons; 6.26–6.06 (m, 2H) H-1′ of two sugar moieties; 5.48 (m, 2H) H-2′ and -3′ of adenosine moiety; 4.60 (m, 3H) H-3′ and -4′ of deoxyguanosine and -4′ of adenosine moiety; 4.15 (m, 2H) H-5′ of deoxyguanosine moiety; 3.66–3.28 (m, 8H) H-5′ of adenosine moiety and -2′ of deoxyguanosine moiety and MTHP; 2.48 (s, 3H) methoxyl of MTHP; 1.64 (m, 4H) MTHP; 1.21 (*s*, 9H) tBB. ^{31}P NMR ($CDCl_3$): −7.93.

*5′-O-(9-Phenylxanthen-9-yl)-6-*N*-*m*-chlorobenzoyl-2′-deoxyadenylyl-(3′→5′)-2′,3′-di-O-acetyl-2-*N-t*-butylguanosine (7).* A mixture of (*2*) (280 mg, 0.30 mmol), *4*) (136 mg, 0.24 mmol) and MS–NT (361 mg, 1.22 mmol) was stirred in dry pyridine (30 ml) solution for 40 min. A standard work-up and a purification by short column chromatography on silica gel using 4 % ethanol–chloroform mixture as an eluent gave the desired compound. Yield 220 mg (69 %) R_f=0.47 (solvent A). ^{1}H NMR ($CDCl_3$): 8.63–6.85 (m, 28H) aromatic; 6.24–5.69 (m, 4H) H-1′ protons of sugar moieties and -2′ and -3′ of guanosine moiety; 5.20 (m, 1H) H-3′ of deoxyadenosine block; 4.56 (m, 3H) H-5′ and -4′ of guanosine block; 4.13 (m, 1H) H-4′ of deoxyadenosine block; 3.22–2.40 (m, 4H) H-5′ and -2′ of deoxyadenosine block; 2.13 and 2.01 (two s, 6H) acetyl groups; 1.27 (*s*, 9H) tBB.

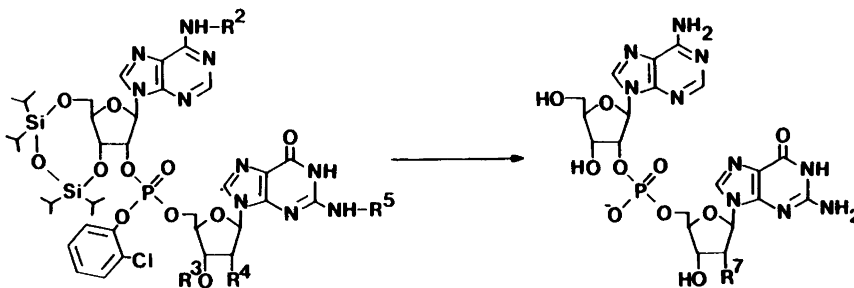
^{31}P NMR ($CDCl_3$): −8.83.

*5′-O-(9-Phenylxanthen-9-yl)-6-*N-m-*chlorobenzoyl-2′-deoxyadenylyl-(3′→5′)-2-*N-t-*butylbenzoyl-2′-deoxyguanosine (8).* A mixture of *2* (300 mg, 0.31 mmol) *4* (112 mg, 0.26 mmol) and MS−NT (387 mg, 1.32 mmol) was stirred in dry pyridine (35 ml) solution for 20 min. A standard work-up procedure and a purification by short column chromatography on silica gel using 5 % ethanol−chloroform mixture gave the title compound. Yield 188 mg (61 %). R_f=0.44 (solvent A). ^{1}H NMR ($CDCl_3$): 8.69 (*s*, 1H) H-8 of 9-adeninyl-; 8.20 (s, 1H) H-2 of 9-adeninyl-; 8.04 (s, 1H) H-8 of 9-guaninyl-; 7.95−7.03 (m, 25 H) aromatic protons; 6.45 (dd, 2H) H-1′ of two sugar residues; 5.36 (m, 1H) H-3′ of deoxyadenosine moiety; 4.42 (m, 3H) H-3′ and -5′ of deoxyguanosine moiety; 4.09 (m, 2H) H-4′ of both deoxysugar moieties; 3.26−2.97 (m, 6H) H-2′ of both deoxysugar moieties and -5′ of deoxyadenosine; 1.27 (s, 9H) tBB.
^{31}P NMR ($CDCl_3$): −7.81.

*3′,5′-O-(1,1,3,3)-Tetraisopropyldisiloxane-1,3-diyl)-6-*N-*benzoyl adenylyl-(2′→5′)-2′,3′-di-O-acetyl-2-*N-t-*butylbenzoylguanosine (10).* A mixture of *9* (317 mg, 0.35 mmol), *3* (165 mg, 0.29 mmol) and MS−NT (343 mg, 1.16 mmol) was stirred in dry pyridine (30 ml) solution for 40 min. A standard work-up, and then a purification by short column chromatography on silica gel using 4 % ethanol-chloroform mixture gave the desired compound. Yield 280 mg (76 %). R_f=0.45 (solvent A). ^{1}H NMR ($CDCl_3$): 8.41 (s, 1H) H-8 of 9-adeninyl-; 8.15−6.88 (m, 15H) aromatic protons; 6.12−5.54 (m, 4H) H-1′ and H-2′ of both sugar moieties; 5.14 (m, 2H) H-3′ of adenosine and guanosine moieties; 4.50 (m, 4H) H-5′ and -4′ of guanosine and -4′ of adenosine moieties; 3.90 (m, 2H) H-5′ of adenosine moiety; 2.13 & 1.92 (two s, 6H) acetyl-; 1.31 (s, 9H) tBB; 1.00−0.76 (m, 26H) disiloxane group, ^{31}P NMR ($CDCl_3$): −8.97.

*3′,5′-O-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-6-*N-*benzoyladenylyl-(2′→5′)-2-*N-t-*butylbenzoyl-2′-deoxyguannosine (11).* A mixture of *9* (250 mg, 0.38 mmol), *4* (99 mg, 0.23 mmol) and MS−NT (340 mg, 1.15 mmol) was stirred in dry pyridine (30 ml) solution for 20 min. A standard work-up and purification by short column chromatography on silica gel using 4 % ethanol−chloroform mixture gave the desired compound. Yield 184 mg (70 %). R_f=0.38 (solvent A). ^{1}H NMR ($CDCl_3$): 8.45 (s, 1H) H-8 of 9-adeninyl-group; 8.06−6.87 (*m*, 15H) aromatic protons; 6.22−6.16 (m, 2H) H-1′of both sugar moieties; 5.57 (m, 1H) H-2′ of adenosine moiety; 5.13 (m, 1H) H-3′ of adenosine moiety; 4.58−4.17 (m, 5H) H-5′ and -4′ of guanosine and -4′ of adenosine and -3′ of deoxyguanosine moieties; 3.91 (m, 2H) H-5′ of adenosine moiety; 2.95 (m, 2H) H-2′ of deoxyguanosine; 1.27 (s, 9H) tBB; 0.98−0.78 (*m*, 26H) disilooxane protons. ^{31}P NMR ($CDCl_3$): −7.39.

Adenylyl-(3′→5′)-guanosine (12). A solution of fully protected dimer (5) (100 mg, 0.071 mmol), *syn*-4-nitrobenzaldoxime (118 mg, 0.71 mmol) and N^1,N^1,N,^{3}, N^3-tetramethylguanidine (81.8 mg, 0.71 mmol) in dioxan-water mixture (1:1, v/v, 8 ml) was stirred at 20 °C. After 24 h aqueous ammonia (d 0.9, 17 ml) was added. After 3 days the solution was evaporated *in vacuo* and the residue was dissolved in 80 % acetic acid (20 ml). The resulting solution was extracted with chloroform (10×10 ml). The aqueous layer was concentrated to a glassy residue *in vacuo,* redissolved in 80 % acetic acid (20 ml) and the reaction mixture was stirred at room temperature for 6 h and then re-extracted with chloroform (10×10 ml).



The aqueous layer was then concentrated *in vacuo* and the residue was co-evaporated with toluene to remove the last traces of acetic acid. The residue was purified by Sephadex DEAE-A25 column (150 mm×20 mm) using an aqueous triethylammonium bicarbonate buffer (pH 7.5, 0.001 M to 0.13 M, linear gradient) as eluent. The elution profile is shown in Fig. 1(a). The main fraction was collected and was found to contain 800 o.d at A_{260} (90 %). R_f=0.53 (solvent B). UV (max=256 nm). The purity of the deblocked dimer (*6*) was checked by HPLC, as shown in Fig. (1b) (Spherisorb ODS 10 μ column, 25 cm×4.1 mm; 0.0005 M tetrapentylammonium phosphate (TPeAmP) in water and 0.0005 M TPeAmP in 20 % acetonitrile–water, linear gradient 0–100 % in 30 min, flow rate 2.0 ml/min); R_t=14.66 min.

^{1}H NMR (CD_3OD); 8.33 (*s*, 1H) H-8 of 9-adeninyl-; 8.20 (s, 1H) H-2 of 9-adeninyl-; 8.04 (s, 1H) H-8 of 9-guaninyl-; 6.02 (d, 5.4 Hz, 1H) H-1′ of a sugar moiety; 5.87 (d, 4.8 Hz, 1H) H-1′ of the second sugar moiety; 5.24; 4.42; 4.19; 3.80.

^{31}P NMR (CD_3OD): −0.1.

Adenylyl-(3′→5′)-2′-deoxyguanosine (13). Fully protected dimer (*6*) (74 mg, 0.059 mmol), *syn*-4-nitrobenzaldoxime (98 mg, 0.59 mmol), N^1,N^1,N^3,N^3-tetramethylguanidine (68 mg, 0.59 mmol) were stirred in dioxan–water mixture (1:1, v/v, 6 ml) for 24 h. Aqueous ammonia (d 0.9, 15 ml) was added and the stirring was continued for a further period of 3 days. Volatile matters were then evaporated *in vacuo*. The residue was subjected to the treatment of 80 % acetic acid (15 ml) for 6h at 20 °C.

The reaction mixture was subsequently extracted with chloroform (10×10 ml). Aqueous phase was concentrated and the residue was purified by a DEAE Sephadex A25 column as described for compound (*12*). The elution profile is shown in Fig. 1(c). The compound that eluted under the main peak was collected to give 620 o.d. units at A_{260} (89 %); R_f=0.55 (solvent B). UV (λ_{max}) 256 nm. The purity of (*13*) was checked by HPLC as shown in Fig. 1(d) using conditions analogous to the procedure described for (*12*). R_t=14.72 min.

^{1}H NMR (CD_3OD): 8.33 (*s*, 1H) H-8 of 9-adeninyl-; 8.16 (*s*, 1H) H-2 of 9-adeninyl-; 7.96 (s, 1H) H-8 of 9-guaninyl-; 6.30 (dd, 7.2 Hz, 1H) H-1′ of deoxyguanosine block; 5.97 (d, 5.4 Hz, 1H) H-1′ of adenosine moiety; 5.36; 4.38–4.13 (m, 5H); 3.78 (brs, 2H). ^{31}P NMR (CD_3OD): −0.6.

2′-Deoxyadenylyl-(3′→5′)-guanosine (14). Fully protected dimer 7 (109 mg, 0.082 mmol), *syn*-4-nitrobenzaldoxime (136 mg, 0.82 mmol), N^1,N^1,N^3,N^3-tetramethylguanidine (95 mg, 0.82 mmol) in dioxan–water mixture (1:1, v/v, 9 ml) for 24 h. Aqueous ammonia (d 0.9, 20 ml) was added and stirred for 3 days. Volatile matters were evaporated and 80 % acetic acid (20 ml) was added. The reaction mixture was then stirred for 15 min at 20 °C and it was then processed as described for compound *12*. The residue was subsequently purified on a DEAE Sephadex A 25 column. The elution profile is shown in Fig. 1(e). The compound that eluted under the main peak was collected to give 900 o.d. units at A_{260} (92 %); R_f=0.52 (solvent B). UV (λ_{max}) 252.5 nm. The purity of the isolated compound was checked by HPLC as for compound (*12*) as shown in Fig. 1(f). R_t=12.98 min.

^{1}H NMR (CD_3OD): 8.24 (*s*, 1H) H-8 of 9-adeninyl-; 8.14 (*s*, 1H) H-2 of 9-adeninyl-; 8.00 (s, 1H) H-8 of 9-guaninyl-; 6.41 (dd, 6.0 Hz, 1H) H-1′ of deoxyadenosine block; 5.86 (d, 6.0 Hz, 1H) H-1′ of guanosine moiety; 4.23 (m, 3H); 3.88 (br*s*, 2H).

^{31}P NMR (CD_3OD): -0.37.

2′-Deoxyadenylyl-(3′→5′)-2′-deoxyguanosine (15). A mixture of fully protected dimer (*8*) (88 mg, 0.069 mmol), *syn*-4-nitrobenzaldoxime (115 mg, 0.69 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (79 mg, 0.69 mmol) was stirred in dioxan-water (1:1 v/v, 7 ml) mixture for 24 h. Aqueous ammonia (d 0.9, 15 ml) was added and stirring was continued for a further period of 3 days. Volatile matter were evaporated *in vacuo*. 80 % acetic acid was added and stirred for 15 min. The reaction mixture was then extracted with chloroform (10×10 ml). The aqueous phase was concentrated *in vacuo*. The residue was subsequently purified by a DEAE Sephadex A 25 column as described for compound (*12*). The elution profile for the purification is shown in Fig. 1(g). The compound under the main peak was collected to give 700 o.d units at A_{260} of desired compound (88 %). R_f=0.51 (solvent B); UV: λ_{max}=253 nm. The purity of (*15*) was checked by HPLC (Fig. 1(h)) using conditions analogous to the procedure described for (*12*). R_t=13.27 min.

^{1}H NMR (CD_3OD): 8.28 (*s*, 1H) H-8 of 9-adeninyl-; 8.14 (s, 1H) H-2 of 9-adeninyl-; 7.98 (s, 1H) H-8 of 9-guaninyl-; 6.41 (dd, 4.8 Hz, 1H) & 6.28 (dd, 6.0 Hz, 1H) H-1′ of

deoxyadenosine and deoxyguanosine; 5.44 (*s*, 1H); 4.17 (m); 3.76 (d).
^{31}P NMR (CD_3OD): −1.10.

Adenylyl-(2′→5′)-guanosine (16). A solution of fully protected dimer (*10*) (60 mg, 0.048 mmol), *syn*-4-nitrobenzaldoxime (80 mg, 0.48 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (55.2 mg, 0.48 mmol) in dioxan−water (1:1 v/v, 5 ml) was stirred for 24 h at 20 °C. Aqueous ammonia (d 0.9, 15 ml) was added. After 3 days the volatile matters were evaporated *in vacuo* and the residue was co-evaporated with dry pyridine and re-dissolved in the same solvent (3 ml), and 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (0.096 ml) was added. After stirring for 24 h at 20 °C, the volatile matters were evaporated and the residue was dissolved in distilled water (15 ml). The lipophilic material was extracted with dichloromethane (10×10 ml). The aqueous layer was concentrated *in vacuo*. The residue, thus obtained, was purified by a DEAE Sephadex A 25 column using triethylammonium bicarbonate aqueous buffer (pH 7.5, 0.001 M to 0.13 M, linear gradient) as eluent. The elution profile is shown in Fig. 1(i). The compound under the main peak was collected to give 460 o.d units at A_{260} (79 %). R_f=0.53 (solvent B). UV: λ_{max}=254 nm. The purity of the deblocked dimer (*16*) was checked by HPLC, in the usual way, as shown in Fig. 1(j). R_t=13.58 min. ^{1}H NMR (CD_3OD): 8.30 (s, 1H) H-8 of 9-adeninyl-; 8.10 (s, 1H) H-2 of 9-adeninyl-; 7.39 (s, 1H) H-8 of 9-guaninyl-; 6.16 (d, 1H) & 5.71 (brs, 1H) H-1′ of adenosine and guanosine moieties. ^{31}P NMR: −0.70.

Adenylyl-(2→5′)-2′-deoxyguanosine (17). Fully protected dimer *11* (111 mg, 0.098 mmol), *syn*-4-nitrobenzaldoxime (162 mg, 0.98 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (112 mg, 0.98 mmol) were dissolved in dioxan-water (1:1 v/v, 10 ml) and stirred for 24 h. Aqueous ammonia (d 0.9, 25 ml) was added and stirred for another 3 days. The volatile matters were evaporated and co-evaporated with pyridine, and the residue was redissolved in the same solvent (6 ml) 1 M TBAF (1.2 ml) was added and the mixture was stirred for 24 h. Volatile matters were evaporated and lipophilic material was extracted from distilled water with dichloromethane. The residue was subsequently purified by Sephadex DEAE-A25 column as described for compound (*12*). The elution profile is shown in Fig. 1(k). The compound under the main peak was collected to obtain 990 o.d units at A_{260} (85 %). R_f=0.51 (solvent B). UV: λ_{max}=254 nm. The purity of (*17*) was checked by HPLC analogously to compound (*12*) as shown in Fig. 1(l). R_t=12.39 min. ^{1}H NMR (CD_3OD): 8.30 (s, 1H) H-8 of 9-adeninyl-; 8.12 (s, 1H) H-2 of 9-adeninyl-; 7.89 (s, 1H) H-8 of 9-guaninyl-; 6.14 (m, 2H) H-1′ of two sugar moieties; 5.34 (m, 1H); 3.85 (m);
^{31}P NMR: −1.07.

Table 5. Relative rates of hydrolysis of fully deprotected dinucleotides, (*12–17*), with crotalus adamanteus snake venom phosphodiesterase, calf spleen phosphodiesterase and 0.1 M sodium hydroxide.

	Snake venom Phosphodiesterase			Calf spleen Phosphodiesterase			0.1 M NaOH
Compound	Substrate (o.d. units)	Enzyme (μg)	$t_{½}$ (min)	Substrate (o.d. units)	Enzyme (μg)	$t_{½}$ (min)	$t_{½}$ (min)
A3′p5′G (*12*)	1.0	20	20	1.0	2.0	15	40
A3′p5′dG (*13*)	1.0	20	20	1.0	2.0	30	45
dA3′p5′G (*14*)	1.0	20	55	1.0	2.0	75	no cleavage
dA3′p5′dG (*15*)	1.0	20	75	1.0	2.0	60	no cleavage
A2′p5′G (*16*)	1.0	20	30	1.0	2.0	no cleavage	15
A2′p5′dG (*17*)	1.0	20	25	1.0	2.0	no cleavage	15

Digestions of deprotected dinucleotides: *12* to *17*

(A) *With Crotalus Adamanteus snake venom phosphodiesterase.* A solution of snake venom phosphodiesterase (20 μg) in tris-hydrochloride buffer (0.1 M, pH 9, 20 μl of 0.01 M $MgCl_2$) was added to a solution of the dinucleotide (1.0 o.d unit at A_{260}) in water (10 μl). The resulting solution was incubated at 37 °C for 24 h.

(B) *With Calf spleen phosphodiesterase.* To a solution of the unprotected dinucleotide (1.0 o.d unit at A_{260}) in water (10 μl) was added calf spleen phosphodiesterase (2.0 μg) in ammonium acetate buffer (20 μl, 0.002 M, pH 7, 0.002 M EDTA and 0.05 % tween 40). The resulting solution was incubated at 37 °C for 24 h.

(C) *With 0.1 M sodium hydroxide.* To a solution of the dinucleotide (1.0 o.d. unit at A_{260}) in water (30 μl) was added sodium hydroxide (0.2 M, 30 μl). The solution was incubated at 37 °C for 24 h. Half-lives of the above digestions have been estimated and are shown in Table 5.

HPLC analysis of digestions. All hydrolyses were analyzed by HPLC using a Spherisorb ODS 10 μ column with 0.0005 M tetraphenylammonium phosphate (TPeAmP) in water (solvent A) and 0.0005 M TPeAmP in 20 % acetonitrile-water mixture (solvent B) in linear gradient mode: 0 to 100 % in 30 min; flow rate: 2.0 ml/min. The calculated and observed ratios between nucleosides and mononucleotides are shown in Tables 2 to 4.

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