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Biologically Stable 2-5A Analogues containing 3′-*O*,4′-*C*-bridged Adenosine as Potent RNase L Agonists

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2′,5′-Oligoadenylate 5′-triphosphate (**1a** in Figure 1), referred to as 2-5A, plays an important role in the 2-5A system that is an interferon-regulated RNA degradation pathway with antiviral, growth-inhibitory, and apoptotic activities in mammalian cells.[1] 2-5A is synthesized from ATP by 2-5A synthetase that is induced by interferons, and is activated by dsRNA that is often produced during viral infection. The 2-5A produced binds and activates 2-5A-dependent endoribonuclease L (RNase L), which cleaves messenger and ribosomal RNA in the 3′-sides of UpNp sequences.[2,3] The RNA degradation results in the inhibition of protein synthesis, thereby inducing cell apoptosis to halt further viral replication.[4,5] In addition, the transfection of 2-5A into cells or the expression of 2-5A synthetase in cells has been found to cause growth arrest and apoptosis.[6,7] Recently, RNase L was identified as a candidate for the hereditary prostate cancer gene, HPC1, by a positional cloning approach.[8] These findings suggest that the direct activation of RNase L by 2-5A might be a novel chemotherapeutic approach both to viral diseases and cancer. Natural 2-5A has a short biological half-life because of its rapid degradation by phosphatase and nuclease activities in cells and in serum.[9] Thus far, a number of 2-5A analogues with modification of the bases, riboses, internucleotide linkages, and a 5′-phosphoryl group have been synthesized to test RNase L activating activity in vitro.[1,10–16] However, no intracellular-effective 2-5A analogue with both sufficient RNase L agonistic activity and high resistance against enzymatic degradation has been reported except for fully phosphorothioated 2-5A.[17] In many cases, the phosphorothioate modification of oligonucleotides seem to bind nonspecifically to protein.[18] Therefore, 2-5A analogues with no phosphorothioate bonds would be desirable as RNase L-specific agonists.

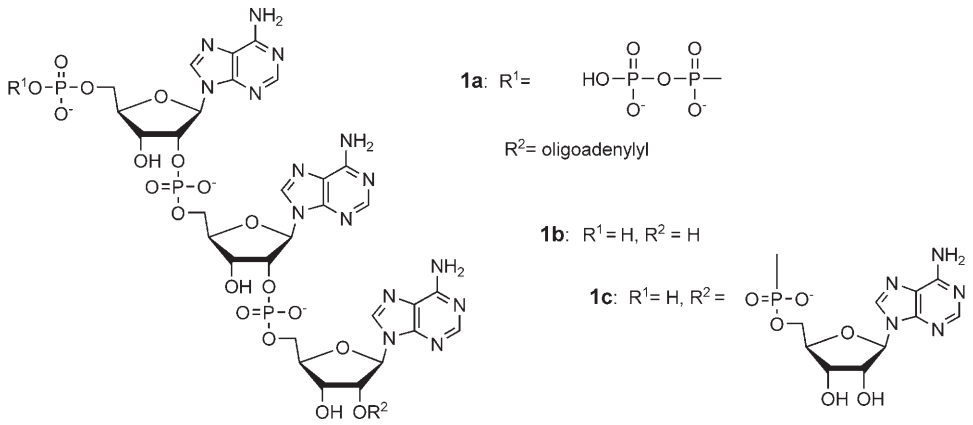

Figure 1. Structures of natural type of 2-5A.

Bridged nucleic acids are modified oligonucleotides which have a fixed sugar conformation resulting from a bridging alkylene linkage in the furanose. We have previously reported the synthesis of oligonucleotides having a 2′-*O*,4′-*C*-methylene or -ethylene linkage and their usefulness as antisense molecules with properties including high affinity to mRNA and extremely high nuclease resistance.[19–21] Also, we have already reported the synthesis of 3′-*O*,4′-*C*-methylene nucleosides (3′,4′-BNA) that are components of oligonucleotides having the 2′,5′-linkage.[22,23] In this study, we applied a bridging strategy to develop novel 2-5A analogues by introducing a 3′-*O*,4′-*C*-alkylene linkage into the adenosine units, and then examining their properties including their RNase L activating ability and their nuclease stability.

For the synthesis of the 2-5A analogues, we prepared three types of bridged adenosine, such as 3′-*O*,4′-*C*-methylene, -ethylene, and -propylene adenosine phosphoramidites. By incorporating these phosphoramidites, we synthesized novel 2-5A analogues using a DNA/RNA synthesizer (see Supporting Information). The activity of RNase L activation by their 2-5A analogues was measured using recombinant human RNase L, as previously reported.[24]

For activating human RNase L, 2-5A must have at least one 5′-phosphoryl group and three or four adenylyl residues with a 2′-5′ linkage.[25] Firstly, to investigate which adenosine residues in 2-5A can be substituted with bridged adenosines for RNase L activation, we synthesized a series of all the possible

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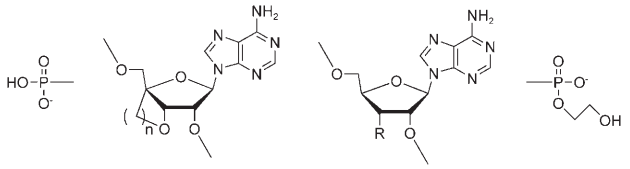
Table 1. Activation of RNase L by 2-5A analogues.

Compd.	Sequence[a]	EC_{50} [nM][b]	C_{rel}[c]
1b	pAAA	3.5	1.0
2a	pEAA	77	22
2b	pAEA	530	150
2c	pAAE	4.9	1.4
2d	pEEA	>1000	>300
2e	pEAE	130	37
2f	pAEE	>1000	>300
2g	pEEE	>1000	>300
3a	pDAAA	9.2	2.6
3b	pMAAA	6.0	1.7
3c	pBAAA	13	3.7
3d	pEAAA	10	2.9
3e	pPAAA	44	13
4a	pAADA	18	5.1
4b	pAAMA	7.8	2.2
4c	pAABA	35	10
4d	pAAEA	36	10
4e	pAAPA	8.1	2.3
5a	pEAAe	130	37
5b	pAAEe	8.8	2.5

[a] All internucleotide linkages are phosphodiester bonds. All compounds are triethylammonium salts. The symbol for 2′,5′-phosphodiester linkages was omitted. The characters indicate the following structures.



[b] EC_{50} was defined as the molarity (M) of 2-5A analogues required to cause a 50% degradation of 0.05 μg μL^{-} of 5S rRNA. Values are the average of two independent experiments. [c] C_{rel} represents the ratio of EC_{50} of analogues as compared to 2-5A (**1b**). The smaller C_{rel}, the more effectively the analogue was able to activate RNase L.

trimer 2-5A analogues in which the adenosine residue has been sequentially replaced by 3′-*O*,4′-*C*-ethylene adenosine (**E**) and evaluated their human RNase L activation ability (**2a**–**g** in Table 1). Whereas compounds **2a** and **2c** with the replacement of the first or third adenosine from the 5′ end with **E** exhibited a moderate activation of RNase L, the activity of compound **2b** with the replacement of the second adenosine with **E** dropped 150-fold. This means that the 3′-hydroxyl group of the second adenosine of 2-5A trimer is important for activating RNase L and is consistent with earlier reports concerning the analogues having 3′-position-modified adenosine, such as 3′-deoxyadenosine (**D**),[26] 3′-*O*-methyladenosine (**M**),[27] and 3′-fluoroadenosine.[28] Also, RNase L activation by compounds **2d**–**g** with two or three **E** residues in 2-5A resulted in a reasonable correlation. That is, only compound **2e** weakly activated RNase L and all others comprising **E** at the second adenosine (**2d**, **2f**, and **2g**) exhibited no activation, indicating the 3′-hydroxy group of the second adenosine was unable to be modified. These results clearly indicated that the first and/or the third adenosine could be substituted with 3′-position-modified adenosines.

Next, to investigate which type of modification at the first and/or the third adenosine would have the potential of RNase L activation, we synthesized 2-5A tetramer with various modifications, such as **D**, **M**, 3′-*O*,4′-*C*-methyleneadenosine (**B**), **E**, and 3′-*O*,4′-*C*-propyleneadenosine (**P**), and evaluated their activities (**3a**–**e** and **4a**–**e** in Table 1). Among compounds **3a**–**e** modified at the first adenosine, compound **3b** with **M** modification showed the highest activity and was almost equal to the natural type of 2-5A, compound **1b**. Compounds **3a**, **3c**, and **3d** modified with **D**, **B**, and **E**, respectively, resulted only in a minimal decrease in activity. Compound **3e** modified with **P** decreased the activity 13-fold. On the other hand, among compounds **4a**–**e** modified with **D**, **M**, **B**, **E**, and **P** at the third adenosine, compounds **4b** and **4e** with **M** and **P** modification, respectively, showed the highest activity. Recently, the crystal structure of the N-terminal ankyrin repeat domain (ANK) of human RNase L complex with natural 2-5A (**1b**) was reported.[29] From the results of that study, it was concluded that the 3′-hydroxy group of the first adenosine moiety does not interact with ANK, but is located close to the surface of ANK, whereas the 3′-hydroxy group of the third adenosine does not interact with ANK and is located toward the extra space in the concavity of ANK. These results might account for why compound **4e** with **P** modification at the third adenosine retained its high activity and compound **3e** with **P** modification of the first adenosine did not. Consequently, the combination of **M** at the first position and **M** or **P** at the third position would be the best modification in terms of RNase L activating ability.

Furthermore, we introduced a 2-hydroxyethylphosphate group at the 2′-end of the 2-5A analogues because we previously demonstrated that the introduction of the 2-hydroxyethylphosphate group at the 3′-end of oligonucleotides promoted a high resistance toward exonucleases.[30] Compounds **5a** and **5b** with the 2-hydroxyethylphosphate group retained RNase L activating activity in comparison with the parent compounds **2a** and **2c**, respectively (Table 1), indicating that the introduction of the 2-hydroxyethylphosphate group to the 2-5A analogues at the 2′-end had little affect on RNase L activation.

To investigate the effects of the modification of the 2-5A analogues on the sensitivity of nuclease, the stability of compounds **4a**–**e** and **5b** toward an exonuclease, snake venom phosphodiesterase (SVPD), was evaluated. The cleavage of the phosphodiester bonds of these compounds from the 2′-end by SVPD was evaluated by quantifying the amount of the residual intact 2-5A analogues, pAAXY, in these reactions by reverse phase HPLC (Figure 2a). Although the unmodified 2-5A **1c** degraded rapidly, compound **4e**, which was modified with **P**, has the highest stability amongst the 2-5A analogues with 3′-*O*,4′-*C*-bridged adenosine, such as **B**, **E**, and **P**. These results suggested that the incorporation of a longer alkylene bridging linkage in the third adenosine gave greater nuclease resistance. Compound **4b** modified with **M** showed more stability than compounds **4c** or **4d** modified with **B** or **E**, probably because the methyl group of **M** at the 3′ position can interfere with the phosphodiesterase more than the conformationally restricted 3′-*O*,4′-*C*-methylene or -ethylene linkage of **B** or **E**. In

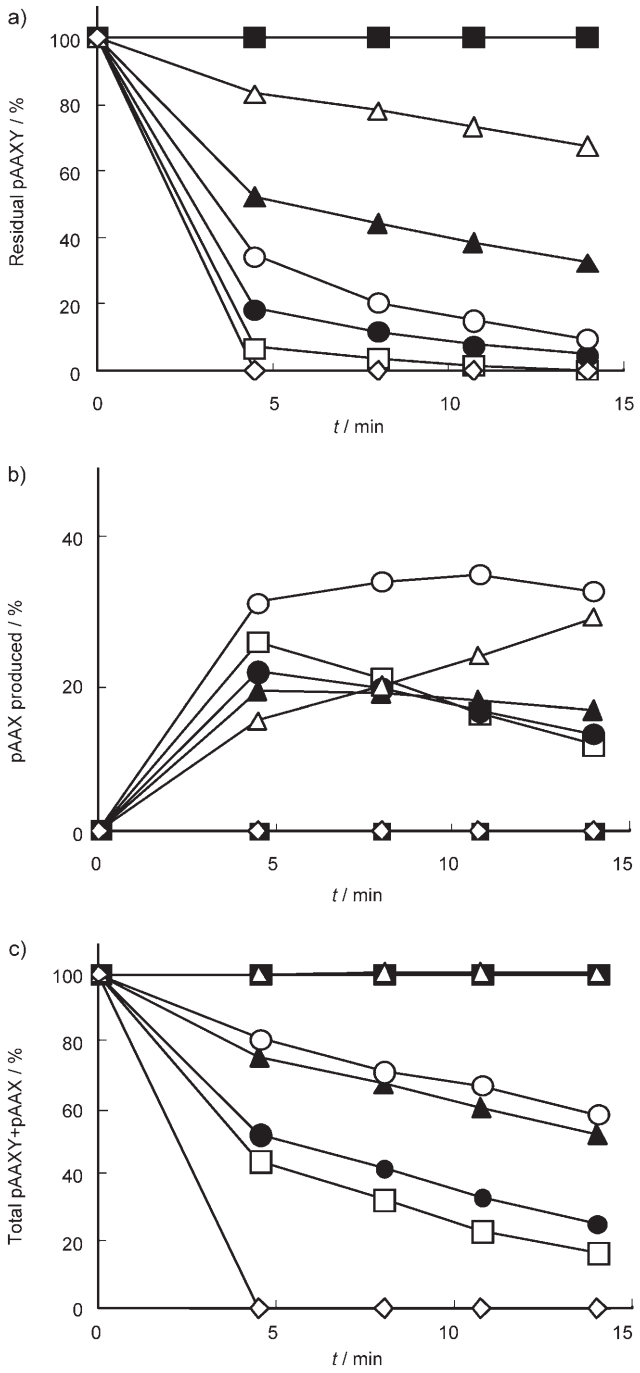


Figure 2. Stability of modified 2-5A analogues, pAAXY, against SVPD. a) residual pAAXY, b) pAAX produced from cleaved pAAXY, c) total amount of pAAXY and pAAX, 2-5A analogue concentration: 5 μM; enzyme concentration: 0.125 μg mL^{-1}; reaction buffer: Tris-HCl (50 mM, pH 8.0), $MgCl_2$ (10 mM) at 37 °C. The amount of each product was quantified by reverse phase HPLC analysis. Open diamonds: compound **1c** (X = **A**, Y = **A**); open squares: compound **4a** (X = **D**, Y = **A**); closed triangles: compound **4b** (X = **M**, Y = **A**); closed circles: compound **4c** (X = **B**, Y = **A**); open circles: compound **4d** (X = **E**, Y = **A**); open triangles: compound **4e** (X = **P**, Y = **A**); closed squares: compound **5b** (X = **A**, Y = **e**). **A**, **B**, **D**, **E**, **M**, **P**, and **e** are shown in Table 1.

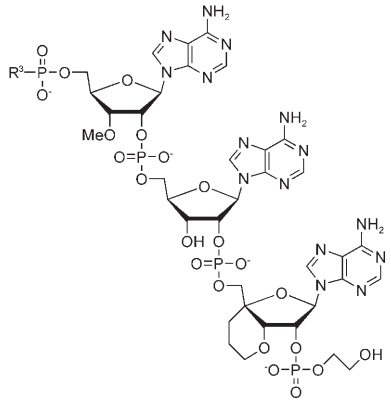
addition, compound **5b**, in which the 2-hydroxyethylphosphate group at the 2′-end was introduced, brought drastically increased stability against exonuclease, as expected. In this exonuclease reaction, we observed the cleaved products (pAAX) where a cleavage reaction occurred only at the third adenosine. Figure 2b shows the time course of these products in the SVPD reaction. Although the amount of pAAX of compounds **4a**–**d** reached maximum after incubation for 5 to 10 min, the accumulation of the trimer produced from compound **4e**, pAA**P**, was uniquely observed. These results, as shown in Figure 2a and b, suggest that the incorporation of **P** into the 2-5A analogue increased not only the nuclease resistance of the phosphodiester bond on the 2′-side of **P**, but also that on the 5′-side of **P**. Figure 2c shows the combined amount of the residual pAAXY and pAAX produced. The combined amount derived from compound **4e** was almost identical to the initial amount of compound **4e**, which clearly indicates that the cleavage stopped on the 5′-side of **P**. The stability on the 5′-side increased by the incorporation of **P** at the third position is favorable, because it is unnecessary to modify the 3′-hydroxyl group of the second adenosine residue which is crucial for high RNase L activation ability. Therefore, the modifications with **M** and **P** as the first and the third adenosine, respectively, and attachment to the 2-hydroxyethylphosphate group at the 2′-end were chosen for designing the biologically active and nuclease-resistant 2-5A analogues without any modification of the second adenosine.

It is thought that the 5′-monophosphate group of 2-5A might be rapidly dephosphorylated by phosphatases into the inactive 2-5A in cells. We have reported that the 5′-monophosphate group of 2-5A can be substituted with the 2-hydroxyethylphosphate group, which forms a phosphodiester bond that is not dephosphorylated by phosphatases.[31] We replaced the 5′-monophosphate group of 2-5A analogues with other various substituted phosphate groups. Their RNase L activation activity is shown in Table 2. Compounds **6a**–**i** bearing various phosphodiester bonds in place of the 5′-monophosphate group exhibited high potential as RNase L activators. Among the tested analogues, compound **6a** with a 2-hydroxyethylphosphate group exhibited the highest activity and was almost equal to the natural 2-5A, compound **1b**. We observed that compound **6a** was not a substrate for one of the phosphases, calf intestine alkaline phosphatase (see Supporting Information). Compounds with longer alkylene groups, such as compounds **6b**–**e**, and the bulky groups, such as compounds **6f** and **6g**, resulted in a decrease in activity. Compound **6h** with an aminoalkyl substituent led to a 31-fold decrease in activity. Compound **6i** with a thiophosphate group was one of the highly active compounds, but was apt to form a disulfide bond yielding a dimerized 2-5A analogue by air-oxidization in solution (data not shown). These results indicate that the 2-hydroxyethylphosphate group in compound **6a** is the most suitable equivalent of the 5′-monophosphate group of the 2-5A.

In conclusion, among the 2-5A analogues we investigated, compound **6a** was the most potent RNase L agonist with RNase L activity as high as native 2-5A and high resistance to enzymatic degradation. However, considering the introduction of the 2-5A analogues into cells, a delivery method including a transfection reagent would be necessary because of their polyanion character. The biological activities of compound **6a** in

Table 2. Activation of RNase L by 2-5A analogues with various 5′-substituted phosphoryl groups.

Compd.	R_3	EC_{50} [nM]	C_{rel}[a]
6a	$-O(CH_2)_2OH$	3.2	0.6
6b	$-O(CH_2)_3OH$	9.6	1.9
6c	$-O(CH_2)_4OH$	12	2.4
6d	$-O(CH_2)_6OH$	24	4.8
6e	$-O(CH_2)_8OH$	12	2.4
6f	$-OCH(CH_2OH)_2$	8.8	1.8
6g	–OPh	22	4.4
6h	$-O(CH_2)_6NH_2$	154	31
6i	–SH	2.7	0.5



[a] C_{rel} represents the ratio of EC_{50} of analogues as compared to 2-5 A (**1b**; EC_{50} = 5.0 nM). The smaller C_{rel}, the more effectively the analogue was able to activate RNase L.

cells and an examination of the delivery method are currently in progress and will be reported in the near future.

Experimental Section

Synthesis of 2-5A analogues

Synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*,4′-*C*-alkylene-6*N*-benzoyladenosine-2′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites is described in the Supporting Information. The building blocks for 2′,5′-adenylate domain, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-6*N*-benzoyladenosine-2′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methyl-6*N*-benzoyladenosine-2′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)-phosphoramidite, were obtained from Chem Genes. The CPG solid support coupled to adenosine (Bz-A-RNA CPG), the phosphorylation reagent for the 5′-terminus (Chemical Phosphorylation Reagent II), 3-(4,4′-Dimethoxytrityloxy)propyl-1-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (Spacer Phosphoroamidite C3), 6-(4-Monomethoxytritylamino)hexyl-1-(2-cyanoethyl-*N*,*N*-diisopropyl)-phosphoramidite (5′-Amino-modifier C6) were from Glen Research. 4-(4,4′-Dimethoxytrityloxy)butyl-1-(2-cyanoethyl-*N*,*N*-diisopropyl)-phosphoramidite (DMT-Butane-Diol Phosphoramidite) and 2-cyanoethyl phosphoramidite derivative of 1,3-bis(4,4′-dimethoxytrityloxy)-2-propanol (Symmetrical Branching CED OP) were from ChemGene. 6-(4,4′-Dimethoxytrityloxy)hexyl-1-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite and 8-(4,4′-Dimethoxytrityloxy)octyl-1-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite were prepared according to the literature.[32,33] 2-(4,4′-Dimethoxytrityloxy)ethyl-1-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite and the CPG solid support bearing 2-hydroxyethyl phosphate were prepared according to the literature.[30,34] Phenyl 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite was prepared according to the literature.[35] DNA/RNA synthesis reagents were purchased from Applied Biosystems.

2-5A analogues were synthesized on a 2.0 μmol scale by solid-phase phosphoramidite chemistry using Applied Biosystems DNA/RNA synthesizer 392. All phosphoramidites were used as 0.1 M solutions in CH_3CN. The coupling of 3′-*O*,4′-*C*-bridged adenosine phosphoramidites and other adenosine phosphoramidites was performed according to standard synthesis cycles except for an elongation of the coupling time (15 min). The coupling step was followed by oxidation and detritylation. After synthesis in the DMTr-on mode on an automated DNA/RNA synthesizer, ABI 394, the CPGs with the attached 2-5A were treated with concentrated aqueous ammonia and EtOH (3:1 *v*/*v*) solution at 60 °C for 5 h. The solutions were separated from the resin by filtration and evaporated on a Speed-vac concentrator. The DMTr-On products were purified by reverse phase HPLC (RP HPLC) with a gradient of CH_3CN (Wakosil DNA, Wako Pure Chemical Industries, 10×250 mm, 0.1 M TEAA (pH 7.0)). In the case of using Chemical Phosphorylation Reagent II, the DMTr group was removed by the treatment with aqueous 80% AcOH for 15 min, then the evaporated residue was treated with the solution of concentrated aqueous ammonia and EtOH (3:1 *v*/*v*) for 10 min to complete the removal of the protection group on the 5′-phosphate. The deprotection of the *tert*-butyldimethylsilyl group was accomplished in acidic conditions (0.01 N HCl, pH 2.0) at 30 °C for 5 h. After neutralization with dilute aqueous ammonia, the crude products were purified by RP HPLC. The purities of all 2-5A analogues were confirmed as >95% by RP HPLC. The molecular weights were detected by negative-ion FAB or negative-ion ESI mass spectrometry. In cases where the 2-5A analogues had the same molecular weights, their structures were further identified by using tandem mass spectrometry.[36]

RNase L activation

The ability of 2-5A analogues to activate RNase L was estimated by monitoring the cleavage of 5S rRNA by the activated RNase L. Recombinant human RNase L was expressed in *Escherichia coli* and purified according to the reported procedure.[37] 2-5A analogue (final concentration, 0.1–1000 nM) was mixed with recombinant human RNase L (final concentration, 60 nM) in a buffer comprising 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 8 mM 2-mercaptoethanol, 90 mM KCl, and 0.1 mM ATP (total 19 μL), and then the mixture was incubated on ice. After 30 min, 1 μL of a solution containing 1 μg of yeast 5S rRNA was added to the mixture (total 20 μL), and then the solution was incubated at 30 °C. After 30 min, the reaction was terminated by the addition of 20 μL of a loading solution containing urea (9 M), EDTA (3 mM), bromophenol blue (0.02%), and xylene cyanol (0.02%). An aliquot (20 μL) of the mixture was electrophoresed on a polyacrylamide (10%)/urea (7 M)/1× TBE gel, followed by staining with ethidium bromide. The RNA signal corresponding to the intact 5S rRNA was quantified using a computer program, NIH Image 1.61.

Nuclease-stability of 2-5A analogues

Snake venom phosphodiesterase (Worthington, 0.1 μg) in 40 μL of H_2O at 37 °C was added to 5 nmol solutions of each 2-5A analogues in 960 μL of reaction buffer (50 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$) . Of the reaction mixture, 200 μL was taken at each sampling time point and immediately heated at 90 °C for 4 min to inactivate the enzyme. The reaction mixture was analyzed by RP HPLC with a gradient of CH_3CN (Inertsil ODS-80A, GL Science, 4.6× 100 mm, 0.1 M TEAA (pH 7.0)). The residual fragment ratio of 2-5A analogues to the initial amount was determined from their peak area ratios.

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Keywords: 2′,5′-oligoadenylate · 3′-*O*,4′-*C*-alkylene adenosine · nuclease resistance · RNase L · structure–activity relationships

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