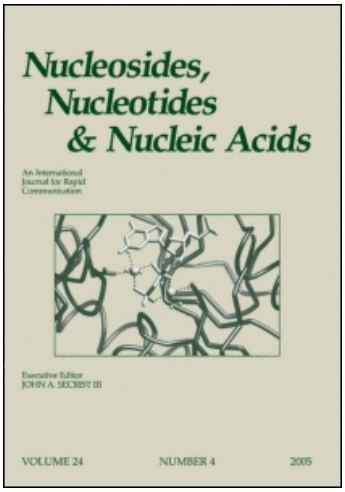

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A 2'←5'-Adenylate Trimer With a Positively Charged 2'(3')-Terminal 8-(4-Aminobunl)Aminoadenosine Residue: Synthesis, Conformation and RNase L Binding

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A 2'→5'-ADENYLATE TRIMER WITH A POSITIVELY CHARGED 2'(3')-TERMINAL 8-(4-AMINOBUTYL)AMINOADENOSINE RESIDUE: SYNTHESIS, CONFORMATION AND RNase L BINDING

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ABSTRACT: An analogue of the 2-5A core trimer containing an 8-(4-aminobutyl)-aminoadenosine (**1**; A*) residue at the 2'(3')-terminus [**2**; (2',5')A_2A*] was synthesized. The conformation of (2',5')A_2A* was studied by ^{1}H, ^{13}C-NMR, and CD spectroscopy. The (2',5')A_2A* exhibits very low binding ability to the RNase L of mouse L cells, but slightly enhanced resistance to digestion by SVPD compared to the parent trimer.

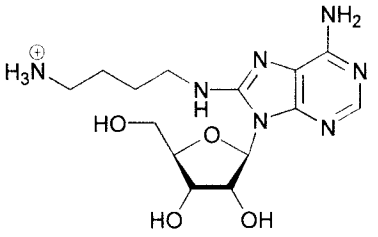
INTRODUCTION

Oligoribonucleotides with aminoalkyl side chains are naturally occurring[1]. Transfer ribonucleic acids contain such residues preferentially at position-5 of pyrimidine bases[1], but also in position-7 of 7-deazapurine residues[1]. These residues influence the stability and the conformation of nucleic acids[2,3]. The present study reports on the synthesis of a (2',5')adenylate trimer with the 8-(4-aminobutyl)aminoadenosine (**1**; A*) 2'(3')-terminus [**2**; (2',5')A_2A*] having a 2',3'-phosphodiester linkage instead of the 3',5'-linkage of the naturally occurring oligonucleotides.

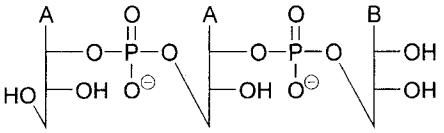
It has been shown that 8-bromo- and 8-methyladenosine replacing the 2'(3')-terminal adenosine moiety of the 2-5A increase RNase L binding. Taking into account that (*i*) both of these nucleosides display preference for *syn* conformation about the glycosyl bond and (*ii*) bromination of the 2'(3')-terminal adenosine give rise to S-type conformation

of its ribofuranose moiety it was suggested that a *syn*/S combination has a positive influence upon RNase L binding[4,5]. Similar observations were made for fluorodeoxy analogues of 2-5A[6,7].

Conformation of 8-(monoalkylamino) derivatives of adenosine and its 5'-monophosphate has been extensively investigated. Thus, ^{1}H NMR data for 8-(methylamino)-adenosine indicated a flexible *syn*/*anti* mixture for this compound in water solution[8]. ^{1}H NMR and CD studies on 8-(2-aminoethyl)amino-AMP (AEA-AMP) and 8-(6-aminohexyl)-amino-AMP (AHA-AMP) also revealed that the conformation of these nucleosides at neutral pH depends upon the length and the nature of 8-substituents[9]: (*i*) in the case of the former, a strong interaction between phosphate and the amino group favors an *anti* conformation range; (*ii*) the latter was proved to be mainly in *syn* conformation. ^{1}H NMR data for 8-(methylamino)adenosine and its 5'-monophosphate pointed to a predominant *anti* conformation about the glycosyl bond and were consistent with the formation of an intramolecular hydrogen bond between the ribose 5'-oxygen and the 8-NH of the base[10,11]. The *anti* conformation about the glycosyl bond of AHA-AMP was found to be the preferred form in aqueous solution on the basis of ^{1}H NMR studies[12]. Based on the above data it stays to proof whether the 4-aminobutyl residue of (2',5')A_2A* (**2**) drives the 2'(3')-terminal nucleoside into the *syn* or *anti* conformation and how this affects the RNase L binding. To our best knowledge such conformational studies on 2',5'-linked oligonucleotides have not been investigated before.



1 (A*)



2 [(2',5')A_2A*]

B = 8-(4-aminobutyl)adenine-9-yl

RESULTS AND DISCUSSION

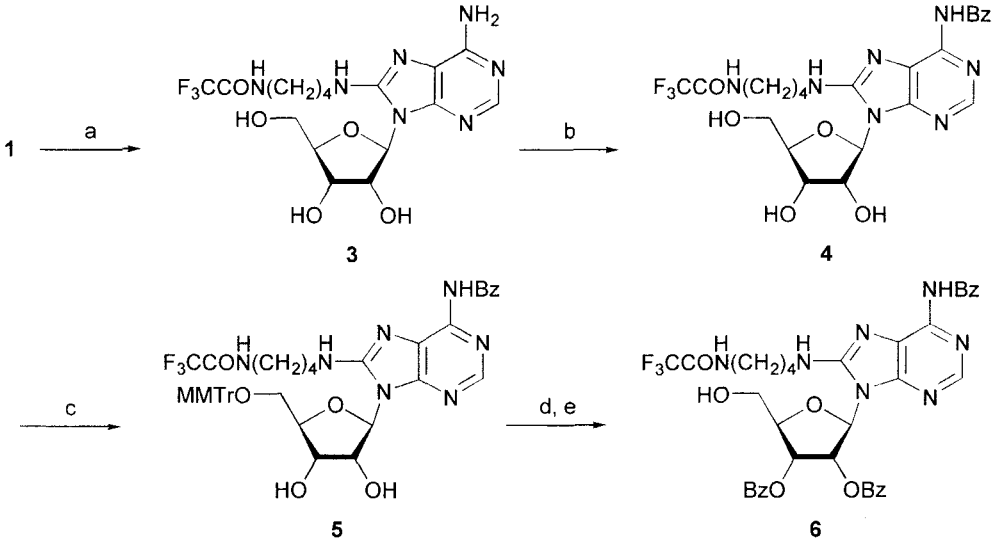
Synthesis and structural assignment.- The synthesis of the 2'(3')-terminal building block 8-[(4-trifluoroacetamido)amino]-N^6, N^6,2'-O,3'-O-tetrabenzoyladenosine (**6**) was achieved in five steps in ≈20% total yield. Treatment of compound **1** (A*)[13] with S-ethyl trifluorothioacetate in MeOH at 20 °C afforded the trifluoroacetamido derivative **3**.

Selective N^6-benzoylation by transient protection[14] yielded the benzoate **4**, 5'-mono-methoxytritylation of which led to compound **5**. Benzoylation of the latter followed by 5'-deprotection gave the desired building block **6** (Scheme 1).

A phosphotriester methodology was applied to synthesize the title trimer **2**, and the phosphodiester building block **7** was prepared essentially as previously described[7,15]. The condensations were performed in chloroform solution using a mixture of 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) and N-methylimidazole (1:3, mol) as condensing reagent.

The synthesis of the trimer was performed by condensation of the monomeric building blocks **6** and **7** to synthesize the 5'-detritylated dinucleoside monophosphate **8**, and then the dimer **8** with phosphodiester **7** to give, after deprotection, the trimer **2**. Deprotection of the trimer was performed by sequential detritylation, deblocking of phosphotriester groups with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and subsequent treatment with saturated methanolic ammonia followed by DEAE-Sephadex A-25 (HCO_3^- form) chromatography using a linear gradient of TEAB buffer to give (2',5')A_2A^* (**2**) (Na^+ salt) in moderate yield (24%) (Scheme 2).

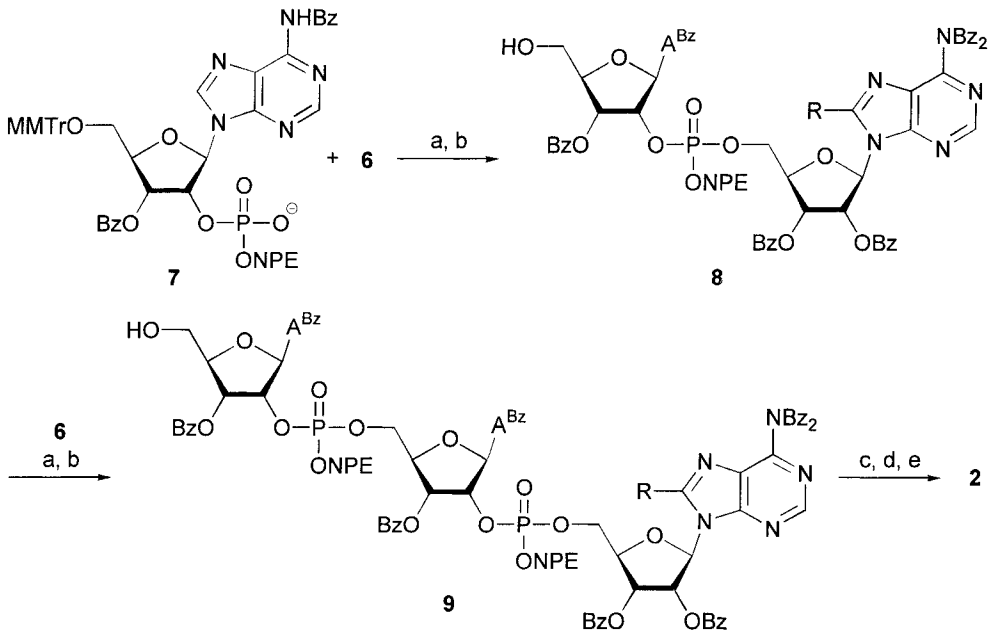
The structure of (2',5')A_2A^* (**2**) was proved by ^1H and ^{13}C NMR spectroscopy thereby the natural trimer, (2',5')A_3[6], and A* (**1**) served as reference compounds. The assignment of most of the sugar protons was achieved by the ^1H-^1H COSY-90 experiment and was confirmed by the 2D ^1H-^{13}C NMR correlation spectrum. As starting points for the interpretation of the ^1H-^1H COSY spectrum, the 1'-H signals of the ribose moieties were used. In the ^1H-^1H COSY spectrum the complete spin-coupling system of ribose protons of the 5'-terminal, A2'p, residue, including 5'-H resonances lacking a coupling to phosphorus, is observed. From the same spectrum the signals of 1'-H, 2'-H, 3'-H, and 4'-H of the middle, p5'A2'p, adenosine could be easily assigned. The 2'-H resonances of both these residues exhibit characteristic couplings to phosphorus atoms, whereas 3'-H signals do not show a proton-phosphorus coupling. The 5'-H resonances of all ribose residues were assigned from the DEPT-135 spectrum, and, as follows from the 2D ^1H, ^{13}C NMR correlation spectrum, the 5''-H resonances of p5'A2'p and A2'p are overlapped. Further, from the ^1H-^1H COSY spectrum the signals of 1'-H, 2'-H (triplet, no proton-phosphorus coupling), and 3'-H (double doublet) of the 2'(3')-terminal, p5'A*, nucleoside could be assigned. The signals of 4'-H and 5'-H of p5'A*, as well as p5'A* 5''-H and p5'A2'p 5'-H are overlapped. The base proton signals of (2',5')A_2A^* (**2**) were assigned by comparison with the data on the (2',5')A_3 and A*(**1**). The data are collected in Table 1.



Scheme 1. (a) CH_3COSEt, MeOH, 20 °C, 30 min (59%); (b) Me_3SiCl, pyridine, 20 °C, 2 h; BzCl, 0→20 °C, 2 h; concd aq NH_4OH, 0 °C, 15 min (67%); (c) MMTrCl, pyridine, 30 °C, 23 h (75%); (d) BzCl, pyridine, 0→20 °C, 12 h; (e) *p*-TsCl, $CHCl_3$-MeOH (7:3, v/v), 20 °C, 5 min (64%).

The assignments of the ^{13}C resonances of the ribose moieties of (2',5')A_2A^* (**2**) is straightforward. The resonances of carbon atoms are spin-coupled to ^{31}P separated by three bonds, depending on the magnitude of the torsion angles[16]. Thus, the signals of the C-1' and C-3' carbons of A2'p and p5'A2'p as well as the C-4' of p5'A2'p and p5'A* residues are assigned. The resonances of C-2' of A2'p and p5'A2'p display characteristic geminal couplings to phosphorus by 4.22 and 5.46 Hz, respectively. FIG. 1 presents ^{13}C resonances of ribofuranose residues of (2',5')A_2A^* (**2**). The correctness of these assignments was further substantiated by means of the 2D ^1H-^{13}C NMR correlation spectrum. The base ^{13}C signals of (2',5')A_2A^* (**2**) were assigned by comparison with the data of the (2',5')A_3 and A*(**1**). The data are summarized in TABLES 2 and 3.

The assignment of the ribosyl protons of A*(**1**) was performed by homonuclear decoupling experiments. The ^{13}C resonances were assigned easily with the aid of gated-decoupled ^{13}C NMR spectra as well as from the 2D ^1H, ^{13}C NMR correlation spectrum. The resonances of C-4 and C-6 carbon atoms are spin-coupled to 2-H ($^3J[^{13}C,H]$) by 11.92 and 11.32 Hz, respectively[17]. Moreover, the resonances of C-4 and C-8 carbons display spin couplings through three bonds to 1'-H by 3.2 and 2.5 Hz, respectively[17,18] (TABLES 1, 2 and 3).



A^{Bz} = N^6-benzoyladenine-9-yl

NPE = $-CH_2CH_2C_6H_4NO_2$ (*p*)

R = $-NH(CH_2)_4NHCOCF_3$

Scheme 2. (a) TPS-Cl, N-methylimidazole, $CHCl_3$, 20 °C, 1 h; (b) TsCl, $CHCl_3$-MeOH (7:3, v/v), 20 °C, 5 min (**8**, Σ 57.3%); (c) 0.5 M DBU in pyridine, 20 °C, 8 h; 1 M AcOH in pyridine, 0→20 °C, 5 min; (d) Saturated at 0 °C methanolic ammonia, 20 °C, 72 h; (e) DEAE-Sephadex A-25 (HCO_3^--form, TEAB buffer (0.005→0.4 M, 2x400 mL)(Σ 24%).

Conformational studies. - The conformation of (2',5')A_2A^* (**2**) in aqueous solution was studied by CD and ^1H and ^{13}C NMR spectroscopy. The hypochromicity value of (2',5')A_2A^* (**2**) was found to be 14%. This value points to the weaker base stacking compared to that of the parent trimer, (2',5')A_3[19], and even weaker than that of adenylyl-(2',5')-8-methyladenosine [(2',5')Ap(me^8A)][20]. However, the CD spectrum of (2',5')A_2A^* (**2**) and to a greater extent the temperature dependence of the intensity of the Cotton effects (FIG. 2) unambiguously suggest considerable base stacking in (2',5')A_2A^* (**2**).

It should be stressed that the temperature dependence is very similar to the corresponding properties of the parent trimer[19] and its 3'-deoxyadenosine [(2',5')(3'dA)$_3$] and 9-(β-D-xylofuranosyl)adenine [(2',5')(*xylo*-A)$_3$][21] analogues as well. At the same time, the band shape of the CD spectrum of (2',5')A_2A^* (**2**) considerably differs from that of the

TABLE 1. ^{1}H NMR Spectral Data of 8-(4-Aminobutyl)aminoadenosine (A*) in DMSO-d_6 and (2',5')ApApA* in D_2O (δ_{TMS}, ppm; J, Hz)

δ ^{1}H	1'-H ($J_{1',2'}$)	2'-H ($J_{2',3'}$) [$^3J_{2',P}$]	3'-H ($J_{3',4'}$)	4'-H ($J_{4',5'}$)	5'-H 5"-H ($J_{5',5''}$)	B - NH - CH_2 - CH_2 - CH_2 - CH_2 - NH_2	2-H	8-H
A(1) (A2'p)	6.03 d (4.68)	4.93 dt (≈4.80) [8.88]	4.50 t (≈4.80)	4.17 br.m (≈2.00)	3.78 dd ≈3.67 m (12.94)		7.95	8.04
A(2) (p5'A2'p)	5.89 d (5.70)	4.77 dt (5.70) [8.36]	4.41 t (≈4.10)	4.07 br.m	≈3.94 m ≈3.67 m		7.80	8.00
A*(3) (p5'A*)	5.67 d (6.47)	4.04 t (6.48)	4.11 dd (4.16)	≈3.99 m	≈3.94 m ≈3.99 m	- ≈2.90 1.54[a] 1.42[a] ≈2.84 -	7.69	-
A* c)	5.84 d (7.5)	4.56 dd (5.1)	4.05 dd (1.4)	3.90 br.s	3.57 br.s	6.98 3.25 1.54[b] 1.35[b] 2.43 ≈3.30 br.s m dt dt m br.s	7.82 s	-

a) and b) The data may be interconvertible.

c) The resonance of 6-NH_2 is at 6.38 br.s.

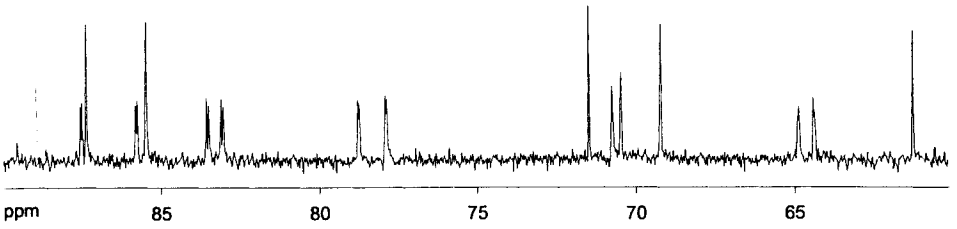


FIG. 1. Proton decoupled ^{13}C NMR spectrum of (2',5')A_2A^* (**2**) recorded in D_2O at 30 °C. Only the ribofuranose region is shown.

parent trimer[19] as well as of those of the above mentioned analogues[21], *viz.*, in the case of (2',5')A_2A^* (**2**) a characteristic bend in the 230-240 nm region is absent. All these data point to a different stacking mode in the (2',5')A_2A^* (**2**) compared to those of the parent trimer and its aforementioned analogues including (2',5')Ap(me^{8}A).

In the two spectra of (2',5')A_2A^* (**2**), most of the ^1H and ^{13}C resonances of the constituent A2'p and p5'A2'p are similar to those of (2',5')A_3[6]. It is noteworthy that the signals of methylene protons of the 8-NH-C$\underline{H}_2$ and -C$\underline{H}_2$-NH_2 fragments are shifted to a lower field by 0.35 ppm and to a higher field by 0.39 ppm *vs.* nucleoside A*(**1**), respectively. Most of the ^{13}C resonances of the A* fragment in the ^{13}C NMR spectrum of (2',5')A_2A^* (**2**) have chemical shifts that are close to the corresponding signals of A*(**1**), with the exception of the signals of C-5' and C-4' resulting from the phosphorylation at the 5'-hydroxyl group. Unexpectedly, ^{13}C resonance of one of the middle methylene group of 8-(4-aminobutyl)amino substituent displays an anomalously high upfield shift by *ca.* 6 ppm and the methylene group of the -C$\underline{H}_2$-NH_2 fragment is also shifted to a higher field by 1.84 ppm. Both latter shifts may be attributed to the influence of the adenine base of the p5'A2'p residue suggesting an *anti* orientation of the p5'A* fragment.

The values of proton-proton couplings of (2',5')A_2A^* (**2**) suggest that the N/S equilibrium of ribofuranose residues of unmodified nucleoside fragments is shifted towards S-type conformers in contrast to those of the parent trimer, (2',5')A_3. Furthermore, the population of S-conformers of the 8-(4-aminobutyl)aminoadenosine residue is reduced in comparison with the nucleoside. The ^3J[2'H,P] couplings show, like those of (2',5')A_3, a strong preference of an *anti* orientation of 2'-H and the phosphorus atom. Overlapping of the 4'-H and 5'-H resonances precluded the evaluation of rotamer populations about the C5'-C4' bonds.

TABLE 2. ^{13}C NMR Spectral Data of 8-(4-Aminobutyl)aminoadenosine (A^*) in DMSO-d_6 and (2',5')ApApA* (represented as the corresponding fragments) in D_2O (δ_{TMS}, ppm; *J*, Hz)

$\delta^{13}C$ [$^1J_{C,H}$]	C-1' {$^3J_{C,P}$}	C-2' {$^2J_{C,P}$}	C-3' {$^3J_{C,P}$}	C-4' {$^3J_{C,P}$}	C-5' {$^2J_{C,P}$}	C-2	C-4	C-5	C-6	C-8
A(1) (A2'p)	87.47 [166.8]	77.85 [152.8]	70.73 [155.6]	85.44 [149.9]	61.28 [144.5]	152.85 [202.2]	148.60	118.87	155.23	140.23 [212.93]
$^3J_{C,H1'}$ $^3J_{C,H}$	{6.8}	{4.2}	{≈2.5}				≈3.0 12.0 (C4;H2)	9.9 (C5,H8)	11.2 (C6,H2)	3.02
A(2) (p5'A2'p)	85.74 [163.8]	78.72 [150.1]	70.46 [153.5]	83.51 [151.4]	64.89 [145.9]	152.20 [202.5]	148.03	118.12	155.21	138.92 [214.80]
$^3J_{C,H1'}$ $^3J_{C,H}$	{7.0}	{5.5}	{≈2.5}	{8.8}	{5.7}		2.9 12.2 (C4,H2)	10.1 (C5,H8)	11.1 (C6,H2)	4.8
A^*(3) (p5'A^*)	87.32 [163.6]	71.47 [150.1]	69.21 [155.1]	83.06 [152.1]	64.39 [150.2]	149.22 [202.9]	148.92	116.40	151.40	151.37
$^3J_{C,H1'}$ $^3J_{C,H}$				{9.75}	{4.38}		<2.0 11.6 (C4,H2)		10.9 (C6,H2)	5.0
A^*	86.43 [162.2]	70.81 [145.9]	71.06 [152.2]	85.72 [140.9]	61.63 [140.9]	148.47 [198.7]	149.87	117.18	152.34	151.45
$^3J_{C,H1'}$							3.2			2.5
$^3J_{C,H2}$							11.9		11.3	

TABLE 3. ^{13}C NMR Spectral Data of the 8-(4-Aminobutyl) Residue of A*(**1**) and (2',5')ApApA* (**2**)

B — NH — CH_2	——— CH_2	——— CH_2	——— CH_2 — NH_2
(2',5')ApApA* (**2**)			
$\delta^{13}C$ 41.73	24.30[a]	25.53[a]	39.33
$\Delta\delta$ [c] -0.63	-6.06	-0.69	-1.84
$^1J_{C,H}$ 138.64	146.92	126.98	147.88
A*			
$\delta^{13}C$ 42.36	30.36[b]	26.22[b]	41.17
$^1J_{C,H}$ 137.09	125.14	123.76	133.32

a) and b) The data (δ and related $\Delta\delta$ and $^1J_{C,H}$) may be interconvertible.

c) $\Delta\delta = \delta^{13}C_{trimer} - \delta^{13}C_{nucleoside}$.

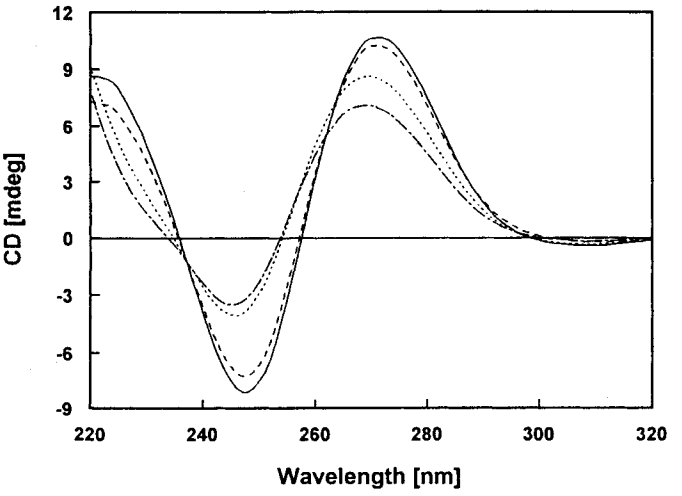


FIG. 2. CD spectra of (2',5')A_2A* (**2**) at 5 °C (——), 20 °C (– – –), 60 °C (·····) and 80 °C (– · –) in 1 M NaCl containing 60 mM Na-cacodylate and 100 mM $MgCl_2$; pH 7.0; concentration 0.5 A_{270}/mL).

The most important information on the conformation of (2',5')A_2A* (**2**) was derived from the ^{13}C NMR experiments. Rotamer populations of the torsion angle C3'-C2'-O2'-P (ε) can be obtained from the vicinal couplings ^3J[C1',P] and ^3J[C3',P]. These couplings point to a strong preference for an *anti* and *gauche* orientation of C-3'/P and C-1'/P atoms, respectively, which is in agreement with the aforementioned ^3J[2'H,P] couplings. Furthermore, the values of ^3J[C4',P] couplings are associated with a very high percentage of an *anti* conformation about the C5'-O5' bond (the torsion angle β). Finally, the vicinal ^3J[C4,1'H] and ^3J[C8,1'H] couplings unambiguously confirm (*i*) the *syn/anti* conformational freedom at the 5'-terminus, and (*ii*) the predominant *anti* conformation about the glycosyl bond of the middle nucleotide and, to a greater extent, of the 2'(3')-terminal modified nucleotide. It appears of interest that the 5'-terminal residue of (2',5')A_3 was shown to exhibit the higher *syn/anti* conformational mobility than two other nucleotides for which a strong preference of an *anti* orientation of the base was suggested on the basis of spin-lattice relaxations time ratios T_1(8-H)/T_1(1'-H)[6]. FIG. 3 presents proton-decoupled and proton-coupled ^{13}C resonances of base residues of (2',5')A_2A* (**2**). We assume that ionic interactions between the 5'-phosphate anion and the 4-amino group of p5'A* residue led to a high population of *anti* base orientation.

The spectral data for A*(**1**) deserve some comments. ^{1}H NMR studies of 8-amino, 8-methylamino, and 8-dimethylamino derivatives of adenosine have led to the conclusion that an *anti*/S-type conformational combination is preferred on the basis of the difference in chemical shifts between 1'-H and 2'-H ($\Delta\delta_{1'/2'}$)[8]. However, we have recently established that the $\Delta\delta_{1'/2'}$ criterion is not an unequivocal indication of a *syn/anti* orientation[22]. In order to gain information on the stereochemistry of A*(**1**), we have carried out an extensive NMR analysis. The $\Delta\delta_{1'/2'}$ value for A*(**1**) was found to be in agreement with that of 8-methylaminoadenosine[8] pointing to a substantial population of *anti* conformers. The proton-proton couplings of the sugar ring are in favor of a strong preference for an S-type conformation (TABLE 1)[9].

On the other hand, the C-2' resonance of A*(**1**) display a large upfield shift (≈4 ppm) in comparison with that of adenosine[17,23]. From ^{13}C NMR studies of various (bromo, methyl, chloro, thiomethyl, and methoxy) 8-substituted purine nucleosides it was concluded that such an upfield shift compared to those of the parent nucleosides is characteristic for a *syn* conformation about the glycosyl bond[23]. The values of vicinal *J*[C4,1'H] and *J*[C8,1'H] couplings give further support to a preferential *syn* orientation of the base[18] in DMSO solution (TABLE 2).

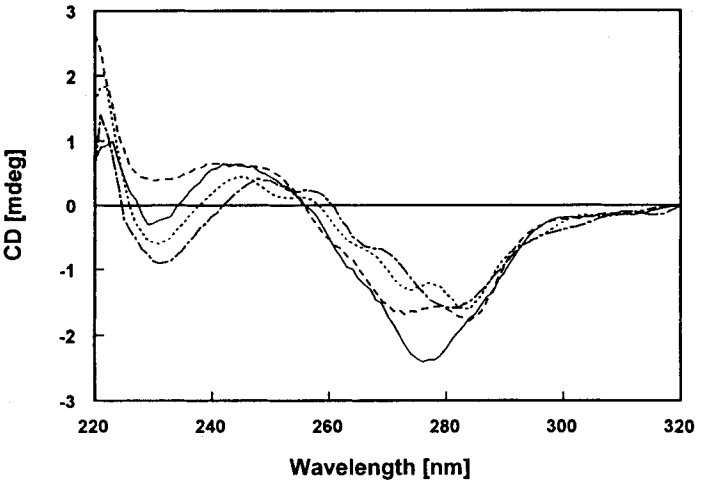


FIG. 3. CD spectra of A* (**1**) at 5 °C (——), 20 °C (– – –), 60 °C (·····) and 80 °C (– · –) in 1 M NaCl containing 60 mM Na-cacodylate and 100 mM $MgCl_2$; pH 7.0; concentration 0.5 A_{280}/mL).

The CD spectrum of A*(**1**) displays, like that of adenosine[24], a negative long-wavelength envelope near 280 nm (B_{2u}) and a positive transition in the 240-250 nm region (B_{1u}) (FIG. 4). Both these CD bands are shifted to the long-wavelength region by about 20 nm compared to adenosine that is in agreement with the UV spectrum of A*(**1**) (λ_{max} at 280.5 nm). Moreover, A*(**1**), in contrast to adenosine, exhibits the CD band near 230 nm which displays a remarkable temperature dependence. The temperature-induced changes in the CD spectrum of A*(**1**) point to predominant population of *anti*-conformers at 5 °C and different blends of *syn*/*anti* conformations by going to higher temperatures.

From the NMR and CD data, it may be concluded that there is a conformational freedom for a base orientation about the glycosyl bond in A*. An intramolecular 8-NH ••• O-5' hydrogen bond may contribute to the stabilization of an *anti*-conformation in aqueous solution (*cf.* with the X-ray data[25] in the solid state of a related compound), but in DMSO solution such a contribution may be diminished.

RNase L binding and conclusion.- The ability of (2',5')A_2A* (**2**) to bind to the RNase L of mouse L cells compared to (2',5')A_3 was examined in a radiobinding assay as described previously[7], displacement curves are illustrated in FIG. 5. Unexpectedly, strong differences in binding ability were observed: the IC_{50} (50% displacement of the

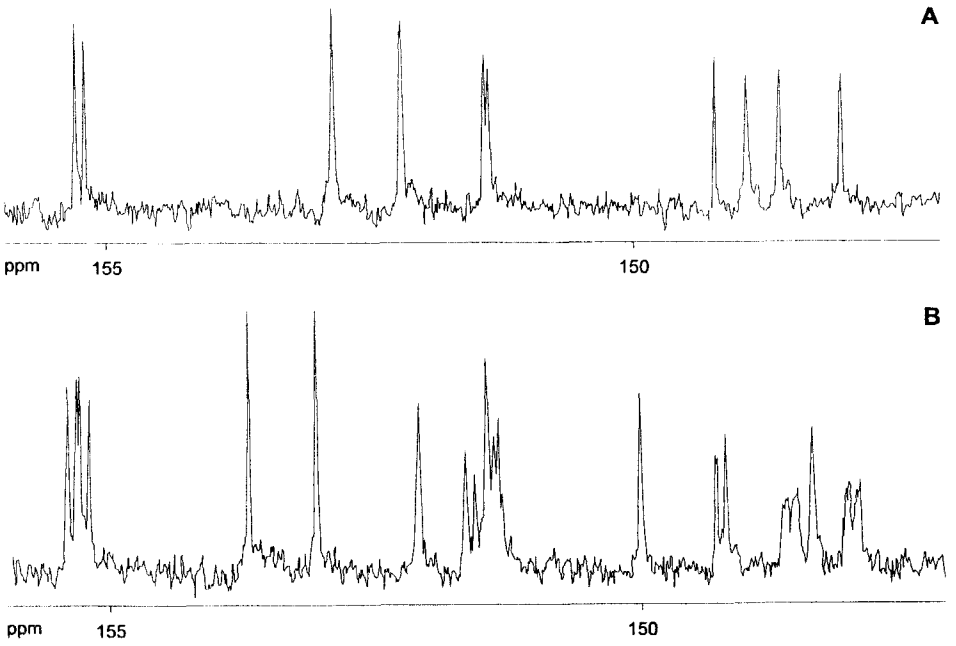


FIG. 4. ^{13}C NMR spectrum of (2',5')A_2A^* (**2**) recorded in D_2O at 30 °C. Detail of the base region. A: Proton-decoupled; B: Proton-coupled.

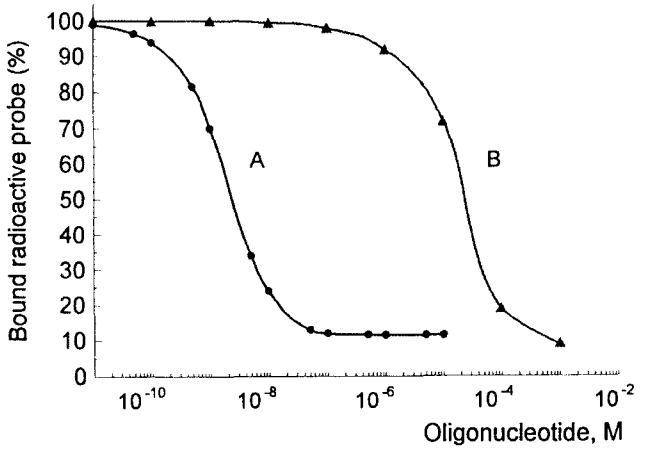


FIG. 5. Ability of (2',5')A_3 (A) and (2',5')A_2A^* (**2**) (B) to inhibit the binding of the radioactive probe to RNase L.

radioactive probe from RNase L) value for (2',5')A_3 was found to be $2.5x10^{-9}$ M whereas (2',5')A_2A^* (**2**) was approximately 10^4 times less effective in this process (IC_{50} $2.0x10^{-5}$ M). In order to exclude the fast degradation of (2',5')A_2A^* (**2**) by phosphodiesterase(s) that may be present in the RNase L preparation (RNase L-nitrocellulose complex), we have studied in detail the digestion of (2',5')A_2A^* (**2**) by snake venom phosphodiesterase (SVPD) (see Experimental; TABLE 4).

Substitution of a hydrogen atom for the 4-(aminobutyl)amino group at C-8 of the 2'(3')-terminus does not lead to an essential alteration of the affinity of the analogue to the enzyme, K_m, but results in an 18-fold reduction of V_{max}. The k_{cat}/K_m ratios for SVPD hydrolysis of the parent trimer, (2',5')A_3, and the analogue (2',5')A_2A^* (**2**), reflecting the specificity of the enzyme for the substrate, were determined to be $2.3 \cdot 10^{-5}$ and $0.037 \cdot 10^{-5}$ $M^{-1} \cdot s^{-1}$, respectively. The decrease in the k_{cat}/K_m value is largely due to a drop in the k_{cat} value. The τ values were found to be 6.95 and 17.3 min for (2',5')A_3 and (2',5')A_2A^* (**2**), respectively, displaying a somewhat lower susceptibility of the latter to the action of the enzyme.

Thus, it may be suggested that the spatial arrangement of (2',5')A_2A^* (**2**) and/or the bulkiness of 8-(aminobutyl)amino substituent at C-8 of the 2'(3')-terminal adenosine cause its very low binding ability to the RNase L. Whether a strong preference of an *anti* orientation of the base of the 2'(3')-terminal residue of (2',5')A_2A^* (**2**) would be maintained upon derivatization of 4-amino group of p5'A* fragment and whether some other factors, *e.g.*, Coulomb repulsion between the aliphatic ammonium group and positively charged amino acid within the binding site of the RNase L contribute to the observed low binding ability remains to be established in the future.

EXPERIMENTAL SECTION

General. UV spectra were recorded on a Specord M-400 instrument (Carl Zeiss, Germany). ^1H and ^{13}C NMR spectra were measured at 200.13 and 50.325 MHz, respectively, at 23 °C on an AC-200 spectrometer equipped with an Aspect 3000 data system (Bruker, Germany), and at 500.14 and 125.76 MHz on an AMX-500 spectrometer with tetramethylsilane (TMS) as an internal standard (s = singlet; d = doublet; t = triplet; m = multiplet; br.s = broad signal); assignments of proton resonances were confirmed, when possible, by selective homonuclear decoupling experiments. *J*-Values are in Hz.

Low resolution FAB mass spectra were obtained on a Kratos MS80 (England) spectrometer from samples dissolved in DMSO with glycerol as matrix under Xe atoms

TABLE 4. Kinetic data for hydrolysis of (2',5')trimers by snake venom phosphodiesterase.

Compd	$K_m \cdot 10^5$ M	V_{max} μmol/min per mg of enzyme	k_{cat} s^{-1}	$k_{cat}/K_m \cdot 10^5$ $M^{-1} \cdot s^{-1}$(%)	τ1/2 min	ΔG_{app} $kJ \cdot mol^{-1}$
(2',5')A_3	2.0	4690	4.63	2.32 (100)	6.95	-
(2',5')A_2A^*	2.7	260	0.1	0.037(1.6)	17.3	-2.1

bombardment (6-8 KeV). CD spectra were obtained on a J-600 (JASCO, Japan) spectropolarimeter in cacodylate buffer (1 M NaCl, 60 mM sodium cacodylate, 100 mM $MgCl_2$; pH 7.0; concentration 0.5 A_{280}/mL and A_{270}/mL for A*(**1**) and (2',5')A_2A^* (**2**), respectively). HPLC was carried out on a Lichrosorb RP-18 column (Merck, 5 μm, 250-10; a linear gradient (0→20%, v/v) of MeCN in buffer $(Et_3NH)OAc$ 0.1 M, pH 7.0; 5% MeCN); flow rate 1 mL/min). Thin layer chromatography (TLC) was carried out on the F 1500 LS 254 silica gel plates (Schleicher & Schuell, Germany). Column chromatography was performed on Silica Gel L (Chemapol, Czech Republic) 100/400 μ and 40/100 μ. Petroleum ether with bp 40-50 °C was used throughout this work. The solutions of compounds in organic solvents were dried with anhydrous Na_2SO_4 for 4 h. Except where otherwise indicated, the reactions were carried out at 20 °C.

The snake venom phosphodiesterase was purchased from Boehringer, cat. No. 108260 (Germany). The hypochromicity of (2',5')A_2A^* (**2**) was measured as described previously.[7]

***8-[(4-Trifluoroacetamidobutyl)amino]adenosine* (3)**. To a suspension of 8-[(4-aminobutyl)amino]adenosine (**1**) (0.212 g, 0.6 mmol) in anhydrous MeOH (7 mL), S-ethyl trifluoroacetate (0.379 mL, 0.474 g, 1.92 mmol) was added; the mixture was stirred for 30 min, and evaporated. The residue was chromatographed on a silica gel column (50 mL), eluting with a linear MeOH gradient (0→20%, v/v; 2 x 300 mL) in $CHCl_3$, then with $CHCl_3$-MeOH (4:1, v/v; 250 mL). The fractions containing individual products were collected and evaporated.

The material of the faster migrating zone was dissolved in acetone (5 mL) and precipitated in petroleum ether (30 mL) to afford pure compound **3** as a colorless amorphous powder (180 mg, 67%), m.p. 113-115 °C; R_f 0.22 ($CHCl_3$-MeOH, 4:1); λ_{max}

(MeOH) nm (lg ε): 215 (4.36) and 278.7 (4.15); *m/z* (FAB) 450 (MH^+); δ_H (DMSO-d_6) 9.46 (1H, br.t, *J* 5.5, δNH), 7.96 (1H, s, 2-H), 7.11 (1H, br.t, *J* 5.5, αNH), 6.83 (2H, br.s, NH_2), 5.90 (1H, d, *J* 7.5, 1'-H), 5.10-5.30 (3H, overlapped signals, OH-2',3', and 5'), 4.62 (1H, m, $J_{2',3'}$ 5.0, 2'-H), 4.11 (1H, m, H-3'), 3.96 (1H, br.m, H-4'), 3.62 (2H, br.s, H-5' and H-5"), 3.20 (4H, overlapped signals, 2 x CH_2-1 and 4), 1.54 (4H, overlapped signals, 2 x CH_2-2 and 3). *Anal.* Calc. for $C_{16}H_{22}F_3N_7O_5$: C, 42.7, H, 4.9, N, 21.8%. Found: C, 42.2, H, 4.6, N, 21.7.

From the slowly migrating zone the starting nucleoside **1** (50 mg; 24%) was recovered.

***N^6-Benzoyl-8-[(4-trifluoroacetamidobutyl)amino]adenosine* (4)**. To a solution of **3** (0.14 g, 0.31 mmol) in anhydrous pyridine (4 mL), trimethylchlorosilane (0.29 mL, 2.32 mmol) was added and the mixture was stirred for 2 h. It was then cooled to 0 °C, benzoyl chloride (0.11 mL, 0.93 mmol) was added and the mixture was stirred for 2 h allowing it to warm to room temperature. The mixture was cooled to 0 °C, water (0.3 mL) was added, stirred for 5 min, then conc. aq. ammonia (0.62 mL) was added and stirring was continued for another 15 min at room temperature. Then the solvent was evaporated, and co-evaporated with toluene (10 mL). The residue was chromatographed on a silica gel column (100 mL), eluting with $CHCl_3$ (500 mL) and then with a linear MeOH gradient (0→ 10%, v/v; 2 x 500 mL) in $CHCl_3$. Two fractions containing individual products were collected and evaporated.

The content of the faster migrating zone was dissolved in acetone (5 mL) and precipitated in petroleum ether (30 mL) to afford compound **4** as a colorless amorphous powder (0.1 g, 59%), m.p. 117-119 °C; R_f 0.76 ($CHCl_3$-MeOH, 4:1) and 0.20 ($CHCl_3$-MeOH, 9:1); λ_{max} (MeOH) nm (lg ε): 235 (4.40) and 311.2 (4.29); *m/z* (FAB) 554 (MH^+); δ_H (DMSO-d_6) 9.44 (1H, br.t, *J* 5.6, δNH), 8.44 (1H; s, 2-H), 8.04-7.28 (5H, m, aromatic H), 7.81 (1H, br.s, αNH), 6.10 (1H, d, *J* 7.5, 1'-H), 4.63 (1H, dd, $J_{2',3'}$ 5.0, 2'-H), 4.15 (1H, br.d, 3'-H), 4.04 (1H, br.s, 4'-H), 3.67 (1H, br.s, 5'-H and 5"-H), 2.98-3.26 (4H, overlapped signals, 2 x CH_2-1 and 4), 1.55 (4H, overlapped signals, 2 x CH_2-2 and 3). *Anal.* Calc. for $C_{23}H_{26}F_3N_7O_6$: C, 49.9, H, 4.7, N, 17.7%. Found: C, 49.5, H, 4.25, N, 17.8.

From the slower migrating zone the starting nucleoside **3** (20 mg; 14.3%) was recovered.

***N^6-Benzoyl-8-[(4-trifluoroacetamidobutyl)amino]-5'-monomethoxytrityl-adenosine* (5)**. Compound **4** (80 mg, 0.14 mmol) was first evaporated with anhydrous pyridine (2 x 10 mL), and then dissolved in anhydrous pyridine (0.9 mL). After addition of

p-monomethoxytrityl chloride (79 mg, 0.25 mmol), the mixture was stirred for 23 h. The reaction mixture was poured into an ice-water mixture (200 mL) and, after the ice had melted, the precipitate formed was filtered off, washed with water and dried. The residue was purified by column chromatography (70 mL) eluting with a linear MeOH gradient (0→ 5%, v/v; 2 x 250 mL) in $CHCl_3$ and then with a $CHCl_3$-MeOH mixture (19:1; 200 mL). The main fraction was collected and evaporated. The residue was dissolved in $CHCl_3$ (3 mL) and precipitated in hexane (200 mL) to give compound **5** as a colorless amorphous powder (90 mg; 75%), m.p. 111-113 °C; R_f 0.16 ($CHCl_3$-MeOH, 9:1); λ_{max} (MeOH) nm (lg ε): 235 (4.42) and 310.3 (4.10); δ_H ($CDCl_3$) 9.02 (1H, br.s, δNH), 8.32 (1H, s, 2-H), 8.04-6.70 (20H, m, aromatic H and αNH), 5.98 (1H, d, *J* 6.5, 1'-H), 5.02 (1H, t, $J_{2',3'}$ 5.0, 2'-H), 4.46 (1H, br.d, 3'-H), 4.34 (1H, br.s, 4'-H), 3.76 (3H, s, OCH_3), 3.56 (1H, dd, $J_{5',4'}$ 3.0, $J_{5',5'}$ 11.0, 5'-H), 3.28 (1H, dd, $J_{5'',4'} \approx 3.0$, 5''-H), 3.24 (4H, overlapped signals, 2 x CH_2-1 and 4), 1.40 (4H, overlapped signals, 2 x CH_2-2 and 3). *Anal.* Calc. for $C_{43}H_{42}F_3N_7O_7$: C, 62.5, H, 5.1, N, 11.9%. Found: C 62.9, H 5.4, N, 11.75.

N^6,N^6,2'-O,3'-O-Tetrabenzoyl-8-[(4-trifluoroacetamidobutyl)amino]-adenosine **(6)**. Compound **5** (72.4 mg, 0.087 mmol) was first evaporated with anhydrous pyridine (5 mL), then dissolved in anhydrous pyridine (3 mL), cooled to 0 °C, and benzoyl chloride (0.08 mL, 0.84 mmol) was added. The mixture was stirred for 12 h allowing it to warm to room temperature, and then poured into an ice-water mixture (200 mL) and, after the ice had melted, the precipitate formed was filtered off, washed with water, dried, and finally co-evaporated with $CHCl_3$. To the oily residue, a 2% solution of *p*-toluenesulfonic acid in $CHCl_3$-MeOH (7:3, v/v; 9.97 mL) was added and the mixture was stirred for 5 min. After dilution with $CHCl_3$ (10 mL), the mixture was shaken with phosphate buffer (pH 7.0; 100 mL), the water layer was separated, and then extracted with $CHCl_3$ (3 x 20 mL). The combined organic phases were dried, filtered, and evaporated to dryness. The residue was chromatographed on a silica gel column (50 mL), by elution successively with hexane-chloroform (1:1, v/v; 200 mL), followed by a linear MeOH gradient (0→2.8%, v/v; 2 x 300 mL) in $CHCl_3$, and finally with $CHCl_3$-MeOH mixture (19:1; 200 mL). The main fraction was collected and evaporated, the residue was dissolved in $CHCl_3$ (2 mL) and precipitated in hexane (150 mL) to give compound **6** as a colorless amorphous powder (48.7 mg; 64%), m.p. 101-103 °C; R_f 0.33 ($CHCl_3$-MeOH, 35:1); λ_{max} (MeOH) nm (lg ε): 231 (4.69) and 312.5 (4.12); δ_H (DMSO-d_6) 9.44 (1H, br.t, *J* 5.5, δNH), 8.30 (1H, s, 2-H), 8.00-7.24 (20H, m, aromatic H + αNH), 6.51 (1H, d, *J* 6.5, 1'-H), 6.20 (1H, t, $J_{2',3'}$ 6.5, 2'-H), 5.89 (1H, dd, $J_{3',4'}$ 2.5, 3'-H), 4.56 (1H, br.d, $J_{4',5'}$ and $J_{4',5''} \approx 2.5$, 4'-H), 3.83 (2H,

m, $J_{5',5''}$ 12.0, H-5' and H-5"), 3.18 (4H, overlapped signals, 2 x CH_2-1 and 4), 1.51 (4H, overlapped signals, 2 x CH_2-2 and 3). *Anal.* Calc. for $C_{44}H_{38}F_3N_7O_9$: C 61.0, H 4.4, N 11.3%. Found: C 60.6, H 4.0, N 11.3.

***Adenylyl-(2',5')-adenylyl-(2',5')-[8-(4-aminobutyl)amino]adenosine* (2).** To a solution of the tetrabenzoate **6** (60.1 mg, 0.079 mmol) and triethylammonium-{N^6,3'-O-dibenzoyl-5'-O-monomethoxytrityladenosine-2'-[2-(4-nitrophenyl)ethyl]-phosphate} (**7**)[5] (82.3 mg, 0.076 mmol) in $CHCl_3$ (0.5 mL), TPS-Cl (126 mg, 0.416 mmol) and N-methylimidazole (99 mg, 1.057 mmol) were added and the reaction mixture was stirred for 1 h. Chloroform (3 mL) was added and the solution was precipitated in hexane (50 mL). The precipitate was filtered off, washed with hexane, and dried. The residue was dissolved in 2% solution of *p*-toluenesulfonic acid in $CHCl_3$-MeOH (7:3, v/v; 9.67 mL) and the mixture was stirred for 5 min. After dilution with $CHCl_3$ (10 mL), the mixture was shaken with phosphate buffer (pH 7.0; 2 x 15 mL), the organic layer was dried, filtered, and evaporated to dryness *in vacuo*. The residue was purified by silica gel (40 mL) column chromatography eluting successively with hexane-chloroform (1:1, v/v; 150 mL), $CHCl_3$ (250 mL), and finally with a linear MeOH gradient (0→5%, v/v; 2 x 300 mL) in $CHCl_3$. The main fraction was collected, evaporated to ~2 mL, and precipitated in hexane (150 mL) to give N^6,3'-O-dibenzoyladenylyl-(2'-{O(P)-[2-(4-nitrophenyl)ethyl]}-5')-N^6,N^6,2'-O,3'-O-Tetrabenzoyl-[8-(4-trifluoroacetamidobutyl)]adenosine (**8**) as a colorless amorphous powder (61.7 mg; 57.3%), m.p. 120-122 °C; R_f 0.67 ($CHCl_3$-MeOH, 24:1); λ_{max} (MeOH) nm (lg ε): 231.4 (4.93) and 275.2 (4.57).

To a solution of the dinucleoside monophosphate **8** (68 mg, 0.044 mmol) and the phosphodiester **7** (51.9 mg, 0.048 mmol) in $CHCl_3$ (0.36 mL), TPS-Cl (79.6 mg, 0.263 mmol) and N-methylimidazole (65 mg, 0.694 mmol) were added and the reaction mixture was stirred for 24 h. Chloroform (5 mL) was added and the solution was precipitated in hexane (150 mL). The precipitate formed was filtered off, washed with hexane, and dried. The residue was dissolved in 2% solution of *p*-toluenesulfonic acid in $CHCl_3$-MeOH (7:3, v/v; 9.67 mL) and the mixture was stirred for 5 min. After dilution with $CHCl_3$ (5 mL), the mixture was shaken with phosphate buffer (pH 7.0; 2 x 10 mL), the organic layer was dried, filtered, and evaporated to dryness. The residue was purified by silica gel (30 mL) column chromatography, eluting successively with ethyl acetate-hexane (1:3, v/v; 100 mL), a linear ethyl acetate gradient (50→75%, v/v; 2 x 120 mL) in hexane (250 mL), and finally with ethyl acetate-hexane (4:1, v/v; 250 mL). The main fraction was collected, evaporated to dryness, and dissolved in 0.5 M solution of DBU in pyridine (11 mL). The

mixture was stirred for 8 h, and then cooled to 0 °C. After addition of a 1 M solution of acetic acid in pyridine (6.6 mL), the mixture was evaporated to dryness and co-evaporated with toluene (3 x 5 mL). The residue was dissolved in MeOH (10 mL), saturated with ammonia at 0 °C, kept for 72 h, and evaporated *in vacuo*. The residue was chromatographed on a DEAE-Sephadex A-25 (HCO_3^--form; 100 mL) column using a linear gradient (0.005→0.4 M, 2 x 400 mL) of TEAB buffer. The product-containing fractions were collected and evaporated *in vacuo*. The deblocked trimer **2** was obtained in the form of the Na^+-salt as an amorphous powder according to Moffatt[26] (13 mg, 24%), R_f 0.41 (2-propanol-25% aq. ammonia-water, 7:1:2); λ_{max} (phosphate buffer, pH 7.0) 261.5 nm(lg ε 4.60); HPLC: retention time 14.85 min.

Kinetic Measurements. The SVPD digestions of (2',5')A_3 and (2',5')A_2A^* (**2**) were performed as described by Itkes *et al.*[27]. The Tris-HCl buffer (0.05 M; pH 8.8) containing $MgCl_2$ (0.005 M) was used throughout this work. The progress of the enzyme-catalyzed hydrolysis was followed using a Specord M-400 spectrphotometer by measuring the change of optical density at 262 nm ($\Delta\varepsilon$) resulting from the disappearance of base-base stacking interactions. The initial rate values were used for calculation of K_m and V_{max}. The best straight lines were obtained in a double-reciprocal plot ($1/\Delta\varepsilon$ *vs.* 1/S). The kinetic parameters are shown in TABLE 4.

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