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## 3-Deazaadenosine analogues of p5'A2'p5'A2'p5'A: synthesis, stereochemistry, and the roles of adenine ring nitrogen-3 in the interaction with RNase L

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Abstract—Sequence-specific 3-deazaadenosine ( $c^3A$ )-substituted analogues of trimeric 2',5'-oligoadenylate, p5'A2'p5'A2'p5'A, were synthesized and evaluated for their ability to activate human RNase L (EC 3.1.2.6) aiming at the elucidation of the nitrogen-3 role in this biochemical process. Substitution of either 5'-terminal or 2'-terminal adenosine with  $c^3A$  afforded the respective analogues  $p5'(c^3A)2'p5'A2'p5'A$  and  $p5'A2'p5'A2'p5'(c^3A)$  that were as effective as the natural tetramer itself as activators of RNase L (EC<sub>50</sub> = 1 nM). In contrast,  $p5'A2'p5'(c^3A)2'p5'A$  showed diminished RNase L activation ability (EC<sub>50</sub> = 10 nM). The extensive conformational analysis of the  $c^3A$ -substituted core trimers versus the parent natural core trimer by the <sup>1</sup>H and <sup>13</sup>C NMR, and CD spectroscopy displayed close stereochemical similarity between the natural core trimer and ( $c^3A$ )2'p5'A2'p5'A and A2'p5'( $c^3A$ ) analogues, thereby strong evidences for the *syn* base orientation about the glycosyl bond of the  $c^3A$  residue of the latter were found. On the contrary, an analogue A2'p5'( $c^3A$ )2'p5'A displayed rather essential deviations from the spatial arrangement of the parent natural core trimer. © 2004 Elsevier Ltd. All rights reserved.

### 1. Introduction

The 2',5'-linked 5'-O-phosphorylated oligoadenylates (2-5A) play a key role in the antiviral action of interferon.<sup>1,2</sup> In the presence of double-stranded RNA, interferon induces in vertebrate cells the production of the enzyme (2',5')oligoadenylate synthetase, which utilize ATP to generate a unique group of 2',5'-phosphodiester-linked oligomers referred to as 2-5A, (pp)p5'A2'(p5'A2')<sub>n</sub>p5'A (n = 1-3; mainly trimer, n = 1) (Scheme 1). These oligomers bind to and subsequently activate RNase L (EC 3.1.2.6), a constitutive, but latent endonuclease that degrades the mRNA of the virus and

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thus inhibits protein synthesis. Moreover, numerous biochemical studies of 2-5A manifested a broad palette of processes that are influenced and/or regulated by these oligomers.<sup>2</sup>

Since the exact chemical structure of 2-5A was established,<sup>1</sup> a number of analogues of 2-5A have been synthesized in order to examine the crucial structural and stereochemical parameters of 2-5A for binding to and activation of RNase L. It was established that diverse functionalities of each individual nucleotide fragment of 2-5A contribute highly specifically to binding to and activation of RNase L. Thus, the role of each purine  $N^7$ atom in both processes was studied with a set of trimeric 2-5A analogues, in which one or more adenosine residues was replaced by 7-deazaadenosine (c<sup>7</sup>A; tubercidin).<sup>3,4</sup> Evaluation of c<sup>7</sup>A analogues of 2-5A for their ability to bind to and activate RNase L of mouse L cells

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Systematic numbering; purine numbering in parenthesis

#### Scheme 1.

showed that there were small changes ( $\leq 10$ -fold) in their ability to bind to RNase L. On the contrary, an essential decrease in the ability to activate RNase L was found upon replacement of either the 5'-terminal (A1, 33-fold) or 2'-terminal (A3, 7-fold) adenosine of 2-5A by c<sup>7</sup>A. Only one analogue, (pp)p5'A2'p5'(c<sup>7</sup>A)2'p5'A, in which the central adenosine residue A2 was replaced by c<sup>7</sup>A, retained activity equivalent to 2-5A itself.<sup>3,4</sup>

Replacement of the adenine base of 2-5A by hypoxanthine allowed to appreciate the role of  $N^6$ -amino/ $N^1$ functionality in the interaction with RNase L of mouse cells.<sup>5,6</sup> The exchange at the 5'-terminus of 2-5A led to a large decrease both in RNase L binding affinity and in activation ability (200-fold). Similarly, adenine  $\rightarrow$ hypoxanthine exchange at the 2'-terminus resulted in dramatic 1000-fold drop in activation ability with little change in RNase L binding ability. On the contrary, the adenine  $\rightarrow$  hypoxanthine replacement in the middle led to decrease in RNase L activation (20-fold loss) without essential deterioration of binding ability (2-3-fold decrease). A further refinement of the role of  $N^6$ -amino/ $N^1$ functionality in the interaction with the recombinant human RNase L was made recently by us employing a set of 2-5A analogues, in which adenosine residues have been successively replaced by 1-deazaadenosine  $(c^{1}A)$ .<sup>7</sup> It was established that the nitrogen-1 of the 5'-terminal adenosine moiety A1 is key for binding to RNase L and its replacement by c<sup>1</sup>A gives rise to a 81-fold decrease in binding affinity. Substitution of A2 or A3 adenine residues with c<sup>1</sup>A resulted in a 9-fold and 15-fold

diminution in affinity to RNase L, respectively. The 5'terminus (A1) c<sup>1</sup>A-substituted analogue,  $p5'(c^1A)2'p5'$ A2'p5'A, showed a 35-fold reduction in RNase L activation ability. The congeners with the A2 or A3 adenosine substitution displayed a slight drop in RNase L activation ability, viz., the 2-fold and 4-fold diminution, respectively. Under similar conditions, the inosinesubstituted analogues p5'I2'p5'A2'p5'A and p5'A2'p5'-A2'p5'I were found to be a 31-fold and 142-fold less efficient in RNase L activation ability. Again, an analogue  $p5'A2'p5'(c^1A)2'p5'A$ , in which the central adenosine residue was replaced by c<sup>1</sup>A, retained activity equivalent to 2-5A itself. Thus, the exocyclic amino group of the 2'-terminal adenosine A3 is critical for RNase L activation.<sup>7</sup>

In order to gain further insight into the role of the adenine nitrogen atoms in binding to and activation of RNase L, we have synthesized 2-5A analogues 1-3 with consecutive replacement of adenosine residues with 3-deazaadenosine ( $c^{3}A$ ; 4) and evaluated their ability to bind to and activate human recombinant RNase L (EC 3.1.2.6).

## 2. Results and discussion

## 2.1. Synthesis of 3-deazaadenosine oligonucleotides 20–22 and their 5'-monophosphates 1–3

They were synthesized essentially as described previously<sup>7,8</sup> by the use a phosphotriester methodology applying the adenosine and 3-deazaadenosine 2'-terminal and phosphodiester building blocks 12/13 and 9/11, respectively. It was previously shown that the 3'-Obenzovl protection in combination with 5'-O-monomethoxytritylation and the 2-(4-nitrophenyl)ethyl (NPE) group for phosphate protection is effective for (2',5')oligonucleotide synthesis.<sup>9–11</sup> By the analogy with the synthesis of 1-deazaadenosine analogues of 2-5A,<sup>7,8</sup> 2-(4-nitrophenyl)ethoxycarbonyl (NPEOC) group<sup>12</sup> was successfully employed for the protection of amino function of c<sup>3</sup>A. Transient protection protocol<sup>13</sup> of hydroxyl groups of c<sup>3</sup>A with TMS followed by the reaction with NPEOC chloride gave, after work-up and silica gel column chromatography, compound 5 in 68% yield. Treatment of the NPEOC derivative 5 with DBU in pyridine at room temperature for 2h resulted in complete deprotection affording  $c^{3}A$  as the only reaction product (Scheme 2).

The method of selective 3'-O-benzoylation<sup>9</sup> using freshly distilled benzoyl chloride (1.065 equiv) in acetonitrile in the presence of Et<sub>3</sub>N and DMAP was now carried out on compound **6** to give the respective 3'-O-benzoylated derivative **8** in a yield of 60%, along with 2',3'-di-Obenzoylated by-product **7** (13%). The former was transformed to the corresponding phosphotriester **10**, and then to the phosphodiester **11** under conventional conditions.<sup>10</sup> Benzoylation of **6** and subsequent detritylation gave the 2'-terminal c<sup>3</sup>A building block **9**. The synthesis of analogous adenosine building blocks **12** and **13** was previously described.<sup>10</sup> The assembly of the trimers was performed by condensing [TPS-Cl/N-methylimidazole, molar ratio 1:3, as an activating agent; CHCl<sub>3</sub> as a solvent (cf., e.g., Ref. 8)] the monomeric building blocks in different successions and combinations in order to synthesize the 5'-detritylated dimers 14-16. These latter dimers were condensed with the phosphodiesters 11 or 13 followed by detritylation to afford the partially blocked trimers 17-19, which were used for the synthesis of the core c<sup>3</sup>A oligonucleotides 20-22 as well as their 5'-monophosphates 1–3. Deprotection of 17–19 was performed by the treatment at room temperature with DBU in pyridine for 2h followed by saturated methanolic ammonia for 20 h to give, after DEAE-Sephadex A-25 (HCO<sub>3</sub><sup>-</sup>-form) chromatography, the corresponding trimers 20-22 in 44–52% overall yield (see Section 4 and Table 1). On the other hand, the treatment of the 5'-detritylated trimers 17–19 with pyrophosphoryl chloride in ethyl acetate<sup>9,14</sup> followed by deprotection and purification as just described for the core trimers afforded the desired 5'monophosphates 1-3 as amorphous Na<sup>+</sup> salts in 35-49% overall yield (see Section 4 and Table 1) (Scheme 3).

### 2.2. Conformational studies

The main goal of these studies was to give further insight into the role of structural and/or stereochemical factors in defining the biochemical properties of (2'-5')oligoadenylates. The conformation of the core trimers **20–22** in aqueous solution was studied by <sup>1</sup>H and <sup>13</sup>C



Table 1. Isolated yields, hypochromicity, and HPLC data of the 3-deazaadenosine core analogues of  $(2',5')A_3$  20–22 and their 5'-monophosphates 1–3

Compd	Isolated yield [purity <sup>a</sup> ] (%)	Hypochromicity <sup>b</sup> (%)	Retention time (min)
$ApAp(c^{3}A)$ (20)	48 [86.1]	25.0	5.72
$Ap(c^{3}A)pA$ (21)	52 [88.4]	21.0	5.35
(c <sup>3</sup> A)pApA ( <b>22</b> )	44 [86.6]	14.5	5.38
$p(c^{3}A)pApA$ (1)	40 [95.7]		8.97
$pAp(c^{3}A)pA(2)$	35 [93.5]		11.28
$pApAp(c^{3}A)$ (3)	49 [69.4]	_	12.08

<sup>a</sup> According to HPLC data for compounds after chromatography (see Section 4); all biochemical and spectral experiments have been performed with compounds that were purified to homogeneity by reverse phase HPLC (purity ≥97%).

<sup>b</sup> Hypochromicity was determined as described previously.<sup>8</sup>



#### Scheme 3.

NMR spectroscopy (Tables 2–5) and CD spectroscopy (Fig. 1). The assignment of all NMR resonances was made by  $2D [^{1}H, ^{1}H]$  and  $[^{1}H, ^{13}C]$  correlation spectra.

The assignment of all the <sup>1</sup>H and <sup>13</sup>C resonances of c<sup>3</sup>A is straightforward. The conformational analysis of the furanose rings of compounds described was performed with the aid of the PSEUROT (version 6.3) program, which calculates the best fits of three <sup>3</sup>*J*<sub>HH</sub> experimental coupling constants [<sup>3</sup>*J*<sub>1',2'</sub>, <sup>3</sup>*J*<sub>2',3'</sub>, and <sup>3</sup>*J*<sub>3',4'</sub>] to the five conformational parameters (phase angles *P* and puckering amplitudes  $\psi_m$  for both the *N*- and the *S*-type conformers and the corresponding molar ratio).<sup>15,16</sup> Furanose ring of c<sup>3</sup>A is in the  $S \leftrightarrow N$  pseudorotational equilibrium with a slight preference for the former [*P*<sub>S</sub> 122.3°, E<sub>1</sub> (C1'-*exo*) conformation]. The <sup>3</sup>*J*<sub>C4,H1'</sub> value of

2.5–3.7 Hz along with the  ${}^{3}J_{C8,H1'} = 3.9$  Hz clearly point to the practically free rotation of the base about the glycosyl bond.<sup>17–20</sup>

There is a close stereochemical similarity between the ribofuranose moieties of adenosine residues in the molecule of natural trimer,  $(2',5')A_3$ ,<sup>21</sup> and analogues, containing c<sup>3</sup>A in the middle position of the chain, A2'p5' (c<sup>3</sup>A)2'p5'A, and at the 2'-terminus, A2'p5'A2'p5'(c<sup>3</sup>A) (Tables 2–5); data for adenosine residues of (c<sup>3</sup>A)2'p5'A2' p5'A are not available owing to the overlap of the relevant <sup>1</sup>H resonances. The sugar rings of all nucleoside residues within the trimers appear to be rather flexible (Table 5). The PSEUROT analyses of the  $S \leftrightarrow N$  pseudorotational equilibrium of the furanose ring of c<sup>3</sup>A fragment within A2'p5'A2'p5'(c<sup>3</sup>A) and the

Compd	Residue	Chemical shifts								
		H-8	H-2	H-3	H-1′	H-2′	H-3′	H-4′	H-5′	H-5″
c <sup>3</sup> A	_	8.33 s	7.75 d	7.04 d	6.00 d	4.63 t	4.40 dd	4.47 m	3.94 dd	3.87 dd
20	A1 A2 c <sup>3</sup> A	8.27 8.14 8.00 $(\Delta \delta = 0.33)$	7.75 s 7.99 s 7.32 d $(\Delta \delta = 0.43)$	${7.00 \text{ d}}$ $(\Delta \delta = 0.04)$	6.11 d 5.97 d 5.84 d	5.07 dt 4.77 m 4.29 t	4.62 t 4.60 t 4.37 dd	4.20 m 4.18 4.28	3.73 dd 3.88 ddd 4.25 m	3.85 dd 4.09 ddd 4.16 ddd
21	A1 c <sup>3</sup> A A3	8.11 7.99 $(\Delta \delta = 0.34)$ 8.08	7.65 s 7.22 d ( $\Delta \delta = 0.53$ ) 8.09 s	$\frac{1}{6.88} \text{ d}$ $(\Delta \delta = 0.16)$	6.04 d 5.90 d 5.84 d	5.00 dt 4.44 ddd 4.48 t	4.55 t 4.33 t 4.32 t	4.20 m 4.07 m 4.22 m	3.68 dd 4.05 m 4.05 m	3.78 dd 3.90 m 4.05 m
22	c <sup>3</sup> A A2 A3	8.10 ( $\Delta \delta = 0.23$ ) 8.00 7.91	7.26 d ( $\Delta \delta = 0.49$ ) 7.74 s 8.26 s	6.91 d ( $\Delta \delta = 0.13$ )	5.95 d 5.98 d 5.74 d	4.98 ddd 4.58 ddd 4.26	4.62 dd 4.28 m 4.15	4.20 m 4.20 m 4.10	3.84 dd n.d. n.d.	3.71 dd n.d. n.d.

**Table 2.** Proton chemical shifts ( $\delta_{TMS}$ , ppm) of 3-deazaadenosine ( $c^{3}A$ ) and analogues of (2-5)A<sub>3</sub> containing  $c^{3}A$  at different positions of the oligonucleotide chain (D<sub>2</sub>O) (purine numbering)<sup>a</sup>

<sup>a</sup> The chemical shifts of the  $\beta$ -D-ribofuranose moieties of adenosine as well as c<sup>3</sup>A residues are in good agreement with those for the corresponding adenosine residue of (2',5')A<sub>3</sub>;<sup>21</sup>  $\Delta \delta = \delta c^3 A$ (free)  $- \delta c^3 A$ (within trimer); n.d.—not determined.

Table 3. Selected coupling constants  $(J, Hz)^a$ 

Compd	Residue	Coupling constants				
		1',2'	2',3'	3',4'	${}^{3}J_{31P,H}$	
c <sup>3</sup> A	_	5.80	5.66	4.29	_	
20	A1 A2 c <sup>3</sup> A	4.42 4.35 6.44	4.95 5.17 5.48	4.95 5.17 3.37	9.01 (P,H-2') 9.0 (P,H-2') ${}^{3}J_{P,H5'}$ 3.84 ${}^{3}J_{P,H5''}$ 3.0 ${}^{3}J_{P,H5''}$ 3.0 ${}^{3}J_{P,H5''}$ 3.78	
21	A1 c <sup>3</sup> A A3	5.07 2.94 5.04	5.10 5.11 5.11	4.66 6.02 4.79	9.06 (P,H-2') 8.03 (P,H-2') <sup>3</sup> J <sub>P,H5'</sub> and <sup>3</sup> J <sub>P,H5''</sub> ca. 3.0	
22	c <sup>3</sup> A A2 A3	3.15 2.33 2.96	5.15 4.77 n.d.	6.00 n.d. n.d.	8.71 (P,H-2') 7.09 (P,H-2') <sup>b</sup>	

<sup>a</sup> The  ${}^{3}J_{2,3}$  values of c<sup>3</sup>A and c<sup>3</sup>A residue within the trimers **20–22** were found to be 5.97, 7.04, 6.99, and 7.05 Hz, respectively.

<sup>b</sup> The  ${}^{3}J_{P,H5'}$  and  ${}^{3}J_{P,H5''}$  were not determined owing to the overlap of the H-5' and H-5'' resonances.

monomeric nucleoside,  $c^{3}A$ , manifest close similarity. On the contrary, the stereochemical behavior of the furanose ring of  $c^{3}A$  residue within  $(c^{3}A)2'p5'A2'p5'A$ and  $A2'p5'(c^{3}A)2'p5'A$  trimers substantially differs from that of the nucleoside itself as well as of adenosine, thereby the pseudorotational parameters of  $c^{3}A$  residue within these trimers are practically identical. Of interest is an observation of a very impressive upfield shift of the H-2 and C-2 resonances of  $c^{3}A$  residues of the trimers in comparison with the monomeric  $c^{3}A$  molecule (Tables 2 and 4). Such remarkable upfield shifts characterize strong stacking interaction and manifest the profound influence of neighboring adenine base onto this fragment of  $c^{3}A$  molecule.

In the  $A2'p5'A2'p5'(c^3A)$  trimer, the available NMR data supply reliable information regarding the spatial

arrangement of this molecule. Stereochemistry of the 3deazaadenosine residue within the trimer is very similar to that of monomeric nucleoside c<sup>3</sup>A as well as of the relevant adenosine residue of the natural trimer  $(2',5')A_3$ (Tables 4 and 5). However, some interesting features are connected with this residue. Thus, a reversed order of the H-2' and H-3', and C-2' and C-3' chemical shifts compared to the parent monomeric c<sup>3</sup>A can be noticed. This fact may be explained by the predominant syn conformation of the c<sup>3</sup>A residue within the trimer. This suggestion is corroborated by the relationship  ${}^{3}J_{C8,H1'} < {}^{3}J_{C4,H1'}$  (Table 4), which points to the predominant population of the *syn* base orientation about the glycosyl bond.<sup>17–20</sup> Moreover, the large upfield shift (3.6 ppm) of the C-2' resonance of  $c^{3}A$  residue of  $A2'p5' A2'p5'(c^{3}A)$ versus the nucleoside c<sup>3</sup>A give further support to a preferential syn orientation of the base (cf. Ref. 17). The extensive <sup>13</sup>C NMR studies by Uesugi and Ikehara of various (bromo, methyl, chloro, thiomethyl, and methoxy) 8-substituted purine nucleosides led to conclusion that such an upfield shift of C-2' resonance compared to those of the parent nucleosides is characteristic for a syn conformation about the glycosyl bond.<sup>22</sup> It is noteworthy that conformational studies of different analogues of 2-5A have led to the hypothesis that the syn base orientation about the glycosyl bond at the 2'-terminus resulted in an enhancement of activating activity for RNase L.23 In the present work we have found that  $p5'A2'p5'A2'p5'(c^3A)$ retained activity equivalent to 2-5A itself (see below).

The NMR data available clearly point to a close stereochemical resemblance of adenosine residues of  $A2'p5'(c^3A)2'p5'A$  to the natural trimer  $(2',5')A_3$ , but rather essential deviations of the ribofuranose ring conformation of  $c^3A$  residue from that of the monomeric nucleoside  $c^3A$  and the middle adenosine residue as well (Tables 2–5) (cf. Ref. 21). The position of the C-2' and C-3' resonances of  $c^3A$  residue within  $A2'p5'(c^3A)2'p5'A$  molecule versus that of  $c^3A$  itself allow to suggest the *anti* base orientation about the glycosyl bond of the middle nucleoside.

**Table 4.** Chemical shifts ( $\delta_{TMS}$ , ppm) of 3-deazaadenosine (c<sup>3</sup>A) and analogues of (2-5)A<sub>3</sub> containing c<sup>3</sup>A at different positions of the oligonucleotide chain (D<sub>2</sub>O) (purine numbering) [some <sup>1</sup>J<sub>C,H</sub> and <sup>3</sup>J<sub>C,H</sub> values are given in brackets]

Compd	Residue	Chemical shift										
		C-6 <sup>a</sup>	C-4	C-2	C-3	C-8 $[{}^{1}J_{C,H}$ and ${}^{3}J_{C8,H1'}]$	C-5 <sup>b</sup>	$C-1' \\ \{{}^{3}J_{C,P}\}$	$\begin{array}{c} \text{C-2'} \\ \{^2 J_{\text{C,P}}\} \end{array}$	$C-3' \{{}^3J_{C,P}\}$	$C-4' \\ \{{}^{3}J_{C,P}\}$	$C-5' \{{}^2J_{C,P}\}$
c <sup>3</sup> A	—	151.3	138.3 <sup>c</sup>	140.3 [179.0] <sup>d</sup>	99.1 [171.3] <sup>e</sup>	141.0 [213.0 and 3.9]	126.6	88.8 [165.2]	73.7 [149.0]	70.0 [152.0]	85.0 [150.3]	61.1 [142.7]
20	$A1^{\rm f}$	155.0	147.9	152.0 [202.8]	_	143.6 [214.0 and 2–3]	118.7	87.5 {6.3}	77.1 {4–5}	69.6	85.0	60.9
	$A2^{f}$	154.5	147.8	152.6 [203.0]		140.4 [212.4 and 4.0]	117.4	86.1 {8.3}	78.4 {4–5}	69.4	82.5 {9.4}	64.1 {4–5}
	c <sup>3</sup> A	148.1	138.3 <sup>g</sup>	129.4 ( $\Delta \delta = 10.9$ )	99.6	138.8 [214.6 and 4.6]	126.3	89.6	70.1	73.7	84.0 {9.7}	64.5 {4–5}
21	$A1^{\rm f}$	155.2	147.8 <sup>h</sup>	152.0	_	142.7	118.8	87.6 {4.9}	77.1 {5.0}	70.3	85.5	61.2
	c <sup>3</sup> A	148.5	137.8	129.6 ( $\Delta \delta = 10.7$ )	99.3	139.1	125.6	88.9 {6.7}	77.5 {5.3}	67.7 {2–3}	81.7 {9.6}	63.0 {4.3}
	A3	155.0	147.6 <sup>h</sup>	152.8		141.1	118.0	87.1	74.1	69.8	83.0 {8.9}	64.6 {4.8}
22	c <sup>3</sup> A	147.9	138.4	129.5 ( $\Delta \delta = 10.8$ )	99.2	138.6	123.6	88.0 {34}	77.8 <sup>h</sup> {5.6}	69.1 {2–3}	83.7	59.8
	$A2^{f}$	155.2	147.9 <sup>h</sup>	152.7		139.2	118.2	86.2 {5–6}	77.5 <sup>h</sup> {5.1}	68.5 {5.0}	82.5 {9.2}	64.0 {4–5}
	A3	154.2	147.7 <sup>h</sup>	152.3		142.2	117.2	87.6	74.8	69.2	81.7 {9.4}	63.7 {2–3}

<sup>a</sup> The  ${}^{3}J_{C6,H2}$  value was found to be ca. 11 and 9 Hz in the case of adenine and c<sup>3</sup>A residues, respectively.

<sup>b</sup> The  ${}^{3}J_{C5,H8}$  and  ${}^{3}J_{C5,H3}$  values of c<sup>3</sup>A were found to be 11–12 and 5–6 Hz, respectively; the  ${}^{3}J_{C5,H8}$  value of adenosine was ca. 11 Hz.

<sup>c</sup> The C-4 resonance appeared as a multiplet with the following <sup>13</sup>C–<sup>1</sup>H couplings: 9.7 Hz ( ${}^{3}J_{C4,H8}$ ), 2.5–3.7 Hz ( ${}^{3}J_{C4,H1'}$ ), and 5.6–6.6 Hz ( ${}^{3}J_{C4,H2}$ ); note that the  ${}^{3}J_{C4,H1'}$  value along with the  ${}^{3}J_{C8,H1'} = 3.9$  Hz clearly point to the practically free rotation of the base about the glycosyl bond.<sup>17–20</sup> The  ${}^{2}J_{C4,H3}$  is less than 2.0 Hz.

<sup>d</sup> The  ${}^{2}J_{C2,H3}$  value is less than 2.0 Hz. An  $\Delta\delta$  is a difference between the C-2 chemical shift of  $c^{3}A$  and  $c^{3}A$  residue within the trimer.

<sup>e</sup> The  ${}^{2}J_{C3,H2}$  was found to be 7.8 Hz in the case of c<sup>3</sup>A and 4.1 Hz for c<sup>3</sup>A residue of ApAp(c<sup>3</sup>A).

<sup>f</sup>The chemical shifts and coupling constants are in fair agreement with those for the corresponding adenosine residue of (2',5')ApAp[8-(4-aminobutyl)amino]adenosine.<sup>17</sup>

<sup>g</sup> The C-4 resonance appeared as a multiplet with the following  ${}^{13}C-{}^{1}H$  couplings: 10.9 Hz ( ${}^{3}J_{C4,H8}$ ) and ca. 6.0 Hz ( ${}^{3}J_{C4,H1'}$  and  ${}^{3}J_{C4,H2}$ ); note that the

 ${}^{3}J_{C4,H1'}$  of ca. 6 Hz along with the  ${}^{3}J_{C8,H1'} = 4.6$  Hz imply the predominant syn base orientation about the glycosidic bond.

<sup>h</sup> The data may be interconvertible.

Table 5. Pseudorotational parameters of the  $\beta$ -p-ribofuranose moieties of 3-deazaadenosine (c<sup>3</sup>A) and analogues of (2-5)A<sub>3</sub> containing c<sup>3</sup>A at different positions of the oligonucleotide chain in D<sub>2</sub>O solutions<sup>a</sup>

Compd	Residue	$P_N$	$\psi_{\mathrm{m}(N)}$	$P_S$	$\psi_{\mathrm{m}(S)}$	% <i>S</i>	
c <sup>3</sup> A	_	-34.3 ( <sup>1</sup> T <sub>2</sub> )	<u>36</u>	122.3 (E <sub>1</sub> )	<u>40</u>	60	
20	$\begin{array}{c} A1^{b} \\ A2^{b} \\ c^{3}A^{b} \end{array}$	-18.1 (E <sub>2</sub> ) -21.8 (E <sub>2</sub> ) -39.5 ( <sup>1</sup> T <sub>2</sub> )	$\frac{38}{38}$ $\frac{36}{36}$	137.0 ( <sub>1</sub> T <sup>2</sup> ) 127.1 (E <sub>1</sub> ) 130.9 (E <sub>1</sub> )	$\frac{38}{38}$ $\frac{38}{43}$	43 43 65	
21	A1 <sup>b</sup> c <sup>3</sup> A <sup>c</sup> A3 <sup>b</sup>	-19.4 (E <sub>2</sub> ) 35.3 ( <sup>3</sup> T <sub>4</sub> ) -18.0 (E <sub>2</sub> )	$\frac{\underline{38}}{\underline{38}}$	133.6 ( <sub>1</sub> T <sup>2</sup> ) 214.8 ( <sup>4</sup> T <sub>3</sub> ) 132.3 ( <sub>1</sub> T <sup>2</sup> )	$\frac{\underline{38}}{\underline{38}}$ $\underline{\underline{38}}$	51 38 51	
22	$c^{3}A^{c}$ A2 A3	38.2 ( <sup>3</sup> T <sub>4</sub> ) n.a. n.a.	<u>38</u>	212.7 ( <sup>4</sup> T <sub>3</sub> )	<u>38</u>	38	

<sup>a</sup> The conformational analysis of the furanose rings of compounds studied was performed by the PSEUROT (version 6.3) program. The underlined values were fixed during the final calculations; the rms deviations and  $|\Delta J_{max}|$  data were found to be 0.00 for all calculated couplings. n.a.—not analyzed owing to the impossibility to measure the necessary H–H coupling constants.

<sup>b</sup> Pseudorotational parameters are in fair agreement with those for the corresponding adenosine residue of  $(2',5')A_3$ .<sup>21</sup>

<sup>c</sup> Pseudorotational parameters display rather essential deviations from those of both the parent  $c^{3}A$  and the corresponding adenosine residue of  $(2',5')A_{3}$ .<sup>21</sup>

The NMR spectral data for  $(c^{3}A)2'p5'A2'p5'A$  trimer are rather scanty, which precludes an elucidation of the

spatial arrangement of this molecule. Nonetheless, the *anti* base orientation about the glycosyl bond of  $c^3A$ 



**Figure 1.** CD spectra of 3-deazaadenosine-substituted analogues of the core trimer A2'p5'A2'p5'A in water at 20 °C.

residue can be suggested taking into account the C-2' and C3' resonances. One common characteristic of trimers **20–21** is a similarity of stereochemistry of the sugar phosphate backbones that can be seen from the  ${}^{3}J_{C1',P}$ ,  ${}^{3}J_{C3',P}$ ,  ${}^{3}J_{C4',P}$ , and  ${}^{3}J_{H2',P}$  values as well.

The CD spectra of trimers **20** and **22** are similar in shape to that of the natural trimer,  $(2',5')A_3$ ,<sup>24,25</sup> albeit display essential differences in amplitudes of the Cotton effects (Fig. 1). Unexpectedly, the CD spectrum of  $A2'p5'(c^3A)2'p5'A$  trimer containing  $c^3A$  in the middle position of the chain shows a mirror-like shape and the long-wave Cotton effect is missing at all. Such CD behavior of the latter points to essential differences in its spatial arrangement compared to two other trimers, which is in agreement with the biochemical data (see below). It is noteworthy that the hypochromism data display remarkable diversity depending on the point of modification of  $(2',5')A_3$  (Table 1), thereby A2'p5'(c<sup>3</sup>A)-2'p5'A trimer occupies the mid-position. It should, however, be stressed that our previous attempts to find out the possible correlations between the NMR, CD, and hypochromicity have failed (cf. Ref. 24).

### 2.3. Biological studies

Although mouse RNase L was first purified to homogeneity,<sup>26</sup> it was the human enzyme, after expression of the cloned enzyme in SF21 cells infected with recombinant baculovirus<sup>27</sup> that was first prepared in sufficient quantity for meaningful enzymological studies. Enzyme used in this study was purified according to such published methods.

Methods of assay of RNase L activity have relied upon cleavage of radiolabeled  $poly(U)^{26}$  or on cleavage of ribosomal RNA (see Ref. 28). A method that would utilize a synthetic RNA of defined length in which cleavages would yield products of discrete lengths has been developed by Carroll et al.<sup>29</sup> Since it was demonstrated earlier that RNase L cleaves preferentially after UU and UA sequences, the sequence chosen for analysis of RNase L was 5'-[<sup>32</sup>P]pC<sub>11</sub>UUC<sub>7</sub>. Detailed procedures have been published for these assays.<sup>27</sup>



Figure 2. RNase L activating ability of analogues of 2-5A. 2-5A and analogues at different concentrations (as indicated) were incubated with human recombinant RNase L on ice for 30 min followed by incubation with  $C_7UUC_{12}$ -[<sup>32</sup>P]pCp at 30 °C for 30 min. An autoradiogram of the dried gel is shown (from left to right): lane 1—control with no activator present; lanes 2–6, 1000–0.1 nM (2',5')pA<sub>4</sub>, respectively; lanes 7–10, 1000–1 nM p(c<sup>3</sup>A)pApA, respectively; lanes 11–14, 1000–1 nM pAp(c<sup>3</sup>A)pA, respectively; lanes 15–18, 1000–1 nM pAp(c<sup>3</sup>A), respectively. Arrows indicate the intact and cleaved RNA,  $C_{12}$ [<sup>32</sup>P]pCp. The graph represents percent RNA cleavage as determined by phosphorimager analysis.

According to the results of Figure 2, parent 2-5A tetramer, p5'A2'p5'A2'p5'A2'p5'A, was able to effect a 50% cleavage of  $pC_7UUC_{12}pCp$  at a concentration of 1 nM. It may be noted that multiple earlier studies have shown that both 2-5A trimer, p5'A2'p5'A2'p5'A and p5'A2'p5'A2'p5'A2'p5'A, are close in bioactivity. Similarly, the trinucleotides, p5'(c<sup>3</sup>A)2'p5'A2'p5'A and  $p5'A2'p5'A2'p5'(c^{3}A)$  were of quite similar potency to parent 2-5A tetramer, effecting substrate cleavages of 60% and 50% at 1 nM, respectively. In distinct contracts, the trimeric congener,  $p5'A2'p5'(c^3A)2'p5'A$  caused only 20% cleavage at 1 nM and 80% cleavage at 10 nM. Thus, this analogue, in which the middle adenosine had been altered to c<sup>3</sup>A, was at least 10-fold less effective than either the 5'- or 2'-terminally modified analogues, thereby testifying to a special influence of this middle 3deazaadenosine nucleoside on the bioactivity of 2-5A.

#### 3. Conclusions

Sequence-specific 3-deazaadenosine-substituted analogues of trimeric 2',5'-oligoadenylate, p5'A2'p5'A2'p5'A, and the respective 5'-dephosphorylated, core trimers were synthesized employing the phosphotriester methodology.

The extensive conformational analysis of the  $c^{3}A$ substituted core trimers versus the parent natural trimer by the <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy and CD displayed close stereochemical similarity between the natural core trimer and  $(c^{3}A)2'p5'A2'p5'A$  and  $A2'p5'A2'p5'(c^{3}A)$ analogues. The NMR data for the latter core trimer,  $A2'p5'A2'p5'(c^{3}A)$ , clearly point to the *syn* base orientation about the glycosyl bond of the  $c^{3}A$  residue. This observation is in a fair agreement with the hypothesis<sup>23</sup> that the *syn* base orientation of the A3 residue of 2-5A contributes to the activation of RNase L, on the one hand, and with the finding that  $p5'A2'p5'A2'p5'(c^3A)$  was nearly as active as the natural tetramer, on the other. An analogue  $A2'p5'(c^3A)2'p5'A$  displayed rather essential deviations from the spatial arrangement of the natural core trimer.

Substitution of either 5'-terminal or 2'-terminal adenosine with c<sup>3</sup>A afforded the respective analogues  $p5'(c^3A)2'p5'A2'p5'A$  and  $p5'A2'p5'A2'p5'(c^3A)$  that were as effective as the natural tetramer itself as activators of RNase L (EC<sub>50</sub> = 1 nM). In contrast,  $p5'A2'p5'(c^3A)2'p5'A$  showed diminished RNase L activation ability (EC<sub>50</sub> = 10 nM). This finding is in harmony with essential stereochemical differences between  $A2'p5'(c^3A)2'p5'A$  and the natural core (2',5')trimer, whereas specific recognition of the nitrogen-3 atom of the middle adenosine A2 seems to be unlikely.

## 4. Experimental

# 4.1. Synthesis of 3-deazaadenosine oligonucleotides 20–22 and their 5'-monophosphates 1–3

4.1.1. General. The UV spectra were recorded on a Specord UV-vis spectrophotometer (Carl Zeiss, Germany). <sup>1</sup>H and <sup>13</sup>C NMR spectra were measured at 200.13 MHz on an AC 200 (blocked derivatives of c<sup>3</sup>A 5-11) and at 500.13 and 125.76 MHz on an Avance 500 DRX (trimers 20–22) spectrometers (Bruker, Germany) with tetramethylsilane as an internal standard (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad signal); the chemical shifts ( $\delta$ ) and coupling constants (J) are given in ppm rel. to external TMS and Hz, respectively. Assignments of proton resonances were confirmed, when possible, by selective homonuclear decoupling experiments and [<sup>1</sup>H,<sup>1</sup>H] correlation spectra as well; assignments of proton and 13-carbon resonances have been proved by [<sup>1</sup>H,<sup>13</sup>C] correlation spectra. The CD spectra were recorded on Jasco J-20 spectropolarimeter (Jasco, Japan) in water solutions. Thin layer chromatography (TLC) was carried out on the 60F<sub>254</sub> silica gel plates (Merck, Germany). As solvent systems were used: CHCl<sub>3</sub>/MeOH, 9:1 (A), CHCl<sub>3</sub>/ MeOH, 4:1 (B), EtOAc/hexane, 4:1 (C), CHCl<sub>3</sub>/MeOH/ Et<sub>3</sub>N, 9:0.3:0.3 (D), *i*-PrOH/NH<sub>3</sub>/H<sub>2</sub>O, 12:1:2 (E). Column chromatography was performed on silica gel Merck 60 (0.040–0.063 mm). Melting points were determined with a Boethius (Germany) apparatus and are uncorrected. The solutions of compounds in organic solvents were dried with anhydrous sodium sulfate for 4 h. The reactions were performed at room temperature, unless stated otherwise. High-performance liquid chromatography (HPLC) was carried out with Waters apparatus with a column Nova-Pac C-18  $(3.9 \times 300 \text{ mm})$ using an isocratic gradient elution with the following buffer: 7% CH<sub>3</sub>CN in 0.05 M KH<sub>2</sub>PO<sub>4</sub>, v/v at a flow rate 0.7 mL/min.

4.1.2. 4-Amino-1-( $\beta$ -D-ribofuranosyl)-1*H*-imidazo[4,5c]pyridine [c<sup>3</sup>A; (4)]. Compound [c<sup>3</sup>A; (4)] was prepared according to Mizuno et al.<sup>30</sup> Yield 34%; mp 225–226 °C (EtOH); TLC (E):  $R_{\rm f}$  0.71; UV (H<sub>2</sub>O),  $\lambda_{\rm max}$ , nm ( $\varepsilon \times 10^{-3}$ ): (pH 1.0 and 7.0) 262 (10.8), (pH 13.0) 265 (10.7); CD (K/Na-phosphate buffer, pH 7.4),  $\lambda_{\rm max}$ , nm ( $\Theta \times 10^{-3}$ ): 218 (0), 220 (-6.97), 230–245 (0), 263 (-2.66), 290 (0).

4.1.3. 4-{[2-(4-Nitrophenyl)ethoxycarbonyl]amino}-N<sup>1</sup>-(β-D-ribofuranosyl)-1*H*-imidazo[4,5-c]pyridine (5) (cf. Ref. 8). Trimethylsilyl chloride (0.9 mL, 7.1 mmol) was added to a solution of 4 (0.25 g, 0.94 mmol) in anhyd pyridine (2.5 mL) and the reaction mixture was stirred for 1.5 h. 2-(4-nitrophenyl)ethylchloroformate Then.  $(1.03 \,\mathrm{g})$ 4.7 mmol) was added and stirring was continued overnight. The reaction mixture was cooled to 0 °C and cold water (1.64 mL) was added under stirring. After 10 min, concd ag ammonia (3.16 mL) was added and stirring was continued for 30 min. The reaction mixture was evaporated and the residue was purified by silica gel column chromatography (80 mL). Elution was performed with a linear MeOH gradient (0-50%, v/v, 1 L) in CHCl<sub>3</sub>. The fractions containing the product were collected, evaporated, and crystallized from EtOH to give 5 as colorless crystals (0.296 g, 68%); mp 112– 114 °C (EtOH); TLC (B):  $R_f$  0.23; UV (H<sub>2</sub>O),  $\lambda_{max}$ , nm  $(\varepsilon \times 10^{-3})$ : 263 (9.0);  $\lambda_{\min}$  232 (1.96); (EtOH),  $\lambda_{\max}$ 271 nm;  $\lambda_{min}$  232 nm. <sup>1</sup>H NMR (DMSO- $d_6$ ): 8.56 (s, 1H, H–C(8)), 8.08 (d, 1H,  $J_{2,3} = 5.5$ , H–C(2)), 7.66 (d, 1H, H-C(3)), 8.16 (d, 2H, J = 8.5; ortho-Ph-NO<sub>2</sub>), 7.60 (d, 2H, J = 8.5; meta-Ph-NO<sub>2</sub>), 5.90 (d, 1H,  $J_{1',2'} = 6.5$ ; H-C(1')), 5.56 (d, 1H,  ${}^{3}J_{2',2'-OH} = 4.5$ ; HO–C(2')), 5.31 (br d, 1H,  ${}^{3}J_{3',3'-OH} = 2.0$ ; HO–C(3')), 5.20 (br t, 1H,  ${}^{3}J_{5',5'-OH} = 2.5; \text{ HO-C}(5')), \approx 4.36 \text{ (m, 1H, H-C}(2')),$  $\approx 4.36$  [br t, 3H (+H-2'), J = 4.0;  $-CH_2-CH_2-PhNO_2$ ], 4.12 (br m, 1H,  $J_{3',4'} = 1.5$ ; H–C(3')), 4.00 (br m, 1H, H– C(4')), 3.67 (br m, 2H, H<sub>2</sub>–C(5'), 3.10 (t, 2H, J = 4.0;  $-CH_2-CH_2-PhNO_2$ ).

Anal. Calcd for  $C_{20}H_{21}N_5O_8$  (459.41): C, 52.29; H, 4.61. Found: C, 52.02; H, 4.62.

4.1.4. 4-{[2-(4-Nitrophenyl)ethoxycarbonyl]amino}-N<sup>1</sup>-[5-O-(4-monomethoxytrityl]-(β-D-ribofuranosyl)-1H-imidazo-[4,5-c]pyridine (6). A solution of compound 5 (70 mg, 0.15 mmol) and 4-monomethoxytrityl chloride (80 mg, 0.25 mmol) in anhyd pyridine (0.5 mL) was stirred for 24 h. The reaction mixture was evaporated, co-evaporated with MeOH (2×10 mL), and the residue was purified by silica gel column chromatography (30 mL). Elution was performed with a linear MeOH gradient (0-10%, v/v, 0.5 L) in CHCl<sub>3</sub>. The fractions containing the product were collected, evaporated. The residue was dissolved in EtOAc (1 mL) and precipitated in hexane (50 mL). The resulting precipitate was collected by filtration and dried in vacuo to give 6 as colorless crystals (80 mg, 73%); mp 136–138 °C; TLC (A):  $R_{\rm f}$  0.33. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.16 (s, 1H, H–C(8)), 8.0– 7.16 (m, 18H,  $2 \times C_6 H_5$  the ortho- and meta-Ph-NO<sub>2</sub>, the ortho protons of MTr group, H-C(2) and H-C(3)), 6.72 (d, 2H,  ${}^{3}J_{meta,ortho} = 8.5$ ; the *meta* protons of MTr group), 5.80 (d, 1H,  $J_{1',2'} = 6.0$ ; H–C(1')), 4.59 (dd, 1H,

 $J_{2',3'} = 3.0; \text{H-C}(2')), \approx 4.50 \text{ (br t, 2H, } J \approx 6.0; -CH_2-CH_2-PhNO_2), 4.52 \text{ (dd, 1H, } J_{3',4'} = 2.5; \text{H-C}(3')), 4.31 \text{ (m, 1H, } J_{4',5'} = J_{4',5''} = 1.5; \text{H-C}(4')), 3.75 \text{ (s, 3H, OCH_3), 3.48 (d, 2H, H_2-C(5')), 3.10 (t, 2H, J = 6.0; -CH_2-CH_2-PhNO_2).}$ 

Anal. Calcd for  $C_{40}H_{37}N_5O_9$  (731.75): C, 65.65; H, 5.10. Found: C, 65.92; H, 5.42.

4.1.5. Benzoylation of 4-{[2-(4-nitrophenyl)ethoxycarbonyl]amino}- $N^1$ -[5-O-(4-monomethoxytrityl]-( $\beta$ -D-ribofuranosyl)-1H-imidazo[4,5-c]pyridine (6) with benzoyl chloride.<sup>8,9,17</sup> To the stirred solution of compound 6 (116 mg, 0.16 mmol) in a mixture of anhyd CH<sub>3</sub>CN (2.1 mL), Et<sub>3</sub>N (0.28 mL), and 4-dimethylaminopyridine (DMAP) (1.4 mg), freshly distilled BzCl (0.02 mL, 24 mg, 0.17 mmol) was added. After stirring for 1 h, the reaction mixture was evaporated, co-evaporated with MeOH (2×20 mL), and the products were purified by silica gel column chromatography (50 mL). Elution was performed with a linear EtOAc gradient (20–80%, v/v, 1 L) in hexane. In order of elution were isolated:

4.1.6. 4-{[2-(4-Nitrophenyl)ethoxycarbonyl]amino}- $N^{1}$ -[(2,3-di-O-benzoyl)-5-O-(4-monomethoxytrityl]-(β-D-ribofuranosyl)-1H-imidazo[4,5-c]pyridine (7). Compound 7 was dissolved, after evaporation of combined fractions, in EtOAc (1 mL) and precipitated from hexane (50 mL) to give a solid (20 mg, 13%); mp 110–112 °C; TLC (C):  $R_{\rm f}$  0.29; UV (EtOH),  $\lambda_{\rm max}$  271 and 231;  $\lambda_{\rm min}$  251 and 225; <sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.16 (s, 1H, H–C(8)), 8.18 [d, 3H, (ortho-Ph-NO<sub>2</sub> and H-8),  $J \approx 7.5$ ], 8.04 and 7.92 (2d, 5H,  $J \approx 7.5$ ; the ortho protons of Bz groups and H-2),  $\approx$ 7.60 (d, 1H,  $J_{2,3} \approx$  6.0; H–C(3)), 7.54–7.16 (m, 21H,  $2 \times C_6 H_5$ , meta-Ph-NO<sub>2</sub> and the ortho protons of Bz groups), 6.82 (d, 2H,  ${}^{3}J_{meta,ortho} = 8.5$ ; the meta protons of MTr group, the meta and para protons of Bz groups), 6.29 (d, 1H,  $J_{1',2'} = 6.0$ ; H–C(1')), 6.19 (br t, 1H,  $J_{2',3'} = 6.0; H-C(2')), 6.03$  (br t, 1H,  $J_{3',4'} \approx 3.0; H-C(2')$ ) C(3')), 4.50 (br t, 2H, J = 6.25;  $-CH_2-CH_2-PhNO_2$ ), 4.60 (br m, 1H, H–C(4')), 3.77 (s, 3H, OCH<sub>3</sub>),  $\approx$ 3.66 (center of m, 2H, H–C(5')), 3.13 (t, 2H, J = 6.25; –CH<sub>2</sub>–  $CH_2$ –PhNO<sub>2</sub>).

Anal. Calcd for  $C_{54}H_{45}N_5O_{11}$  (939.96): C, 69.00; H, 4.83. Found: C, 69.22; H, 5.12.

4.1.7. 4-{[2-(4-Nitrophenyl)ethoxycarbonyl]amino}- $N^{1}$ -[(3-O-benzoyl)-5-O-(4-monomethoxytrityl]-(β-D-ribofuranosyl)-1H-imidazo[4,5-c]pyridine (8). Compound 8 was dissolved, after evaporation of combined fractions, in EtOAc (1 mL) and precipitated from hexane (50 mL) to give a solid (80 mg, 60%); mp 110–112 °C; TLC (C):  $R_{\rm f}$ 0.14; UV (EtOH),  $\lambda_{\rm max}$  270;  $\lambda_{\rm min}$  249. <sup>1</sup>H NMR (CDCl<sub>3</sub>): 8.20–8.00 (m, 6H, *ortho*-Ph–NO<sub>2</sub>, the *ortho* protons of Bz group, H–C(8) and H–C(2)), 7.73 (d, 1H, J = 5.5; H– C(3)), 7.60–7.14 (m, 17H, 2×C<sub>6</sub>H<sub>5</sub>, *meta*-Ph–NO<sub>2</sub>, the *ortho* protons of MTr group and Bz group, the *meta*-Ph–NO<sub>2</sub>, the *meta* and *para* protons of Bz group), 6.77 (d, 2H, <sup>3</sup>J<sub>meta.ortho</sub> = 8.5; the *meta* protons of MTr group), 5.94 (d, 1H,  $J_{1',2'} = 6.0$ ; H–C(1')), 4.98 (br t, 1H,  $J_{2',3'} = 4.0$ ; H–C(2')), 5.72 (br t, 1H,  $J_{3',4'} = 1.5$ ; H–C(3')), 4.51 (br m, 1H,  ${}^{3}J_{4',5'} = {}^{3}J_{4',5''} = 2.0$ ; H–C(4')), 4.38 (br t, 2H, J = 4.25; –CH<sub>2</sub>–CH<sub>2</sub>–PhNO<sub>2</sub>), 3.74 (s, 3H, OCH<sub>3</sub>),  $\approx 3.56$  (center of m, 2H,  ${}^{gem}J_{5',5''} = 10.0$ ; H–C(5')), 3.04 (t, 2H, J = 4.25; –CH<sub>2</sub>–CH<sub>2</sub>–PhNO<sub>2</sub>).

Anal. Calcd for  $C_{47}H_{41}N_5O_{10}$  (835.86): C, 67.54; H, 4.94. Found: C, 67.91; H, 4.62.

 $4-\{[2-(4-Nitrophenyl)ethoxycarbonyl]amino\}-N^{1}-$ 4.1.8. [(2,3-di-O-benzoyl)-(β-D-ribofuranosyl)]-1H-imidazo[4,5-c]pyridine 9. Compound 9 was prepared by standard detritylation<sup>12</sup> of 7 (94 mg, 0.1 mmol) and isolated as an amorphous powder; yield 60 mg (90%); mp 103-104 °C; TLC (A): R<sub>f</sub> 0.45. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): 9.74 (br s, 1H, H-N(6)), 8.68 (s, 1H, H-C(8)), 8.12 (m, 3H, ortho-Ph-NO<sub>2</sub> and H–C(2)), 8.04 and 7.80 (2dd, 4H,  ${}^{3}J_{ortho.meta} =$ 7.5,  ${}^{3}J_{para,ortho} \approx 1.0$ ; the ortho protons of Bz groups), 7.76-7.32 (m, 9H, the meta and para protons of Bz groups, H-C(3) and meta-Ph-NO<sub>2</sub>), 6.64 (d, 1H,  $J_{1',2'} = 7.0; \text{H-C}(1')), 5.96 \text{ (dd, 1H, } J_{2',3'} = 5.0; \text{H-C}(2')),$ 5.84 (dd, 1H,  $J_{3',4'} = 1.5$ ; H–C(3')), 5.70 (t, 1H,  $J_{5',5'-OH} =$ 2.5; HO–C(5')), 4.56 (br m, 1H,  $J_{4',5'} = J_{4',5''} = 2.0$ ; H– C(4'), 4.34 (br t, 2H, J = 6.25;  $-CH_2-CH_2-PhNO_2$ ), 3.88 (center of m, 2H, H<sub>2</sub>–C(5')), 3.06 (t, 2H, J = 6.25;  $-CH_2-CH_2-PhNO_2$ ).

Anal. Calcd for  $C_{34}H_{29}N_5O_{10}$  (667.62): C, 61.17; H, 4.38. Found: C, 61.22; H, 4.63.

 $4-\{[2-(4-Nitrophenyl)ethoxycarbonyl]amino\}-N^1-$ 4.1.9. {2-O-[2-(4-nitrophenylethyl)-phosphato]-[3-O-benzoyl-5-O-(4-monomethoxytrityl)-β-D-ribofuranosyl]-1H-imidazo-[4,5-c]pyridine (11). Compound 11 was prepared as described earlier.<sup>9-12</sup> Phosphorylation of 8 (0.11 g,0.132 mmol) with 2-chlorophenyldi(triazolido)phosphate followed by the treatment with 2-(4-nitrophenyl)ethanol afforded the triester 10 as oil (0.112 g, 73%); TLC (C):  $R_f$  0.47. The latter (0.13 g, 0.11 mmol) was treated with *p*-nitrobenzaldoxime in a mixture of triethylamine/pyridine/water (1:1:1, vol) followed by workup to give the diester 11 (triethylammonium salt) as an amorphous powder (91 mg, 71%). TLC (D):  $R_{\rm f}$  0.27. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): 9.78 (br s, 1H, H–N(6)), 8.56 (s, 1H, H-C(8)), 8.10-7.18 (m, arom. H), 6.78 (d, 2H,  $J_{meta,ortho} = 7.5$ ; the meta protons of MTr group), 6.38 (d, 1H,  $J_{1',2'} = 7.5$ ; H–C(1')), 5.72 (br s, 1H, H–C(2')), 5.40 (br s, 1H, H-C(3')), 4.40 (br s, 1H, H-C(4')), 4.36 (br t, 1) $2H, J = 6.0; -CH_2 - CH_2 - PhNO_2), 3.06 (t, 2H, J = 6.0; CH_2-CH_2-PhNO_2$ ; resonances of the second -CH<sub>2</sub>-CH<sub>2</sub>-PhNO<sub>2</sub> group are overlapped by an intense resonance of OH group and DMSO-d<sub>5</sub>; resonances of  $H_2$ –C(5') are overlapped by an intense resonance of OH group.

4.1.10.  $N^6, N^6, O^{2'}, O^{3'}$ -Tetrabenzoyladenosine (12) and  $N^6, O^{3'}$ -dibenzoyl-5'-O-(4-monomethoxytrityl)-2'-O-[2-(4-nitrophenylethyl)-phosphato]adenosine (triethylammo-nium salt) (13). Compounds 12 and 13 have been prepared as described previously.<sup>9,10,17</sup>

4.1.11. Synthesis of dimers 14–16, trimers 17–19 and deprotected core (2',5')trimers 20–22. Synthesis of dimers (14–16) and then trimers (17–19) was performed according to the previously described methodology<sup>7–10,17</sup> consisting of (i) condensation of either phosphodiester 11 with 2'-terminal building block 12, or 13 with 9, or 12 with 13 and subsequent detritylation, and (ii) condensation of individual dimer obtained with phosphodiesters 11 or 13, and subsequent detritylation.

To a solution of the appropriate phosphodiester (0.11 mmol) and the 5'-terminal building block or dimer (0.1 mmol) in CHCl<sub>3</sub> (0.5 mL), 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl, 0.3 mmol) and Nmethylimidazole (0.9 mmol) were added and the reaction mixture was stirred for 20 min. The reaction mixture was poured into hexane (200 mL), the resulting precipitate was collected by filtration, dried in vacuo, and then dissolved in 2% solution of p-toluenesulfonic acid in CH<sub>2</sub>Cl<sub>2</sub>/MeOH (7:3, v/v, 15mL). After stirring for 10 min (in the case of 14), 20 min (15), 30 min (16), and 55 min (in the case of trimers 17–19), the solution was diluted with CHCl<sub>3</sub> (15 mL) and washed with 0.05 M phosphate buffer, pH 7.0 (2×30 mL). The organic layer was separated, dried, evaporated, and purified by silica gel column chromatography (60 mL). The product was eluted with a linear methanol gradient (0-5%, v/v, 2×300 mL) in CHCl<sub>3</sub>. Appropriate fractions were collected, evaporated to a volume of 2mL and precipitated into hexane (200 mL). The compounds were obtained as amorphous solids.

Deprotection of trimers 17-19 was performed by the sequence deblocking of phosphate group and subsequent treatment with methanol, saturated with ammonia at 0 °C. A trimer (0.1 mmol) was dissolved in 0.5 M solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in pyridine (30 mL) and stirred for 2 h. After the addition of 1 M solution of acetic acid in pyridine (15 mL), the mixture was evaporated and then coevaporated with pyridine  $(2 \times 10 \text{ mL})$ . The residue was dissolved in saturated methanolic ammonia (20 mL), kept for 20h and evaporated. The residue was chromatographed on a DEAE-Sephadex A-25 (HCO<sub>3</sub><sup>-</sup>form, 100 mL) column using a linear gradient (0-0.8 M,  $2 \times 500 \,\mathrm{mL}$ ) of TEAB buffer. The product containing fractions were collected and evaporated. Deblocked core (2',5') trimers (20–22) were obtained in the form of Na<sup>+</sup>salts as amorphous powders according to Moffatt.<sup>31</sup> The isolated yields and some physico-chemical properties are given in Table 1.

**4.1.12.** 5'-O-Phosphoryladenylyl( $2' \rightarrow 5'$ )-3-deazaadenylyl( $2' \rightarrow 5'$ )adenosine, sodium salt (2). To a solution of the trimer 18 (30 mg, 0.0137 mmol) in anhyd EtOAc (0.3 mL) pyrophosphoryl chloride (0.247 mmol, 63 mg, 0.034 mL) was added and the reaction mixture was stirred for 3 h at 0 °C. The reaction was stopped by the addition of ice, neutralized with cold 0.5 M TEAB (4 mL), evaporated and co-evaporated with MeOH (2×30 mL) and pyridine (2×10 mL). The residue was dissolved in 0.5 M solution of DBU in pyridine (20 mL)

and stirred for 18 h. After the addition of 1 M solution of acetic acid in pyridine (12 mL), the mixture was evaporated and then co-evaporated with pyridine ( $2 \times 5$  mL). The residue was dissolved in aq 25% solution of ammonia, kept for 18 h, evaporated, and purified by column chromatography as described above for core trimers **20–22**. Deblocked 5'-monophosphate (**2**) was obtained in form of Na<sup>+</sup>-salt as an amorphous powder.

**4.1.13.** 5'-O-Phosphoryladenylyl( $2' \rightarrow 5'$ )adenylyl( $2' \rightarrow 5'$ )-**3-deazaadenosine, sodium salt (3).** In a similar way, starting from the trimer 17 (30 mg, 0.015 mmol) the compound 3 was obtained as an amorphous powder.

**4.1.14.** 5'-O-Phosphoryl-3-deazaadenylyl( $2' \rightarrow 5'$ )adenylyl-( $2' \rightarrow 5'$ )adenosine, sodium salt (1). In a similar way, starting from the trimer 19 (30 mg, 0.014 mmol) the compound 1 was obtained as an amorphous powder.

The isolated yields and some physico-chemical properties are given in Table 1.

## 4.2. Biochemical studies

**4.2.1. General.** Pure recombinant human RNase L was prepared by a modification<sup>28</sup> of a previously described procedure.<sup>27</sup> The 5'-[<sup>32</sup>P]pCp (specific activity 3000 Ci/mmol) was purchased from DuPont/NEN (Wilmington, DE, USA). The synthetic oligonucleotide 5'-[<sup>32</sup>P]pC<sub>11</sub>UUC<sub>7</sub> was prepared according to Carroll et al.<sup>29</sup>

4.2.2. RNase L activation assays of 3-deazaadenosine analogues 1-3 of 2-5A. Assay for activation of RNase L was done using <sup>32</sup>P-labeled C<sub>7</sub>UUC<sub>12</sub> as an RNA substrate.<sup>29</sup> The synthetic RNA, C<sub>7</sub>UUC<sub>12</sub>, which was prepared on an ABI model 380 DNA synthesizer, was labeled at its 3'-terminus with [5'-<sup>32</sup>P]pCp (3000 Ci/ mmol) with T4 RNA ligase. Different 2-5A analogues at  $1-1000 \,\mathrm{nM}$ , compared with  $0.1-1000 \,\mathrm{nM}$  of pA4  $[pA(2'p5'A)_3]$  as a positive control, were incubated on ice for 30 min with 0.2 mg/reaction of RNase L expressed from a baculovirus vector in insect cells.<sup>27,28,32</sup> Reaction mixtures were further incubated with the RNA substrate, 80 nM of C7UUC12[32P]pCp, for 30 min at 30 °C. RNA was analyzed in 20% acrylamide/7 M urea gels and the extent of degradation was measured with a phosphorimager (Amersham Biosciences).

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