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Synthesis and resistance to enzymic hydrolysis of stereochemically-defined phosphonate and thiophosphate analogues of P^1,P^4-bis(5′-adenosyl) tetraphosphate

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ABSTRACT

Novel analogues of P^1,P^4-bis(5'-adenosyl) tetraphosphate, Ap_4A (1), have been prepared with sulphur substituents at P^1 and P^4 and either oxygen or methylene bridges at the P^2,P^3-position. Separation of three isomers of the $Ap_spCH_2pp_sA$ species has been achieved by a combination of mplc and hplc and the R_p,R_p, R_p,S_p, and S_p,S_p diastereoisomers identified on the basis of selective enzymatic hydrolysis using snake venom phosphodiesterase. Each of these three isomers is a strong competitive inhibitor of the specific Ap_4Aase from *Artemia* and is highly resistant to the asymmetric cleavage normally catalysed by this enzyme.

INTRODUCTION

Although first detected over 20 years ago as a by-product of amino acid activation by lysyl-tRNA synthetase,[1] P^1,P^4-bis(5'-adenosyl) tetraphosphate (1), Ap_4A, has successfully defied a precise definition of its biological function. It has been shown to vary in intracellular concentration directly with cellular growth rate[2] and in several cases to increase by anything up to three orders of magnitude, from 0.01 to 10µM, during the G_0 to S-phase progression of the cell cycle[3-6] although reports to the contrary also exist.[7-9] Although it is synthesised, presumably in the cytoplasm, by several aminoacyl-tRNA synthetases[10,11] it appears to concentrate in the nucleus[12] and to bind there to at least one nuclear target protein.[13] One such protein is the replicative DNA polymerase-α holoenzyme. DNA polymerase-α from calf thymus[14] and HeLa cells[15] possesses a specific Ap_4A-binding subunit. This subunit may also be found in a free form.[16,17] When bound, Ap_4A is able to serve as a primer for the DNA polymerase *in vitro* and this ability depends on the presence of the Ap_4A-binding subunit.[18] Such an activity *in vivo* would conflict with the known role of primase in the synthesis of conventional RNA primers[19] and the suggestion that Ap_4A may be involved in the initiation of DNA synthesis is not universally accepted.[20] A specific

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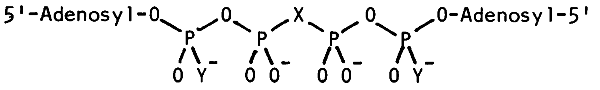
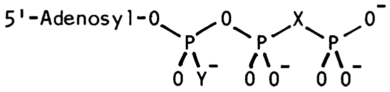
role for this nucleotide in priming eukaryotic replication origins is more appealing but requires substantiation.



(1) X = O, Y = O
(2) X = O, Y = S
(3) X =CH_2, Y = S

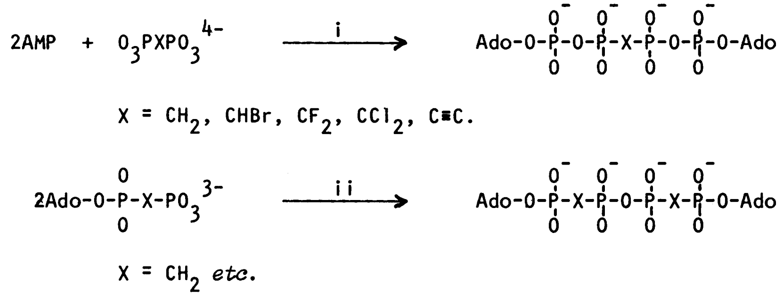


(4) X = CH_2, Y = S

Ap$_4$A also binds to other proteins including histone H1,[21] poly(ADP-ribose) polymerase,[22] and a protein kinase[23] and may therefore have alternative or additional roles. The suggestion by Varshavsky[24] that Ap$_4$A and related bis(5'-nucleosidyl) oligophosphates such as Ap$_3$A and Ap$_4$G may serve as alarmones to signal the onset of metabolic stress received considerable support from the discovery of large increases of these nucleotides upon exposure of *Escherichia coli* and *Salmonella typhimurium* to oxidative stresses and to heat shock.[25,26] More modest increases have been observed in eukaryotic systems subjected to similar stresses[8,27-29] but the possibility that they could serve as inducing signals for the expression of stress proteins seems unlikely since significant increases are only observed after extreme, lethal metabolic stress[28,30] and the normally greater cell-cycle variations do not by themselves lead to the induction of stress proteins.[29] Once again, the role of Ap$_4$A and related nucleotides in the stress response remains unclear.

A major problem in assessing the true function of Ap$_4$A is its great metabolic lability. It is turned over rapidly *in vivo*[2] and Ap$_4$A supplied exogenously to experimental cellular and subcellular systems is also rapidly degraded by non-specific phosphodiesterases in the serum component of mammalian cell growth media and by the highly specific cellular bis(5'-adenosyl) tetraphosphatases (Ap$_4$Aases) which have been demonstrated in several systems. These enzymes operate by a variety of processes and generally have K_m values for Ap$_4$A in the micromolar range. Thus, symmetrical cleavage by an Ap$_4$Aase from *E.coli*[31,32] and *Physarum*[33] breaks the $P^2{:}P^3$ linkage to form ADP. Asymmetrical cleavage by Ap$_4$Aases from *Artemia*,[34] lupin seeds,[35] or mammalian cells[36-39] between P^1 and P^2 gives AMP and ATP. Lastly, phosphorolysis of the $P^1{:}P^2$ bridge by an enzyme from yeast[40] leads to ATP and ADP. This lability may explain difficulties experienced in reproducing the original observation that Ap_4A could cause premature initiation of DNA replication when added to permeabilised mammalian cells. The very existence of these specific degradative enzymes and the fact that they are inhibited by low concentrations of Zn^{2+} ions while Ap_4A synthesis is strongly stimulated by zinc ions[10,11,42] suggest a precise biological role for Ap_4A. The continuing difficulties encountered in determining this role point to a clear need for an analogue of Ap_4A which closely resembles it in shape and in ionic character but resists all of these different patterns of cleavage.

As a first step towards this objective, we[43,44] and Tarussova[45] have made bisphosphonate analogues of Ap_4A having P-C-P bridges in place of P-O-P links. When these are located in the $P^2{:}P^3$ position they block the symmetrical enzyme cleavage while in the $P^1{:}P^2{;}P^3{:}P^4$ positions there is inhibition of the asymmetrical cleavage (Scheme 1).



$$2\mathrm{AMP} + O_3PXPO_3{}^{4-} \xrightarrow{i} \mathrm{Ado{-}O{-}}\overset{O^-}{\underset{O}{P}}\mathrm{{-}O{-}}\overset{O^-}{\underset{O}{P}}\mathrm{{-}X{-}}\overset{O^-}{\underset{O}{P}}\mathrm{{-}O{-}}\overset{O^-}{\underset{O}{P}}\mathrm{{-}O{-}Ado}$$

$X = CH_2, CHBr, CF_2, CCl_2, C{\equiv}C.$

$$2\mathrm{Ado{-}O{-}}\overset{O}{\underset{O}{P}}\mathrm{{-}X{-}}PO_3{}^{3-} \xrightarrow{ii} \mathrm{Ado{-}O{-}}\overset{O^-}{\underset{O}{P}}\mathrm{{-}X{-}}\overset{O^-}{\underset{O}{P}}\mathrm{{-}O{-}}\overset{O^-}{\underset{O}{P}}\mathrm{{-}X{-}}\overset{O^-}{\underset{O}{P}}\mathrm{{-}O{-}Ado}$$

$X = CH_2$ *etc.*

Reagents: i, carbonyldi-imidazole/pyridine;
ii, dicyclohexylcarbodi-imide/pyridine.

SCHEME 1

While it is obvious that a tris-carbon-bridged species, such as $P{-}CH_2{-}P{-}CH_2{-}P{-}CH_2{-}P$, would necessarily resist *both* the symmetrical *and* the asymmetrical cleavage processes, we considered that the conformational perturbation associated with the replacement of <u>three</u> oxygens by CH_2 or equivalent groups might cause too large a shape change for the manifestation of normal biological activity by the Ap_4A analogue thus generated. Mindful of the success of thiophosphonate analogues of nucleotides in a wide variety of enzyme applications[46-48] we therefore chose to restrict the symmetrical cleavage by the incorporation of a single $P^2{-}C{-}P^3$ carbon bridge at the same time as impeding hydrolytic attack at P^1 and P^4 by the incorporation of a sulphur atom in place of oxygen at these loci. We here describe the synthesis of some such species, separation of diastereoisomers, characterisation of stereoisomers, and enzyme inhibition studies for the asymmetrical Ap_4Aase

from *Artemia*. A preliminary account of part of this work has been published elsewhere.[49]

EXPERIMENTAL

Phosphorus NMR spectra were recorded at 32.4MHz using Bruker WP80SY and at 162MHz using Bruker WP400 instruments with 85% phosphoric acid as external reference. Proton NMR were recorded at 250MHz using a Bruker AM250 instrument. Mass spectra were obtained on a Kratos MS80 machine fitted with a FAB source and in the negative ion mode.

For the preparation of analogues of Ap_4A, MPLC was performed with DEAE Sephadex A-25 (Pharmacia) and HPLC analyses were carried out using a Nucleosil 5C-18 column (Technicol Ltd.) Isocratic elution was with Varian 2000 series equipment and gradient elution employed Waters 6000A equipment with peak monitoring by UV absorption at 265nm. All nucleotides showed characteristic absorption spectra for adenosine.

Snake venom phosphodiesterase from *C.durissus* (EC 3.1.16.1) was purchased from Boehringer Mannheim and asymmetrical bis(5'-adenosyl) tetraphosphatase was purified from embryonic cysts of the brine shrimp *Artemia* by a procedure to be published elsewhere,[50] which involves ammonium sulphate precipitation and chromatography on Ultrogel AcA 44, Mono Q, and high performance hydroxyapatite columns. One unit of Ap_4Aase activity is that amount which hydrolyses Ap_4A at a rate of 1 μmol/min under standard assay conditions. Ap_4A was obtained from Sigma Chemicals.

Preparation of the Diastereoisomeric Mixture (R_p,S_p)-Diadenosine 5',5'''-P^1,P^4--$(P^1,P^4$-dithio)-tetraphosphate, Ap_sppp_sA (2).

Adenosine 5'-*O*-monothiophosphate triethylammonium salt[51] (1.175g, 2mmole) and trioctylamine (740μl, 2.10mmole) were shaken with methanol (15ml) until dissolved and the solution evaporated *in vacuo* to a dry foam. The mixture was rendered anhydrous by repeated evaporation from anhydrous pyridine and finally dried at 0.5mm pressure overnight. Dry dioxan (14ml) followed by dry tri-n-butylamine (800μl) were added followed by diphenyl phosphorochloridate (δ_P -5.3 in $CDCl_3$)(600μl, 2.9mmole). The mixture was stirred 3h under nitrogen when the initially cloudy solution became clear. This solution was then evaporated *in vacuo* and the resulting syrup washed with dry ether (50ml) and left 1h at 4°C. The ether was decanted and the residue dried *in vacuo*.

A solution of pyridinium pyrophosphate (from 124mg pyrophosphoric acid, 0.7mmole) and tri-n-butylamine (370mg, 2.0mmole) in anhydrous pyridine (10ml) was added to the activated AMPS. (If any precipitate formed at this stage, it was rendered soluble by the addition of further dry tri-n-butylamine). The clear solution was stirred 3h then evaporated *in vacuo*, and washed with dry ether. The residual gum was taken up in aqueous triethylammonium bicarbonate solution, TEAB (20ml, 0.1M, pH7.6) and methanol (2ml).

The crude product was chromatographed on DEAE Sephadex (60x150mm) with a linear gradient (2.51,0.1M TEAB pH7.6 to 2.51,0.35M TEAB) then (1.31,0.35M to 1.31,0.6M TEAB) and finally TEAB (0.7M,11). The following peaks were obtained (elution volume): adenosine 5'-thiophosphate, AMPS (2 1-2.51) δ_P 43.2; adenosine 5'-α-thiotriphosphate, ATPαS (4.6-5.41) δ_P 43.28 and 43.01 (d, J_{12} 27Hz, P_α), -4.97 (d, J_{23}21.4Hz, P_γ), and -21.66 (dd, J_{12}27Hz, J_{23}21.4 Hz, P_β); and $\mathrm{Ap_sppp_sA}$ (6.8-8.01).

The appropriate fractions containing $\mathrm{Ap_sppp_sA}$ were combined and evaporated *in vacuo*. The mixed isomers were converted into their sodium salts using NaI in acetone in the usual way and were chromatographed through a short column of Chelex (Sigma) to remove paramagnetic species: δ_P (D_2O) +43.54 (35%), +43.34 (65%) (P^1,P^4) and -23.39 (P^2,P^3) ($^2J_{1,2}$26Hz, $^2J_{2,3}$16Hz, and $^4J_{1,3}$ 0.2Hz); δ_H (D_2O) 8.47 (35%) and 8.39 (65%) (s, H^8), 7.93 (s, H^2), 5.98 (d, 5Hz, $H^{1'}$), 4.75 (m, $H^{2'}$), 4.59 (m, $H^{3'}$), 4.4 (m, $H^{4'}$, $H^{5'}$, $H^{5''}$). M/z 955 ($M-H^+$),(46%), 933 ($M-Na^+$),(100%); $C_{20}H_{24}N_{10}Na_4O_{17}P_4S_2$ requires M/z 956.

Preparation of the Diastereoisomeric Mixture (R_p,S_p)-Diadenosine 5',5'''-P^1,P^4--(P^1,P^4-dithio-P^2,P^3-methylene)-tetraphosphate, $\mathrm{Ap_spCH_2pp_sA}$ (3).

AMPS triethylammonium salt[51] (0.582mg, 1mmole) was brought into reaction with methylenebisphosphonic acid (61.6mg, 1mmole) as described above. The gummy product was chromatographed on DEAE Sephadex (60x150mm) using linear gradients of TEAB (2.51, 0.1M to 2.51, 0.35M) followed by (2.51, 0.35M to 2.51, 0.6M) and collecting aliquots (20ml). Peaks were collected as follows.

AMPS (21 to 2.71) δ_P +43.2.

$\mathrm{Ap_spCH_2p}$, α-Thio-β,γ-methylene adenosine 5'-triphosphate (4), (94mg as its sodium salt) δ_P +41.83 (d, $J_{\alpha\beta}$34.2Hz, P^α), +10.65 (dd, J34.2Hz, 6.2Hz, P^β) and +12.25 (d, $J_{\beta\gamma}$6.2Hz, P^γ); the signal at +41.83δ showed partial separation for the two diastereoisomers; M/z 608 ($M-H^+$),(10%), 586 ($M-Na^+$),(35%), 564 ($M-2Na^++H^+$),(100%), 542 ($M-3Na^++2H^+$),(50%), and 520 ($M-4Na^++3H^+$),(6%); $C_{11}H_{14}N_5Na_4O_{11}P_3S$ requires M/z 609; δ_H 8.62 (45%) and 8.60 (55%) (s, H^8), 8.15 (s, H^2), 6.10 (d, J5Hz, $H^{1'}$), 4.80 (t, J5Hz, $H^{2'}$), 4.55 (m, $H^{3'}$), 4.48 (m, $H^{4'}$), 4.38 (m, $H^{5'}$,$H^{5''}$), and 2.34 (dd, $^2J_{PH}$21Hz, 19Hz, PCH_2P).

$\mathrm{Ap_spCH_2pp_sA}$ (3) was collected as a broad peak eluting from 6.61 to 8.41. The early fractions contained largely one of the three diastereoisomers (**3A**) while the later fractions contained the two remaining isomers (**3B** and **3C**) essentially free from (**3A**).

Isomer 3A (6.61 to 7.21) δ_P +42.0 ($J_{1,2}$34.2Hz) and +6.72;
Isomers 3B and **3C** (7.21 to 8.41) δ_P +41.3 ($J_{1,2}$34.2Hz) and +5.78; δ_H (Na salt in D_2O) 8.5 (m, H^8), 8.00 (s, H^2), 6.04 (d, 5Hz, $H^{1'}$), 4.75-4.35 (m, $H^{2'}$, $H^{3'}$, $H^{4'}$, $H^{5'}$, and $H^{5''}$), and 2.86 (m, PCH_2P).

Separation of the Isomers of $Ap_SpCH_2pp_SA$ (3A, 3B, and 3C).

The fractions of the above eluate at *ca.*0.4M to 0.45M TEAB were pooled and evaporated to give 120mg of the mixed isomers. This material was further chromatographed on the same column using a linear gradient of TEAB (2l, 0.2M to 2l, 1M). Early fractions of this eluate were then subjected to HPLC purification on a C18 column (5x150mm) with isocratic elution using 5% MeOH/TEAB (0.1M). Repetition of this HPLC provided the pure diastereoisomer (**3A**). The later fractions from the DEAE column eluting at *ca.*0.45 to 0.53M TEAB were similarly purified by HPLC chromatography on the same column using first 15% then 12% MeOH/TEAB (0.1M) to give isomers (**3B**) and (**3C**), shown by HPLC analysis to be greater than 90% homogeneous. These three isomers were then finally purified immediately prior to use in enzyme assays by HPLC on Partisil 10-SAX anion exchange resin (5x250mm) eluting with a gradient of 5% to 50% ammonium phosphate (1M, pH5.7) in ammonium phosphate (0.05M, pH5.2).

^{31}P NMR spectra were obtained for these isomers as their triethylammonium salts and mass spectral data were recorded using sodium salts obtained in the usual way with NaI in acetone.

Isomer (**3A**) (S_P,S_P) δ_P +41.8 (P^1,P^4) and +6.80 (P^2,P^3) (doublets, J35Hz); M/z 953 ($M-H^+$),(25%), 931 ($M-Na^+$),(100%), and 909 ($M-2Na^++H^+$),(60%).
Isomer (**3B**) (R_P,R_P) δ_P +41.6 and +6.70 (doublets, J34Hz); M/z 953 (30%), 931 (100%), and 909 (65%).
Isomer (**3C**) (R_P,S_P) δ_P +42.0 and +6.75 (doublets, J35.4Hz), M/z 953 (50%), 931 (100%), and 909 (55%); $C_{21}H_{26}N_{10}Na_4O_{16}P_4S_2$ requires M/z 954.

Incubation of Ap_4A and $Ap_SpCH_2pp_SA$ (3) with Snake Venom Phosphodiesterase.

Standard assay solutions were prepared containing Hepes-NaOH (pH7.75, 30mM), magnesium acetate (5mM), nucleotide, (0.2mM), and phosphodiesterase, SVP (1 g, 1.5units) in 250μl. The solutions were incubated at 28° for periods of 0, 10, 20, and 40min and aliquots (20μl) injected onto an analytical HPLC column (Partisil 10-SAX 5x250mm) and eluted with a gradient of 5% to 50% ammonium phosphate (1M, pH7.5) in ammonium phosphate (0.05M, pH 5.2) at 1.5ml min^{-1} for 15min. Peak areas were integrated to determine the rate of breakdown.

Incubation of Ap_4A (1) and $Ap_spCH_2pp_sA$ with P^1,P^4-bis(5'-adenosyl) tetraphosphatase (Ap_4Aase).

Assays contained Hepes-NaOH (42mM, pH 7.75), magnesium acetate (7mM), nucleotide (0.2mM), and 0.6mU (with Ap_4A as substrate) or 6mU (with analogues as substrates) of *Artemia* Ap_4Aase in a final volume of 25µl. After incubation at 30° for various times, 20µl samples were injected onto Partisil 10-SAX and analysed as described above.

Luminescence Assay for Ap_4Aase.

Ap_4Aase from *Artemia* embryos[50] (0.04mU) was pre-incubated at room temperature for 15min with 3µM or 15µM Ap_4A analogue and then added to an assay mixture which contained the following in a final volume of 125µl: 42mM Hepes-NaOH (pH7.75), magnesium acetate (7mM), ATP-monitoring reagent (25µl, LKB), Ap_4A analogue (3µM or 15µM), and Ap_4A (concentrations between 0.5µM and 50µM). The increase in luminescence was monitored at 30° over a period of 5min with an LKB Luminometer. K_m and K_i values were determined from double reciprocal plots.

RESULTS AND DISCUSSION

The condensation of adenosine 5'-phosphate or 5'-thiophosphate with either pyrophosphate or one of a range of methylenebisphosphonates can be carried out using a variety of condensing agents including dicyclohexylcarbodi-imide, DCCD, and carbonyldi-imidazole, CDI. Tarussova[52] has also made use of carbonyldibenzimidazole and carbonyldi-(1,2,4-triazole) for the same purpose. In this work we have found that diphenyl phosphorochloridate in pyridine as solvent was the most satisfactory activating agent and gave acceptable yields of the desired dinucleoside dithiotetraphosphates. These products can be well separated from AMPS and ATPαS by ion exchange chromatography on DEAE Sephadex where they elute at higher ionic strength than the other nucleotides.

The presence of a sulphur substituent at P^1 and P^4 means that there must be three diastereoisomeric products, R_p,R_p, R_p,S_p, and S_p,S_p. These are not adequately resolved by DEAE-Sephadex chromatography where even for the most favourable cases only partial enrichment of diastereoisomers was attained. Consequently, further purification had to be sought using HPLC procedures where reversed-phase chromatography with a C18 column led to a viable separation of three isomers of $Ap_spCH_2pp_sA$ (3A, 3B, and 3C respectively) in small amounts. The extent of this separation varies from analogue to

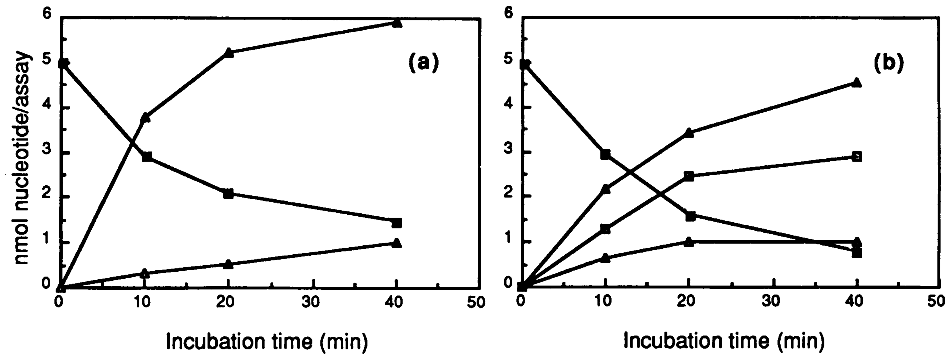


Figure 1: Sensitivities of (a) isomer (3B) and (b) isomer (3C) of $Ap_spCH_2pp_sA$ to hydrolysis by snake venom phosphodiesterase. Incubations were carried out as described in the Experimental Section. Retention times of product nucleotides are as in legend to Figure 2.
(a): (■) $Ap_spCH_2pp_sA$ (3B) + Ap_spCH_2p; (▲) AMPS; (△) AMP.
(b): (■) $Ap_spCH_2pp_sA$ (3C); (□) Ap_spCH_2p; (▲) AMPS; (△) AMP.

analogue and, for example, the P-CF_2-P dithiophosphate analogue of (3) cannot be fully resolved into discrete stereoisomers in this way.

There is, without doubt, a need to develop an alternative synthesis which offers either partial or complete stereospecificity. Such might, for example be based on the condensation of one resolved diastereoisomer of Ap_spXp with AMPS thereby leading to a mixture of only two diastereoisomers, *e.g.* R_p,R_p and R_p,S_p isomers. Alternatively, the self-condensation of a single diastereoisomer of Ap_sXp would provide a single diastereoisomer of the dimer Ap_sXppXp_sA.

The presence of both R_p and S_p diastereoisomers at $P^1(P^4)$ in a mixture is manifest in both the proton and the phosphorus NMR spectra of the analogues of Ap_4A (1). Shift differences of up to 0.1ppm for the adenine-H^8 (but not the H^2) signals are associated with the alternative configurations at the proximate phosphorus centre. Likewise, phosphorus chemical shift differences of up to 0.1ppm are seen for $P^1(P^4)$ but with much smaller changes at $P^2(P^3)$ - which are frequently only resolvable at high field (162MHz). The analysis of these spectra is complicated by the fact that they are generally of the XAA'X' variety[53] and give up to twelve lines per isomer (rather than the four lines expected for an AX spectrum). The two-bond coupling constants are generally in the range 25-35Hz for P^1-P^2. The coupling between P^2 and P^3 is also large (*ca.* 16Hz) in oxygen-bridged species but can become vanishingly small for carbon-bridged species (3). The four-bond coupling between P^1 and P^3 is rarely larger than 0.4Hz.

Such small differences in NMR spectra between the different diastereoisomers at phosphorus have proved, in our hands, to be of little value for their separate identification. By contrast, there are clear-cut differences in the HPLC retention times of the isomers which are, moreover, very reproducible.

The use of Fast Atom Bombardment, FAB, ionisation techniques in the negative ion mode provides excellent mass spectra of the tetrasodium salts of these sulphur-containing analogues of Ap_4A and ATP. On the other hand, results of little value were obtained from spectra of the tetrakis-triethylammonium salts. FAB mass spectra typically show anions derived by loss of either a proton or of one or more sodium ions from the parent molecule and the $M-Na^+$ peak invariably provides the base peak for the high mass range of these spectra. (On occasion, weaker signals are observed at masses in excess of $(M-H^+)$ and originate from glycerol capture from the matrix. There appear to be no significant differences between the spectra of different stereoisomers of (3).

For the P^1,P^4-dithio-P^2,P^3-methylene analogue (3) of Ap_4A, intensive and repetitive MPLC and HPLC permitted the isolation of milligramme quantities of the discrete diastereoisomers. These were subjected to individual incubation with both snake venom phosphodiesterase, SVP, and the Ap_4Aase from *Artemia*. The former enzyme is known to be selective for the R_p isomer of Ap_spp[54,55] while the latter enzyme cleaves Ap_4A asymmetrically to give AMP and ATP, presumably by nucleophilic attack at $P^1(P^4)$.

When the three stereoisomers of (3) were incubated with SVP, isomer (3A) proved to be highly resistant to digestion (0.3nmol/min/mg protein) under conditions which led to the complete cleavage both of Ap_4A (1) itself and of both of the other two stereoisomers of (3). Accordingly, it is assigned the S_p,S_p configuration, By contrast, both isomer (3B) and (3C) were cleaved to mononucleotides, though both at a rate some 40 times slower than that of (1), which was cleaved at a rate of 12µmol/min/mg protein under identical conditions (Figure 1). Isomer (3B) was completely hydrolysed to AMPS (along with a small amount of AMP produced as a result of SVP action on AMPS). On the other hand, isomer (3C) was hydrolysed to give equal amounts of AMPS and a second nucleotide which had the same retention time on analytical HPLC as an authentic sample of Ap_spCH_2p (4), which was synthesised and characterised fully. We therefore conclude that isomer (3B) is the R_p,R_p species while isomer (3C) is the R_p,S_p isomer that should be cleaved by SVP only at the R_p centre to give AMPS and the S_p diastereoisomer of Ap_spCH_2p (4). Corresponding experiments with SVP and the mixed diastereoisomers of Ap_sppp_sA (2) suggest the presence of three isomers which are hydrolysed at rates similar to those shown for the separated isomers of (3).

For these analogues to be of practical value in the elucidation of Ap_4A function, it is essential that they be recognised as structurally similar to the parent compound (1) as well as be resistant to hydrolysis by the enzymes primarily responsible for Ap_4A catabolism *in vivo*. The Ap_4Aase from *Artemia* was employed to test both of these requirements. It is representative of the major class of highly specific Ap_4Aases found in mammaliam cells and in other higher eukaryotes which cleave Ap_4A asymmetrically to yield AMP and ATP.

Using a sensitive bioluminescence assay,[56] we determined the K_m for Ap_4A with the *Artemia* Ap_4Aase to be 4.2μM (Table 1). This is close to the value of 2μM determined previously for the partially purified enzyme. All three stereoisomers of (3) behaved as competitive inhibitors for this enzyme, with K_i values of 1μM for the R_p,R_p (3B) and S_p,S_p (3A) stereoisomers and of 1.5μM for the R_p,S_p isomer (3C). The unresolved mixed stereoisomers of Ap_sppp_sA (2) also inhibited this Ap_4Aase with an apparent K_i of 2.5μM (Table 1). All of these analogues of Ap_4A appear to undergo a slow conformational rearrangement prior to binding to the *Artemia* Ap_4Aase since, without pre-incubation of these analogues with the Ap_4Aase, the inhibition of the enzyme reaction rate slowly increased and achieved a constant level only after some 10min in each case.

Because the bioluminescence assay is dependent upon the formation of ATP, it could not be used to determine the rate of breakdown of the analogues by Ap_4Aase. Instead, their degradation was monitored by HPLC. It can be seen (Figure 2a) that Ap_4A is rapidly degraded to yield equimolar amounts of AMP and ATP, as expected. In contrast, all three isomers of $Ap_spCH_2pp_sA$ (3) proved highly resistant to degradation (Figure 2b-d). In order to achieve a measurable degree of breakdown, incubations for 5h were necessary with these substrates. Fortunately, the *Artemia* Ap_4Aase proved to be remarkably stable in the presence of the analogues (3): the rate of breakdown of the R_p,S_p isomer, which is the most sensitive, measured after 5h was still 82% of the rate after 1h. A similar stability of the enzyme in the presence of the other isomers was shown in experiments where a large excess of Ap_4A was added after 5h incubation of the enzyme with one of the analogues (3) and comparing

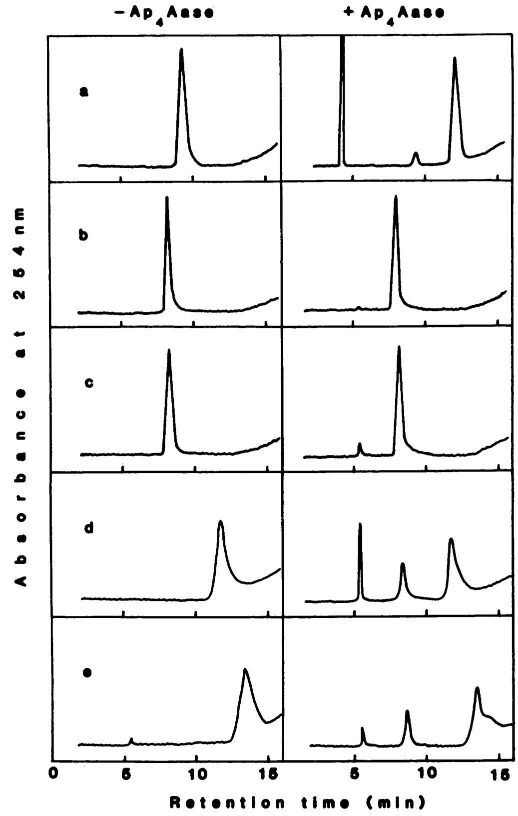


Figure 2: Sensitivities of Ap_4A and Ap_4A analogues to hydrolysis by *Artemia* Ap_4Aase. Incubations were carried out as described in the Experimental section. Substrates, enzyme concentrations, and assay times were respt.: (a) Ap_4A, 0.6mU, 10min; (b) S_p,S_p $Ap_spCH_2pp_sA$ (3A), 6mU, 5h; (c) R_p,R_p $Ap_spCH_2pp_sA$ (3B), 6mU, 5h; (d) R_p,S_p $Ap_spCH_2pp_sA$ (3C), 6mU, 5h; (e) *mixed* Ap_sppp_sA (2), 6mU, 3h. Retention times (minutes) were: AMP,4.2; ADP,7.1; ATP,12.2; Ap_4A (1),9.4; AMPS,5.6; Ap_sp,8.8; Ap_spp,14.1; *mixed* Ap_sppp_sA (2),13.4; (3A),8.3; (3B),8.3; (3C),11.7; and *mixed* Ap_spCH_2p (4),8.3.

the rate of its hydrolysis with that for the unincubated enzyme. This showed only a slight loss of enzyme activity which could be allowed for to determine rates of hydrolysis for the R_p,R_p, S_p,S_p, and R_p,S_p isomers of (3) as 2.1, 0.5, and 7.0 nmol/min/unit respectively and compared to the standardised rate of 1µmol/min/unit for Ap_4A. The rates of hydrolysis relative to that for Ap_4A are shown in Table 1. In the case of the R_p,R_p and S_p,S_p stereoisomers, these rates were estimated from the rate of appearance of AMPS because the

Table 1: Kinetic parameters of *Artemia* $\mathrm{Ap_4Aase}$ with $\mathrm{Ap_4A}$ and $\mathrm{Ap_4A}$ analogues

NUCLEOTIDE	K_m (μM)	K_i (μM)	k_{rel}
ApppppA (1)	4.2	—	2,500
$(S_p,S_p)\mathrm{Ap_spCH_2pp_sA}$ (3A)	—	1.0	1
$(R_p,R_p)\mathrm{Ap_spCH_2pp_sA}$ (3B)	—	1.0	5
$(R_p,S_p)\mathrm{Ap_spCH_2pp_sA}$ (3C)	—	1.5	17
Mixed $\mathrm{Ap_sppp_sA}$ (2)	—	2.5	85

isomers of $\mathrm{Ap_spCH_2p}$ (4) co-chromatographed with the corresponding $\mathrm{Ap_4A}$ analogues (Figure 2b,c). With the R_p,S_p isomer, which eluted very much later than the other two isomers, the formation of equimolar amounts of AMPS and $\mathrm{Ap_spCH_2p}$ (4) could be seen (Figure 2d). The possible stereospecificity of this cleavage was not determined.

An interesting observation emerged when the mixed stereoisomers of $\mathrm{Ap_sppp_sA}$ (2) were incubated with the *Artemia* $\mathrm{Ap_4Aase}$. A new nucleotide peak appeared at a rate of 35 nmol/min/unit of enzyme which had a retention time identical to that of authentic adenosine 5'-α-thiodiphosphate (Boehringer) (Figure 2e). This result suggests that the specificity of this enzyme for the P^α-O-P^β bridge is not absolute and that the presence of P^1 and P^4 thiophosphates can force a symmetrical cleavage of the $\mathrm{Ap_sppp_sA}$ for at least one of the diastereoisomers of (2). The presence of the P^2:P^3 methylene bridge in (3) affords at least a further five-fold resistance to hydrolysis. In other prelimiary experiments, we have also observed a similar relaxation of regio-specificity of hydrolysis in the case of the *E.coli* $\mathrm{Ap_4Aase}$. This enzyme cleaves $\mathrm{Ap_4A}$ symmetrically to give ADP. However, after prolonged incubation, the R_p,R_p isomer (3B) is cleaved to yield AMPS and $\mathrm{Ap_spCH_2p}$ (4), *i.e.* an asymmetric cleavage (data not shown).

In conclusion, the $\mathrm{Ap_4A}$ analogues reported here will be of undoubted value as probes in two areas of interest: (1) in mechanistic studies of the stereospecificity and regiospecificity of hydrolysis of $\mathrm{Ap_4A}$ by symmetrical and asymmetrical $\mathrm{Ap_4Aases}$, and (2) in establishing the true biological function(s) of $\mathrm{Ap_4A}$. The combination of phosphonate and thiophosphate chemistry has proved to be particularly worthwhile in view of the apparent "wobble" in bridge specificity of $\mathrm{Ap_4Aases}$. The high resistance to degradation by both non-specific and specific hydrolytic enzymes shown especially by the S_p,S_p diastereoisomer of $\mathrm{Ap_spCH_2pp_sA}$ (3A) will make it a particularly valuable agent in these latter studies.

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