Acta Biochimica Polonica

Vol. 50 No. 4/2003

947–972

QUARTERLY

Review

Analogs of diadenosine tetraphosphate (Ap_4A)✪

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Received: 03 September, 2003; revised: 26 November, 2003; accepted: 04 December, 2003

Key words: diadenosine tetraphosphate, Ap_4A, dinucleoside polyphosphates, Ap_4A analogs, biological effects of Ap_4A analogs

This review summarizes our knowledge of analogs and derivatives of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A), the most extensively studied member of the dinucleoside 5′,5‴-P^1,P^n-polyphosphate (Np_nN) family. After a short discussion of enzymes that may be responsible for the accumulation and degradation of Np_4N′s in the cell, this review focuses on chemically and/or enzymatically produced analogs and their practical applications. Particular attention is paid to compounds that have aided the study of enzymes involved in the metabolism of Ap_4A (Np_4N′). Certain Ap_4A analogs were alternative substrates of Ap_4A-degrading enzymes and/or acted as enzyme inhibitors, some other helped to establish enzyme mechanisms, increased the sensitivity of certain enzyme assays or produced stable enzyme:ligand complexes for structural analysis.

Dinucleoside 5′,5‴-P^1,P^n-polyphosphates (Np_nN′s; where N and N′ are nucleosides and n represents the number of phosphate residues in the polyphosphate chain that links N with N′) have been found in various organisms (Garrison & Barnes, 1992). Their normal, submicromolar levels increase dramatically during cellular stress, reaching submillimolar in some cases (Lee *et al.*, 1983; Coste *et al.,* 1987; Pálfi *et al.,* 1991). Diadenosine tri- and tetraphosphates appear to be the most prominent but diadenosine penta- (Pintor *et al.*, 1992a), hexa- (Pintor *et al.*, 1992b) as well as di- (Luo *et al.*, 1999) and

✪This work was supported by grant PBZ-KBN 059/T09/04 from the State Committee for Scientific Research (KBN, Poland)

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heptaphosphates (Jankowski *et al.*, 1999) have been detected in some mammalian cells. *In vitro s*tudies suggest that the following enzymes from various phyla may be responsible for the accumulation of the adenine-containing Np_nN's *in vivo*: certain aminoacyl-tRNA synthetases (EC 6.1.1.x) (Zamecnik *et al.*, 1966), Ap_4A phosphorylase (EC 2.7.7.53) (Guranowski *et al.*, 1988), firefly luciferase (EC 1.13.12.7) (Guranowski *et al.,* 1990), acyl-CoA synthetase (EC 6.2.1.8) (Fontes *et al.*, 1998), HIV-1 reverse transcriptase (EC 2.7.7.49) (Meyer *et al.*, 1998), DNA ligases (EC 6.5.1.1) (Madrid *et al.*, 1998; McLennan, 2000; Günther Sillero *et al.*, 2002), RNA ligase (EC 6.5.1.3) (Atencia *et al.*, 1999), nonribosomal peptide synthetase (Dieckman *et al.*, 2001) and, in plants, coumarate-CoA synthetase (EC 6.2.1.12) (Pietrowska-Borek *et al.*, 2003). A GTP:GTP guanylyltransferase (EC 2.7.7.45) also exists in certain organisms and may be responsible for the synthesis of Gp_nNs, i.e. guanine-containing Np_nN's (Wang & Shatkin, 1984; Liu & McLennan, 1994). These enzymes are particularly effective in the synthesis of dinucleoside tetraphosphates: diadenosine tetraphosphate (AppppA or Ap_4A) and diguanosine tetraphosphate (GppppG or Gp_4G), respectively. They are also able to synthesize Np_nN's in which n>4. Not all of them, however, can produce Np_3N's. For example, firefly luciferase and coumarate-CoA synthetase are incapable of Ap_3A synthesis. Finally, it has been suggested (Guranowski *et al.*, 1990) that dinucleotides such as Ap_4U and Ap_4C, found among the pyrimidine-containing Np_4N's in *Escherichia coli* and *Saccharomyces cerevisiae* (Coste *et al.*, 1987), can be formed by enzymatic transfer of UMP- and CMP-moieties onto ATP from appropriate intermediates involved in carbohydrate and lipid metabolism (e.g. UDP-glucose and CDP-choline, respectively). In fact, the uridine triphosphate:glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) from *S. cerevisiae* has been recently found to synthesize Up_nNs, including Ap_4U and Ap_5U, by transfer of the UMP residue from UDP-glucose on to ATP and p_4A, respectively. (Guranowski *et al.*, 2004).

The biological roles of Np_nN's are rather obscure. Some data suggest that they act as signalling molecules in, for example, regulation of the cell cycle (Grummt, 1978; Nishimura, 1998). Under certain circumstances, e.g. when competing with ATP in ATP-dependent reactions (Rotllan & Miras-Portugal, 1985; Pype & Slegers, 1993) and/or binding with nucleotide receptors (Pintor *et al.*, 1991), Np_nN's can be detrimental to the organism (McLennan, 2000). The levels of Np_nN's can be precisely regulated by numerous degradative enzymes (Guranowski, 2000). In addition to the non-specific ones, like nucleotide pyrophosphatases/phosphodiesterases (Jakubowski & Guranowski, 1983; Bartkiewicz *et al.*, 1984; Cameselle *et al.*, 1984; Gasmi *et al.*, 1998; Vollmayer *et al.*, 2003), for which Np_nN's are very good substrates, there are various specific enzymes. In higher eukaryotes (animals and plants) there is a dinucleoside triphosphatase (EC 3.6.1.29) that preferentially converts Np_3N's to nucleoside mono- and diphosphates, NMP + N′DP and/or N′MP + NDP, and a dinucleoside tetraphosphatase (EC 3.6.1.17) that asymmetrically hydrolyzes Np_4N's to either NTP + N′MP or N′TP + NMP. In lower eukaryotes — fungi (yeast) and protozoa (*Euglena*) — dinucleoside tetraphosphates are degraded phosphorolytically, either to N′DP + NTP or to NDP + N′TP, by Ap_4A phosphorylases (EC 2.7.7.53). In bacteria, Np_4N's are hydrolyzed symmetrically to NDP + N′DP by a specific Co^{2+}-dependent Ap_4A hydrolase (EC 3.6.1.41). Recently, however, an asymmetrically-acting Np_4N-ase related to the higher eukaryotic enzyme has been detected in several bacteria (Conyers & Bessman, 1999; Cartwright *et al.*, 1999; Bessman *et al.*, 2001; Lundin *et al.*, 2003). The *asymmetrical* Np_4N-ases belong to the "nudix" protein family, comprising enzymes that hydrolyze nucleotides in which a **nu**cleoside **di**phosphate is attached to one of

various groups assigned as **x** (Bessman *et al.*, 1996; 2001). These nudix proteins have a conserved amino+acid sequence that directly participates in catalysis (Harris *et al.*, 2000; Maksel *et al.*, 2001). A search for nudix proteins in various genomes has led to the discovery of hydrolases that prefer Ap_5A and/or Ap_6A as substrates in budding yeast (*S. cerevisiae*) (Cartwright & McLennan, 1999), fission yeast (*Schizosaccharomyces pombe*) (Ingram *et al.*, 1999) and humans (Safrany *et al.*, 1999). One approach to understanding the biological roles of Np_nN's is through the use of structural analogs in biochemical and physiological studies. This review focuses on analogs of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (**1**)* (Ap_4A), the most widely investigated member of the Np_nN's, and presents our current knowledge of both chemically and enzymatically produced nucleotides. Particular attention is paid to compounds that have been useful in studies of enzymes involved in the metabolism of Ap_4A. Analogs that behave either as alternative substrates of Ap_4A-degrading enzymes and/or as enzyme inhibitors have helped to establish the mechanism of action of these enzymes. They have also increased the sensitivity of some enzyme assays, or, by forming stable enzyme:ligand complexes, allowed an analysis of the substrate-binding site in the three-dimensional structures of certain hydrolases. This work partially updates two earlier reviews (Blackburn *et al.*, 1992; Guranowski, 2000) that presented some chemical and biological aspects of both Ap_4A and Ap_3A analogs.

CHEMICALLY SYNTHESIZED ANALOGS OF Ap_4A

Organic chemists have produced a wide variety of Ap_4A analogs differing from Ap_4A either in the oligophosphate chain (thus being Ap_4A homologs), in the base, and/or in the sugar moieties. Np_nN's, including Ap_4A, were observed by John Moffatt and co-workers in 1965 as highly stable by-products of ATP dismutation reactions (Reiss & Moffatt, 1965). Subsequently, procedures directed towards the syntheses of various homo- (N = N′) and hetero- (N ≠ N′) Np_nN's were developed in several laboratories (Feldhaus *et al.*, 1975; Tarussova *et al.*, 1986; Ng & Orgel, 1987; Fukuoka *et al.*, 1995). Other laboratories focused on syntheses that yielded analogs modified in the polyphosphate chain. These comprised: (i) replacement of the P^2-P^3 (**2**), P^1-P^2 (**3**) or P^1-P^2 and P^3,P^4 (**4**) bridging oxygen(s) with methylene (**2**–**4**), halomethylene- (**5**), ethylene- or acetylene- group(s) (Tarussova *et al.*, 1983; 1985; Blackburn *et al.*, 1987a); (ii) replacement of the oxygen(s) with imido group(s) (Shumiyanzeva & Poletaev, 1984); (iii) attachment of adenylate moieties to methanetrisphosphonate (**10**) (Liu *et al.*, 1999); (iv) adenylylation of polyalcohols such as glycerol, erythritol (**11**) and pentaerythritol (Baraniak *et al.*, 1999) and (v) adenylylation of the hydroxymethyl groups of di(hydroxymethyl)phosphinic acid (Baraniak *et al.*, 1999). Yet another group of chemically generated Ap_4A analogs comprised mono- (**6**–**7**) (Haikal *et al.*, 1989; Puri *et al.*, 1995) and diphosphorothioates either of Ap_4A (**8**) (Blackburn *et al.*, 1987b), or of the aforementioned Ap_4A analogs with methylene or halomethylene group(s) (**9**) (Blackburn & Guo, 1990), or of the derivatives of polyols (**12**–**14**) (Baraniak *et al.*, 1999) and di(hydroxymethyl)phosphinic acid (Baraniak *et al.*, 1999; Walkowiak *et al.*, 2002). Recently, di(adenosine-5'-*O*-phosphorodithioate),di(hydroxymethyl)phosphonic acid (**15**) was synthesized (Walkowiak *et al.*, 2002).

The list of Ap_4A analogs can be further extended by modifications of the adenine or sugar moieties. Treatment of Ap_4A with 2-chloroacetaldehyde yields the fluorescent mono- and di(1, N^6-etheno)derivatives, εAp_4A

*Numbers in bold refer to compounds whose structures are presented in Table 1.

Table 1. Selected analogs of diadenosine tetraphosphate

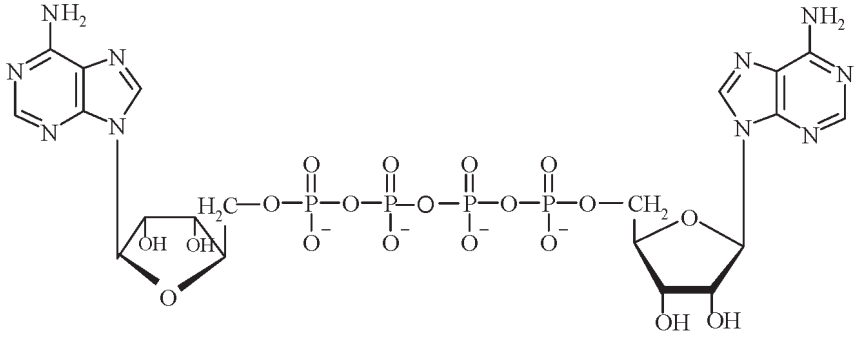
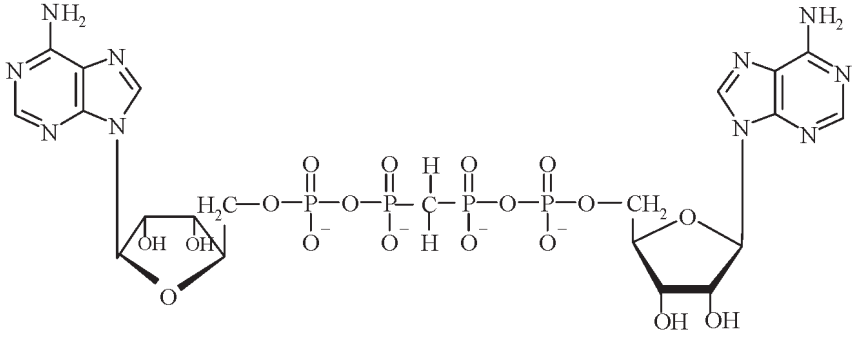
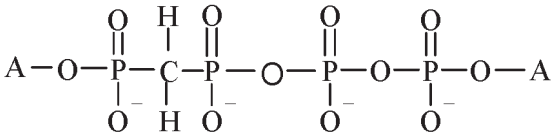
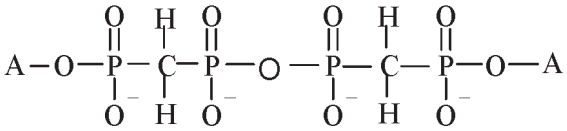
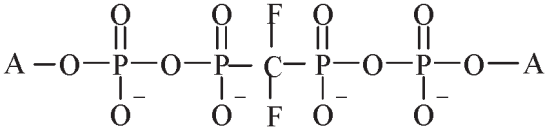
No.	Formula	Selected refs.
1	 AppppA	Reiss & Moffatt, 1965; Zamecnik *et al.*, 1966
	Analogs Modified in the Polyphosphate Chain	
	Methylene- and Halomethylene Analogs	
2	 AppCH$_2$ppA	Tarussova *et al.*, 1983; Guranowski *et al.*, 1987 Bailey *et al.*, 2002
3	 ApCH$_2$pppA	Guranowski *et al.*, 1987
4	 ApCH$_2$ppCH$_2$pA	Tarussova *et al.*, 1983; Guranowski *et al.*, 1987
5	 AppCF$_2$ppA	McLennan *et* al., 1989; Guranowski *et al.*, 1989

Table 1. Continued

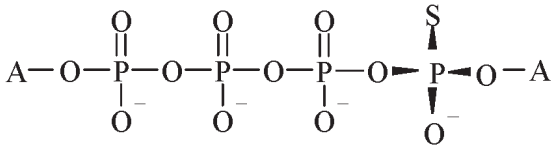
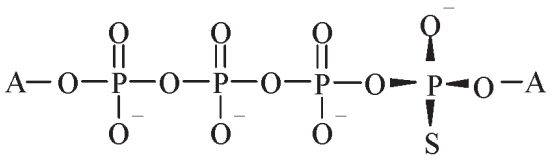
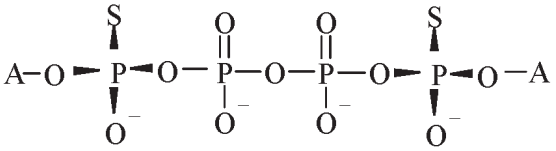
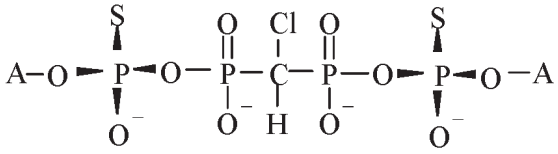
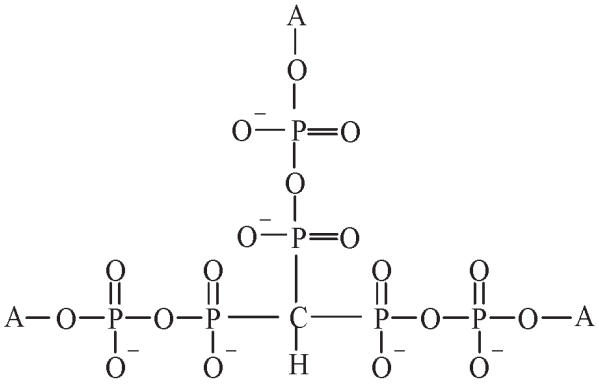
	Phosphorothioate Analogs	
6	 (*Sp*)AppppAαS	Haikal *et al.*, 1989; Łażewska & Guranowski, 1990
7	 (*Rp*)AppppAαS	Łażewska & Guranowski, 1990
8	 (*Sp,Sp*)Ap_spppp_sA	Blackburn *et al.*, 1987b
9	 $Ap_spCHClpp_sA$	Blackburn & Guo, 1990; Chen *et al.*, 1997
	Adenylated Methanetrisphosphonates	
10	 Triadenylated-methanetriphosphonate	Liu *et al.*, 1999

Table 1. Continued

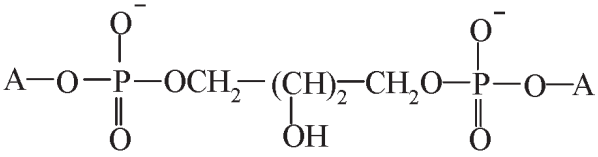
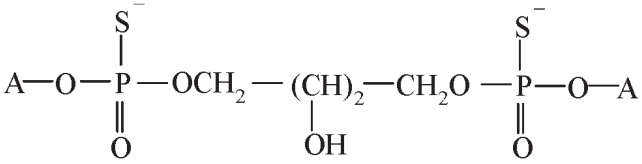
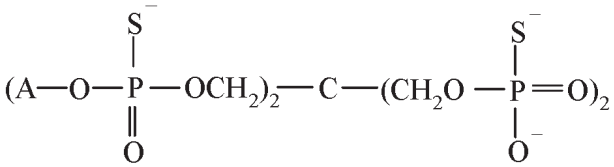
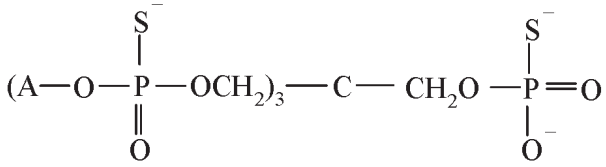
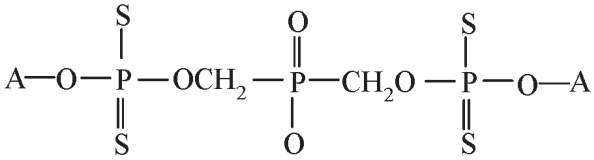
	Adenosine-phosphorylated and Adenosine-phosphorothioylated Polyols	
11	 1,4-Di(adenosine-5′-*O*-phospho)erythritol	Baraniak *et al.*, 1999; Guranowski *et al.*, 2003b
12	 1,4-Di(adenosine-5′-*O*-phosphorothio)erythritol	Baraniak *et al.*, 1999; Guranowski *et al.*, 2003b
13	 Di(adenosine-5′-*O*-phosphorothio), di(phosphorothio)penthaerythritol	Baraniak *et al.*, 1999; Guranowski *et al.*, 2003b
14	 Tri(adenosine-5′-*O*-phosphorothio), phosphorothio, penthaerythritol	Baraniak *et al.*, 1999; Guranowski *et al.*, 2003b
	Di(Thio)Adenylated Di(hydroxymethyl)phosphonic acid	
15		Walkowiak *et al.*, 2002
	Ap_4A Analogs Modified in Aglycone(s); Hybrids	

Table 1. Continued

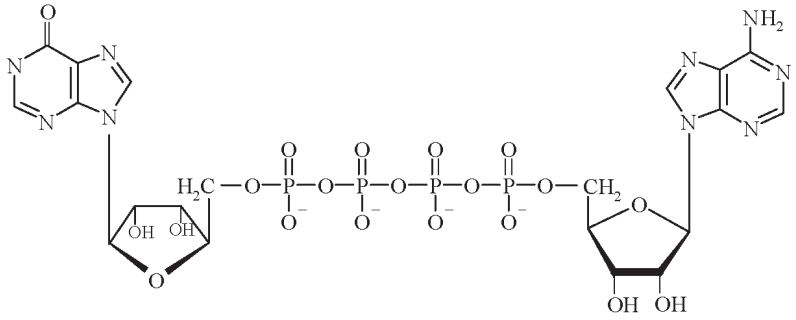
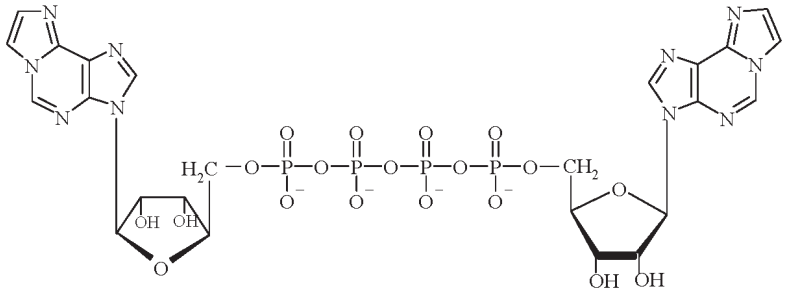
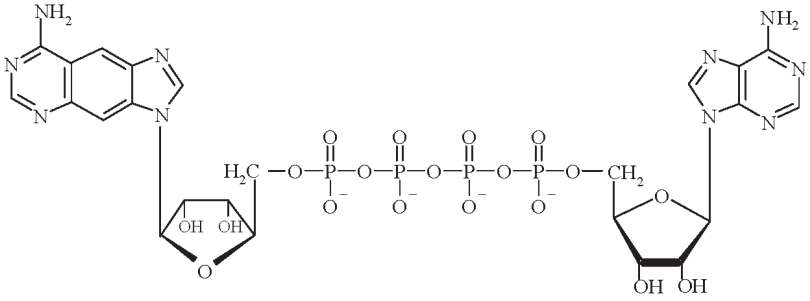
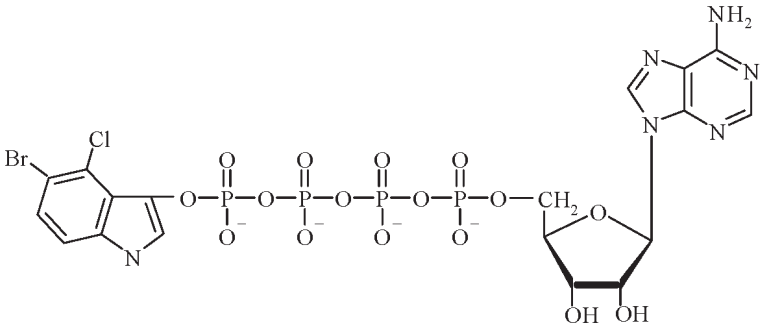
16	 IppppA	Guranowski *et al.*, 1995;
17	 εAppppεA	Wierzchowski *et al.*, 1985; Rotllán *et al.*, 1991
18	 *lin*-benzo-AppppA	VanDerLijn *et al.*, 1979
19	 5Br-4Cl-3-indolyl-ppppA	Garrison *et al.*, 1993

Table 1. Continued

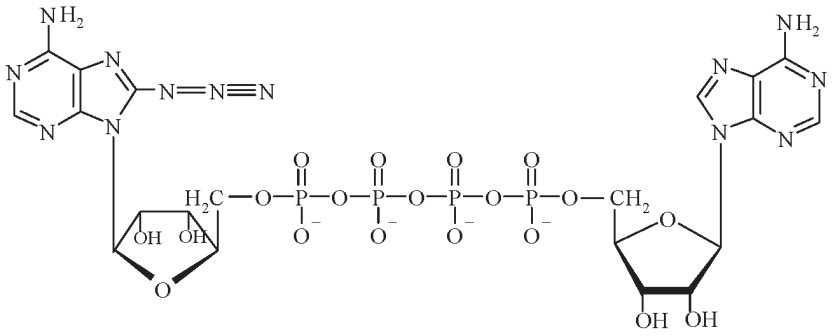
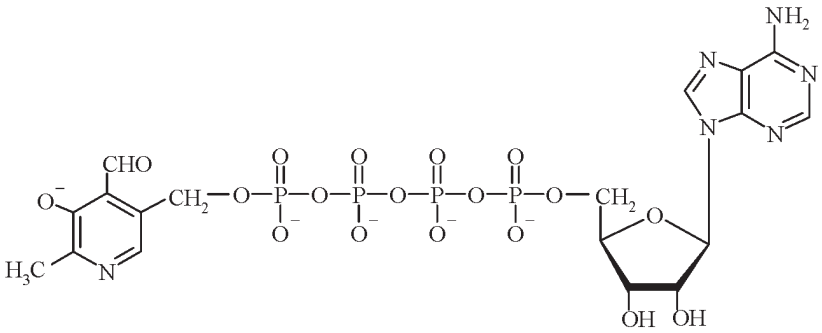
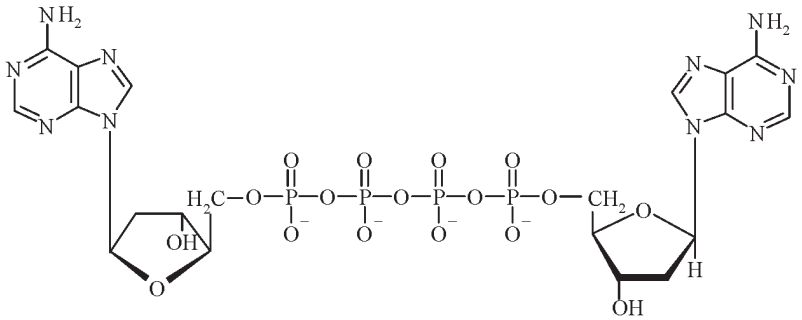
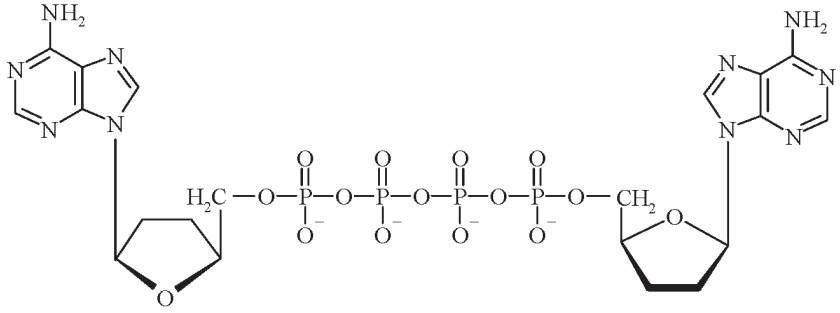
20	 8-azido-AppppA	Prescott & McLennan, 1990; Baxi *et al.*, 1994; Chavan & Haley, 1994
21	 pyridoxal-ppppA	Tagaya & Fukui, 1986; Yagami *et al.*, 1988; Dominici *et al.*, 1988; Hibi *et al.*, 1993;
	Ap_4A Analogs Modified in Sugar(s)	
22	 dAppppdA	Randerath *et al.*, 1966; Pietrowska-Borek *et al.*, 2003
23	 ddAppppddA	Günther Sillero *et al.*, 1997

Table 1. Continued

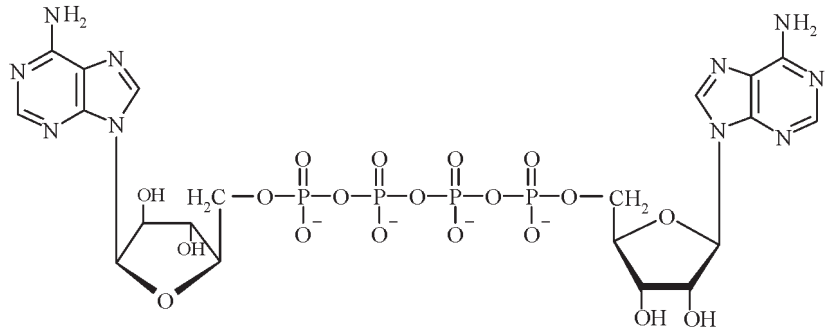
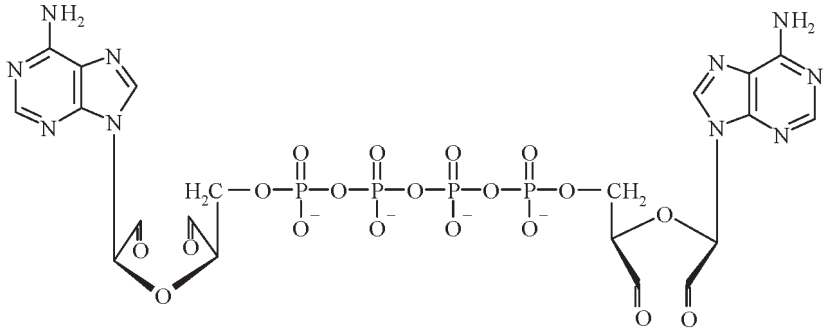
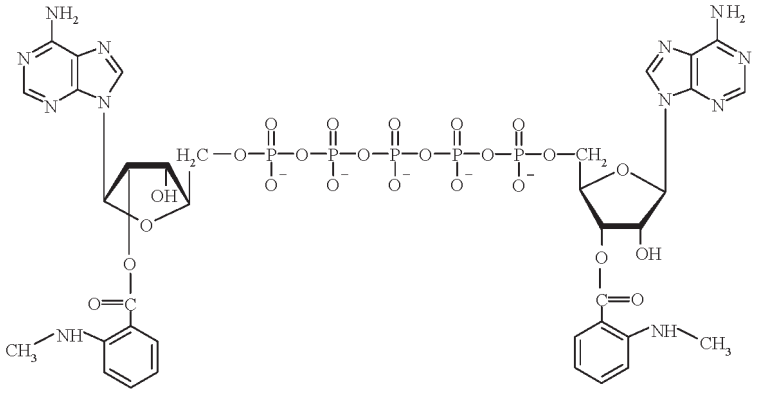
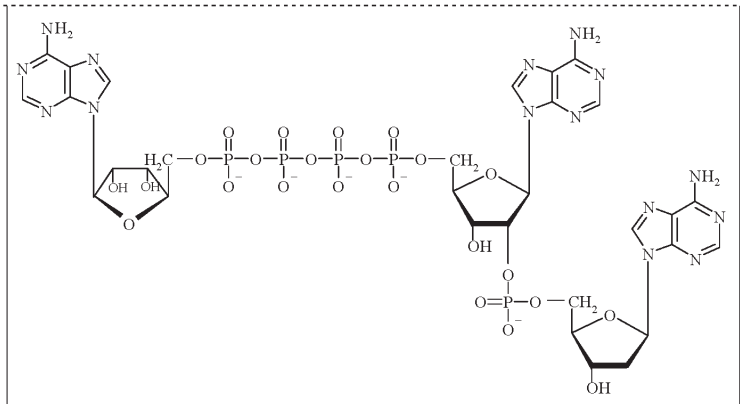
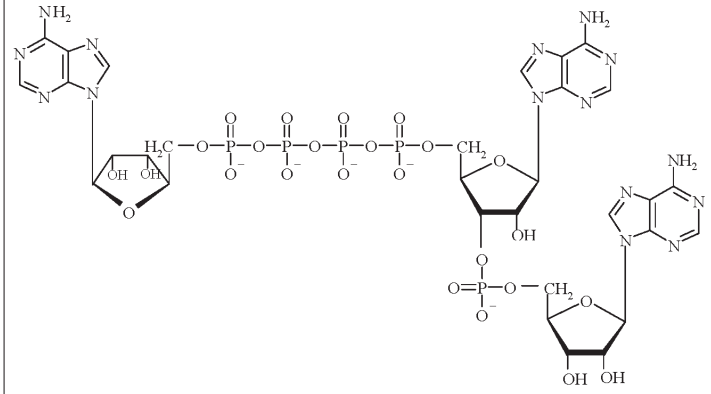
24	 (ara)AppppA	Theoclitou *et al.*, 1994
25	 oAppppoA (where oA represents adenosine-2′,3′-dialdehyde)	Grummt *et al.*, 1979
Ap_4A Derivatives Substituted in Ribose(s)		
26	 *m*Apppppm*A* (where *m* = *N*-methylantraniloyl-)	Reistein *et al.*, 1990

Table 1. Continued

27	 (2′-pdA)AppppA	Guranowski *et al.*, 2000
28	 (3′-pA)AppppA	Günther Sillero *et al.*, 2001; Guranowski *et al.*, 2003a

and εAp$_4$εA (**17**) (Wierzchowski *et al.*, 1985; Rotllán *et al.*, 1991). Periodate converts Ap$_4$A into its (bis)2′,3′-dialdehyde derivative, oAp$_4$oA (**25**), which can serve as an affinity label (Grummt *et al.*, 1979). A chromogenic analog, in which 5-bromo-4-chloro-3-indolyl phosphate is coupled to ATP (**19**) (Garrison *et al.*, 1993), another fluorescent analog, *lin*-benzoAp$_4$A (**18**) (VanDerLijn *et al.*, 1979), and two analogs for affinity labeling, 8-azido-Ap$_4$A (**20**) (Prescott & McLennan, 1990; Baxi *et al.*, 1994) and pyridoxal-p$_4$A (**21**) (Yagami *et al.*, 1988), have also been synthesized.

So far, only three Ap$_4$A analogs modified in the sugar (ribose) moieties have been synthesized. These are the dinucleotides containing fluorescent *N*-methylanthraniloyl group(s) (abbreviated here as *m*) bound to the 2′- or 3′-hydroxyl of the ribose(s) *via* an ether linkage: *m*Ap$_5$A and *m*Ap$_5$*m*A (**26**) (Reinstein *et al.*, 1990) and *m*Ap$_5$T (Lavie *et al.*, 1998).

ENZYMATICALLY PRODUCED Ap$_4$A ANALOGS

Enzymatically produced Ap$_4$A analogs can be synthesized as a result of the broad substrate specificity of some ligases, transferases and firefly luciferase. This ability is conferred principally by the adenylate-binding sites of these enzymes. Certain aminoacyl-tRNA synthetases (Randerath *et al.*, 1966; Plateau & Blanquet, 1982; Jakubowski, 1983; Theoclitou *et al.*, 1994), yeast Ap$_4$A phosphorylase (Brevet *et al.*, 1987; Guranowski *et al.*, 1988; Łażewska & Guranowski,

1990), firefly luciferase (Günther Sillero *et al.*, 1991; Ortiz *et al.*, 1993; Günther Sillero *et al.*, 1997) and coumarate:CoA ligase (Pietrowska-Borek *et al.*, 2003) can therefore be employed for the synthesis of different Ap_4Ns using ATP (or ADP in the case of the Ap_4A phosphorylase) as an adenylate donor and various NTPs as adenylate acceptors. In addition to NTPs, ADP can also act as an adenylate acceptor (but only in the case of aminoacyl-tRNA ligases) to yield Ap_3A, while p_4A or p_4G give rise to Ap_5A and Ap_5G, respectively. ATPαS used as an adenylate acceptor yields a monophosphorothioate derivative of Ap_4A ($Ap_4A\alpha S$ or Ap_spppA) (**6**,**7**) and $\beta,\gamma[CH_2]$ATP and $\alpha,\beta[CH_2]$ATP allow the synthesis of $AppCH_2ppA$ (**2**) and $ApCH_2pppA$ (**3**), respectively. εATP used as the only NTP in the reaction mixture yields $\varepsilon Ap_4\varepsilon A$ (**17**), while ATPγS, being a poor adenylate acceptor, yields only small amounts of $Ap_4A\beta S$ (App_sppA) (Günther Sillero *et al.*, 1991). Some sugar-modified NTPs such as dATP, ddATP and araATP have also been used as adenylate acceptors yielding dAppppA, ddAppppA and araAppppA (**24**) (Zamecnik *et* al., 1966; Plateau & Blanquet, 1982; Theoclitou *et al.*, 1994; Günther Sillero *et al.*, 1997). It has been shown that dATP and ddATP, used as the sole NTP in the reaction mixture, act both as NMP-donors and NMP-acceptors yielding dAppppdA (**22**) (Randerath *et al.,* 1966; Plateau & Blanquet, 1982; Günther Sillero *et al.*, 1991; 1997; Pietrowska-Borek *et al.*, 2003) and ddAppppddA (**23**) (Günther Sillero *et al.*, 1997), respectively. As mentioned previously, Ap_4U can be also formed by yeast UTP:glucose-1-phosphate uridylyltransferase, which catalyzes transfer of UMP from UDP-glucose to ATP (Guranowski *et al.*, 2004). Another family of enzymatically produced Ap_4A analogs, such as Ap_4ddA or Gp_4ddA, can be formed by HIV-1 reverse transcriptase (Meyer *et al.*, 1998). In pyrophosphorolysis-like reactions, this transferase carries out nucleotide-dependent removal of a dideoxynucleotide residue from the 3′-end of a chain-terminated DNA primer through production of a dinucleoside polyphosphate.

Other Ap_4A derivatives modified on the sugar moiety can be obtained from reactions supported by 2′,5′-poly(A) synthase, which introduces (deoxy)adenylate residue(s) onto the 2′-(and 2‴-) position(s) of Ap_4A (**27**) (Cayley & Kerr, 1982; Guranowski *et al.*, 2000) while the recently reported poly(A) polymerase from *E. coli* can monoadenylate Ap_4A at one of its two 3′-positions (**28**) (Günther Sillero *et al.*, 2001; Guranowski *et al.*, 2003).

The adenine-containing analogs can be successively converted into their hypoxanthine-containing counterparts, Ip_nNs, such as Ip_4A (**16**) and Ip_4I by treatment with adenosine-phosphate deaminase (EC 3.5.4.17) from the snail *Helix pomatia* or the fungus *Aspergillus oryzae* (Guranowski *et al.*, 1995).

Finally, truncated Ap_4A derivatives, such as adenosine(5′)tetraphospho(5′)ribose and ribose(5′)tetraphospho(5′)ribose, can be produced by ATP *N*-glycosidase from the marine sponge *Axilla polypoides*. This unusual enzyme catalyzes hydrolysis of the *N*-glycosidic bond in any compound containing an adenosine-5′-diphosphoryl moiety (Reintamm *et al.*, 2003). In practice, the quantities of Ap_4A analogs synthesized enzymatically are much lower than those obtained by chemical procedures. Representative structures of chemically and enzymatically generated Ap_4A analogs are shown in Table 1.

ISOTOPICALLY LABELED Ap_4A ANALOGS AND THEIR APPLICATION

[U-^{14}C]Ap_4A or [^{3}H]Ap_4A, which can either be produced enzymatically from appropriately labeled ATP (Jakubowski & Guranowski, 1983; Baril *et al.*, 1983) or purchased from radiochemical suppliers (Guranowski *et al.*, 1983; Guranowski & Blanquet, 1985; Guranowski *et al.,* 1987), have been routinely

used as substrates for various Ap_4A-degrading enzymes in quantitative assays. [^{3}H]Ap_4A has also been used to study the distribution of Ap_4A binding sites in rat brain (Rodriquez-Pascual *et al.*, 1997) and the mechanism by which Ap_4A mediates stimulation of gluconeogenesis in isolated rat proximal tubules (Edgecombe *et al.*, 1997). [^{3}H]oAppppoA was used to identify the Ap_4A-binding subunit of calf thymus DNA polymerase α (EC 2.7.7.7) (Grummt *et al.*, 1979), and [^{3}H]Ap_4A and [^{32}P]Ap_4A allowed identification of the Ap_4A-binding subunit of a multiprotein form of HeLa cell DNA polymerase α (Baril *et al.*, 1983). Ap_4A labeled with the stable oxygen isotopes ^{17}O and ^{18}O, (R_p,R_p)-P^1,P^4-bis(5'-adenosyl)-1[^{17}O,$^{18}O_2$],4[^{17}O,$^{18}O_2$]tetraphosphate, was prepared by Dixon & Lowe (1989) and used in their elegant stereochemical analysis of the hydrolytic mechanism of the (*asymmetrical*) Ap_4A hydrolase from yellow lupin seeds. Another labeled analog, [^{32}P]-P^1-(adenosyl-5′)-P^4-(8-azido-adenosyl-5′)tetraphosphate ([α'-^{32}P]8-N_3-Ap_4A), was synthesized chemically from [α-^{32}P]ATP and 8-N_3-AMP by the water-soluble carbodiimide method and used as a photoaffinity label to detect Ap_4A-binding proteins in cell extracts (Prescott & McLennan, 1990; Baxi *et al.*, 1994). [β-^{32}P]8-N_3-Ap_4A was also synthesized by Chavan & Haley (1994) to study its interaction with acidic fibroblast growth factor. [^{3}H]Apppp$_s$As, (S_p) [^{3}H]$Ap_4A\alpha$S and (R_p) [^{3}H]$Ap_4A\alpha$S, all synthesized enzymatically from [^{3}H]ATP and the appropriate stereoisomers of ATPαS, were used as alternative substrates for various Ap_4A-degrading enzymes (Łażewska & Guranowski, 1990). A 2'-deoxyadenylated derivative of Ap_4A labeled on both adenines, [^{3}H]Apppp[^{3}H]A(2′-pdA), was used to demonstrate the cleavage preference of this asymmetric substrate by the (*asymmetrical*)Ap_4A hydrolases, yielding either [^{3}H]ATP + [^{3}H]AMP(2′-pdA) or [^{3}H]ATP(2′-pdA) + AMP (Guranowski *et al.*, 2000; Maksel *et al.*, 2001). Similarly, AppppA(3′-[^{32}P]pA) has recently been synthesized (Günther Sillero *et al.*, 2001) and used to study the susceptibility of this new type of Ap_4A derivative to enzymatic cleavage catalyzed by several specific Ap_4A- and/or Ap_3A-degrading enzymes (Guranowski *et al.*, 2003a). Studies of these two sets of Ap_4A derivatives also showed that the (*asymmetrical*)Ap_4A hydrolases tolerate a bulky substituent such as a nucleotide at the 2′ or 3′ position of their potential substrates. The (*symmetrical*)Ap_4A hydrolase from *E. coli* also cleaved 3′-pA(Apppp)A (to ADP and ADP(3′-pA)) but the yeast Ap_4A phosphorylase and Ap_3A hydrolases did not recognize this compound as a substrate (Guranowski *et al.*, 2003a).

APPLICATION OF SOME OTHER Ap_4A ANALOGS; THEIR INTERACTION WITH DIFFERENT PROTEINS

Ap_4A homologs

So far, Ap_5A has been the most useful of the Ap_4A homologs. Acting as a bisubstrate analog it strongly inhibits adenylate (EC 2.7.4.3) and adenosine (EC 2.7.1.20) kinases. The lowest K_i values, around 30 nM, were estimated for the adenylate kinases from rabbit (Lienhard & Secemski, 1973) and pig skeletal muscle (Feldhaus *et al.*, 1975). Based on this, an Ap_5A-Sepharose affinity resin was prepared and successfully used for the isolation of adenylate kinase from vertebrate muscle (Feldhaus *et al.*, 1975). Ap_6A was a much poorer inhibitor, with K_i values of 450 nM for the porcine adenylate kinase (Feldhaus *et al.*, 1975) and 55 nM for the rabbit muscle enzyme (Bone *et al.*, 1986b). Co-crystallization of Ap_5A with adenylate kinases from pig muscle (Pai *et al.*, 1977), baker's yeast (Egner *et al.*, 1987) and *E. coli* (Müller & Schulz, 1988) has allowed the three-dimensional structures of these enzymes to be studied. The K_i values for Ap_5A acting as a competitive inhibitor of MgATP binding were 73 nM and 400 nM, respectively, for the adenosine kinase from human liver (Bone *et al.*, 1986b) and bovine adrenal me-

dulla (Rotllan & Miras-Portugal, 1985). Ap_5A and Ap_6A also inhibited calf thymus terminal deoxynucleotidyl transferase (EC 2.7.7.31) with K_i values of 1.5 μM and 1.3 μM, respectively. These two compounds were found to be more effective than the diadenosine polyphosphates containing 2-, 3- or 4-phosphate groups. However, only Ap_5A seems to span both the substrate and primer binding site domains of the enzyme (Pandey *et al.*, 1987; Pandey & Modak, 1987). Ap_5A and Ap_6A also inhibit the nucleotide-depleted mitochondrial F_1-ATPase (EC 3.6.1.34) and have been employed in studies of the orientation of the catalytic and non-catalytic sites of this enzyme (Vogel & Cross, 1991). Finally, Ap_5A has been shown to inhibit carbamoyl phosphate synthetase (glutamine hydrolyzing) (EC 6.3.5.5), indicating that this enzyme has two separate binding sites for ATP (Powers *et al.*, 1977).

Ap_5A and Ap_6A also affect some physiological processes. Of the various $Ap_{2-6}As$ studied, Ap_5A appeared to be the strongest inhibitor of ADP-induced human platelet aggregation (Harrison *et al.*, 1977) while both Ap_5A and Ap_6A, which occur naturally in platelets, act as vasopressors (Schlüter *et al.*, 1994).

Another naturally occurring homolog of Ap_4A, Ap_3A, is the preferred Np_nN substrate for various dinucleoside triphosphatases (EC 3.6.1.29) (Lobatón *et al.*, 1975; Jakubowski & Guranowski, 1983; Barnes *et al.*, 1996; Guranowski *et al.*, 1996). These specific hydrolases always liberate AMP (NMP) from their substrates. In addition to Ap_3A, they hydrolyze Ap_4A (to AMP and ATP) and Ap_5A, which is slowly degraded to AMP and p_4A (Jakubowski & Guranowski, 1983; Brevet *et al.*, 1991; Prescott *et al.*, 1992; Barnes *et al.*, 1996).

Hybrid analogs

Hybrid dinucleoside tetraphosphates, Ap_4Ns (where N ≠ A), have been tested as alternative substrates for different Ap_4A-degrading enzymes (Jakubowski & Guranowski, 1983; Plateau *et al.*, 1985; Brevet *et al.*, 1987; Prescott *et al.*, 1989) and for Ap_3A hydrolase (Barnes *et al.*, 1996). Their use has revealed the asymmetry of the Np_4N'-binding sites of such enzymes as yeast Ap_4A phosphorylase (Brevet *et al.*, 1987), asymmetrically acting Ap_4A hydrolase from *Artemia* (Prescott *et al.*, 1989), and the human Fhit protein, which is a typical dinucleoside triphosphatase (Barnes *et al.*, 1996). In each case, a degree of preferential bond cleavage was observed for these hybrid molecules rather than random degradation. For example, phosphorolysis of Ap_4G by yeast Ap_4A phosphorylase yielded over 7-fold more ATP + GDP than GTP + ADP while hydrolysis of Ap_4G by the *Artemia* Ap_4A hydrolase yielded a 4.5-fold excess of AMP + GTP over GMP + ATP. The Fhit protein degraded Ap_4G to AMP + GTP (85%) and GMP + ATP (15%).

In addition, hybrid Ap_4A analogs have been used as typical bisubstrate analogs in studies of various nucleoside and nucleotide kinases. Ap_4U was shown to be an effective inhibitor of uridine kinase from Ehrlich ascites tumor cells (Cheng *et al.*, 1986) and analogs with N = dN were used as probes for distinguishing between kinetic mechanisms of the appropriate kinases: Ap_4dT for thymidine kinase (EC 2.7.1.21) (Bone *et al.*, 1986a), and Ap_4dC, Ap_4dG and Ap_4dA for deoxycytidine (EC 2.7.1.74), deoxyguanosine (EC 2.7.1.113) and deoxyadenosine (EC 2.7.1.76) kinases (Ikeda *et al.*, 1986). Ap_4dT, Ap_5dT and Ap_6dT were found to be inhibitors of thymidylate kinase (EC 2.7.4.9) from peripheral blast cells of patients with acute myelocytic leukemia (Bone *et al.*, 1986b) and Ap_5dT and/or P^1-(5′-adenosyl)-P^5-(5‴-(3″-azido-3″-deoxythymidine)pentaphosphate, AZT-p_5A, were used in studies of the same kinase from yeast (Lavie *et al.*, 1998a) and *E. coli* (Lavie *et al.*, 1998b). The crystal structures of the latter enzyme complexed with these compounds have been solved to 2.0-Å and 2.2-Å resolution. Davies and co-workers (1988) tested Ap_3dT, Ap_4dT,

Ap_5dT and Ap_6dT plus their analogs with a methylene group α,β to the thymidine residue, e.g. $ApppCH_2pdT$, as potential inhibitors of thymidine kinase, thymidylate kinase and ribonucleotide reductase (EC 1.17.4.1). Ap_5dT was the best inhibitor of the thymidine kinase and both Ap_5dT and Ap_6dT strongly inhibited the thymidylate kinase and were potent inhibitors of CDP reduction catalyzed by the ribonucleotide reductase from L1210 cells. 8-Azido-Ap_4A (**20**) was employed to covalently label acidic fibroblast growth factor (FGF-1) (Chavan & Haley, 1994) and sugar-modified analogs, dAp_4dA and dAp_4dT, were shown to be a new type of substrates for several DNA polymerases of human, bacterial and viral origin. The strongest activity of those compounds was observed for HIV reverse transcriptase (Victorova *et al.*, 1999).

Fluoro- and chromogenic analogs

εAp_4A and $\varepsilon Ap_4\varepsilon A$ (**17**) can serve as convenient fluorescent substrates of various Ap_4A-degrading enzymes. Degradation of the latter results in a 6-fold increase in fluorescence intensity at 410 nm (Wierzchowski *et al.*, 1985; Rotllán *et al.*, 1991). 5-Bromo-4-chloro-3-indolyl-tetraphospho-5′-adenosine (**19**) was used as a chromogenic substrate for three different types of Ap_4A-degrading enzymes in alkaline phosphatase-coupled reactions (Garrison *et al.*, 1993). P^1-(*lin*-Benzo-5′-adenosyl)-P^5-(5′-adenosyl)pentaphosphate and P^1-(*lin*-benzo-5′-adenosyl)-P^4-(5′-adenosyl)tetraphosphate (**18**), both potent inhibitors of porcine muscle adenylate kinase, were used to study the active site of this enzyme. An increase in fluorescence intensities and fluorescence lifetimes of both inhibitors upon binding to adenylate kinase resulted from disruption of the intramolecular stacking interactions that prevail when these ligands are free in solution, suggesting that they bind to the enzyme in an "open" or "extended" form (VanDerLijn *et al.*, 1979).

Another fluorogenic analog, mAp_5mA (**26**), was used to measure the binding constants of adenylate kinase–ligand complexes. It was specially designed for the *E. coli* enzyme, which has no tryptophan residues and therefore no strong intrinsic fluorescence signal to report ligand binding. Moreover, εAp_5A produces no significant fluorescence enhancement with this enzyme, in contrast to mammalian cytosolic adenylate kinase. However, mAp_5mA produced an exceptionally high fluorescence enhancement upon binding to *E. coli* adenylate kinase (about 300%) (Reinstein *et al.*, 1990).

Other Ap_4A derivatives

Pyridoxal-tetraphospho-5′-adenosine (**21**) has been used to explore the topography of the catalytic sites of the following enzymes. Acting as an affinity label it modified the active site of rabbit muscle lactate dehydrogenase (EC 1.1.1.27) (Tagaya & Fukui, 1986) and glutathione synthetase (EC 6.3.2.3) (Hibi *et al.*, 1993) and inactivated rabbit muscle adenylate kinase (Yagami *et al.*, 1988) and sheep brain pyridoxal kinase (Dominici *et al.*, 1988).

Diinosine polyphosphates (Ip_nIs) have been shown to act as selective antagonists at a diadenosine polyphosphate receptor identified in rat brain synaptic terminals. The best was Ip_5I, which was 6000-fold more selective for the P4 dinucleotide receptor than for the ATP receptor (Pintor *et al.*, 1997). Recently, Ip_5I and Ip_6I were proposed as valuable tools for diabetes research. They antagonize Ap_5A-mediated inhibition of insulin release from insulin-secreting (INS-1) cells (Verspohl *et al.*, 2003).

Ap_4A analogs modified in the polyphosphate chain

Various methylene and halomethylene analogs of Ap_4A have been assayed with specific and non-specific Ap_4A-degrading en-

zymes, acting as substrates and/or potent inhibitors. Chronologically, the first were $AppCH_2ppA$ (**2**), AppCHBrppA, $ApCH_2pppA$ (**3**) and $ApCH_2ppCH_2pA$ (**4**) (Guranowski *et al.*, 1987). None was hydrolyzed by the (*symmetrical*)Ap_4A hydrolase from *E. coli* but all were strong inhibitors of this enzyme, with K_i values ranging from 3-fold (for $ApCH_2p$-pCH_2pA) to 15-fold (for AppCHBrppA) lower than the K_m for Ap_4A (25 μM). The (*asymmetrical*)Ap_4A hydrolase from yellow lupin did hydrolyze those analogs with one methylene or halomethylene group. $AppCH_2ppA$ competitively inhibited the hydrolysis of Ap_4A with a K_i 4-fold lower than the K_m for Ap_4A (0.25 μM *versus* 1 μM). The same three analogs were substrates for the non-specific phosphodiesterase from yellow lupin. Finally, of the analogs tested, only $ApCH_2pppA$ was a substrate of the Ap_4A phosphorylase from yeast. It was degraded 40-fold more slowly than Ap_4A and was also the strongest inhibitor of this enzyme, with a K_i of 24 μM *versus* the K_m of 60 μM for Ap_4A. Similar measurements were subsequently performed with the same and other $\beta\beta'$- and $\alpha\beta,\alpha'\beta'$-disubstituted phosphonate analogs of Ap_4A. $AppCF_2ppA$ (**5**) and $AppCCl_2ppA$ were as potent as $AppCH_2ppA$ and AppCHBrppA as inhibitors of lupin Ap_4A hydrolase but were weaker when tested against the *E. coli* hydrolase (Guranowski *et al.*, 1989). McLennan and coworkers (1989) studied a set of 13 phosphonate Ap_4A analogs with the (*asymmetrical*)Ap_4A hydrolase from *Artemia* and established that the substrate efficiency of $\beta\beta'$-substituted compounds decreased with decreasing substituent electronegativity ($O>CF_2>CFH>CCl_2>CClH>CH_2$). These compounds were competitive inhibitors of this enzyme with K_i values that generally also decreased with electronegativity from 12 μM for $AppCF_2ppA$ to 0.4 μM for $AppCH_2ppA$ (K_m for Ap_4A was 33 μM). Disubstituted analogs were generally less effective inhibitors. However, they displayed a low and unexpected rate of symmetrical cleavage by the *Artemia* enzyme. Both sets of analogs were also competitive inhibitors of *E. coli* Ap_4A hydrolase with K_i values ranging from 7 μM for $AppCH_2ppA$ to 250 μM for $ApCH_2CH_2ppCH_2CH_2pA$. The only disubstituted analog to be hydrolyzed by the *E. coli* enzyme was $ApCF_2ppCF_2pA$ at 0.2% of the rate of Ap_4A; however, several of the $\beta\beta'$-substituted compounds showed a limited degree of asymmetrical cleavage. These results were interpreted in terms of a "frameshift" model for substrate binding in which the oligophosphate chain can position itself in the active site of the enzyme with either P^{α} or P^{β} adjacent to the attacking nucleophile (water) depending on the electronegativity of the substituent.

Due to their ability to bind tightly to Ap_4A hydrolases, some of the methylene and halomethylene analogs were used to determine the three-dimensional structures of the enzyme-substrate complexes: a non-degradable analog with two types of modification, $Ap_spCHClpp_sA$ (**9**), was complexed with the enzyme from *Lupinus angustifolius* (Swarbrick *et al.*, 2000) and $AppCH_2ppA$ with the hydrolase from *Caenorhabditis elegans* (Bailey *et al.*, 2002). In physiological assays with INS-1 cells, $Ap_spCH_2pp_sA$ and $Ap_spCHClpp_sA$ inhibited insulin release to the same degree as Ap_4A (Verspohl *et al.*, 2003). $AppCH_2ppA$, AppCHFppA and AppCHClppA were quite potent inhibitors of rat brain adenosine kinase while $AppCCl_2ppA$ was approximately 5-times less potent (Delaney *et al.*, 1997). Different methylene and halomethylene analogs of Ap_4A, εAp_4A, $\varepsilon Ap_4\varepsilon A$, as well as an imido-analog (AppNHppA) were examined as effectors of the ADP-ribosylation reaction of histone H1 catalyzed by purified bovine thymus poly(ADP-ribose)transferase (EC 2.4.2.30). Of the compounds tested, $ApCH_2pppA$ and εAp_4A were shown to be the most effective inhibitors of the enzyme. The imido analog was the least effective (Suzuki *et al.*, 1987). To the best of my knowledge, there are no other reports of effects exerted by the imido analog.

The monophosphorothioates (S_p)$Ap_4A\alpha S$ (**6**) and (R_p)$Ap_4A\alpha S$ (**7**) were tested as alternative substrates for three specific Ap_4A-degrading enzymes (Łażewska & Guranowski, 1990); the asymmetrically degrading Ap_4A hydrolase from yellow lupin seeds, symmetrically acting Ap_4A hydrolase from *E. coli* and the Ap_4A phosphorylase from yeast. Generally, the R_p isomer was a better substrate for all three enzymes than the S_p one. Interestingly, the S_p analog was cleaved randomly by the yeast phosphorylase yielding four reaction products: ATP, ATPαS, ADP and ADPαS. Since the latter product retained its configuration at the α-phosphorus, this confirmed formation of a covalent NMP-enzyme intermediate as previously postulated (Guranowski *et al.*, 1988). Analysis of the regiospecificity of diadenosine polyphosphate hydrolysis catalyzed by three specific hydrolases using $H_2{}^{18}O$ and Ap_spppA showed that, for the (*symmetrical*)Ap_4A hydrolase from *E. coli*, attack by water took place only at the β-phosphorus at the unmodified end of the molecule, producing [β-^{18}O]ADP and unlabeled ADPαS (Guranowski *et al.*, 1994). Regardless of their configuration, diphosphorothioates (Ap_sppp_sA, **8**) were highly resistant to the (*asymmetrical*)Ap_4A hydrolase from *Artemia* while the presence of the P^2:P^3 methylene bridge in $Ap_spCH_2pp_sA$ afforded at least a further five-fold increase of resistance to hydrolysis. Moreover, it was noticed that the presence of P^1 and P^4 thiophosphates can force a symmetrical cleavage of Ap_sppp_sA for at least one of the diastereoisomers to yield ADPαS (Blackburn *et al.*, 1987b). Another Ap_4A analog with dual modifications, $Ap_spCHClpp_sA$ (**9**), proved to be a promising anti-platelet aggregation agent (Chen *et al.*, 1997). Recently, strong inhibition of ADP-triggered blood platelet aggregation was also reported for the analog containing a central di(hydroxymethyl)phosphonic acid moiety in which both non-bridging oxygens of the phosphates linked directly to each terminal adenosyl residue were replaced with sulfur atoms (compound **15**) (Walkowiak *et al.*, 2002).

Adenylated derivatives of methanetrisphosphonate, so called "supercharged" analogs of Ap_4A, have been shown to be effective inhibitors of Ap_3A hydrolases (Liu *et al.*, 1999). They were, however, poor inhibitors of the human and narrow-leafed lupin (*asymmetrical*)Ap_4A hydrolases (Maksel *et al.*, 1999); only the triadenylated compound, App-CH(ppA)-ppA (**10**), exerted a significant effect, with IC_{50} values estimated in the presence of 50 μM Ap_4A of 80 μM for the human and 40 μM for the lupin enzyme.

A novel family of Ap_4A analogs in which adenylate or adenosine-5'-phosphorothioate residues are chemically attached to polyols such as glycerol, erythritol and pentaerythritol (Baraniak *et al.*, 1999) have been tested in my laboratory as potential substrates and/or inhibitors of the following enzymes: Ap_3A hydrolase from yellow lupin seeds, human Ap_3A hydrolase (Fhit protein), yeast Ap_4A phosphorylase, (*symmetrical*)Ap_4A hydrolase from *E. coli* and two (*asymmetrical*)Ap_4A hydrolases, from narrow-leafed lupin and humans. All the compounds were resistant to the action of these enzymes. However, as inhibitors, they behaved differently. Generally, the adenosine-5'-*O*-phosphorothioylated polyols were much more potent inhibitors of these enzymes than their adenosine-5'-*O*-phosphorylated counterparts. Yeast Ap_4A phosphorylase was the most refractory to inhibition. The Ap_3A hydrolases were inhibited quite effectively but the estimated K_i values did not exceed the range of K_ms for Ap_3A; i.e. they were not lower than 10^{-6} M. The strongest inhibitory effects were observed for some of these analogs with the Ap_4A hydrolases (Guranowski *et al.*, 2003b): 1,4-di(adenosine-5'-*O*-phosphorothio)erythritol (**12**) appeared to be the strongest inhibitor of the (*asymmetrical*)Ap_4A hydrolase from lupin and humans (K_i values of 0.15 μM and 1.5 μM, re-

spectively). Of eight adenosine-5'-*O*-phosphorylated compounds, the same enzyme was inhibited only by 1,4-di(adenosine-5'-*O*-phospho)erythritol (**11**). Di(adenosine-5'-*O*-phosphorothio), di(phosphorothio)pentaerythritol (**13**) and tri(adenosine-5'-*O*-phosphorothio), thiophosphoro-pentaerythritol (**14**) were the most powerful inhibitors of the (*symmetrical*)Ap_4A hydrolase from *E. coli* ever reported (K_i values of 0.04 μM and 0.08 μM, respectively). For these two types of enzymes the K_i values were lower than the K_ms for Ap_4A. A comparison of the inhibitory effects exerted by di(adenosine-5'-*O*-phosphorothio)erythritol towards three different (*asymmetrical*)Ap_4A hydrolases — lupin, human and nematode (*C. elegans*), — showed that each enzyme was inhibited to a different extent, probably due to small differences in the shape (topography) of the active sites of these hydrolases. Interestingly, the homologous compound di(adenosine-5′-*O*-phosphorothio)glycerol was not inhibitory. To further pursue this structure-activity relationship, two di(adenosine-5'-*O*-phosphorothio)diols, 1,4-di(adenosine-5'-*O*-phosphorothio)butanediol and 1,5-di(adenosine-5'-*O*-phosphorothio)pentanediol, were synthesized. These two compounds inhibited neither the lupin nor the human (*asymmetrical*)Ap_4A hydrolase. Thus, one can conclude that not only the distance between the adenosine-5'-*O*-phosphorothio-residues matters but also the presence of hydroxyls in the chain linking the two nucleosides.

The strongest inhibitors of the (*symmetrical*)Ap_4A hydrolase from *E. coli* were also the strongest when tested with the equivalent symmetrically acting enzyme from *Salmonella typhimurium*, although the K_i values for the latter enzyme were markedly higher from those found for the *E. coli* counterpart: 0.2 μM *versus* 0.04 μM for di(adenosine-5'-*O*-phosphorothio), di(phosphorothio)pentaerythritol and 1.7 μM *versus* 0.08 μM for tri(adenosine-5'-*O*-phosphorothio), phosphorothio-pentaerythritol. This result also points to small differences in the binding sites of these two similar enzymes.

Of the enzymes that specifically hydrolyze dinucleoside tri- or tetraphosphates, only the (*symmetrical*)Ap_4A hydrolase has not yet had its three-dimensional structure determined. The above analogs may help this goal to be achieved.

CONCLUDING REMARKS AND PERSPECTIVES

As has been shown in this review, Ap_4A analogs helped to increase our knowledge about the enzymes involved in the metabolism of Ap_4A and other nucleotides. In particular, the hybrid analogs, Ap_4Ns, proved to be alternative substrates of different Ap_4A-degrading enzymes. The preference of cleavage of these asymmetrical compounds as well as other asymmetrically modified Ap_4A derivatives, e.g. 2'- or 3'-adenylylated Ap_4As, showed that the active sites of the Ap_4A hydrolases and Ap_4A phosphorylases are also asymmetric. Various Ap_4Ns and an Ap_4A homolog, Ap_5A, were applied as useful tools in studies of different nucleoside and/or nucleotide kinases. Ap_4A analogs modified in the oligophosphate chain, particularly the non-degradable ones, such as $AppCH_2ppA$ and $Ap_spCHClpp_sA$, form stable complexes with (*asymmetrical*)Ap_4A hydrolases and this allowed the determination of the three-dimensional structures of these enzymes from the nematode *C. elegans* (Bailey *et al.*, 2002) and higher plant *L. angustifolius* (Swarbrick *et al.*, 2000), respectively. The very strong inhibition of (*symmetrical*)Ap_4A hydrolases by some adenylylated and adenosine-5'-phosphorothioated polyols (Guranowski *et al.*, 2003b) encourages one to use these analogs for determination of the three-dimensional structure of a (*symmetrical*)Ap_4A hydrolase.

P^{α}-chiral analogs of Ap_4A allowed the mechanism of Ap_4A degradation catalyzed by (*asym-*

metrical)Ap_4A hydrolase (Dixon & Lowe, 1989) and Ap_4A phosphorylase (Łażewska a& Guranowski, 1990) to be elucidated.

The extracellular, physiological effects of Ap_nAs (Lüthje & Ogilvie, 1988; Miras-Portugal *et al.*, 1999; Campbell *et al.*, 1999; Hoyle *et al.*, 2001) call for more detailed studies on the interaction of these compounds with purine-nucleoside receptors (P1), purine-mononucleotide receptors (P2X, P2Y) and specific receptors termed dinucleotide or P4 receptors, and on the enzymes located on the cell surface, such as ecto-nucleotide pyrophosphatases/phosphodiesterases, NPP1, NPP2 and NPP3 (Vollmeyer *et al.*, 2003). Ap_4A analogs should help to discriminate between these proteins and to better understand the role of Ap_4A and other Np_nN's as signal molecules.

Finally, I anticipate studies on new Ap_nA-derivatives, adenosine(5')polyphospho(5')riboses and ribose(5')polyphospho(5')riboses, that can be produced by the use of a novel enzyme, ATP *N*-glycosidase (Reintamm *et al.*, 2003). Testing of these truncated Ap_4A derivatives as potential substrates and/or inhibitors of different Ap_4A-degrading enzymes will shed new light on the requirements of those enzymes with respect to the structure of their substrates.

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