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Synthesis of boranoate, selenoate, and thioate analogs of AZTp$_4$A and Ap$_4$A

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ABSTRACT

We report efficient, one-flask procedures for the synthesis of a family of 14 analogs of AZTp$_4$A and Ap$_4$A containing BH_3, S, or Se, along with two bisphosphonate analogs of Ap$_4$A. These compounds should slow unwanted enzymatic hydrolysis and have the potential to create unique binding interactions in biochemical and structural studies of the excision reaction responsible for resistance of HIV-1 to AZT, as well as assist in drug design.

1. Introduction

Resistance by human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) to the nucleoside inhibitor 3′-azido-3′-deoxythymidine (AZT) has been a serious problem for many years.[1–3] After the chain-terminating AZT is incorporated into the DNA strand, it can be excised through nucleophilic attack by cellular adenosine triphosphate (ATP), thereby unblocking the DNA while creating the dinucleoside tetraphosphate, 5′-5″-AZTp$_4$A.[4,5] This excision occurs at the catalytic center of RT, where mutations that are known to enhance this reaction create a new hydrophobic pocket that binds the adenine ring of ATP. AZTp$_4$A has recently been shown to be a potent chain-terminating inhibitor of RT, and mutant RTs that had become AZT-resistant showed stronger inhibition of excision than wild-type RT.[6] Because RT continues to mutate with chronic drug treatment, a continuing stream of new drug candidates is needed, and the AZTp$_4$A analogs described here may provide useful therapeutic leads.

As part of our program in developing new synthetic methods for dinucleoside polyphosphates and their analogs,[7,8] we now report the synthesis of a series of AZTp$_x$pppA analogs, **6**–**8**, containing a single modified phosphate adjacent to the AZT: both diastereomers of a boranoate, **6**, a thioate, **7**, and a selenoate, **8**, as well as **16** with a thioate adjacent to the adenosine. We had previously described a series of related hydrolysis-resistant analogs with thioates adjacent to each nucleoside and a methylene or difluoromethylene in place of the central phosphate.[8] Preliminary experiments, however, suggest that analogs with bisphosphonates do not appear to be substrates for HIV-1 RT.[9] Fourteen of the 16 new compounds reported here retain the four bridging phosphate oxygen atoms that may be essential for coordination to Mg^{2+} ions in the RT active site, but represent a range of phosphate modifications adjacent to the AZT that might result in improved inhibition. We also report the related Ap$_x$pppA analogs. These compounds were important in defining the stereochemistry of the AZTp$_x$pppA analogs, as described below, and are of interest themselves because Ap$_4$A has been proposed as a signaling and regulatory molecule for many biological functions.[10]

2. Results and discussion

Nucleic acid boranophosphates were pioneered by Shaw,[11] and they are receiving increasing attention for therapeutics and diagnostics.[12–14] Thiophosphates are well known to display altered interactions with other species, including metals.[15] Selenophosphates[16] are less well known, but have recently been used for nucleic acid structure determination.[17] Eckstein originally reported the synthesis of nucleoside triphosphates and α-thio-nucleoside triphosphates by hydrolysis or sulfurization, respectively, of

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a trimetaphosphate intermediate.[18] Shaw later modified this method to prepare α-borano-nucleoside triphosphates,[19] and more recently, α-thio, seleno-, and borano-nucleoside diphosphates.[20] α-Borano- and seleno nucleoside triphosphates containing further modifications have also been made using trimetaphosphate intermediates.[21–23] In our original method for synthesis of unmodified dinucleoside tetraphosphates,[7] a protected nucleoside (**1**) was phosphitylated with 2-chloro-4*H*-1,3,2-benzo-dioxaphosphorin-4-one in DMF to give **2** (Scheme 1). This intermediate was then reacted with pyrophosphate as the tri-*n*-butylammonium salt to form **3**, followed by oxidation with I_2/H_2O to give the trimetaphosphate (**4**), a sequence first described by Eckstein.[18] Instead of subsequent hydrolysis as in Eckstein's method, we then added a nucleoside 5′-monophosphate (**5**) in DMF with $ZnCl_2$ as a catalyst to give unmodified dinucleoside tetraphosphates in 50–85% yields after removal of the protecting groups.

We report here a series of 16 modified dinucleoside tetraphosphates, listed in Table 1, that were made by expanding our approach as shown in Scheme 1 and described below. Synthetic procedures are detailed in the Experimental section with full spectral characterizations for all compounds given in Supplementary data.

Table 1
Analogs of $AZTp_4A$ and Ap_4A

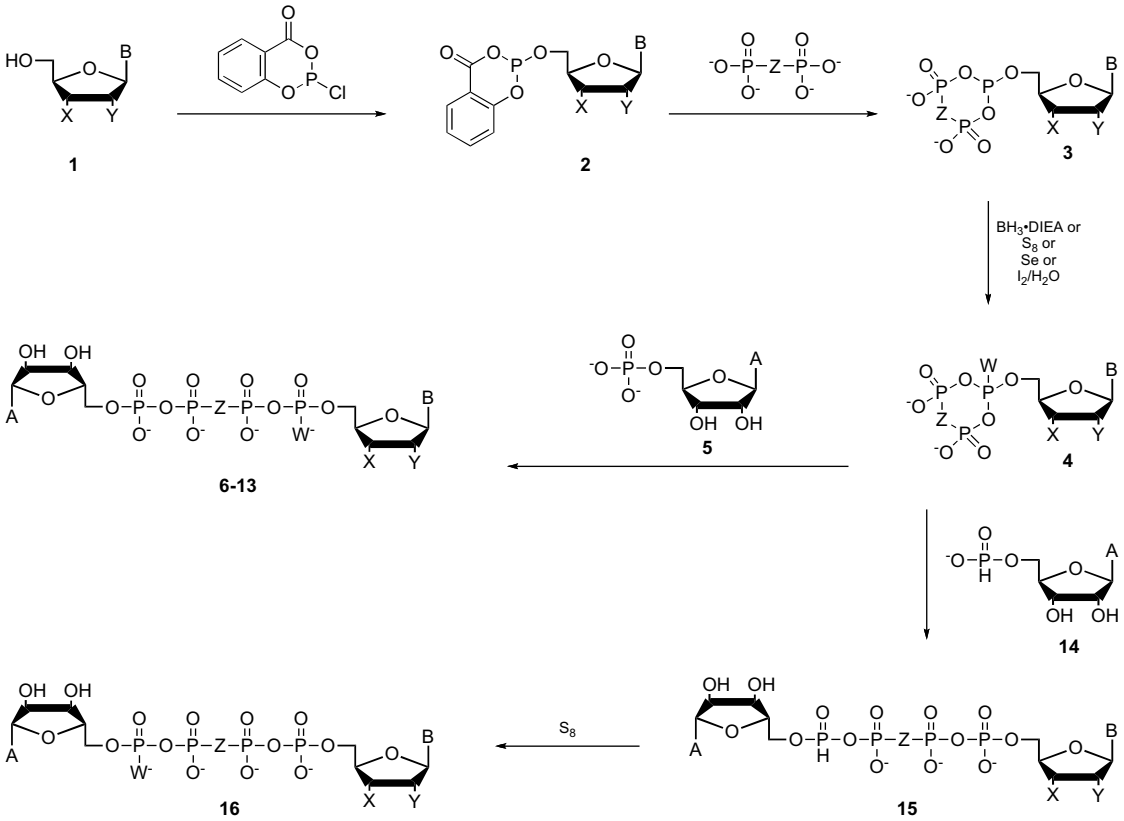
	B	X	Y	Z	W	%[a]	%[b]
[***R***]-**6a**	T	N_3	H	O	BH_3	36	66
[***S***]-**6b**	T	N_3	H	O	BH_3	30	
[***S***]-**7a**	T	N_3	H	O	S	39	75
[***R***]-**7b**	T	N_3	H	O	S	36	
[***S***]-**8a**	T	N_3	H	O	Se	29	57
[***R***]-**8b**	T	N_3	H	O	Se	28	
[***R***]-**9a**	A	OH	OH	O	BH_3	40	66
[***S***]-**9b**	A	OH	OH	O	BH_3	26	
[***S***]-**10a**	A	OH	OH	O	S	36	66
[***R***]-**10b**	A	OH	OH	O	S	30	
[***S***]-**11a**	A	OH	OH	O	Se	21	38
[***R***]-**11b**	A	OH	OH	O	Se	17	
12	A	OH	OH	CH_2	O	—	80
13	A	OH	OH	CF_2	O	—	82
[***S***]-**16a**	T	N_3	H	O	S	33	76
[***R***]-**16b**	T	N_3	H	O	S	43	

A=adenine; T=thymine; %.
[a] Yield of individual diastereomer; %.
[b] Combined yield of both diastereomers.

2.1. Boranoates, thioates, and selenoates

As originally described by Eckstein,[18] the oxidation of **3** can employ elemental sulfur (S_8) rather than I_2/H_2O. The resulting sulfurized trimetaphosphate (**4**, W=S) is stable in the absence of water, and we find that it reacts readily with adenosine monophosphate (**5**, AMP) in good yield to form a modified dinucleoside tetraphosphate with a single thiophosphate adjacent to the nucleoside derived from the trimetaphosphate, AZT. By this method, we have obtained $AZTp_spppA$ (**7**) in 75% yield. We are able to completely separate the two diastereomers by reverse-phase chromatography and identify their stereochemistry, as described in a later section. The analog $AZTpppp_sA$ (**16**) was made by oxidation of **3** using a minimal amount of I_2/H_2O, followed by coupling with adenosine H-phosphonate (**14**) and sulfurization.

We have found that we can introduce Se or a BH_3 by a similar reaction of the AZT trimetaphosphate (**3**), although the reaction requires longer time. The selenization step using elemental



A = adenine; B = adenine or thymine; W = BH_3 or S or Se or O; X = N_3 or OH; Y = H or OH; Z = O or CH_2 or CF_2

Scheme 1. Synthesis of $AZTp_4A$ and Ap_4A analogs.

selenium[20] in toluene requires 7 h, and subsequent reaction with AMP gives AZTp$_{Se}$pppA (**8**) in 57% yield. Boranation using a borane-*N*,*N*-diisopropylethylamine complex (BH_3·DIPEA)[19,21,22] requires 16 h, even with 17 equiv of reagent. Subsequent reaction with AMP gives AZTp$_{BH3}$pppA (**6**) in 66% yield. Reaction of the boranoated trimetaphosphate (**4**) with AMP appears to give better results using $MgCl_2$ as a catalyst compared to $ZnCl_2$, while the other trimetaphosphates all give higher yields with $ZnCl_2$. We have separated the diastereomers in each set by reverse-phase chromatography.

The retention times on reverse-phase chromatography for these tetraphosphates with single modifications increase in the order O<S<Se∼BH_3. Further, the ^{31}P NMR resonances of the modified phosphorus atoms move downfield in the order O (∼−11 ppm)<Se (∼34 ppm)<S (∼43 ppm)<BH_3 (∼87–80 ppm), with the boranoate phosphorus resonances being quadrupole-broadened.

The boranoates may be of particular interest, since in general, they are known to display greater resistance to hydrolysis by cellular enzymes than thioates.[11] Because the borano group is isoelectronic with oxygen and pseudoisoelectronic with sulfur, it is negatively charged like phosphates and thioates. However, its properties are altered in several ways. It does not have lone pairs of electrons that can participate in hydrogen bonding or metal interactions; it increases hydrophobicity; and the P–B bond is less polarized than P–O and P–S bonds.[11] Canard has shown that the *R* diastereomer (shorter retention time) of α-borano-AZT triphosphate not only inhibits reverse transcription, but also decreases the excision rate.[13] A group from Biota, Inc. extended this work by synthesizing α-borano-β,γ-difluoromethylene-AZT triphosphate.[22] They showed that its *R* diastereomer, but not the *S*, was as effective an RT inhibitor as *R*-α-borano-AZT triphosphate, while it had considerably more stability in serum and cell extracts.[22] They found that the diastereomer with shorter retention time of α-seleno-β,γ-difluoromethylene-AZT triphosphate was a potent inhibitor of HIV-RT as well.[23]

We have also made a series of Ap_4A analogs with a single boranoate, Ap$_{BH3}$pppA (**9**) in 66% yield, thioate, Ap$_S$pppA (**10**) in 66% yield, and selenoate, Ap$_{Se}$pppA (**11**) in 38% yield. A compilation of a number of Ap_4A analogs has recently been reported.[24] Blackburn had previously synthesized a large group of bisphosphonate analogs of Ap_4A.[25]

2.2. Bisphosphonates

Analogs containing a central bisphosphonate, App$_{CX2}$ppA (**12**, X=H; **13**, X=F), were made in similar fashion in good yields using either methylenediphosphonate ($O_3PCH_2PO_3^{4-}$) or difluoromethylenediphosphonate ($O_3PCF_2PO_3^{4-}$)[26] in place of pyrophosphate. This variation of the trimetaphosphate approach had previously been demonstrated for triphosphate analogs.[22] The ^{31}P NMR chemical shifts of the phosphonate resonances are ∼8 ppm with CH_2 and ∼−6 ppm with CF_2, compared to ∼−22 ppm with oxygen (Supplementary data).

2.3. Assignment of stereochemistry

We have assigned the configurations of the diastereomers based on their relative retention times on reverse-phase chromatography and, where possible, their relative susceptibility to hydrolysis by snake venom phosphodiesterase. Eckstein had originally demonstrated that the *R* diastereomer of α-thioadenosine triphosphate was cleaved significantly faster than the *S* diastereomer.[27] Further, he found that the *R* diastereomer had a longer retention time on reverse-phase HPLC. Blackburn and McLennan used this selective enzymatic hydrolysis approach to assign configurations of the three diastereomers of Ap$_S$p$_{CH2}$pp$_S$A.[28] Canard has used X-ray crystallography to determine the absolute configuration of α-boranothymidine diphosphate complexed with nucleoside diphosphate kinase,[13] and Shaw has correlated this assignment with relative retention time.[29] Because the group priorities around the chiral phosphorus of a boranophosphate are opposite of those around thiophosphates, the designation of *R* and *S* are reversed for a given absolute configuration in these two families of analogs. Thus the *R* diastereomer of α-boranothymidine diphosphate has a shorter retention time than the *S* version.[29] Shaw[20] and the Biota Inc. group[22] have used this correlation of retention time and stereochemistry to make strong arguments for assignments of other boranoate and selenoate analogs.

The monosubstituted analogs containing AZT (**6**–**8** and **16**) all gave poorly resolved chromatograms upon enzymatic digestion with snake venom phosphodiesterase due to the broad peak of phosphorylated AZT. Therefore, for these cases we have assigned the configurations of their diastereomers based on retention time. For the thioates (**7** and **16**) as well as the selenoate (**8**) we assign the diastereomers that elute first on both analytical and preparative reverse-phase chromatography (listed first in Table 1), as *S*. For the boranoate (**6**) the diastereomer that elutes first (listed first in Table 1) has the same absolute configuration as the *S* thioates/selenates but is assigned *R*, because of the opposite priorities of boron with respect to sulfur/selenium, as described above.

The assignments described above were strongly supported by enzymatic degradation results for the closely related monosubstituted analogs of Ap_4A (**9**–**11**), which gave sufficient resolution of intermediates on analytical HPLC to allow the cleavage to be monitored. We digested 0.63 mM samples of each diastereomer of **9**–**11** with 0.2 mg of snake venom phosphodiesterase. These compounds were all quickly cleaved at their unmodified outer phosphates to form modified adenosine triphosphates that retained the chiral center at the α modifications. For these cases, we plotted the % triphosphate remaining, determined by HPLC, as a function of time (Fig. 1). The results were in agreement with the assignments based on HPLC retention time.

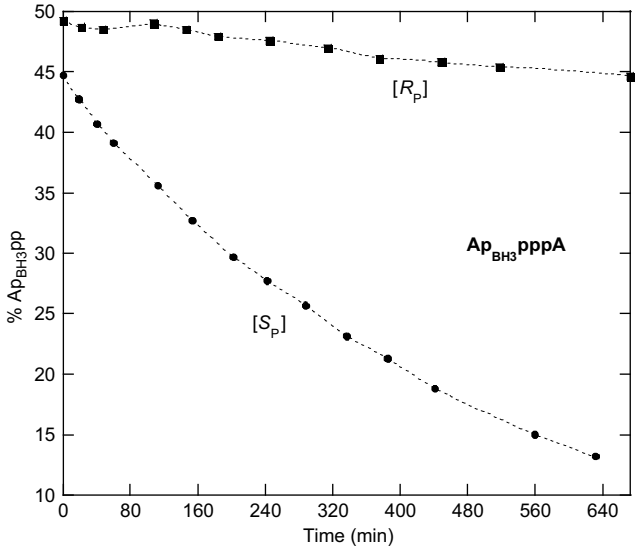


Figure 1. Disappearance of Ap$_{BH3}$pp with enzymatic degradation of Ap$_{BH3}$pppA (**9**).

3. Conclusion

The syntheses we describe here, together with the routes we previously reported,[7,8] complete our elaboration of a general and convenient one-flask approach to the preparation of dinucleoside tetraphosphates and analogs. At least some of these compounds

will be useful in further study of the AZT excision reaction, signaling, and drug design.

4. Experimental section

4.1. General procedures

Methylenediphosphonic acid, 2-chloro-4*H*-1,3,2-benzo-dioxaphosphorin-4-one, tri-*n*-butylamine, adenosine monophosphate monohydrate, elemental selenium, and borane-*N*,*N*-diisopropylethylamine complex were purchased from Aldrich. Elemental sulfur was purchased from J.T. Baker. AZT was obtained from Transgenomic. Bis(tri-*n*-butylammonium) pyrophosphate was prepared according to the procedure described by Eckstein,[30] bis(tri-*n*-butylammonium) difluoromethylenediphosphonate was prepared by the procedure reported by Boyle,[31] and adenosine 5′-*H*-phosphonate was prepared by a procedure developed in the Jones Lab.[32] The preparative reverse-phase HPLC was performed on a PrepPak system with three Waters Delta-Pak PrepPak® cartridges (C18, 300 Å, 40 mm×100 mm, 15 μm) using acetonitrile and 0.1 M ammonium bicarbonate (pH 7.5) or water. The analytical samples were analyzed on a Waters Atlantis™ analytical column (C18 column, 100 Å, 4.6 mm×50 mm, 3.0 μm) using acetonitrile and 0.1 M triethylammonium acetate (TEAA) buffer (pH 6.8), and the flow rate is 1.0 mL/min.

4.2. NMR spectra

The NMR spectra were acquired on Varian 300 or 400 spectrometers. The samples were all converted to the sodium form and dissolved in D_2O. The ^1H and ^{13}C spectra were referenced to 3-(trimethylsilyl)-1-propane-sulfonic acid, sodium salt. Signals in the ^{13}C spectra at 57.1, 21.8, 17.7, and 0.0 are from this standard. The ^{31}P spectra were referenced to 10% phosphoric acid in D_2O. The ^{19}F spectra were referenced to hexafluorobenzene. In the ^1H NMR spectra, water suppression diminishes the adjacent resonances. UV spectra were acquired on a Varian Cary 4000 or an Aviv 14 UV spectrophotometer, and the MS acquired on a Waters/Micromass Platform LCZ mass spectrometer.

4.3. [R_P] and [S_P] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^4-borano)-tetraphosphate (6a,b)

To a solution of 3′-azido-3′-deoxythymidine (0.087 g, 0.33 mmol) in 2 mL of anhydrous *N*,*N*-dimethylformamide (DMF) was added 2-chloro-4*H*-1,3,2-benzo-dioxaphosphorin-4-one (0.106 g, 0.36 mmol, 1.1 equiv). The solution was stirred for 15 min at room temperature under N_2. A 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in anhydrous DMF (1.3 mL, 0.65 mmol, 2.0 equiv) was vortexed with tri-*n*-butylamine (0.60 mL, 2.5 mmol, 7.6 equiv) and immediately added to the reaction mixture. After 20 min, borane-*N*,*N*-diisopropylethylamine complex (1.0 mL, 5.8 mmol, 18 equiv) was added. After 16 h, adenosine monophosphate monohydrate, in the proton form (0.68 g, 1.9 mmol, 5.8 equiv), was dissolved in a 40% tetrabutylammonium hydroxide aqueous solution (2.5 mL, 3.8 mmol, 12 equiv), dried by evaporation of pyridine and DMF, and added to the reaction with stirring. Immediately $MgCl_2(H_2O)_6$ (0.40 g, 2.0 mmol, 5.9 equiv) that had been dried by evaporation of pyridine and DMF was added. The reaction was stirred for 4 h and then 20 mL of water was added. The resulting precipitate was removed by filtration, and the filtrate was washed with 3×30 mL of CH_2Cl_2. The aqueous solution was concentrated and applied to a column of sodium cation-exchange resin (50WX2, 10 mL, 6.0 mmol). The mixture of two diastereomers was concentrated, purified, and separated by reverse-phase HPLC to give 0.11 g of [R_P]-**6a** (0.12 mmol, 36%) and 0.088 g of [S_P]-**6b** (0.098 mmol, 30%) as the ammonium salts. The products eluted in the order listed. LC–MS in negative mode, with *m*/*z* (M−1) 833.37 for [R_P]-**6a** and 833.31 for [S_P]-**6b** (calculated for $C_{20}H_{30}BN_{10}O_{18}P_4^-$: 833.08). [$R_P$]-**6a**: ^{31}P NMR (D_2O, 121 MHz): δ 86.00–81.00 (br, 1P), (−)10.40–(−)11.07 (m, 1P), (−)21.75–(−)22.81 (m, 2P). [S_P]-**6b**: ^{31}P NMR (D_2O, 121 MHz): δ 86.75–79.77 (br, 1P), (−)10.78–(−)11.15 (m, 1P), (−)22.36–(−)23.13 (m, 2P).

4.4. [S_P] and [R_P] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^4-thio)-tetraphosphate (7a,b)

Starting with 3′-azido-3′-deoxythymidine (0.087 g, 0.33 mmol), intermediate **3** was prepared by the same procedure described for **6a**,**b**. After 20 min excess S_8 (0.037 g, 1.2 mmol, 3.6 equiv) that had been dried by evaporation of pyridine and DMF was added. After 15 min, a mixture of AMP, in the proton form (0.44 g, 1.2 mmol, 3.6 equiv), and zinc chloride (0.43 g, 3.2 mmol, 9.7 equiv) that had been dried together by evaporation of pyridine and DMF was added with stirring. After 16 h, 10% aqueous ammonia (20 mL, 118 mmol) was added, and the precipitated sulfur was removed by filtration. The basic filtrate was applied to a column of sodium cation-exchange resin (50WX2, 10 mL, 6.0 mmol) to remove Zn^{2+}. The eluate was concentrated and the two diastereomers were purified and separated by reverse-phase HPLC to give 0.12 g of [S_P]-**7a** (0.13 mmol, 39%) and 0.11 g of [R_P]-**7b** (0.12 mmol, 36%) as the ammonium salts. The products eluted in the order listed. LC–MS in negative mode, with *m*/*z* (M-1) 851.54 for [S_P]-**7a** and 851.54 for [R_P]-**7b** (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02). [$S_P$]-**7a**: ^{31}P NMR (D_2O, 121 MHz): δ 43.29 (d, *J*=24.33 Hz, 1P), −10.98 (d, *J*=18.07 Hz, 1P), −22.74 (t, *J*=17.35 Hz, 1P), −23.68 (dd, *J*=24.32 Hz, *J*=16.44 Hz, 1P). [R_P]-**7b**: ^{31}P NMR (D_2O, 121 MHz): δ 43.20 (d, *J*=25.87 Hz, 1P), −10.97 (d, *J*=18.07 Hz, 1P), −22.73 (t, *J*=17.33 Hz, 1P), −23.71 (dd, *J*=25.80 Hz, *J*=16.45 Hz, 1P).

4.5. [S_P] and [R_P] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^4-seleno)-tetraphosphate (8a,b)

Starting with 3′-azido-3′-deoxythymidine (0.081 g, 0.30 mmol), **8a**,**b** were prepared by the same procedure described for **7a**,**b**, except that a dry solution of Se (0.072 g, 0.91 mmol, 3.0 equiv) in 30 mL of toluene was used to oxidize **3** (7 h) instead of S_8. The reaction solution was concentrated to remove toluene before the ammonia treatment (20 mL, 118 mmol) and was filtered to remove excess Se after the ammonia treatment. The mixture of two diastereomers was concentrated, purified, and separated by reverse-phase HPLC to give 0.085 g of [S_P]-**8a** (0.088 mmol, 29%) and 0.082 g of [R_P]-**8b** (0.085 mmol, 28%) as the ammonium salts. The products eluted in the order listed. LC–MS in negative mode, with *m*/*z* (M−1) 899.34 for[S_P]-**8a** and 899.47 for [R_P]-**8b** (calculated for $C_{20}H_{27}N_{10}O_{18}P_4Se^-$: 898.96). ^{31}P NMR (D_2O, 121 MHz): δ 33.73 (d, *J*=28.70 Hz, 1P), −10.98 (d, *J*=18.27 Hz, 1P), −22.73 (t, *J*=17.08 Hz, 1P), −24.18 (dd, *J*=29.07 Hz, *J*=16.32 Hz, 1P). [R_P]-**8b**: ^{31}P NMR (D_2O, 121 MHz): δ 33.64 (d, *J*=31.09 Hz, 1P), −10.98 (d, *J*=18.19 Hz, 1P), −22.77 (t, *J*=17.31 Hz, 1P), −24.25 (dd, *J*=31.08 Hz, *J*=16.57 Hz, 1P).

4.6. [R_P] and [S_P] diadenosine-5′,5‴-P^1,P^4-(P^1-borano)-tetraphosphate (9a,b)

Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.12 g, 0.30 mmol), **9a**,**b** were prepared by the same procedure described for **6a**,**b**. The two diastereomers were purified and separated by reverse-phase HPLC to give 0.11 g of [R_P]-**9a** (0.12 mmol, 40%) and 0.070 g of [S_P]-**9b** (0.078 mmol, 26%) as the ammonium salts. The products eluted in the order listed. LC–MS in negative mode, with *m*/*z* (M−1) 833.37 for [R_P]-**9a** and 833.31 for [S_P]-**9b** (calculated for

$C_{20}H_{30}BN_{10}O_{18}P_4^-$: 833.08). ^{31}P NMR ($D_2O$, 121 MHz): δ 86.03–80.61 (br, 1P), (−)10.54–(−)11.16 (m, 1P), (−)22.24–(−)23.15 (m, 2P). [**S**$_P$]-**9b**: ^{31}P NMR (D_2O, 121 MHz): δ 86.54–81.39 (br, 1P), (−)10.61–(−)11.26 (m, 1P), (−)22.30–(−)23.10 (m, 2P).

4.7. [S_P] and [R_P] diadenosine-5′,5‴-P^1,P^4-(P^1-thio)-tetraphosphate (10a,b)

Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.13 g, 0.33 mmol), **10a**,**b** were prepared by the same procedure described for **7a**,**b**. The two diastereomers were purified and separated by reverse-phase HPLC to give 0.11 g of [**S**$_P$]-**10a** (0.12 mmol, 36%) and 0.094 g of [**R**$_P$]-**10b** (0.10 mmol, 30%) as the ammonium salts. The products eluted in the order listed. LC–MS in negative mode, with *m*/*z* (M−1) 851.48 for [**S**$_P$]-**10a** and 851.41 for [**R**$_P$]-**10b** (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02). [**S**$_P$]-**10a**: ^{31}P NMR ($D_2O$, 121 MHz): δ 43.67 (d, J=25.3 Hz, 1P), −10.85 (d, J=17.8 Hz, 1P), −22.60 (t, J=17.1 Hz, 1P), −23.62 (dd, J=25.3 Hz, J=16.5 Hz, 1P). [**R**$_P$]-**10b**: ^{31}P NMR (D_2O, 121 MHz): δ 43.51 (d, J=25.1 Hz, 1P), −10.97 (d, J=18.1 Hz, 1P), −22.74 (t, J=17.4 Hz, 1P), −23.67 (dd, J=25.0 Hz, J=16.7 Hz, 1P).

4.8. [S_P] and [R_P] diadenosine-5′,5‴-P^1,P^4-(P^1-seleno)-tetraphosphate (11a,b)

Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.12 g, 0.30 mmol), **11a**,**b** were prepared by the same procedure described for **8a**,**b**. The two diastereomers were purified and separated by reverse-phase HPLC to give 0.060 g of [**S**$_P$]-**11a** (0.062 mmol, 21%) and 0.050 g of [**R**$_P$]-**11b** (0.052 mmol, 17%) as the ammonium salts. The products eluted in the order listed. LC–MS in negative mode, with *m*/*z* (M−1) 899.28 for [**S**$_P$]-**11a** and 899.60 for [**R**$_P$]-**11b** (calculated for $C_{20}H_{30}BN_{10}O_{18}P_4^-$: 898.96). [**S**$_P$]-**11a**: ^{31}P NMR ($D_2O$, 121 MHz): δ 33.92 (d, J=30.0 Hz, 1P), −10.93 (d, J=18.0 Hz, 1P), −22.80 (t, J=17.4 Hz, 1P), −24.31 (dd, J=30.1 Hz, J=16.8 Hz, 1P). [**R**$_P$]-**11b**: ^{31}P NMR (D_2O, 121 MHz): δ 33.90 (d, J=30.4 Hz, 1P), −11.00 (d, J=18.2 Hz, 1P), −22.54 (t, J=17.5 Hz, 1P), −24.34 (dd, J=30.4 Hz, J=16.9 Hz, 1P).

4.9. Diadenosine-5′,5‴-P^1,P^4-(P^2,P^3-methylene)-tetraphosphate (12)

To a solution of 2′,3′-*O*-6-*N*-triacetyladenosine (0.12 g, 0.30 mmol) in 2 mL of anhydrous DMF was added 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one (0.12 g, 0.59 mmol, 2.0 equiv). The solution was stirred for 15 min at room temperature under N_2. Tri-*n*-butylamine (0.60 mL, 2.5 mmol, 8.3 equiv) and methylenediphosphonic acid (0.073 g, 0.41 mmol, 1.4 equiv) were added. After 20 min, a solution of iodine (0.18 g, 0.70 mmol, 2.3 equiv) in 1.5 mL of pyridine and 0.01 mL of water was added. After 15 min, a mixture of AMP, in the proton form (0.39 g, 1.1 mmol, 3.7 equiv), and zinc chloride (0.44 g, 3.2 mmol, 11 equiv) that had been dried together by evaporation of pyridine and DMF was added with stirring. After 20 h, 10% aqueous ammonia (20 mL, 118 mmol) was added, and the deprotection was complete after 1 h. The dilute basic solution was applied to a column of sodium cation-exchange resin (50WX2, 10 mL, 6.0 mmol) to remove Zn^{2+}. The product was concentrated and purified by reverse-phase HPLC to give 0.22 g of **12** as the ammonium salt (0.24 mmol, 80%). LC–MS in negative mode, with *m*/*z* (M−1) 833.24 (calculated for $C_{21}H_{29}N_{10}O_{18}P_4^-$: 833.06). ^{31}P NMR ($D_2O$, 121 MHz): δ 8.33 (d, J=25.6 Hz, 2P), −10.41 (d, J=25.5 Hz, 2P).

4.10. Diadenosine-5′,5‴-P^1,P^4-(P^2,P^3-difluoromethylene)-tetraphosphate (13)

Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.13 g, 0.33 mmol), **13** was prepared by the same procedure described for **12**, except that a 0.2 M solution of bis(tri-*n*-butylammonium) difluoromethylenediphosphonate in anhydrous DMF (3.0 mL, 0.60 mmol, 1.8 equiv) was vortexed with tri-*n*-butylamine (0.60 mL, 2.5 mmol, 7.6 equiv) and immediately added to the reaction mixture of **2** to make intermediate **3**, instead of adding tri-*n*-butylamine and methylenediphosphonic acid. The product was purified by reverse-phase HPLC to give 0.25 g of **13** as the ammonium salt (0.27 mmol, 82%). LC–MS in negative mode, with *m*/*z* (M−1) 869.65 (calculated for $C_{21}H_{27}F_2N_{10}O_{18}P_4^-$: 869.04). ^{31}P NMR ($D_2O$, 121 MHz): δ −5.90 (tm, J=82.8 Hz, 2P), (−)10.12–(−)11.02 (m, 2P).

4.11. [S_P] and [R_P] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^1-thio)-tetraphosphate (16a,b)

To a solution of 3′-azido-3′-deoxythymidine (0.081 g, 0.30 mmol) in 2 mL of anhydrous DMF was added 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one (0.078 g, 0.39 mmol, 1.3 equiv). The solution was stirred for 15 min at room temperature under nitrogen. A 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in anhydrous DMF (1.3 mL, 0.65 mmol, 2.2 equiv) was vortexed with tri-*n*-butylamine (0.60 mL, 2.5 mmol, 8.3 equiv) and immediately added to the reaction mixture. After 20 min a solution of iodine (0.092 g, 0.36 mmol, 1.2 equiv) in 2.0 mL of pyridine and 0.01 mL of water was added. After 15 min, a mixture of 0.16 M of adenosine 5′-*H*-phosphonate, **14**, in the tri-*n*-butylammonium form (6.0 mL, 0.96 mmol, 3.2 equiv), S_8 (0.056 g, 1.7 mmol, 5.7 equiv) and zinc chloride (0.36 g, 2.64 mmol, 8.8 equiv) that had been dried together by evaporation of pyridine and DMF was added with stirring. After 21 h, 10% aqueous ammonia (20 mL, 118 mmol) was added to keep the zinc in solution, and the dilute basic solution was washed with methylene chloride to remove the excess sulfur and DMF, and then applied to a column of sodium cation-exchange resin (50WX2, 10 mL, 6.0 mmol, 20 equiv) to remove Zn^{2+}. The product was concentrated and purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.094 g of [**S**$_P$]-**16a** (0.10 mmol, 33%) and 0.12 g of [**R**$_P$]-**16b** (0.13 mmol, 43%) as the ammonium salts. The products eluted in the order listed. LC–MS in negative mode, with *m*/*z* (M−1) 851.40 for [**S**$_P$]-**16a** and 851.46 for [**R**$_P$]-**16b** (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02). [**S**$_P$]-**16a**: ^{31}P NMR ($D_2O$, 162 MHz):

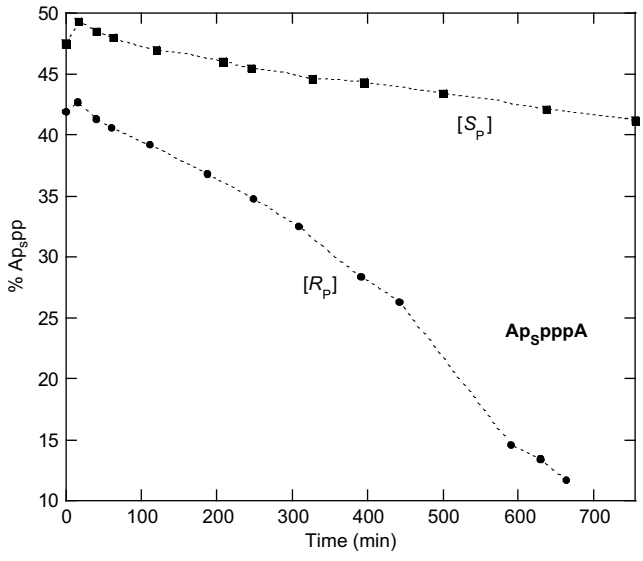


Figure 2. Disappearance of Ap$_S$pp with enzymatic degradation of Ap$_S$pppA (**10**).

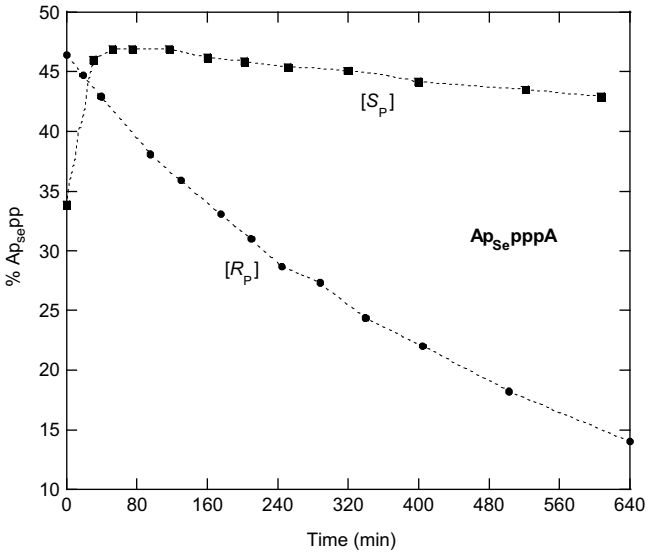


Figure 3. Disappearance of Ap$_{Se}$pp with enzymatic degradation of Ap$_{Se}$pppA (**11**).

δ 44.32 (d, J=25.7 Hz, 1P), −10.57 (d, J=17.6 Hz, 1P), −21.94 (t, J=16.8 Hz, 1P), −23.00 (dd, J=25.7 Hz, J=16.1 Hz, 1P). [$\boldsymbol{R_P}$]-**16b**: ^{31}P NMR (D_2O, 162 MHz): δ 44.18 (d, J=26.0 Hz, 1P), −10.59 (d, J=17.9 Hz, 1P), −22.00 (t, J=17.3 Hz, 1P), −23.00 (dd, J=25.9 Hz, J=16.4 Hz, 1P).

4.12. Identification of configurations of the diastereomers of Ap$_{BH3}$pppA (9a,b), Ap$_S$pppA (10a,b), and Ap$_{Se}$pppA (11a,b)

To a solution of 0.32 μmol of one diastereomer from each pair in 500 μL of 100 mM Tris·HCl buffer (pH=8.7), 2 mM $MgCl_2$, was added 4 μL of snake venom phosphodiesterase in water (50 μg/μL, Type IV from *Crotalus atrox*). The solution was maintained at 37 °C and analyzed by LC–MS (20 μL each time, λ=280 nm) after various time intervals. The percentages of Ap$_W$pp (W=BH_3, S or Se) (relative to the total nucleoside-containing peaks) were monitored and plotted against time (Figs. 1–3). The other diastereomer was assayed following the same procedure.

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Supplementary data

Spectra (UV, MS, and ^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR) for **6**–**13** and **16**. Supplementary data associated with this article can be found in online version, at doi:10.1016/j.tet.2009.07.079.

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