# Activation of Murine RNase L by Isopolar 2′-Phosphonate Analogues of 2′,5′ Oligoadenylates

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To determine the influence of methylene group insertion in the internucleotide linkage on the binding process of 2′,5′-oligoadenylates to RNase L, a series of 2′-phosphonate-modified trimers and tetramers were synthesized from appropriate monomeric units and evaluated for their ability to bind to murine RNase L. Tetramers pAA***X***A modified by *ribo*-, *arabino*-, or *xylo*-2′-phosphonate unit ***X*** in the third position were capable of binding to RNase L in nanomolar concentrations. The replacement of the first residue (p***X***AAA), or both the first and the third residues (p***X***A***X***A), was also tolerated by the enzyme. In contrast, in all cases, the replacement of the second residue (pA***X***AA) resulted in the significant decrease of binding ability. Additionally, no more than two phosphonate modifications in the tetramer were allowed to retain the binding affinity to the enzyme. Although all three tetramers pAA***X***A were found to be potent enzyme binders, only tetramers modified by *ribo*- and *xylo*-2′-phosphonate unit ***X*** activated the RNase L-catalyzed cleavage of the RNA substrate. Surprisingly, tetramer pAA***X***A, modified by *arabino*-2′-phosphonate unit ***X***, did not activate the enzyme and can be considered a potent antagonist. In comparison with their natural counterpart, the phosphonate analogues of the $pA_4$ exhibit superior resistance toward nucleases present in the murine spleen homogenate.

## Introduction

The short 2′,5′-oligoadenylates (2-5A) play an important role in the interferon-induced antiviral defense mechanism of cells.[1,2] In the presence of double-stranded RNA, interferon induces the expression of the 2′,5′-oligoadenylate synthetase utilizing ATP as a substrate for the synthesis of the 5′-phosphorylated 2-5As, (pp)p5′Ap(Ap)$_n$A2′ (where $n = 1-8$, but mainly 3 or 4).[3] These oligonucleotides bind to RNase L, a latent endoribonuclease and, subsequently, activate it. This enzyme, in its active form, is capable of cleaving single stranded RNA and thus preventing the expression of viral or bacterial proteins. The activity of RNase L is terminated/regulated by a specific 2′,5′-exonuclease that cleaves 2-5A to AMP and ATP. It has been speculated that 2-5A may also play an additional role in the regulation of cell growth and differentiation and apoptosis.[2,4]

The effort to develop 2-5A analogues capable of activating RNase L, at least as potent as the natural 2-5A itself, has led to the definition of enzyme structural requirements related to the particular parts of the 2-5A. It was found that every functionality of each individual nucleotide moiety of 2-5A contributed highly specifically to the binding to RNase L and its activation. Thus, the conformation of ribose moieties, the nature of the adenine-based nucleobase, the 2′,5′-phosphate backbone, and the presence of 5′-(poly)phosphate group were determined the most important factors influencing RNase L activity.[2,5] Recently, the resolution of the crystal structure of the ankyrin domain of RNase L in a complex with 2-5A trimers provided more detailed information about the mode of the binding process and confirmed some experimentally obtained structure−activity relationships.[6]

Surprisingly, among a number of prepared oligoadenylate analogues, there are only two types of 2-5As with modified internucleotide linkage. Among these, chiral phosphorothioate 2-5A trimers[7−10] are capable of binding to RNase L and activating the enzyme in the order of pA($R_P$)A($R_P$)A > pA($S_P$)A($R_P$)A > pA($R_P$)A($S_P$)A, with an efficiency comparable to that of natural pAAA. However, the pA($S_P$)A($S_P$)A trimer as well as a nonchiral phosphorodithioate trimer[11] bind to RNase L but do not activate it ($R_P$ and $S_P$ denote the configuration of the phosphorus atom of phosphorothioate internucleotide linkage).

The biological application of an antisense oligonucleotide covalently linked with 2′,5′-oligoadenylate remarkably increased the antisense effect of the oligonucleotide. Under these conditions, target viral mRNA was cleaved with RNase L, the cleaving activity of which was stimulated by the 2-5A part of the oligonucleotide chimeras. In contrast to the RNase H mechanism of antisense oligonucleotide action, there is no limitation concerning the structure of the antisense oligonucleotide.[12,13] These anticancer and antiviral oligonucleotide chimeras demonstrated the possibility of the successful application of the antisense strategy combined with the recruitment and activation of RNase L to the destruction of an antisense oligonucleotide-selected RNA. In particular, the potent inhibition of RSV and tumor proliferation was reported.[5,14−18]

Any successful biological application of oligoadenylates would depend on the stability of these compounds in cells, in fact, on the resistance of internucleotide linkage against nuclease cleavage. In connection with this requirement, the modified oligonucleotides bearing the O−P−C and C−P−O internucleotide linkages were shown to offer absolute protection against nucleases releasing the 3′(or 2′-)- and 5′-nucleotide units, respectively. These compounds containing structurally diverse types of isosteric, nonisosteric, or chiral phosphonate linkages have attracted our attention for many years.[19−25]

This work deals with the role of the one type of *C*-phosphonate internucleotide linkage in 2-5A analogues in the process of binding to and the activation of murine RNase L. We synthesized three sets of trimeric and tetrameric 2-5A

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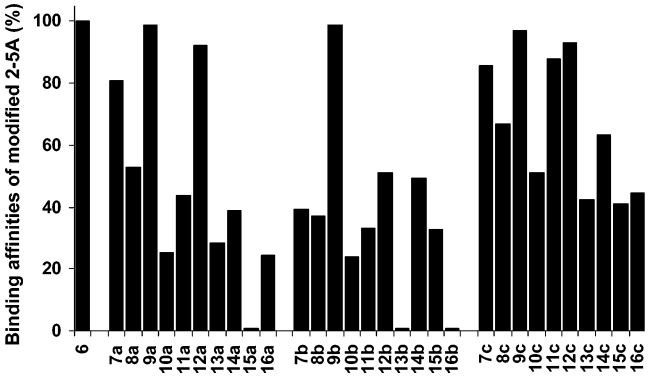



**Figure 1.** Binding affinities of modified oligoadenylates (% at 1 $\mu$M concentration) to murine RNase L related to natural pA$_4$ (**6**). (For values see Table 1.)

**Table 1.** Binding Affinities (% at 1 $\mu$M concentration) of Phosphonate Oligoadenylates to Murine RNase L Related to Natural pA$_4$ (**6**)

| 2′-5′ oligoadenylate | | binding affinity (%) unit ***X*** sugar configuration | | |
|---|---|---|---|---|
| no. | abbreviation | *ribo*-(**a**) | *arabino*-(**b**) | *xylo*-(**c**) |
| **6** | pAAAA | **100** | | |
| **7** | p***X***AAA | **81** | 40 | **86** |
| **8** | pA***X***AA | 53 | 37 | 67 |
| **9** | pAA***X***A | **99** | **99** | **97** |
| **10** | p***XX***AA | 25 | 24 | 51 |
| **11** | pA***XX***A | 44 | 33 | **88** |
| **12** | p***X***A***X***A | **92** | 51 | **93** |
| **13** | p***XXX***A | 28 | 0 | 43 |
| **14** | p***X***AA | 39 | 49 | 64 |
| **15** | pA***X***A | 0 | 33 | 41 |
| **16** | p***XX***A | 25 | 0 | 45 |

analogues in which one or more adenylate residues were replaced by either *ribo*-, *arabino*-, or *xylo*-adenosine-2′-phosphonate unit ***X*** (**a**−**c**) (Figure 1, Table 1). The newly formed 2′-5′ linkage, isopolar to the phosphodiester one but nonisosteric to it, contains an extra methylene group inserted between the phosphorus atom and the 2′-oxygen atom of the sugar residue. The presence of the bridging methylene group makes the linkage one atom longer in comparison with phosphodiester one, and these compounds thus exhibit more conformational flexibility than natural pA$_4$. Moreover, the introduction of phosphonate linkages into the 2-5A chain was seen as a factor for the remarkable increase in stability of the modified 2-5As against nucleases.

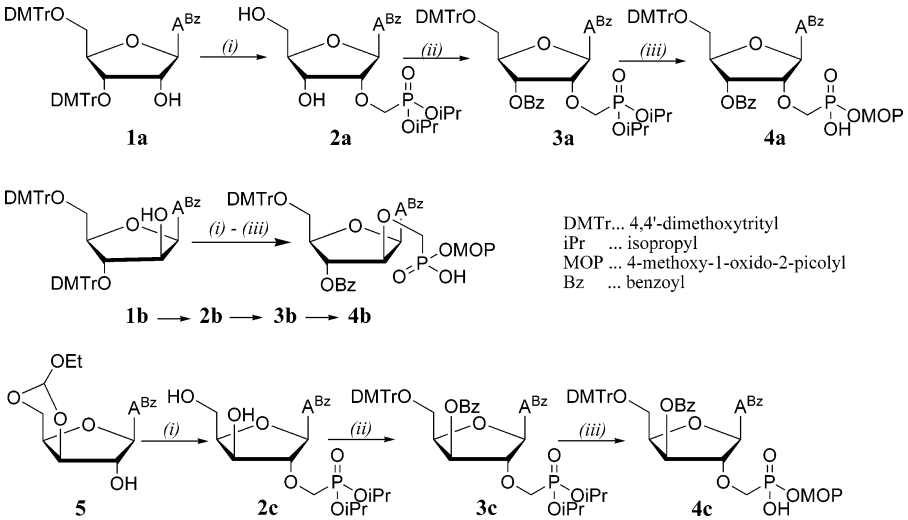
## Results and Discussion

**Preparation of Monomers.** Phosphonate monomers **4a**−**c** (Scheme 1) were prepared by multistep synthesis from 6-*N*-benzoyl derivatives of adenosine (*ribo*-A), arabinofuranosyladenine (*ara*-A), and xylofuranosyladenine (*xylo*-A). The dimethoxytritylation of *ribo*-A and *ara*-A derivatives resulted in a mixture of 2′(3′),5′-bis-*O*-dimethoxytrityl derivatives, which afforded, after resolution on silica gel,[26] approximately 40% of the desired 3′,5′-bis-*O*-dimethoxytrityl derivatives **1a** (ribo) and **1b** (arabino), respectively, as the starting compounds for the phosphonylation reaction. In the xylo series, the more readily accessible 3′,5′-*O*-ethoxymethylene derivative **5** was prepared from *xylo*-A and triethyl orthoformate. The protected nucleosides with free 2′-hydroxy groups **1a**, **1b**, and **5** were phosphonylated with diisopropyl-tosyloxymethylphosphonate. Upon acidic deprotection, they provided the *N*-protected 2′-phosphonates **2a**−**c**. 5′-*O*-Dimethoxytritylation of these compounds followed by benzoylation of the 3′-hydroxy group yielded the fully protected phosphonates **3a**−**c**. The deprotection of isopropyl ester groups with bromotrimethylsilane and subsequent esterification of free phosphonic acids with 4-methoxy-1-oxido-2-pyridylmethanol in the presence of DCC afforded appropriate *ribo*-**4a**, *arabino*-**4b**, and *xylo*-**4c** monomers.

**Synthesis of Oligoadenylates.** The synthesis of the library of 2-5A analogues was performed by sequential replacement of adenosine 2′-phosphate units in oligoadenylates pA$_4$ and pA$_3$ by *ribo* (***X*a**)-, *arabino* (***X*b**)-, or *xylo* (***X*c**)-2′-phosphonate units (Figure 1, Table 1).

The oligonucleotides were synthesized from the 2′- to the 5′-end on CPG-*ribo*A$_{5'-OH}$ (loading 25 $\mu$mol/g) on a 0.5 $\mu$mol scale using the GenSyn V02 DNA/RNA synthesizer. The protected adenosine 2′-phosphoramidite and the appropriate 2′-phosphonates **4a**−**c** were introduced into the oligonucleotide chain by phosphoramidite and phosphotriester methods, respectively. The 5′-terminal phosphate group was introduced by 2-[2-(4,4′-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (DESEP). Protocols for the synthesis and deprotection procedure are provided in the

**Scheme 1**[a]




[a] (i) (a) TsOCH$_2$PO(OiPr)$_2$, NaH, DMF, r.t.; (b) 80% AcOH, r.t. (ii) (c) DMTr-Cl, py, r.t; (d) BzCN, Et$_3$N, CH$_3$CN, r.t. (iii) (e) Me$_3$SiBr, 2,6-lutidine, CH$_3$CN, r.t.; (f) 4-methoxy-1-oxido-2-pyridylmethanol, DCC, py, r.t.

**Table 2.** $EC_{50}$ Values of the Most Potent Phosphonate-Modified Oligoadenylates

| 2′-5′ oligoadenylate | | |
|---|---|---|
| no. | abbreviation | $EC_{50}$[a] (nM) |
| **6** | pAAAA | 0.9 |
| **7a** | p***X***AAA | 396 |
| **7c** | | 421 |
| **9a** | pAA***X***A | 8.3 |
| **9b** | | 3.6 |
| **9c** | | 1.3 |
| **11c** | pA***XX***A | 393 |
| **12a** | p***X***A***X***A | 267 |
| **12c** | | 323 |

[a] $EC_{50}$ represents the concentration of the oligoadenylate analogue that gives a 50% displacement of radioactive $pA_4$[$^{32}$P]pCp; for details, see the Experimental Section.

Experimental Section and the Supporting Information. All prepared oligoadenylates **6**−**16** were purified by HPLC, and the purity was checked by RP HPLC in 0.1 M TEAA and 0.1% TFA. The molecular weights of the products were confirmed by MALDI-TOF MS.

**Binding of Modified Oligoadenylates to Murine RNase L.** As was mentioned above, one of the aims of the presented study was to define the role of stereochemical factors influencing the process of binding of oligoadenylates to RNase L, especially those associated with internucleotide linkage. Thus, we introduced the ribo, arabino, and xylo-configured 2′-phosphonate units ***X*** (**a**−**c**) (Figure 1) into individual positions of oligoadenylates. The introduction of these units into oligoadenylates variously changed the length of the internucleotide linkage, the conformation of the sugar−phosphate backbone, and the conformation of the sugar part of the respective phosphonate unit.

The affinities of natural 2-5A and its phosphonate-modified analogues to RNase L were examined in a radiobinding assay according to Silverman et al.[27] This assay is based on the ability of modified oligoadenylates to compete with a [$^{32}$P]-labeled $pA_4$pCp probe for the specific binding to RNase L. The assay was performed with a 126 000*g* supernatant prepared from the murine spleen homogenate. The results are illustrated in Figure 1 and summarized in Table 1. For the eight most potent compounds, we determined the $EC_{50}$ values (Table 2).

The data shows that the binding affinity of modified 2-5A to murine RNase L is dependent more on the position and the number of modified units ***X*** (**a**−**c**) than on the conformation and/or configuration of the appropriate sugar part. Phosphonate tetramers pAA***X***A (**9a**−**c**) modified in the third position are able to bind to RNase L with an efficiency comparable to that of natural pAAAA (**6**) (Table 2). Also, tetramers p***X***AAA (**7a**, **7c**), pA***XX***A (**11c**), and p***X***A***X***A (**12a**, **12c**) containing modifications (i) at the first position (**7a**, **7c**), (ii) both at the second and the third positions (**11c**), and (iii) both at the first and the third positions (**12a**, **12c**) are still efficient competitors of the natural pAAAA (**6**).

However, tetramers pA***X***AA (**8a**−**c**), p***XX***AA (**10a**−**c**), pA***XX***A (**11a**, **11b**), and p***XXX***A (**13a**−**c**), bearing modification at the second position, exhibited a binding affinity below 80%, except for pA***XX***A (**11c**) (Table 1). Under experimental conditions, the 80% binding affinity at 1 $\mu$M concentration approximately corresponds to the $EC_{50}$ value, which is three orders of magnitude higher than the $EC_{50}$ value of potent RNase L activators (data not shown).

What is the explanation for this low binding affinity to the enzyme? The second adenylate unit seems to be crucial for the binding process. The only data in the literature related to the modification of the sugar component of the second unit concerns the 3′-hydroxy group, which is the essential structural motif for binding of the oligoadenylates to RNase L.[2,6] The removal of the 3′-hydroxyl or its replacement with fluoro or methoxy moieties were found to result in the inability of the respective oligoadenylates to bind to and activate RNase L.[28−31] The changes in the sugar conformation of the second unit exerted a similar effect. This finding could explain the low affinity of the xylo tetramer pA***X***AA (**8c**) in which the configuration (spatial orientation) of the 3′-hydroxyl differs from that of the ribo configuration. Surprisingly, the ribo tetramer pA***X***AA (**8a**) was also found inactive, despite the correct configuration on the C3′ atom bearing the hydroxy group. In addition, the NMR study aimed at the determination of the C3′-*endo* and C2′-*endo* conformations and performed with the modified ribo dimer (2′,5′)-***X***A and the unmodified ribo dimer (2′,5′)-AA revealed the conformational identity of both dimers.[32] These findings suggest that the mutual orientation of the second and third units as well as the spatial shift of the charged phosphorus moiety in the pA***X***AA tetramer, given by the conformation of the methylene-extended internucleotide linkage C2′-*O*-***CH$_2$***-*P*-*O*-C5″, also plays an important role in the binding process. In addition, the hydroxyls of the phosphodiester group connecting the second and third units of 2-5A interact highly specifically with amino acid residues in the binding pocket.[6] The spatial shift of the charged phosphorus moiety may disrupt these interactions, and this effect may thus result in the decrease of the binding affinity to the enzyme. We also cannot exclude a steric conflict of the 2′-*O*-methylene group with amino acid residues in the RNase L binding pocket. In this respect, interesting results could be obtained with oligoadenylates with a ribo-configured second unit but with regioisomeric phosphonate internucleotide linkage C2′-*O*-*P*-***CH$_2$***-*O*-C5″ connecting the second and third units. Finally, the reasons for the low binding activity in the case of arabino tetramer pA***X***AA (**8b**) could be seen both in the changed conformation of the sugar component of the second unit that may distort the 3′-hydroxy group and in the geometry of the modified internucleotide linkage in which the phosphorus moiety is oriented in the direction opposite to that in the ribo compound. No more than two phosphonate modifications in the oligoadenylate can be made to retain its binding affinity to the enzyme. Fully modified oligoadenylates p***XXX***A (**13**) and p***XX***A (**16**) showed considerably reduced binding ability. In addition, trimeric oligoadenylates **14**−**16** were unable to compete effectively with labeled $pA_4$pCp.

All modified oligoadenylates without the 5′-terminal phosphate exhibited considerably reduced binding to RNase L (structures and data not shown). This finding is consistent with an earlier conclusion that at least one 5′-terminal phosphate group is necessary for the effective activation of RNase L.[33,34]

**RNase L-Catalyzed Cleavage of 5′-[$^{32}$P]-r($C_{11}U_2C_7$).** The activation of murine RNase L by selected binders (**6**, **7a**, **7c**, **9a**−**c**, **11c**, **12a**, **12c**) was determined by monitoring the ability of the enzyme to cleave the specific radiolabeled substrate 5′-[$^{32}$P]-r($C_{11}U_2C_7$). For the cleavage assay, we modified the procedure described by Silverman.[35] In our methodology, streptavidin-coated magnetic beads modified by biotinylated $pA_4$ **17** were used as an affinity matrix for the selective immobilization and purification of RNase L from the murine spleen homogenate.

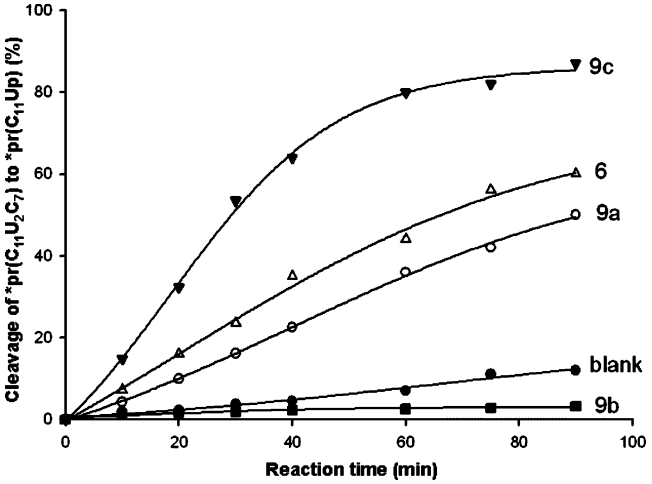



**Figure 2.** The time-dependent cleavage of 5′-[$^{32}$P]-r($C_{11}U_2C_7$) to 5′-[$^{32}$P]-r($C_{11}$Up) by murine RNase L in the presence of 1 $\mu$M $pA_4$ (**6**) and 1 $\mu$M modified tetramers pAA***X***A (**9a**−**c**) and in the absence of the added activator (blank).

We found that the incubation of magnetic bead-bound RNase L with the 5′-[$^{32}$P]-r($C_{11}U_2C_7$) substrate in the absence of free oligoadenylate resulted in only 15% cleavage (see blank, Figure 2) of the substrate after 90 min. However, a considerable enhancement of specific RNase L cleaving activity was achieved in the presence of 1 $\mu$M natural activator $pA_4$ **6** (Figure 2). Also, tetramers pAA***X***A **9a** and **9c** modified by ribo- and xylo-phosphonate units, respectively, were found to be potent activators of RNase L-catalyzed cleavage. Moreover, tetramer **9c** seems to be a more potent activator than natural $pA_4$ (Figure 2). Surprisingly, the closely related arabino tetramer **9b** was found to be completely inactive in the assay (the extent of substrate cleavage did not surpass a blank experiment; Figure 2), and therefore, tetramer **9b** can be considered an inhibitor of RNase L. In all of these cases, the only product of cleavage was 5′-[$^{32}$P]-r($C_{11}$Up). This finding is in agreement with that obtained with the human enzyme as reported by Carroll et al.[36]

Tetramers pAA***X***A **9a**−**c** differ from natural oligoadenylates in the conformation of the sugar component of the third unit and in stereochemical arrangement of the internucleotide linkage. Thus, it seems that these stereochemical changes in the third position could be responsible for the properties that classify modified oligoadenylates as either agonists or antagonists in the activation process of RNase L. Concerning compound **9b**, it is very probably a potent antagonist. Other selected binders (**7a**, **7c**, **11c**, **12a**, **12c**) with $EC_{50}$ values of about 300 nM were unable to activate RNase L under experimental conditions (data not shown).

**Nuclease Stability of Modified Oligoadenylates.** A rapid degradation of phosphodiester oligonucleotides by nucleases in body fluids is one of the most important limitations to their in vivo use. The increase of the half-life values of these compounds could thus bring the remarkable benefit of a prolonged antiviral effect. In connection with this, the stability of 2-5A phosphonate analogues against the nucleases of the murine spleen supernatant was examined under the conditions of the binding assay. The course of cleavage was monitored by HPLC, and the results are summarized in Figure 3. Phosphodiester-linked pAAAA (**6**) and oligoadenylates p***X***AAA (**7a**, **7c**) are rapidly degraded in the murine spleen supernatant (half-life for $pA_4$ is approximately 10 h at 4 °C). In contrast, the most potent oligoadenylates pAA***X***A (**9a**−**c**) and p***X***A***X***A (**12a**, **12c**) (see Table 2), with $EC_{50}$ values in the nanomolar range, exhibited superior nuclease

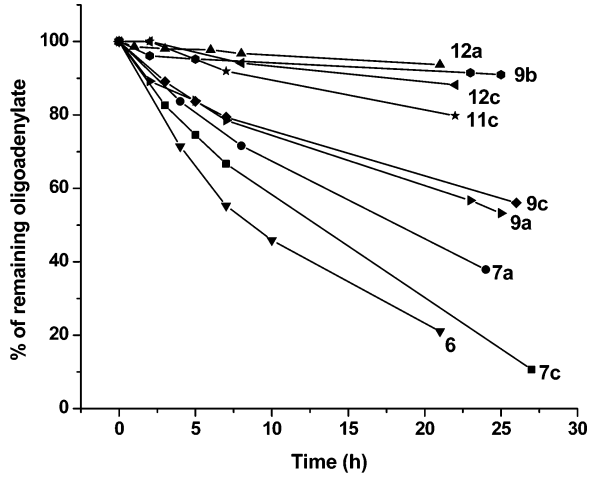



**Figure 3.** Degradation of 2-5A and its analogues in the murine spleen supernatant.

resistance in the murine spleen supernatant. No cleavage of the internucleotide linkages in compounds **9a**−**c** and **12a**, **12c** was observed. In this case, only slow 5′-dephosphorylation caused by a phosphomonoesterase took place. These findings suggest that under experimental conditions an exonuclease releasing 5′-mononucleotides from the 3′-end of oligonucleotide is probably responsible for the cleavage of compounds **7a**, **7c** and **6**. This exonuclease did not cleave oligoadenylates **9a**−**c** and **12a**, **12c** because of the presence of the noncleavable C2′-*O*-***CH*$_2$**-***P*-*O*-C5″ internucleotide linkage in the cleavage site (the enzyme cannot cleave off of the first 5′-mononucleotide).

## Conclusion

In summary, we prepared a series of phosphonate-modified oligoadenylate trimers and tetramers and examined their binding affinity to murine RNase L. We found that the replacement of the first (p***X***AAA **7a**, **7c**), the third (pAA***X***A **9a**−**c**), and both the first and the third natural residues (p***X***A***X***A **12a**, **12c**) by the modified ones preserved the binding affinity to RNase L. In this study, we have selected several potent binders of murine RNase L. We also examined the ability of these selected compounds to activate RNase L. Tetramers pAA***X***A **9a** and **9c** modified by *ribo*- and *xylo*-2′-phosphonate units, respectively, activated RNase L with an efficiency comparable to that of natural $pA_4$. Surprisingly, tetramer pAA***X***A **9b** modified by the *arabino*-2′-phosphonate unit was unable to activate RNase L and seems to be an inhibitor of the enzyme. The binders with $EC_{50}$ values of about 300 nM were unable to activate RNase L under experimental conditions. The introduction of the phosphonate internucleotide linkage of the C2′-*O*-***CH*$_2$**-***P*-*O*-C5″ type remarkably increased the stability of the modified oligoadenylates against the nucleases of the murine spleen homogenate in comparison with natural 2-5A. The findings achieved in this work seem to be a good starting point for further research in this area. We have already started the work on the synthesis of modified oligoadenylates containing the regioisomeric C2′-*O*-***P*-*CH*$_2$**-*O*-C5″ type of phosphonate internucleotide linkage, which differs from the one described here in the position of the extra methylene group within the linkage. Our attention will be focused on the modification of the 5′-terminal phosphate group of oligoadenylates to eliminate its instability against phosphomonoesterases.

## Experimental Section

The course of the reactions was checked by TLC on silica gel HPTLC, Alufolien Kieselgel 60 F254 (Merck) foils, and the

products were detected by UV light or by spraying with 5% ethanolic solution of sulfuric acid and subsequent heating (detection of *O*-DMTr derivatives, deep orange spots). Preparative column chromatography (PLC) was carried out on 40−60 $\mu$m spherical silica gel (Fluka). The sorbent was neutralized with $Et_3N$ (0.1%, v/v). Elution was performed at the rate of 40 mL/min. PLC and TLC were carried out with the following solvent systems (v/v): chloroform-ethanol 9:1 (C-1), ethyl acetate-toluene 1:1 (T-1), ethyl acetate−acetone−ethanol−water 4:1:1:1 (H-1), and ethyl acetate−acetone−ethanol−water 12:2:2:1 (H-2). HPLC analysis was performed on a column of reverse-phase (4.6 × 150 mm) Nucleosil 100-5 C18 (Macharey-Nagel), either isocratically at various concentrations of methanol in 0.1 M TEAA or by the gradient of methanol in the same buffer. Mass spectra were recorded on a ZAB-EQ (VG Analytical) instrument, using the FAB (ionization by Xe, accelerating voltage 8 kV) technique with glycerol and thioglycerol as matrixes. MALDI TOF MS was performed on a Reflex IV (Bruker-Daltonics) instrument. $^1$H and $^{13}$C NMR spectra were measured on a Varian UNITY-500 instrument ($^1$H at 500 MHz, $^{13}$C at 125.7 MHz) in $d_6$-DMSO or $CDCl_3$ and were referenced either to TMS as internal standard ($^1$H in $CDCl_3$) or to the solvent signal using the following relationships: $\delta_H$(DMSO) = 2.50, $\delta_C$-(DMSO) = 39.70, and $\delta_C(CDCl_3)$ = 77.00.

**General Procedures. Method A. Preparation of 5′-*O*-Dimethoxytrityl 2′-phosphonates.** Dimethoxytrityl chloride (1.1 mmol) was added under stirring to a solution of 2′-phosphonate (**2a**−**c**) (1.0 mmol) in pyridine (10 mL), and the mixture was stirred overnight at r.t. (TLC in system C-1). The reaction was quenched by the addition of $Et_3N$ (1 mL) and methanol (5 mL), and the mixture was concentrated under reduced pressure. The residue was dissolved in chloroform (50 mL) and extracted with water (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated. The product was purified by chromatography on silica gel (elution with a gradient of 0−8% ethanol in chloroform).

**Method B. Preparation of 3′,5′-*O*-Bis-dimethoxytrityl Nucleosides 1a, b.** Dimethoxytrityl chloride (1.1 mmol) was added under stirring to a solution of 6-*N*-benzoyl adenosine (1.0 mmol) in pyridine (10 mL), and the mixture was stirred overnight at r.t. (TLC in system C-1). After that, additional dimethoxytrityl chloride (1.3 mmol) and $Et_3N$ (2.0 mmol) were added, and the mixture was set aside for 1 day at r.t. (TLC in system T-1) to produce a mixture of 2′,5′- and 3′,5′-bis-dimethoxytrityl derivatives. The reaction was quenched by methanol (5 mL), and the mixture was concentrated under reduced pressure. The residue was dissolved in chloroform (50 mL) and extracted with water (3 × 20 mL), and the organic layer was dried over anhydrous sodium sulfate and evaporated. The product was chromatographed on silica gel (elution with a gradient of 0−50% ethyl acetate in toluene) to afford the 2′,5′- and 3′,5′-bis-dimethoxytrityl derivatives as faster and slower TLC compounds, respectively. Only the 3′,5′-regioisomer was used for further reactions.

**Method C. Preparation of 2′-*O*-Diisopropyl-phosphonomethyl Nucleosides 2a, b.** Sodium hydride (3.0 mmol) was added at 0 °C to a stirred solution of 3′,5′-bisdimethoxytrityl derivative (**1a**, **b**) (1.0 mmol) and diisopropyl-tosyloxymethylphosphonate (2.0 mmol) in DMF (10 mL), and the reaction mixture was left to warm gradually to r.t. and then stirred overnight (HPLC in the system with 75%−100% MeOH). The reaction was quenched by the addition of glacial acetic acid (0.11 mL; 2.0 mmol) at 0 °C, and the mixture was concentrated under reduced pressure. The product was purified by chromatography on silica gel (elution with a gradient of 0−50% ethyl acetate in toluene).

**Method D. Preparation of 3′-*O*-Benzoyl Nucleosides 3a−c.** Benzoyl cyanide (1.5 mmol) was added under stirring to a solution of 5′-dimethoxytrityl phosphonate derivative (1.0 mmol) and triethylamine (0.2 mmol) in acetonitrile (10 mL), and the mixture was stirred overnight at r.t. (TLC in system T-1). The reaction was quenched by methanol (5 mL), and the mixture was concentrated under reduced pressure. The product was purified by chromatography on silica gel (elution with a gradient of 0−50% ethyl acetate in toluene).

**Method E. Removal of Isopropyl Ester Groups.** Bromotrimethylsilane (4 mmol) was added to a solution of phosphonate diester (**3a**−**c**) (1.0 mmol) and 2,6-lutidine (8 mmol) in acetonitrile (10 mL). The reaction mixture was left aside overnight at r.t. and then concentrated under reduced presure (TLC in system H-1). The residue was treated with 2 M TEAB (5 mL) and methanol (10 mL), and the solution was evaporated and the residue co-distilled with ethanol and toluene. The crude nucleoside phosphonic acids were used for subsequent steps without further purification.

**Method F. Preparation of MOP Monoesters 4a−c.** DCC (5.0 mmol) was added to a solution of nucleoside phosphonic acid (1.0 mmol) and 4-methoxy-1-oxido-2-pyridylmethanol (3.0 mmol) in pyridine (5 mL), and the reaction mixture was stirred for 3 days at r.t. (TLC in system H-1). The reaction mixture was diluted with water (3.3 mL) to achieve a 60% concentration of pyridine, heated overnight at 50 °C to cleave off one ester group, and concentrated under reduced pressure. The product was purified by chromatography on silica gel (elution gradient from ethyl acetate to 100% H-2 and then from H-2 to 100% H-1) and freeze-dried from dioxane.

**Method G. Removal of 3′,5′-*O*-Bis-dimethoxytrityl and 3′,5′-Ethoxymethylene Protecting Groups**. Bis-dimethoxytrityl and ethoxymethylene derivatives (1.0 mmol) were treated with 80% acetic acid (10 mL) for 12 and 48 h, respectively, at r.t. (TLC in system C-2) and then concentrated under reduced presure. The product was purified by chromatography on silica gel (elution with a linear gradient of 0−10% ethanol in chloroform).

**6-*N*-Benzoyl-9-(3,5-*O*-Bis-dimethoxytrityl-β-D-ribofuranosyl)adenine (1a).** Compound **1a** was prepared using method B from 6-*N*-benzoyl-9-(β-D-ribofuranosyl)adenine (3.5 g; 9.5 mmol). Chromatography on silica gel afforded 4.8 g (52%) of the faster TLC compound (2′,5′-derivative) and 3.4 g (36%) of the slower TLC compound (3′,5′-derivative).

**Diisopropyl-[1-(6-*N*-benzoyladenin-9-yl)-β-D-ribofuranos-2-*O*-yl]methylphosphonate (2a).** Compound **2a** was prepared using method C from 6-*N*-benzoyl-9-(3,5-*O*-bis-dimethoxytrityl-β-D-ribofuranosyl)adenine (**1a**) (1.6 g; 1.6 mmol) followed by method G. Yield: 0.67 g (75%).

**Diisopropyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-dimethoxytrityl-β-D-ribofuranos-2-*O*-yl]methylphosphonate (3a).** Compound **3a** was prepared using method A from diisopropyl-[1-(6-*N*-benzoyladenin-9-yl)-β-D-ribofuranos-2-*O*-yl]methylphosphonate (**2a**) (0.67 g; 1.2 mmol) followed by method D. Yield: 0.98 g (85%).

**4-Methoxy-1-oxido-2-picolyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-dimethoxytrityl-β-D-ribofuranos-2-*O*-yl]methylphosphonate (4a).** Compound **4a** was prepared using method E from diisopropyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-dimethoxytrityl-β-D-ribofuranos-2-*O*-yl]methylphosphonate (**3a**) (0.88 g; 0.9 mmol) followed by method F. Yield: 0.54 g (59%).

**6-*N*-Benzoyl-9-(3,5-*O*-bis-dimethoxytrityl-β-D-arabinofuranosyl)adenine (1b).** Compound **1b** was prepared using method B from 6-*N*-benzoyl-9-(β-D-arabinofuranosyl)adenine (6.7 g; 18.0 mmol). Chromatography on silica gel afforded 4.9 g (28%) of the faster TLC compound (3′,5′-derivative) and 8.3 g (47%) of the slower TLC compound (2′,5′-derivative).

**Diisopropyl-[1-(6-*N*-benzoyladenin-9-yl)-β-D-arabinofuranos-2-*O*-yl]methylphosphonate (2b).** Compound **2b** was prepared using method C from 6-*N*-benzoyl-9-(3,5-*O*-bis-dimethoxytrityl-β-D-arabinofuranosyl)adenine (**1b**) (3.8 g; 3.9 mmol) followed by method G. Yield: 1.6 g (76%).

**Diisopropyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-dimethoxytrityl-β-D-arabinofuranos-2-*O*-yl]methylphosphonate (3b).** Compound **3b** was prepared using method A from diisopropyl-[1-(6-*N*-benzoyladenin-9-yl)-β-D-arabinofuranos-2-*O*-yl]methylphosphonate (**2b**) (1.6 g; 2.9 mmol) followed by method D. Yield: 2.3 g (82%).

**4-Methoxy-1-oxido-2-picolyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-dimethoxytrityl-β-D-arabinofuranos-2-*O*-yl]methylphosphonate (4b).** Compound **4b** was prepared using method E from diisopropyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-

dimethoxytrityl-β-D-arabinofuranos-2-*O*-yl]methylphosphonate (**3b**) (2.2 g; 2.3 mmol) followed by method F. Yield: 1.5 g (65%).

**Diisopropyl-[1-(6-*N*-benzoyladenin-9-yl)-β-D-xylofuranos-2-*O*-yl]methylphosphonate (2c).** Toluenesulfonic acid (2.5 mL of 10% solution in dioxane) was added under stirring to a solution of 6-*N*-benzoyl-9-(β-D-xylofuranosyl)adenine (0.9 g; 2.5 mmol) and triethyl orthoformate (0.5 mL; 3.0 mmol) in DMF (20 mL), and the mixture was stirred for 5 h at r.t. (TLC in system C-1). The reaction was quenched by the addition of 2 M TEAB (5 mL), and the mixture was concentrated under reduced pressure. The residue was dissolved in chloroform (100 mL) and extracted with saturated sodium bicarbonate (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated. Crude 3′,5′-ethoxymethylene derivative **5** was then converted, according to method C followed by method G, to phosphonate **2c**. Yield: 0.8 g (60%).

**Diisopropyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-dimethoxytrityl-β-D-xylofuranos-2-*O*-yl]methylphosphonate (3c).** Compound **3c** was prepared using method A from diisopropyl-[1-(6-*N*-benzoyladenin-9-yl)-β-D-xylofuranos-2-*O*-yl]methylphosphonate (**2c**) (0.8 g; 1.5 mmol) followed by method D. Yield: 1.1 g (76%).

**4-Methoxy-1-oxido-2-picolyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-dimethoxytrityl-β-D-xylofuranos-2-*O*-yl]methylphosphonate (4c).** Compound **4c** was prepared using method E from diisopropyl-[3-*O*-benzoyl-1-(6-*N*-benzoyladenin-9-yl)-5-*O*-dimethoxytrityl-β-D-xylofuranos-2-*O*-yl]methylphosphonate (**3c**) (1.1 g; 1.1 mmol) followed by method F. Yield: 0.7 g (64%).

**Synthesis and Deprotection of Oligoadenylates 6−16.** Oligoadenylates **6**−**16** were synthesized using the oligoadenylate solid-phase synthesis protocol (Supporting Information). The 5′-terminal phosphate was introduced by 2-[2-(4,4′-dimethoxytrityloxy)-ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (DESEP).

The deprotection of **6**−**16** was achieved in three steps. The 4-methoxy-1-oxido-2-picolyl (MOP) ester group of phosphonate moieties was removed by treatment with thiophenol in DMF (PhSH−$Et_3N$−DMF 1:1.4:2) at r.t. for 6 h. The removal of the 2-cyanoethyl protecting groups of the phosphodiester residues, 2-(2-hydroxyethylsulfonyl)ethyl ester group of the 5′-phosphate moiety, *N*-benzoyl groups of adenine residues as well as the release of oligomers from CPG was achieved in 8 M ethanolic methylamine at r.t. for 16 h. The 2′-*O*-silyl protecting groups were cleaved by treatment with 1 M TBAF in THF at r.t. for 16 h. Desalting and prepurification on Oligopure cartridges (Hamilton) gave deprotected oligoadenylates, which were finally purified by the RP HPLC using a linear gradient of acetonitrile (0−10%, 50 min) in 0.1 M TEAB or TEAA and freeze-dried.

The purity of prepared oligonucleotides **6**−**16** was checked by RP HPLC in 0.1 M TEAA and 0.1% TFA and found to be higher than 95% (based on an HPLC chromatogram).

**Preparation of Soluble Mouse Spleen Homogenate.** Male inbred BALB/c mice weighing approximately 22−27 g were decapitated, and their spleens were perfused with cold saline, excised, and immediately frozen at −72 °C. All of the following procedures were carried out at 4 °C. When needed, mouse spleens were thawed, minced with scissors, and homogenized in 25 mM HEPES/NaOH (pH 7.6), 5 mM $Mg(OAc)_2$, 100 mM KCl, 5 mM DTT, 1 mM PMSF, 25 mg/mL of pepstatin, 100 mg/mL of leupeptin A, and 1.2 mM ATP (1.5 mL per one spleen) using a Teflon-glass homogenizer. The homogenate was sonicated twice on ice for 15 s. The homogenate was centrifuged at 600*g* for 20 min. The pellet was discarded, and the supernatant was centrifuged at 17 000*g* for 20 min. The pellet was discarded again, and the supernatant was ultracentrifuged at 126 000 *g* for 90 min. The protein concentration in the supernatant was measured by the procedure of Bradford[37] using bovine serum albumin as a standard. The resulting supernatant was supplemented with glycerol (10% v/v), stored at −72 °C, and later used as a source of RNase L.

**Synthesis of p-5′-(2−5)$A_4$-pC-3′-p.** The synthesis of nonlabeled p-5′-(2−5)$A_4$-pC-3′-p was performed according to Silverman et al. with several modifications.[27] Thus, 2.5 nmol of $pA_4$, 10 nmol of pCp, and 2.5 nmol of ATP were mixed with 30 U (0.75 μL) of $T_4$ RNA ligase (Amersham Biosciences) in a total volume of 50 μL of 100 mM HEPES/NaOH (pH 7.6), 15 mM $MgCl_2$, 6.6 mM DTT, and 20% (v/v) DMSO (ligation buffer). The reaction proceeded at 4 °C, and after 15 h, additional 10 nmol of pCp, 2.5 nmol of ATP, and 20 U of $T_4$RNA ligase in 8 μL of ligation buffer were added. The reaction was checked on a Luna C18 column (5 μm, 150 × 4.6 mm, Phenomenex) at 260 nm using a linear gradient of acetonitrile in aqueous 0.1 M TEAB (0−12% in 70 min) at a flow rate of 1 mL/min. The same conditions were used for the isolation of the product ($pA_4$pCp). The molecular weight of the products was confirmed by MALDI-TOF MS.

**Synthesis of p-5′-(2−5)$A_4$-[$^{32}$P]pC-3′-p.** The synthesis of $^{32}$P-labeled, radioactive $pA_4$-[$^{32}$P]pCp was also performed according to Silverman et al. with several modifications.[27] Thus, 0.275 nmol of $pA_4$, 0.275 nmol of ATP, and 22 U of T4 RNA ligase in a total volume of 17 μL of ligation buffer were added to 83 pmol (250 μCi, 9.25 MBq) of freeze-dried [$^{32}$P]pCp (Amersham Biosciences). The reaction proceeded at 4 °C for 3 days, and the radiolabeled product was purified by HPLC using the conditions described above. The product was freeze-dried, dissolved in water, and stored at −70 °C. The purity of the product was checked using the HPLC system with radiodetection.

**Binding Affinity Assay.** The soluble mouse spleen homogenate (20 μL), prepared as described above, was mixed in a final volume of 25 μL with $pA_4$ or its analogue and $pA_4$-[$^{32}$P]pCp to give about 20 000 dpm per test tube (about 0.4 nM $pA_4$-[$^{32}$P]pCp). The final concentration of $pA_4$ or its analogue was set to 1 μM for the determination of percent inhibition. The competition curves for the determination of the $EC_{50}$ values of the oligoadenylate analogues were measured at several different analogue concentrations at otherwise identical conditions. The samples were allowed to stand at 4 °C for 60 min and then filtered through nitrocellulose filters. The filters were thoroughly washed with 20 mM Tris/HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, and 5% glycerol (5 × 1 mL). The adsorbed radioactivity was measured, after drying, using a toluene scintillation cocktail. All samples were assayed in triplicate, and the values were reproducible within ±15%.

**Modification of Magnetic Beads.** The modification of magnetic beads (Sigma) was performed according to MagSelect SA instructions. The resin slurry (250 μL) was transferred to a sample tube and washed with PBS at pH 7.4 (5 × 1 mL) using a magnetic separator. After that, the magnetic beads were incubated with biotinylated $pA_4$ **17** (60 nmol) in PBS (0.5 mL) under shaking at r.t. for 2 h and finally washed with PBS (3 × 1 mL). The measurement of absorbance of the supernatant at 260 nm revealed an almost quantitative binding yield (>95%). The modified beads were stored in PBS with 0.01% $NaN_3$ as a preservative (final volume 750 μL) at 2 °C.

**RNase L Purification and Cleavage Activity Assay.** Prior to the assays, a slurry of $pA_4$-modified beads (6 μL, about 0.5 nmol of bound $pA_4$) was diluted with the unmodified resin slurry (10 μL) and washed 3 times with the buffer A (0.2 mL, 25 mM HEPES/NaOH at pH 7.6, 100 mM KCl, 5 mM $Mg(OAc)_2$, 5 mM DTT, 1.2 mM ATP, 1 mM PMSF, 25 μg/mL of pepstatin, and 100 μg/mL of leupeptin).

Then, the murine spleen homogenate (0.3 mL, 7.4 mg of proteins per ml) was added, and the suspension of beads was shaken at 5 °C for 2 h. Afterward, the magnetic beads with bound RNase L were washed 10 times with ice-cold buffer A (0.5 mL) and finally resuspended in 90 μL of buffer A containing BSA (0.1 mg/mL). The cleavage assay (in a final volume of 100 μL) was started by the addition of $pA_4$ or its modified analogue (final concentration 1 μM) and the 5-[$^{32}$P]-r($C_{11}U_2C_7$) substrate (specific activity 3 Ci/mmol, suplemented with r($C_{11}U_2C_7$) to the final concentration of 10 μM). The reactions were shaken at 37 °C for 90 min. Aliquots (10 μL) of the reaction mixture were taken after 0, 10, 20, 30, 40, 60, 75, and 90 min, and the reaction was stopped by adding 10 μL of the Maxam−Gilbert loading buffer (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue) and boiling for 5 min. In a blank assay, the $pA_4$ was omitted.

The samples were electrophoresed in a denaturing 20% polyacrylamide gel (7 M urea, 0.09 M Tris-borate at pH 8.3, 2 mM EDTA, and 20% acrylamide). The gels were dried and analyzed using a Phosphor Imager (Molecular Dynamics). The scanned gels were quantitated with ImageQuant software (Molecular Dynamics).

**Stability of $pA_4$ and Its Analogues in Soluble Mouse Spleen Homogenate.** The resistance of oligonucleotides toward degradation by nucleases from soluble mouse spleen homogenate was checked on a Luna C18 column (5 $\mu$m, 150 × 4.6 mm, Phenomenex) at 260 nm, using a linear gradient of acetonitrile in aqueous 0.1% (v/v) TFA (0−12% in 70 min) at a flow rate of 1 mL/min. The soluble mouse spleen homogenate (9 $\mu$L) was mixed with 2.5 nmol (1 $\mu$L) of $pA_4$ or its analogue. The reaction mixtures were kept at 4 °C and periodically checked by HPLC (after mixing the sample with 10 $\mu$L of aqueous 0.1% (v/v) TFA and centrifugation at 14 000*g* for 10 min). The peaks of oligonucleotides and their degradation products were integrated using CSW 1.6 software (Data Apex, Prague).

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**Supporting Information Available:** $^{1}H$, $^{13}C$ NMR, and HR FAB data for compounds **1**−**4**, MALDI-TOF MS, purity data, and retention time data ($t_R$) for oligoadenylates **6**−**16**, protocol for the synthesis of oligoadenylates, synthesis of biotinylated $pA_4$ **17**, and the time-dependent profiles of 5′-[$^{32}$P]-r($C_{11}U_2C_7$) cleavage by murine RNase L. This material is available free of charge via the Internet at http://pubs.acs.org.

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