Heteroatom Chemistry
Volume 2, Number 1, 1991

Application of Phosphonate and Thiophosphate Analogues of Nucleotides to Studies of Some Enzyme Reactions

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Received 28 June 1990.

ABSTRACT

The enzymatic cleavage of a scissile P—O bond can be blocked by recourse to phosphonate analogues of biological phosphate esters. α-Fluorophosphonates have an enhanced electronegativity at the bridging carbon, which, in many cases, makes them superior to simple methylene phosphonates for the study of enzyme reactions. Thus, the β,γ-difluoro-methylene analogue of ATP is a good substrate for the interferon-induced (2→5)A_n synthetase, which converts it into a (2→5)A_4 species having a 5′-β,γ-difluoromethylene-triphosphate. This binds strongly to RNase L but does not activate it. The unsymmetrical Ap_4Aases from Artemia *and Lupin are strongly inhibited by* P^1,P^4-*dithiophosphate analogues of diadenosyl-5′,5‴-*P^1,P^4-*tetraphosphate although nonregiospecific cleavage of certain* P^2,P^3-*methylene analogues can be observed. Some of these analogues are remarkably effective inhibitors of platelet aggregation and are effective inhibitors in vivo of arterial blood-clotting in rabbits. Separation of all diastereoisomers of* P^1,P^4-*dithiophosphate analogues of Ap_4A is achieved cleanly using reverse-phase hplc chromatography and this provides direct access to β,γ-CHF-bridged analogues of ATP with resolved stereochemistry at the CHF center. Lastly, growing cells of* Dictyostelium discoideum *not only tolerate a range of substituted methylene bisphosphonates in their growth medium but actually incorporate them into nucleotide analogues of ATP and Ap_4A.*

The opportunities for the application of phosphonate analogues of biological phosphate esters are as many and varied as the enzymes that operate on phosphate esters. Their successful application in agriculture and in medicine leaves no doubt about the scientific and industrial importance of these species. Thus, glyphosate (**1**) is arguably the world's most successful herbicide, with sales in excess of $1 \times 10^9$$ per annum, a range of substituted methylene bisphosphonates, e.g. hydroxyethylidenebisphosphonate (**2**), are important for the control of osteoporosis and related problems, phosphonomycin (**3**) is a useful broad-spectrum antibiotic, while phosphonoacetic acid (**4**) remains the standard test compound for evaluation of inhibitors of the HIV reverse transcriptase (Scheme 1).

Rational analysis shows that phosphonate analogues of phosphate monoesters (**5a**) can either replace one of the nonbridging oxygens, as in a meth-

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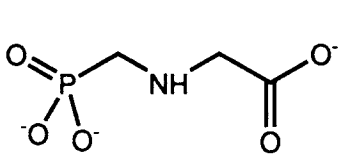
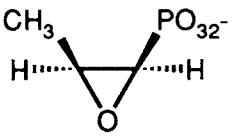


(1)



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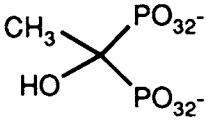

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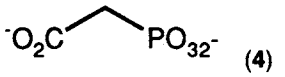
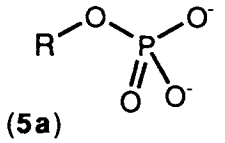


(4)

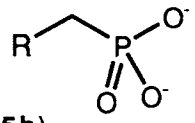
SCHEME 1

ylphosphonate ester (**6**), or the bridging oxygen by a methylene group, as in **5b**. An instructive example of the application of such analogues is found in studies of the enzyme 3-dehydroquinate synthetase, which is strongly inhibited by the phosphonate analogue **7b** of D-*arabino*-heptulosonic acid 7-phosphate **7a**. Unexpectedly, the nonisosteric phosphonate analogue **7b** binds thirtyfold tighter [1, 2] than the substrate **7a** (K_i/K_m = 0.06) while the isosteric methylene-phosphonate analogue [2] (**7c**) is virtually noninhibitory of this enzyme. Similar differences between isosteric and nonisosteric phosphonate inhibitors of this enzyme are found [3] in the series of cyclohexane inhibitors (**8**) where simple inhibition of the enzyme is most strongly exhibited by the attenuated phosphonate analogue **8a**, binding 5000 times tighter than the substrate **7a**, while the isosteric analogue **8b** binds only marginally (2.5 times) better than the parent phosphate **7a**. It is rather significant that **8a** cannot interact with NAD^+ in the first, oxidative stage of the enzyme reaction while analogue **8b** and the rigid *E*-isomer of the ethylenephosphonate **8c** are substrate analogues that participate in the first redox process. Such problematic behavior is typical of the fickleness in performance of isosteric and nonisosteric phosphonate analogues of biological phosphates that has engendered the development of isoelectronic phosphonate analogues of phosphates as enzyme inhibitors [4] (Scheme 2).

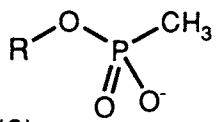
At the same time, growing emphasis on the need for rational design of enzyme inhibitors paves the way for creative thinking about phosphonates. A timely example concerns the role of phosphonates as natural products. Recent exploration [5,6] of one of the key steps in the biosynthesis of phosphonates, the enzymatic isomerization of phosphoenolpyruvate (**9**) into 3-phosphopyruvic acid (**10**) has led to the unexpected finding that the equilibrium position for the process is 99% in favor of PEP **9**. This



(5a)

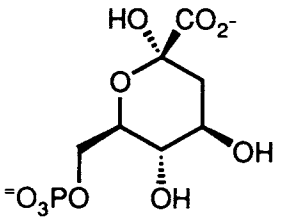


(5b)

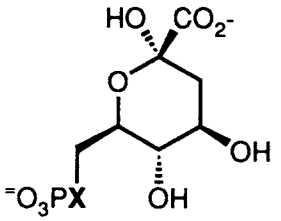


(6)

PHOSPHONATE INHIBITORS FOR DEHYDROQUINATE SYNTHASE

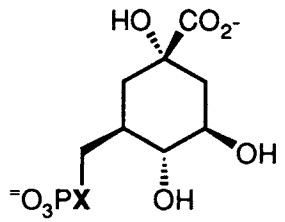


(7a)



(7b) X = −

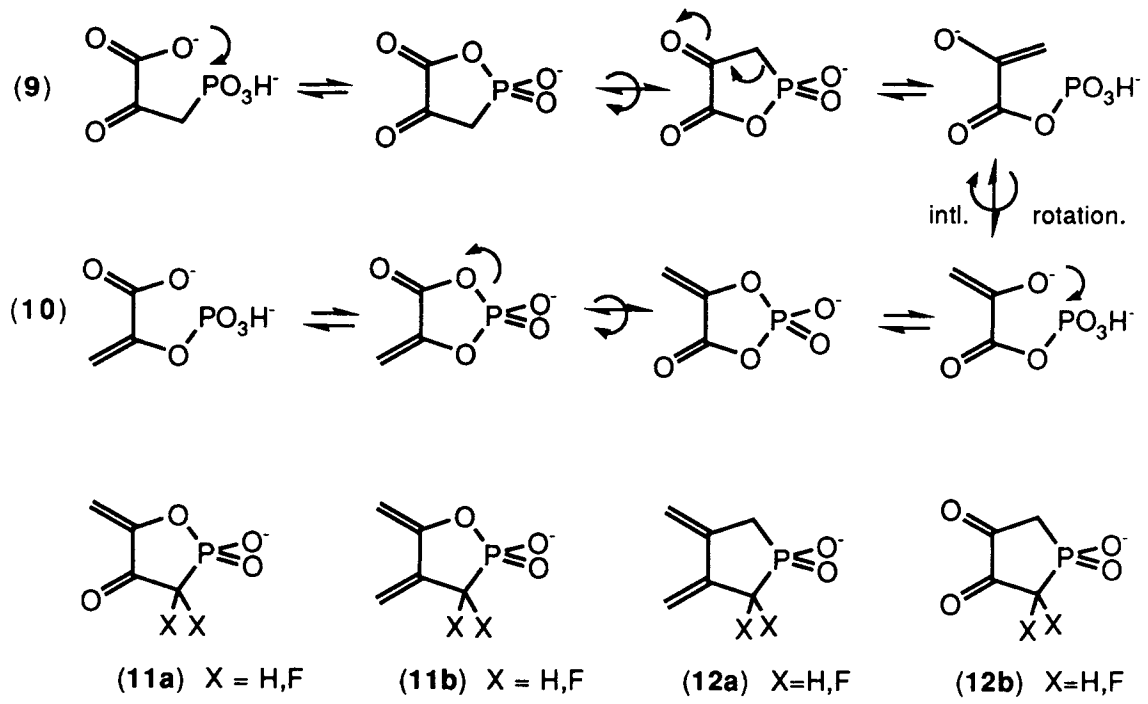
(7c) X = CH_2



(8a) X = -

(8b) X = CH_2

SCHEME 2



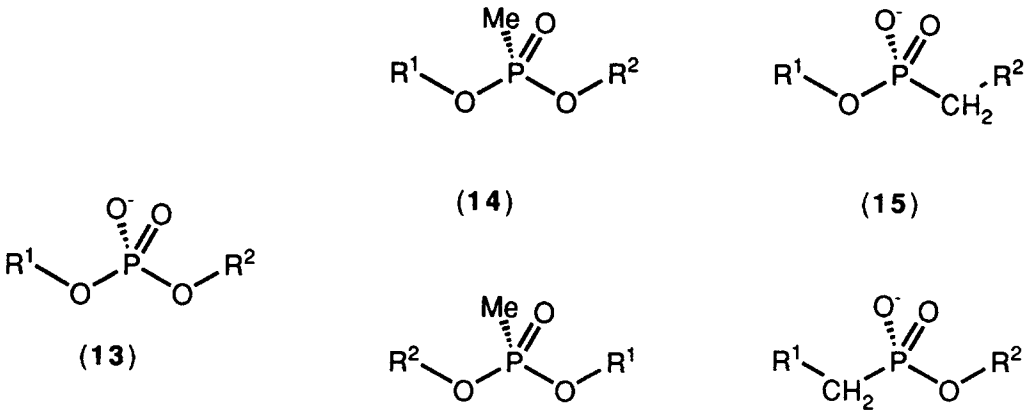
SCHEME 3

means that 3-phosphopyruvic acid is seemingly some 3 kcal mole^{-1} less stable than PEP, and makes this enzymatic interconversion of even greater significance. It is relatively profitable to speculate on the design of phosphonate inhibitors that might be used to identify the course of the reaction at a time when its mechanism is uncertain. One possible pathway (Scheme 3) whose bondmaking/bond-breaking processes via sequential 5-membered cyclic intermediates are clearly Westheimer-influenced [7], which takes advantage of a suitably-located carboxyl function [8], and which is compatible with the recently established [5] retention stereochemistry for intramolecular phosphoryl transfer, involves a series of pseudo-isosteric transformations that might reasonably be accommodated by a single enzyme binding/activating pocket that permits dissociation-rotation-reassociation processes of intermediates in a fashion that is reminiscent of the mode of action of enzymes such as aconitase (Scheme 3). Such a process might well be strongly inhibited by cyclic phosphonates such as **11** or phosphinic acids such as **12**, and it is feasible to envisage suicide-substrates based on such a geometry.

For the design of phosphonate monoester analogues of phosphate diesters, the possibilities are even more varied. In the general case of an unsymmetrical diester (**13**), one can replace either of the nonbridging oxygens by a carbon function, thereby creating a pair of stereoisomeric phosphonate diesters. The attractiveness of such species as, for example, nonionic, methylphosphonate diester analogues (**14**) of oligonucleotides has resulted in the application of great skill to the control of their stereochemistry [9]. Alternatively, one can substitute either of the bridging oxygens of a phosphate diester by carbon functions to create a pair of isomorphous phosphonate monoesters (**15**), both of which are isosteric and isocoulombic with the prototype phosphate **13** (Scheme 4).

It is in this area that we have been able to explore the particular features of phosphonate analogues of nucleotides that have accrued, especially from the replacement of a bridging oxygen between adjacent phosphorus atoms, in di-, tri-, and tetrapolyphosphate derivatives of nucleosides. At the same time as preserving the isoteric character of such analogues, which we have found to be essential for their proper coordination to metals such as magnesium [10], we have also developed isoelectronic phosphonate analogues by the incorporation of electronegative halogen atoms on bridging methylene groups to provide second-generation analogues of pyrophosphate, notably the fluoromethylene- and difluoromethylene-bisphosphonic acids [11].

In physical terms, such α-fluorophosphonates essentially correct the three 'static' mismatch parameters of geometry, acidity, and electronic shielding at phosphorus. The excellent LFE relationship between pK_a2 and ^{31}P NMR chemical shift established for a range of halomethylphosphonic acids [12] can now be set against the close geometrical relationship between an α-difluoroalkylphosphonate and the corresponding phosphate ester as shown by X-ray crystallographic analysis [13].

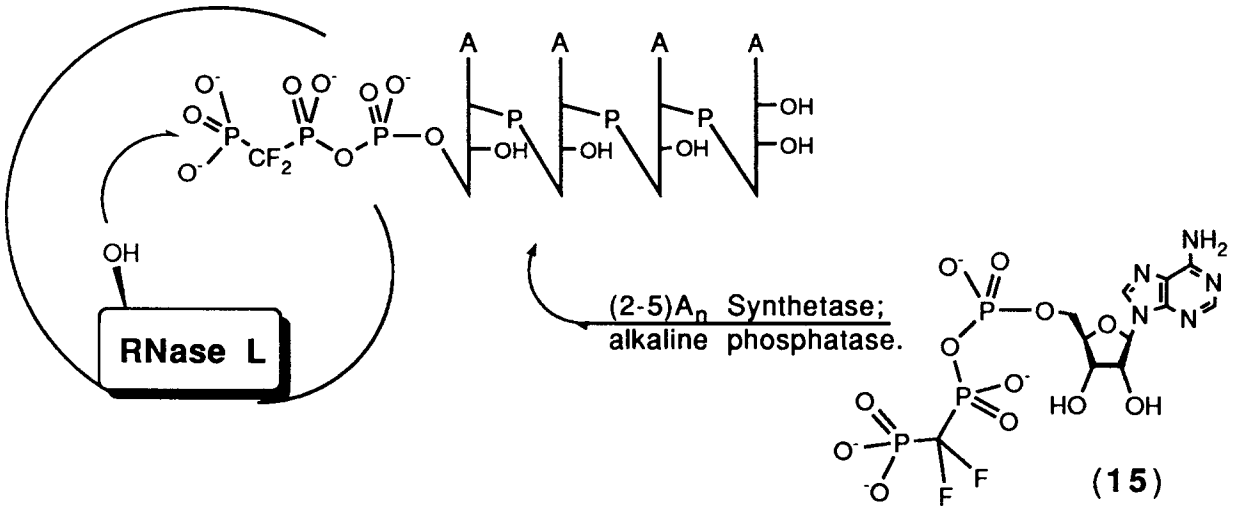


SCHEME 4

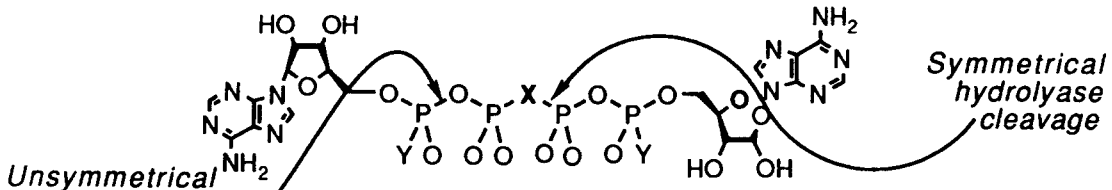
Moreover, α-fluorination also corrects the "dynamic" mismatch in which $S_N2(P)$ displacement at a phosphoryl center proceeds with retention of configuration for a thiophosphate, with inversion for the related phosphonothioate, but again with retention for the corresponding α-difluorophosphonothioate [14].

In terms of acceptability as analogues for enzymes substrates, the α-fluorination of phosphonates can have significantly beneficial effects. These also can be analyzed in static or dynamic terms. Following our demonstration of the improved performance of the β,γ-difluoromethylene analogue of ATP over the methylene species as a substrate for DNA-dependent RNA polymerase [10], we deployed this species (**15**) as a substrate for the interferon-induced $(2\rightarrow5)A_n$ polymerase where it proved to work at some 10% of V_{max} for ATP. The product, **16**, proved to be resistant to degradation by alkaline phosphatase to such an extent that a phosphatase digestion was introduced into the purification procedure! In an equally satisfactory manner, the analogue proved to bind as tightly to the RNase L as any analogue of $(2\rightarrow5)A_4$. However, rather than activate this RNase, the analogue **16** proved [15] to be an antagonist, this result supports the proposal that the biological role of $(2\rightarrow5)A_n$ is not simply that of an allosteric activator for RNase L but rather that a phosphorylation process is involved in the activation of this endonuclease, as adequately depicted in Scheme 5.

The topic to which we have applied the greatest range of applications of phosphonate analogues of nucleotides is in the investigation of the key minor nucleotide, 5′,5‴-diadenosyl P^1,P^4-tetraphosphate, Ap_4A, coupled to a study of the inhibition of its degradation by specific hydrolases and the study of its biological role. This species can be cleaved symmetrically by enzymes, such as that obtained from *E. coli*, to give two molecules of ADP, or it can be cut unsymmetrically by enzymes, as isolated from Lupin or *Artemia* species, to give ATP and AMP [16–18]. These enzymes are not only extremely specific, with K_m values in the micromolar range, they appear to be under kinase control to regulate the concentration of Ap_4A through the cell cycle [19]. Accordingly, their controlled inhibition provides a

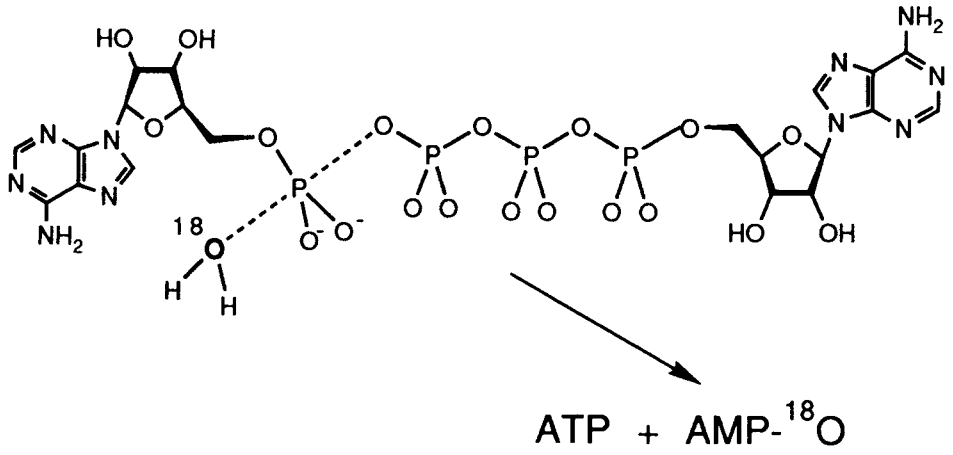


SCHEME 5



Diadenosyl Tetraphosphate X=Y=O (**17**)
Ap_4A analogues: $X=CH_nF_{2-n}$; Y=O,S (**18a,b**)
X=CHCl; Y=O (**18c,d**)

SCHEME 6



SCHEME 7

direct means for the exploration of the cellular function of Ap_4A (Scheme 6).

Our early studies identified the fact that P^1,P^4-dithio-P^2,P^3-methylene analogues of Ap_4A could inhibit enzymes catalyzing either the symmetrical or unsymmetrical cleavage [16]. However, when we explored the action of the *Artemia* enzyme on P^2,P^3-bridged Ap_4A analogues, we discovered not only that they behaved as substrates, but that their relative V_{max} data correlated linearly [17] with the pK_a of the leaving group, giving a Brønsted coefficient, β, of 0.5. In conjunction with the knowledge that the related Lupin enzyme cleaves Ap_4A at P-1 with inversion of configuration [20], indicating the absence of any covalently bound enzyme intermediate, it is clear that the transition state for this enzyme involves approximately 50% bond-breaking from P-1 to the bridging (P-2)-oxygen, and is consistent with an associative [21] transition state in an $S_N2(P)$ process (Scheme 7).

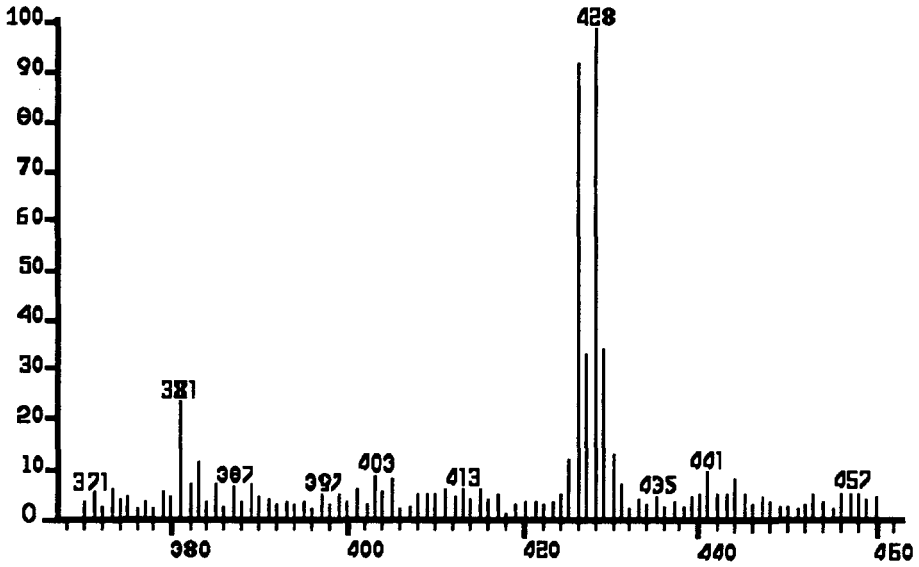
The active site for the Lupin enzyme is known to have the following preferences:

1. It requires adenosine nucleotides.
2. It prefers phosphate at the site of cleavage rather than thiophosphate [22, 23].
3. It accepts the (S_p) thiophosphate preferentially to its (R_p) diastereoisomer [23].
4. It does not cleave Ap_3A [24].

We decided to explore this picture further by investigation of the course of enzyme cleavage of Ap_4A and Ap_5A in water 50% enriched with 18-oxygen. Isolation of the resultant nucleotides by reverse phase hplc permitted investigation of their isotope content using positive ion FAB MS using a matrix of *p*-toluenesulphonic acid in glycerol. As a result, the nucleotide product(s) containing the phosphorus attacked by water in the cleavage reaction provide doublets for the parent ions (M + 1 and M + 3), shown in Scheme 8 for the ADP produced from Ap_5A.

The results are clearcut. The lupin hydrolase cleaves Ap_4A with incorporation of 18-oxygen exclusively into AMP while it cleaves Ap_5A with oxygen entering ADP but not ATP. Taken together with the inertness of Ap_3A, the picture of the binding site that emerges is shown in Scheme 9. It appears that:

1. The enzyme recognizes an adenosine 5′-polyphosphate ($n > 3$).
2. In the "inside" 5′-position, it prefers a phosphate to an (S_P)-thiophosphate.



Positive ion FAB MS of the ADP hydrolysis product from Ap_5A cleavage by Lupin Ap_4A hydrolase in 50%-enriched 18-oxygen water.

Matrix: glycerol / *p*TSA.

SCHEME 8

3. It "measures" four phosphates from the bound adenosine.
4. It cuts at that locus with water and the cleaved AMP (ADP) in an "outside" position.

Accordingly, it is able to accept Ap_5A and Ap_6A as substrates while Ap_3A cannot simultaneously satisfy the binding requirements both for the "inside" 5′-adenylate and for a phosphate in the fourth position (Scheme 9). Ap_3A is thus not cleaved.

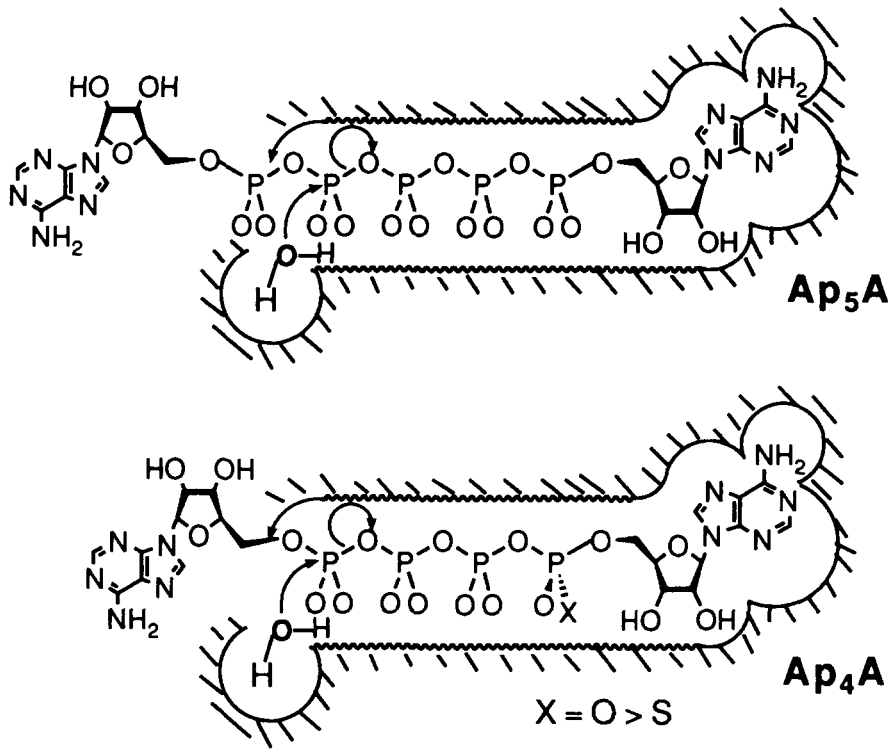
It remains likely that there is some flexibility in the recognition process. In cognate investigations [17] of the cleavage of the $P^1,P^2:P^3,P^4$-bis(monofluoromethylene) analogue of Ap_4A using the *Artemia* unsymmetrical hydrolase, we found a small but significant (5.5%) rate of cleavage to give β,γ-monofluoromethylene ADP, necessarily involving attack of water at P-2(3). Further analysis of this behavior is clearly required.

We have carried out related experiments using the symmetrical Ap_4A hydrolase from *E. coli*. It also manifests anomalous cleavage in the case of the β,β'-methylene analogue of Ap_4A, which gives AMP and β,γ-methylene ATP, though at only 4% of the rate of cleavage for Ap_4A. While this could involve adjacent attack of water at P-2(3), it seems much more likely to involve attack at P-1(4). The nucleotide products of hydrolysis of Ap_nA in 50% 18-oxygen water have been identified and show that the isotope resides in the ADP produced from Ap_4A, from Ap_5A, and from Ap_3A. While the stereochemistry of this process has not been determined, given the assumption that it involves inversion of configuration, the picture of the recognition site that emerges is that shown in Scheme 10.

The utility of these analogues of Ap_4A as enzyme inhibitors is of more than mere academic value. Paul Zamecnik has found them to be good inhibitors of platelet aggregation in vitro, and a survey for more than a dozen analogues with carbon bridges at $P^1,P^2:P^3,P^4$ or P^2,P^3 has identified the β,β'-monochloromethylene Ap_4A analogue as one of the most effective inhibitors. When used in vivo in the millimolar range, this substance is capable of arresting the stimulated formation of a blood clot in the carotid artery of the rabbit in at least 70% of animals. Moreover, the analogue cannot be degraded to ADP (which stimulates clotting!) and survives cleavage in the blood serum in vivo for a period of over two hours [25].

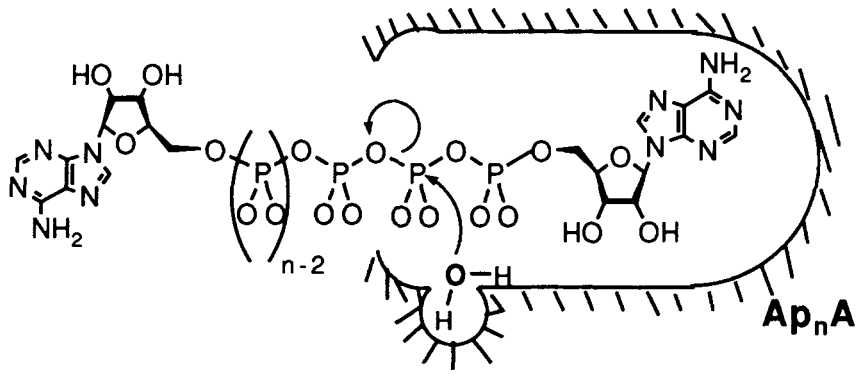
These enterprises have led us to establish means for the general synthesis of stereochemically-defined P^1,P^4-dithiophosphate analogues of Ap_4A, and this is most expediently accomplished by the coupling of adenosine 5′-thiophosphoromorpholidate with pyrophosphate or with various methylenebisphosphonates [26]. The multiple diastereoisomers can be efficiently separated in milligram quantities using reverse-phase fplc and they can be characterized in standard fashion by examination of their cleavage products with snake venom phosphodiesterase. In the case of the P^1,P^4-dithio-P^2,P^3-monofluoromethylene analogue of Ap_4A, there are four diastereoisomers. Two of these are only semisus-

Proposed Mechanism of Action of Lupin Ap_4A Hydrolase on Ap_nAs



SCHEME 9

Ap_nA Hydrolysis by *E.coli* Ap_4A Hydrolase



SCHEME 10

ceptible to SVPase and can thus be identified as the (R_p,S_p') and (S_p,R_p') species, which differ only in their stereochemistry at the CHF center. Preparative cleavage of these species provides two diastereoisomeric β,γ-monofluoromethylene ATPαS nucleotides and it is a simple process to desulphurize these using one of the standard cleavage systems (styrene oxide, NBS, etc.) to provide the first stereochemically controlled synthesis of the two β,γ-monofluoromethylene analogues of ATP [26]. Such compounds offer enormous potential for the application of ^{19}F NMR to explore the binding of nucleotide analogues to ATP-utilizing enzymes.

Finally, it is significant to record that there is an in vivo role for bisphosphonate analogues of Ap_4A. The innovative discovery by Klein and Satre that growing cells of the slime mould, *Dictyostelium discoideum*, not only tolerate millimolar concentra-

tions of methylenebisphosphonic acid but actually incorporate it into ATP (as β,γ-methylene ATP) and into Ap_4A (as β,β'-methylene Ap_4A) is a dramatic result [27]. We have elaborated on that initiative to explore a wide range of bisphosphonates. It is clear from a combination of ^{31}P NMR and fplc analysis that monofluoromethylenebisphosphonic acid is also incorporated into nucleotide products in the cell but that the range of acceptable species is rather narrowly defined. Some evidence suggests that the charge density might be an important factor while other results indicate that biosynthetic incorporation of analogues such as difluoromethylenebisphosphonate may be accompanied by efficient enzymatic cleavage of the nucleotide products, leading to no net incorporation!

In summary, we have demonstrated that there is an enormous potential for the exploitation of phosphonate analogues of nucleotides as probes of biochemical processes in vitro and in vivo. Some of the opportunities may well be of profound medical significance.

ACKNOWLEDGMENT

We wish to thank the SERC for financial support for this work and the Nuffield Foundation for a Travel Grant (to A.G.)

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