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

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Supporting Information

ABSTRACT: Adenosine and uridine 5′-tetra- and 5′-pentaphosphates were synthesized from an activated tetrametaphosphate ([PPN]2[P4O11]), [PPN]2[P1], PPN = bis(triphenylphosphine)iminium) and subsequently tested for inhibition of the enzymatic activity of ribonuclease A (RNase A). Reagent [PPN]2[P1] 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 (p4A, p4U) in 92% and 85% yields, respectively, from the starting nucleoside. Treatment of ([PPN]2[P1]) with AMP or UMP yields nucleoside-monophosphate tetrametaphosphates (cp,pA, cp,pU) having limited aqueous stability. Ring opening of these ultraphosphates with [TBA][OH] yields p5A and p5U 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 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 p5A binds in the P1 enzymic subsite and forces the oligophosphate to adopt a convoluted conformation, while the oligophosphate of p5U 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 and neurotoxic activities,5 and targeted inhibitors of these enzymes may have human therapeutic potential.6 RNase A binds its substrates in enzymic subsites that interact with phosphoryl groups and nucleobases (Figure 1).8,9

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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In addition to canonical nucleoside mono-, di-, and triphosphates, nucleosides bearing longer oligophosphate chains are potent signaling molecules in biology.14,15 These and other related morphologies, such as dinucleotide oligophosphates,19–21 have been implicated in a variety of biological processes and ailments including hypertension22 and bacterial accumulation of polyphosphate.23

The activated tetrametaphosphate, [PPN]2[P4O12H]38, is synthesized by protonation of tetrametaphosphate and subsequent dehydration.38 Treatment of adenosine or uridine with [PPN]2[P4O12H] results in selective phosphorylation of the 5′ position. No satisfactory purification could be found for the resulting nucleoside-substituted tetrametaphosphates (cp,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 (pA, Table 1, entry 18, 93% yield) and uridine tetraphosphate (pU, 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,N, 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,N with excess [TBA][OH] results in selective ring opening to p,N in 24 h. The products were again purified by HPLC in triethylammonium acetate buffer, providing pA (Table 1, entry 19, 58% yield) and pU (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,N and p,N, activating [PPN]2[P4O12H]38 in situ with dicyclohexylcarbodiimide (DCC) to form reagent 1. These reagents are bench stable, and this methodology can be utilized.

| entry | compound | Kᵢ (µM) | ref |
|-------|----------|---------|-----|
| 1     | dUppAp   | 0.12    | 7   |
| 2     | pdUppAp  | 0.027   | 7   |
| 3     | TppAp    | 4       | 7   |
| 4     | pdTppAp  | 0.041   | 7   |
| 5     | ApAp     | 29      | 10  |
| 6     | ApAp     | 2.6     | 10  |
| 7     | ApAp     | 0.23    | 10  |
| 8     | Pₖ       | 4600    | 40  |
| 9     | Pₙ       | 170     | 40  |
| 10    | Pₙ       | 23 ± 1  | this work |
| 11    | Pₙ       | 6.8 ± 0.2 | this work |
| 12    | cPₙ      | 960 ± 80 | this work |
| 13    | cPₙ      | 30 ± 0.8 | this work |
| 14    | cPₙ      | 6.2 ± 0.1 | this work |
| 15    | pA       | 170 ± 6 | this work |
| 16    | pₙA      | 1.1     | 41  |
| 17    | pₙA      | 0.86    | 10  |
| 18    | pₙA      | 2.1 ± 0.2 | this work |
| 19    | pₙA      | 1.4 ± 0.06 | this work |
| 20    | cpₙpₙA   | 0.48 ± 0.03 | this work |
| 21    | ppAp     | 0.24    | 7   |
| 22    | pU       | 4000    | 42  |
| 23    | pₙU      | 650     | 43  |
| 24    | pₙU      | 8.3 ± 0.3 | this work |
| 25    | pₙU      | 1.8 ± 0.1 | this work |
| 26    | pₙU      | 0.068 ± 0.007 | this work |
| 27    | cpₙpₙU   | 0.98 ± 0.07 | this work |

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

Figure 2. Synthesis of nucleoside tetraphosphates by Kowalska13 and Taylor14 compared to this synthesis of nucleoside tetra- and pentaphosphates.
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 previously39 to assess the binding of oligophosphates to RNase A. In addition to the synthesized molecules, pN and pN, 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 (P, Table 1, entry 8) and pyrophosphate (P, Table 1, entry 9), we tested P and P (Table 1, entries 10–11). The measured Ki values decrease for longer phosphates with a value of 23 μM for P. Although a longer inorganic oligophosphate may be a more potent inhibitor, each subsequent phosphoryl group has a diminished impact on lowering the Ki value. We similarly tested inorganic tri- (P, Table 1, entry 12), tetra- (P, Table 1, entry 13), and hexametaphosphate (P, 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 Ki values are given in Table 1, entries 15–19 for adenosine S′-oligophosphates ranging from monophosphate (PA) to pentaphosphate (pA). Inhibition increases from PA to the strongest inhibitor of this series, pA, with a Ki value of 0.86 μM. pA and pA are somewhat less effective inhibitors with Ki 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 pApA and found that it was a superior inhibitor with a Ki value of 0.25 μM (Table 1, entry 20), suggesting that this unusual phosphate geometry is better able to target the active site.

In contrast to S′-adenosine nucleotides, RNase A inhibition by S′-uridine nucleotides follows a simple trend analogous to inorganic phosphates. In the series PuU to PuU (Table 1, entries 22–26), inhibition increases successively with longer oligophosphate chains, and the strongest inhibitor is PuU with a Ki 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 pU is, however, a weaker inhibitor with a Ki value of 0.98 μM (Table 1, entry 27). Finally, we assessed the salt dependence of inhibition by pU. Compared to a DNA tetramer AUAA, which exhibits a dlogK/dlog[Na+]

![Figure 3](image3.png)

Figure 3. (A) i. [PPN]2[1] (1.5 equiv) and triethylamine (2 equiv) (DMF, N2 atmosphere, 48 h); ii. [TBA][OH] (7.5 equiv) (DMF/H2O, 2 h) followed by HPLC (50 mM triethylammonium acetate (TEAA)). (B) i. [PPN]2[1] (1.1 equiv) (DMF, N2 atmosphere, 30 min); ii. [TBA][OH] (4.5 equiv) (DMF/H2O, 24 h) followed by HPLC (50 mM TEAA).

![Figure 4](image4.png)

Figure 4. Interactions between nucleotides and the active site of RNase A. The structures are depicted as described in Figure 1. (A) PA and pA bind the active site by positioning the terminal phosphate group in the P1 subsite with the adenosine base in the B2 subsite (1z6s, 2w5g). (B) pA binds similarly to shorter adenosine nucleotides (6pvy). (C) pA binds similarly to pA but more efficiently targets Lys7 of the P2 subsite (6pvv). (D) Two molecules of pU bind RNase A; however, only the B1 subsite is efficiently targeted (3dhk). (E) Chain A of the RNase A-pU complex is similar to pU (6pvy). (F) In chain B, pU only binds in the B1 subsite and alternatively targets the P1 and P2 subsites (6pvy).
suggested 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 pU over other adenosine nucleotides is illuminated by crystallography. In structures of both pU (2w5g, Figure 4A) and pA (6pvx, Figure 4B), the terminal phosphoryl group of the oligophosphate chain binds in the P1 sub site with hydrogen bonds to His12 and His119. For the longer oligophosphate in pU 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 cp,pA is attributable in part to more efficient targeting of Lys7 in this unusual phosphate geometry (6pvw, Figure 4C).

In S'-uridine nucleotides, longer oligophosphate chains are not detrimental to inhibition of RNase A. Pyrimidine nucleobases are preferred in the B1 sub site, but a previous structure of pU revealed occupancy of both the P0/B1 and P1/B2 sub sites by a pair of these ligands (3dxh, Figure 4D). Chain A of the structure of pU bound to RNase A (6pvx, Figure 4E) shares this mode of recognition; however, only a single pU binds the P1/B2 sub sites in chain B (Figure 4F). In both chains, the oligophosphates extend well beyond the phosphoryl group-binding sub sites 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 S'-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 under increasing study. Here, we contribute to the synthetic nucleobases are preferred in the B1 subsite, but a previous 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**

2 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, 6pvw; RNase A·cp4A complex, 6pvx; RNase A·p5SU complex, 6pvx.

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**Notes**

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

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**REFERENCES**

(1) Lu, L.; Li, J.; Moussaoui, M.; Boix, E. Immune modulation by human secreted RNases at the extracellular space. *Front. Immunol.* 2018, 9, 1012.

(2) Sheng, J.; Xu, Z. Three decades of research on angiogenin: a review and perspective. *Acta Biochim. Biophys. Sin.* 2016, 48, 399–410.

(3) Cuchillo, C. M.; Nogués, M. V.; Raines, R. T. Bovine pancreatic ribonuclease: fifty years of the first enzymatic reaction mechanism. *Biochemistry* 2011, 50, 7835–7841.

(4) Marshall, G. R.; Feng, J. A.; Kuster, D. J. Back to the future: ribonuclease A. *Biopolymers* 2008, 90, 259–277.

(5) Olson, K. A.; Fett, J. W.; French, T. C.; Key, M. E.; Vallee, B. L. Angiogenin antagonists prevent tumor growth in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 1995, 92, 442–446.

(6) Gleich, G. J.; Loegering, D. A.; Bell, M. P.; Checkel, J. L.; Ackerman, S. J.; McKeen, D. J. Biochemical and functional similarities between human eosinophil-derived neurotoxin and eosinophil cationic protein: homology with ribonuclease. *Proc. Natl. Acad. Sci. U. S. A.* 1986, 83, 3146–3150.

(7) Russo, N.; Shapiro, R. Potent inhibition of mammalian ribonucleases by 3′,5′-pyrophosphate-linked nucleotides. *J. Biol. Chem.* 1999, 274, 14902–14908.

(8) Fisher, B. M.; Ha, J.-H.; Raines, R. T. Coulombic forces in protein-RNA interactions: binding and cleavage by ribonuclease A and variants at Lys7, Arg10, and Lys66. *Biochemistry* 1998, 37, 12121–12132.

(9) Fontecilla-Camps, J. C.; de Llorens, R.; Le Du, M.; Cuchillo, C. M. Crystal structure of ribonuclease A-d(ApTpApGpG) complex. Direct evidence for extended substrate recognition. *J. Biol. Chem.* 1994, 269, 21526–21531.

(10) Kumar, K.; Jenkins, J. L.; Jardine, A. M.; Shapiro, R. Inhibition of mammalian ribonucleases by endogenous adenosine dinucleotides. *Biochem. Biophys. Res. Commun.* 2003, 300, 81–86.

(11) Leonidas, D. D.; Shapiro, R.; Irons, L. I.; Russo, N.; Acharya, K. R. Toward rational design of ribonuclease inhibitors: High-resolution
crystal structure of a ribonuclease A complex with a potent 3′,5′-pyrophosphate-linked dinucleotide inhibitor. *Biochemistry* **1999**, *38*, 10287–10297.

(12) Fisher, B. M.; Grilley, J. E.; Raines, R. T. A new remote subsite in ribonuclease A. *J. Biol. Chem.* **1998**, *273*, 34134–34138.

(13) Strenkowski, M.; Wanat, P.; Ziemniak, M.; Jemioly, J.; Kowalska, J. Preparation of synthetically challenging nucleotides using cyanoethyl P-imidazolides and microwaves. *Org. Lett.* **2012**, *14*, 4782–4785.

(14) Mohamady, S.; Taylor, S. D. Synthesis of nucleoside tetraphosphates and dinucleoside pentaphosphates via activation of cyclic trimetaphosphate. *Org. Lett.* **2013**, *15*, 2612–2615.

(15) R. Kore, A.; Yang, B.; Srinivasan, B.; Conrad, R. Chemical and enzymatic synthesis of nucleoside tetraphosphates. *Curr. Org. Chem.* **2014**, *18*, 1621–1650.

(16) Jankowski, V.; Tolle, M.; Vanholder, R.; Schonfelder, G.; van der Giet, M.; Henning, L.; Schlotter, H.; Paul, M.; Zidek, W.; Jankowski, J. Uridine adenosine tetraphosphate: a novel endothermally derived vasorestrictive factor. *Nat. Med.* **2005**, *11*, 223.

(17) Han, Q.; Gaflney, B. L.; Jones, R. A. One-flask synthesis of dinucleoside tetra and pentaphosphates. *Org. Lett.* **2006**, *8*, 2075–2077.

(18) Sundaralingam, M. Stereochemistry of nucleic acids and their constituents. IV. Allowed and preferred conformations of nucleosides, nucleoside mono-, di-, tri-, tetraphosphates, nucleic acids and polynucleotides. *Biopolymers* **1969**, *7*, 821–860.

(19) Jovanovic, A.; Jovanovic, S.; Mays, D. C.; Lipsky, J. J.; Terzić, A. Diadenosine 5′,5′-P2O7-P5′-pentaphosphate harbors the properties of a signaling molecule in the heart. *FEBS Lett.* **1998**, *423*, 314–318.

(20) Luo, J.; Jankowski, V.; Gungar, N.; Neumann, J.; Schmitz, W.; Zidek, W.; Schlotter, H.; Jankowski, J. Endogenous diadenosine tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate in human myocardial tissue. *Hypertension* **2004**, *43*, 1055–1059.

(21) Miras-Portugal, M. T.; Gualix, J.; Pintor, J. The neurotransmitter role of diadenosine polyphosphates. *FEBS Lett.* **1998**, *430*, 78–82.

(22) Matsumoto, T.; Goulopoulou, S.; Taguchi, K.; Tostes, R. C.; Kobayashi, T. Constrictor prostanoids and uridine adenosine tetraphosphate: vascular mediators and therapeutic targets in hypertension and diabetes. *Br. J. Pharmacol.* **2015**, *172*, 3980–4001.

(23) Mordhorst, S.; Singh, J.; Mohr, M. K.; Hinkelmann, R.; Keppler, M.; Jessen, H. J.; Andexer, J. N. Several polyphosphate kinase inhibitors catalyze the production of adenosine 5′-polyphosphates. *ChemBioChem* **2019**, *20*, 1019–1022.

(24) Bhandari, R.; Sairadi, A.; Ahmadibeni, Y.; Snowman, A. M.; Resnick, A. C.; Kristiansen, T. Z.; Molina, H.; Pandey, A.; Werner, J. K.; Juluri, K. R.; Xu, Y.; Prestwich, G. D.; Parang, K.; Snyder, S. H. Protein pyrophosphorylation by inositol pyrophosphates is a protein modification with key roles in cellular signaling. *Org. Lett.* **2015**, *17*, 1041–1044.

(25) Azevedo, C.; Sanghvi, S.; Steck, N.; Hofer, A.; Ripp, A.; Captain, I.; Keller, M.; Wender, P. A.; Bhandari, R.; Jessen, H. J. A phosphoramidite analogue of cyclotriphosphophate enables iterative polyphosphorylations. *Angew. Chem. Int. Ed.* **2019**, *58*, 3928–3933.

(31) Fernandes-Cunha, G. M.; McKinlay, C. J.; Vargas, I. R.; Jessen, H. J.; Waymouth, R. M.; Wender, P. A. Delivery of inorganic polyphosphate into cells using amphiphatic oligocarbonate transporters. *ACS Cent. Sci.* **2018**, *4*, 1394–1402.

(32) Azevedo, C.; Singh, J.; Steck, N.; Hofer, A.; Ruiz, F. A.; Singh, T.; Jessen, H. J.; Sairadi, A. Screening a protein array with synthetic biotinylated inorganic polyphosphate to define the human polyP-ome. *ACS Chem. Biol.* **2018**, *13*, 1958–1963.

(33) Ko, H.; Carter, R. L.; Cosyn, L.; Petrelli, R.; de Castro, S.; Besada, P.; Zhou, Y.; Cappellacci, L.; Franchetti, P.; Grimantini, M.; Calenbergh, S. V.; Harden, T. K.; Jacobson, K. A. Synthesis and potency of novel uracil nucleotides and derivatives as P2Y2 and P2Y6 receptor agonists. *Bioorg. Med. Chem.* **2008**, *16*, 6319–6332.

(34) Zuberek, J.; Jemioly, J.; Jablonowska, A.; Stepinski, J.; Dadlez, M.; Stolarski, R.; Dzarnyikiewicz, E. Influence of electric charge variation at residues 209 and 159 on the interaction of eIF4E with the mRNA S′ terminus. *Biochemistry* **2004**, *43*, 5370–5379.

(35) Guranowski, A.; Siller, M. G.; Siller, A. Adenosine S′tetraphosphate and adenosine S′-pentaphosphate are synthesized by yeast acetyl coenzyme A synthetase. *J. Bacteriol.* **1994**, *176*, 2986–2990.

(36) Guranowski, A.; de Diego, A.; Siller, A.; Günther Siller, M. A. Uridine S′-polyphosphates (pU and pU) and uridine(S′)-polyphospho(S′)nucleotides (Up, Ns) can be synthesized by UTP: glucose-1-phosphate uridylyltransferase from *Saccharomyces cerevisiae*. *FEBS Lett.* **2004**, *561*, 83–88.

(37) Nakajima, H.; Tomioka, I.; Kitabatake, S.; Dombou, M.; Tomita, K. Facile and selective synthesis of diadenosine polyphosphates through catalysis by leucyl tRNA synthetase coupled with ATP regeneration. *Agric. Biol. Chem.* **1989**, *53*, 615–623.

(38) Jiang, Y.; Chakarawet, K.; Kohout, A. L. M.; Nava, A.; Marino, N.; Cummins, C. C. Dihydropyrimidinase tetraphosphate, [P 4O12H2]2− synthesis, solubilization in organic media, preparation of its anhydride [P 4O12]− and acidic methyl ester, and conversion to tetraphosphate metal complexes via protonolysis. *J. Am. Chem. Soc.* **2014**, *136*, 11894–11897.

(39) Kelemen, B. R.; Klink, T. A.; Behike, M. A.; Eubanks, S. R.; Leland, P. A.; Raines, R. T. Hypersensitive substrate for ribonucleases. *Nucleic Acids Res.* **1997**, *25*, 3696–3701.

(40) Anderson, D. G.; Hammes, G. G.; Walz, F. G. Binding of phosphate ligands to ribonuclease A. *Biochemistry* **1968**, *7*, 1637–1645.

(41) Russo, N.; Shapiro, R.; Valle, B. L. S′-Diphosphoadenosine 3′ phosphate is a potent inhibitor of bovine pancreatic ribonuclease A. *Biochem. Biophys. Res. Commun.* **1997**, *231*, 671–674.

(42) Tsirkone, V. G.; Dossi, K.; Drakou, C.; Zographos, S. E.; Kontou, M.; Leonidas, D. D. Inhibitor design for ribonuclease A: the binding of two S′-phosphate uridine analogues. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **2009**, *65*, 671–677.

(43) Dossi, K.; Tsirkone, V. G.; Hayes, J. M.; Matoušek, J.; Poučková, P.; Soucek, J.; Zadínová, M.; Zographos, S. E.; Leonidas, D. D. Mapping the ribonucleolytic active site of bovine seminal ribonuclease. The binding of pyrimidyl phosphonucleotide inhibitors. *Eur. J. Med. Chem.* **2009**, *44*, 4496–4508.

(45) Holloway, D. E.; Chavali, G. B.; Leonidas, D. D.; Baker, M. D.; Acharya, K. R. Influence of naturally-occurring 5′-pyrophosphate-linked substrates on the binding of adenylic inhibitors to ribonuclease A: an X-ray crystallographic study. *Biopolymers* **2009**, *89*, 1005–1008.