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Nucleoside Tetra- and Pentaphosphates Prepared Using a Tetraphosphorylation Reagent Are Potent Inhibitors of Ribonuclease A

Scott M. Shepard,[†] Ian W. Windsor,[†] Ronald T. Raines,[*] and Christopher C. Cummins[*]

Department of Chemistry, Massachusetts Institute of Technology, Cambridge Massachusetts 02139, United States

Supporting Information

ABSTRACT: Adenosine and uridine 5′-tetra- and 5′-pentaphosphates were synthesized from an activated tetrametaphosphate ($[PPN]_2[P_4O_{11}]$, $[PPN]_2[\mathbf{1}]$, PPN = bis(triphenylphosphine)iminium) and subsequently tested for inhibition of the enzymatic activity of ribonuclease A (RNase A). Reagent $[PPN]_2[\mathbf{1}]$ reacts with unprotected uridine and adenosine in the presence of a base under anhydrous conditions to give nucleoside tetrametaphosphates. Ring opening of these intermediates with tetrabutylammonium hydroxide ([TBA][OH]) yields adenosine and uridine tetraphosphates ($\mathbf{p_4A}$, $\mathbf{p_4U}$) in 92% and 85% yields, respectively, from the starting nucleoside. Treatment of ($[PPN]_2[\mathbf{1}]$) with AMP or UMP yields nucleoside-monophosphate tetrametaphosphates ($\mathbf{cp_4pA}$, $\mathbf{cp_4pU}$) having limited aqueous stability. Ring opening of these ultraphosphates with [TBA][OH] yields $\mathbf{p_5A}$ and $\mathbf{p_5U}$ in 58% and 70% yield from AMP and UMP, respectively. We characterized inorganic and nucleoside-conjugated linear and cyclic oligophosphates as competitive inhibitors of RNase A. Increasing the chain length in both linear and cyclic inorganic oligophosphates resulted in improved binding affinity. Increasing the length of oligophosphates on the 5′ position of adenosine beyond three had a deleterious effect on binding. Conversely, uridine nucleotides bearing 5′ oligophosphates saw progressive increases in binding with chain length. We solved X-ray cocrystal structures of the highest affinity binders from several classes. The terminal phosphate of $\mathbf{p_5A}$ binds in the P_1 enzymic subsite and forces the oligophosphate to adopt a convoluted conformation, while the oligophosphate of $\mathbf{p_5U}$ binds in several extended conformations, targeting multiple cationic regions of the active-site cleft.

Secretory ribonucleases (RNases) are a diverse family of enzymes that catalyze the cleavage of RNA to elicit biological functions ranging from cell signaling to innate immunity.[1,2] Fundamental knowledge generated by studying RNase A, which derives from the bovine pancreas, has shaped the fields of enzymology and protein chemistry.[3,4] Furthermore, mammalian RNases have been shown to have angiogenic[5] and neurotoxic activities,[6] and targeted inhibitors of these enzymes may have human therapeutic potential.[7] RNase A binds its substrates in enzymic subsites that interact with phosphoryl groups and nucleobases (Figure 1).[8,9]

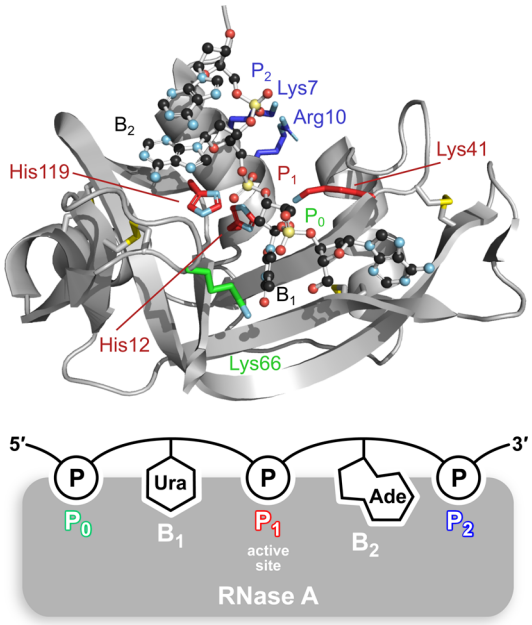

Figure 1. Cocrystal structure of RNase A bound to an AUAA DNA tetramer revealed the subsites that recognize nucleobases and phosphoryl groups (1rcn, top). The mainchain of RNase A is traced with a cartoon, key active-site and cysteine residues are shown as sticks, and ligands are shown as balls-and-sticks. Residues in subsites are colored blue (P_2), red (P_1), and green (P_0). A cartoon representation of the RNase A active site showing the preferred binder for each subsite (bottom). For simplicity, the P_{-1} subsite[12] is not shown.

Atypical nucleotides are among the best small-molecule inhibitors of RNase A. Diadenosine oligophosphates (Table 1, entries 5−7) are micromolar to submicromolar inhibitors that exhibit increasing affinity with longer phosphate chain lengths.[10] Additionally, the highest affinity small-molecule inhibitors of RNase A, pyrophosphate-linked dinucleotides (Table 1, entries 1−4), have enhanced inhibition activity upon further phosphorylation.[11] These observations prompted us to ask: can a simple oligophosphate on its own or appended to a single nucleoside serve as an effective small-molecule inhibitor of RNase A?

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Table 1. Inhibition Constants of Inorganic Phosphates and Nucleotides for RNase A

entry	compound	K_i (μM)[a]	ref
1	**dUppAp**	0.12	7
2	**pdUppAp**	0.027	7
3	**TppdA**	4	7
4	**pdTppAp**	0.041	7
5	**Ap$_3$A**	29	10
6	**Ap$_4$A**	2.6	10
7	**Ap$_5$A**	0.23	10
8	**P$_i$**	4600	40
9	**P$_{2i}$**	170	40
10	**P$_{3i}$**	23 ± 1	this work
11	**P$_{4i}$**	6.8 ± 0.2	this work
12	**cP$_{3i}$**	960 ± 80	this work
13	**cP$_{4i}$**	30 ± 0.8	this work
14	**cP$_{6i}$**	6.2 ± 0.1	this work
15	**pA**	170 ± 6	this work
16	**p$_2$A**	1.2	41
17	**p$_3$A**	0.86	10
18	**p$_4$A**	2.1 ± 0.2	this work
19	**p$_5$A**	1.4 ± 0.06	this work
20	**cp$_4$pA**	0.48 ± 0.03	this work
21	**ppAp**	0.24	7
22	**pU**	4000	42
23	**p$_2$U**	650	43
24	**p$_3$U**	8.3 ± 0.3	this work
25	**p$_4$U**	1.8 ± 0.1	this work
26	**p$_5$U**	0.068 ± 0.007	this work
27	**cp$_4$pU**	0.98 ± 0.07	this work

[a]Values from this work are reported ± the standard error of fitting a one-site binding equation.

In addition to canonical nucleoside mono-, di-, and triphosphates, nucleosides bearing longer oligophosphate chains are potent signaling molecules in biology.[14−18] These and other related morphologies, such as dinucleotide oligophosphates,[19−21] have been implicated in a variety of biological processes and ailments including hypertension[22] and bacterial accumulation of polyphosphate.[23−26] Nonetheless, the synthesis of oligophosphorylated compounds typically increases in difficulty with longer phosphate chains. Recently, methods have been developed to efficiently couple a triphosphate chain in one operation from trimetaphosphate ($P_3O_9^{3-}$).[14,27−32] Here, we extend this methodology to the tetraphosphorylation of biomolecules, utilizing tetrametaphosphate ($P_4O_{12}^{4-}$) to synthesize nucleoside tetraphosphates (**p$_4$N**) and nucleoside pentaphosphates (**p$_5$N**). Previous syntheses of **p$_4$N** have suffered from extremely low yields,[33] requiring nucleoside triphosphates as starting materials,[34] or iterative syntheses to add each additional phosphoryl group.[13,23] The state-of-the-art synthesis of **p$_4$N** involves coupling of trimetaphosphate and nucleoside monophosphates.[14] Here, we describe the facile synthesis of **p$_4$N** and **p$_5$N** by coupling tetrametaphosphate with nucleosides and nucleoside monophosphates, respectively (Figure 2). Utilizing tetraphosphorylation reagent **1** permits unprotected nucleosides to be converted to the corresponding **p$_4$N** efficiently in a single operation. This is in contrast to the method of Taylor,

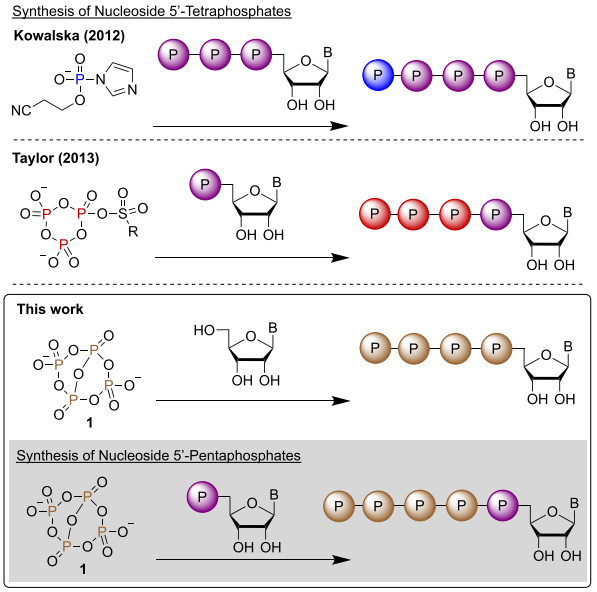

Figure 2. Synthesis of nucleoside tetraphosphates by Kowalska[13] and Taylor[14] compared to this synthesis of nucleoside tetra- and pentaphosphates.

which requires nucleoside monophosphates as the substrate.[14] While enzymatic methods have been reported,[35−37] the few reported chemical syntheses of **p$_5$N** have been limited in scope and low yielding.[23,33]

The activated tetrametaphosphate, [PPN]$_2$[**1**], is synthesized by protonation of tetrametaphosphate and subsequent dehydration.[38] Treatment of adenosine or uridine with [PPN]$_2$[**1**] under rigorously anhydrous conditions leads to selective phosphorylation of the 5′ position. No satisfactory purification could be found for the resulting nucleoside-substituted tetrametaphosphates (**cp$_4$N**, Figure 3), but treatment with [TBA][OH] results in ring opening to the linear tetraphosphates. HPLC purification in triethylammonium acetate buffer of the resulting mixtures gives adenosine tetraphosphate (**p$_4$A**, Table 1, entry 18, 93% yield) and uridine tetraphosphate (**p$_4$U**, Table 1, entry 25, 85% yield) as pure triethylammonium salts.

Nucleoside 5′-pentaphosphates were obtained similarly by treatment of [PPN]$_2$[**1**] with the anhydrous TBA salts of **pA** and **pU**. The intermediate nucleoside-monophosphate substituted tetrametaphosphates, **cp$_4$pN**, could be isolated in reasonable purity and were found to be stable in aqueous solution for several hours at room temperature before hydrolyzing to a mixture of nucleoside monophosphate, tetrametaphosphate, and nucleoside pentaphosphate. Treatment of **cp$_4$pN** with excess [TBA][OH] results in selective ring opening to **p$_5$N** in 24 h. The products were again purified by HPLC in triethylammonium acetate buffer, providing **p$_5$A** (Table 1, entry 19, 58% yield) and **p$_5$U** (Table 1, entry 26, 70% yield) as triethylammonium salts (Figure 3).

Reagent **1** is highly moisture sensitive and must be prepared, stored, and utilized in an anhydrous environment, ideally inside a glovebox. We therefore developed a second phosphorylation methodology for the syntheses of both **p$_4$N** and **p$_5$N**, activating [PPN]$_2$[$P_4O_{12}H_2$][38] *in situ* with dicyclohexylcarbodiimide (DCC) to form reagent **1**. These reagents are bench stable, and this methodology can be utilized

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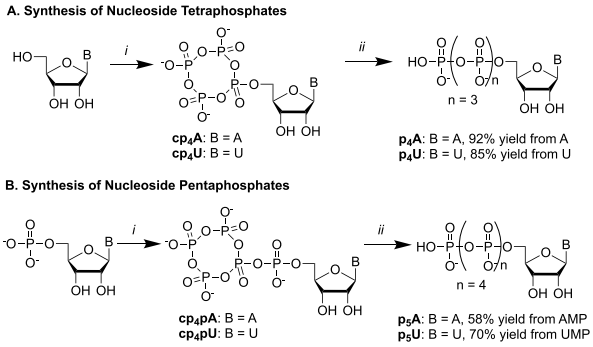

Figure 3. (A) *i.* $[PPN]_2[\mathbf{1}]$ (1.5 equiv) and triethylamine (2 equiv) (DMF, N_2 atmosphere, 48 h); *ii.* [TBA][OH] (7.5 equiv) (DMF/H_2O, 2 h) followed by HPLC (50 mM triethylammonium acetate (TEAA)). (B) *i.* $[PPN]_2[\mathbf{1}]$ (1.1 equiv) (DMF, N_2 atmosphere, 30 min); *ii.* [TBA][OH] (4.5 equiv) (DMF/H_2O, 24 h) followed by HPLC (50 mM TEAA).

conveniently with a Schlenk line, although it suffers from lower yields (SI Sections 2.2 and 2.5).

We performed inhibition kinetics using a fluorogenic substrate as described previously[39] to assess the binding of oligophosphates to RNase A. In addition to the synthesized molecules, $\mathbf{p_4N}$ and $\mathbf{p_5N}$, we assessed inhibition kinetics for a variety of inorganic phosphates to evaluate our hypothesis that longer oligophosphate chains increase binding affinity. Complementing previous reports of weak RNase A inhibition by orthophosphate ($\mathbf{P_i}$, Table 1, entry 8) and pyrophosphate ($\mathbf{P_{2i}}$, Table 1, entry 9), we tested $\mathbf{P_{3i}}$ and $\mathbf{P_{4i}}$ (Table 1, entries 10−11). The measured K_i values decrease for longer phosphates with a value of 23 μM for $\mathbf{P_{4i}}$. Although a longer inorganic oligophosphate may be a more potent inhibitor, each subsequent phosphoryl group has a diminished impact on lowering the K_i value. We similarly tested inorganic tri- ($\mathbf{cP_{3i}}$, Table 1, entry 12), tetra- ($\mathbf{cP_{4i}}$, Table 1, entry 13), and hexametaphosphate ($\mathbf{cP_{6i}}$, Table 1, entry 14) as these cyclic phosphates have been largely ignored in biological systems despite their indefinite stabilities near neutral pH. A similar trend was observed with increased inhibition of RNase A for longer oligophosphates and diminishing returns for each additional phosphoryl group. The metaphosphates are modestly less effective inhibitors than the corresponding linear phosphate.

Inhibition of RNase A by adenosine nucleotides does not follow the same simple trend as inorganic phosphates. Reported K_i values are given in Table 1, entries 15−19 for adenosine 5′-oligophosphates ranging from monophosphate (**pA**) to pentaphosphate ($\mathbf{p_5A}$). Inhibition increases from **pA** to the strongest inhibitor of this series, $\mathbf{p_3A}$ with a K_i value of 0.86 μM. $\mathbf{p_4A}$ and $\mathbf{p_5A}$ are somewhat less effective inhibitors with K_i values of 2.1 and 1.4 μM, respectively, indicating that the role of the oligophosphate chain in binding is not reducible simply to a Coulombic interaction. Furthermore, we tested the hydrolytically sensitive ultraphosphate $\mathbf{cp_4pA}$ and found that it was a superior inhibitor with a K_i value of 0.48 μM (Table 1, entry 20), suggesting that this unusual phosphate geometry is better able to target the active site.

In contrast to 5′-adenosine nucleotides, RNase A inhibition by 5′-uridine nucleotides follows a simple trend analogous to inorganic phosphates. In the series **pU** to $\mathbf{p_5U}$ (Table 1, entries 22−26), inhibition increases successively with longer oligophosphate chains, and the strongest inhibitor is $\mathbf{p_5U}$ with a K_i value of 0.068 μM. This mononucleotide approaches the potency of the best pyrophosphate-linked dinucleotide inhibitors of RNase A (Table 1, entries 2 and 4).[11] The ultraphosphate species $\mathbf{cp_4pU}$ is, however, a weaker inhibitor with a K_i value of 0.98 μM (Table 1, entry 27). Finally, we assessed the salt dependence of inhibition by $\mathbf{p_5U}$. Compared to a DNA tetramer AUAA, which exhibits a $\delta \log K_d/\delta \log[Na^+]$ of 2.3,[8] $\mathbf{p_5U}$ has a greater value of 3.2 (Figure S30), indicating a greater dependence on Coulombic interactions. Nonetheless, $\mathbf{p_5U}$ is among the most potent small-molecule inhibitors reported of RNase A and maintains high affinity at physiological salt concentrations.

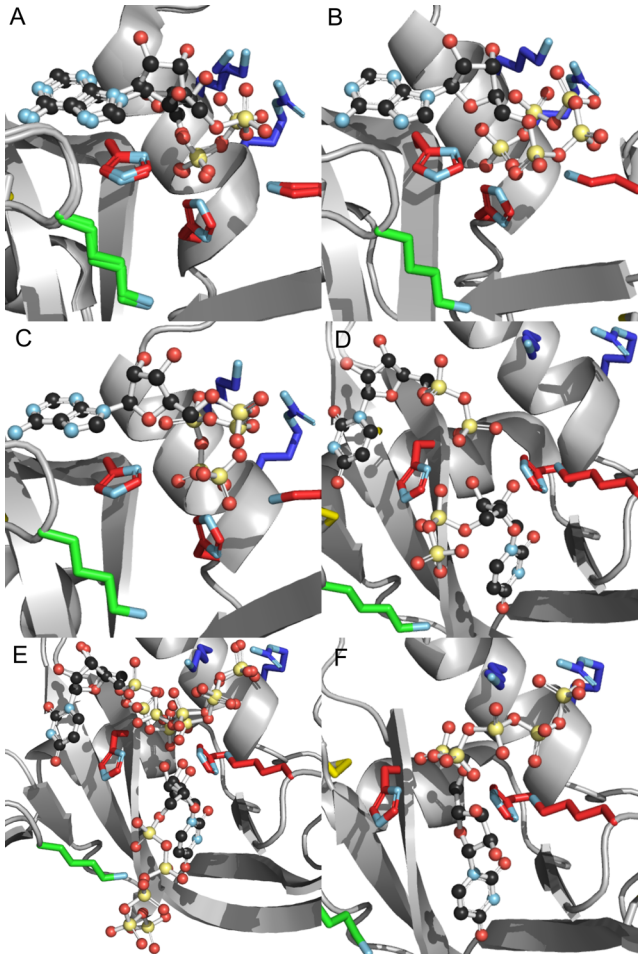

Figure 4. Interactions between nucleotides and the active site of RNase A. The structures are depicted as described in Figure 1. (A) **pA** and $\mathbf{p_3A}$ bind the active site by positioning the terminal phosphate group in the P_1 subsite with the adenosine base in the B_2 subsite (1z6s, 2w5g). (B) $\mathbf{p_5A}$ binds similarly to shorter adenosine nucleotides (6pvv). (C) $\mathbf{cp_4pA}$ binds similarly to $\mathbf{p_5A}$, but more efficiently targets Lys7 of the P_2 subsite (6pvw). (D) Two molecules of $\mathbf{p_2U}$ bind RNase A; however, only the B_1 subsite is efficiently targeted (3dxh). (E) Chain A of the RNase A·$\mathbf{p_5U}$ complex is similar to $\mathbf{p_2U}$ (6pvx). (F) In chain B, $\mathbf{p_5U}$ only binds in the B_1 subsite and alternatively targets the P_1 and P_2 subsites (6pvx).

To understand the differing trends in inhibition of RNase A by adenosine and uridine nucleotides, we solved X-ray cocrystal structures of several of these ligands bound to RNase A. The structure of bound $\mathbf{cP_{6i}}$, which is the first protein crystal structure to contain a metaphosphate, shows the ligand bound primarily in the P_1 subsite (Figure S31A). This is analogous to structures of bound $\mathbf{P_i}$ (5rsa)[44] and $\mathbf{P_{2i}}$ (2w5m),[45]

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suggesting that the binding of these inorganic phosphates is largely conserved. Differences in inhibition are attributable to Coulombic interactions and slight variations in hydrogen bonding.

The enhanced inhibition of $\mathbf{p_3A}$ over other adenosine nucleotides is illuminated by crystallography. In structures of both $\mathbf{p_3A}$ (2w5g, Figure 4A)[45] and $\mathbf{p_5A}$ (6pvv, Figure 4B), the terminal phosphoryl group of the oligophosphate chain binds in the P_1 subsite with hydrogen bonds to His12 and His119. For the longer oligophosphate in $\mathbf{p_5A}$ to target the same site requires the phosphate chain to form a loop. Apparently, the thermodynamic penalty associated with this constrained geometry is sufficient to overcome the greater Coulombic attraction between the enzyme and the more highly charged pentaphosphate, resulting in weaker inhibition. Furthermore, the strong inhibition by $\mathbf{cp_4pA}$ is attributable in part to more efficient targeting of Lys7 in this unusual phosphate geometry (6pvw, Figure 4C).

In 5′-uridine nucleotides, longer oligophosphate chains are not detrimental to inhibition of RNase A. Pyrimidine nucleobases are preferred in the B_1 subsite, but a previous structure of $\mathbf{p_2U}$ revealed occupancy of both the P_0/B_1 and P_1/B_2 subsites by a pair of these ligands (3dxh, Figure 4D). Chain A of the structure of $\mathbf{p_5U}$ bound to RNase A (6pvx, Figure 4E) shares this mode of recognition; however, only a single $\mathbf{p_5U}$ binds the P_1/B_1 subsites in chain B (Figure 4F). In both chains, the oligophosphates extend well beyond the phosphoryl group-binding subsites and can efficiently target several cationic regions of the active-site cleft. Thermodynamically unfavorable constraints are not imposed on the polyphosphate chain; therefore, longer, more highly charged oligophosphates could improve affinity of 5′-uridine nucleotides.

The role of polyphosphates in biological systems has come under increasing study. Here, we contribute to the synthetic methodology to create these species as well as perform analyses of their function. The activated tetrametaphosphate reagent $[PPN]_2[\mathbf{1}]$ is a useful synthetic tool for synthesizing polyphosphate chains of four units or longer by reaction with suitable anhydrous nucleophiles. Furthermore, intermediates containing substituted metaphosphates can, in some cases, be isolated as pure compounds that possess modest aqueous stability. We showed that binding of polyphosphorylated compounds to the active-site cleft of RNase A generally follows the simple trend that affinity increases with oligophosphate length, with 5′-adenosine oligophosphates being the notable exception. The limit of affinity enhancement conferred by lengthening the phosphate chain of 5′-uridine nucleotides remains to be determined, and we are working toward synthesizing $\mathbf{p_6U}$ as well as exploring phosphorylation on 3′-nucleotide positions. Furthermore, this work presents the first crystal structures of a metaphosphate (Figure S31A) or an ultraphosphate (Figure 4C) bound to a protein. This demonstrates that complex polyphosphate morphologies that have been largely excluded from consideration in aqueous media may in fact be relevant to biological systems, opening new avenues for biochemical studies and drug development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b09760.

Synthetic details, spectra, kinetic data, and crystallographic data collection and refinement statistics (PDF)

Accession Codes

Structure data for the new compounds are available from the Protein Database under the following PDB codes: RNase A·cP6i complex, 6pvu; RNase A·p5A complex, 6pvv; RNase A·cp4pA complex, 6pvw; RNase A·p5U complex, 6pvx.

■ AUTHOR INFORMATION

Corresponding Authors

*rtraines@mit.edu
*ccummins@mit.edu

ORCID

Scott M. Shepard: 0000-0001-8619-1300
Ian W. Windsor: 0000-0002-6289-6928
Ronald T. Raines: 0000-0001-7164-1719
Christopher C. Cummins: 0000-0003-2568-3269

Author Contributions

†S.M.S. and I.W.W. contributed equally.

Notes

The authors declare the following competing financial interest(s): The tetraphosphorylation reagent is covered in patent US10017388B2.

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