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TWO HYDROLASE RESISTANT ANALOGUES OF DIADENOSINE 5′,5‴-*P*^{1},*P*^{3}-TRIPHOSPHATE FOR STUDIES WITH FHIT, THE HUMAN FRAGILE HISTIDINE TRIAD PROTEIN†

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Abstract

The design and synthesis of analogues of diadenosine 5′,5‴-*P*^{1},*P*^{3}-triphosphate that are resistant to pyrophosphate hydrolysis is described in relation to their role in signaling and tumorigenesis involving the Fhit protein, the human fragile histidine triad protein, which is a novel Ap3A binding/cleaving protein.

Dinucleoside polyphosphates (DNPPs) occur ubiquitously in both prokaryotic and eukaryotic cells.[1] Naturally occurring species have linear polyphosphate chains ranging from 3 to 6 phosphate residues, among which the major forms are the dinucleoside triphosphates (Np3Ns) and dinucleoside tetraphosphates (Np4Ns) with adenosine as the dominant nucleoside. The precise biological function of these nucleotides has been a subject of conjecture and exploration since the discovery of the diguanosine polyphosphates, Gp_nGs, by Warner[2] in 1963 and of the diadenosine polyphosphates, Ap_nAs, by Zamecnik[3] and coworkers in 1966. *Inter alia*, it has been observed that levels of DNPPs rise markedly *in vivo* under condition of cell stress, especially for Ap4A, which has excited strong interest and has been well studied.[4]

It has been suggested that levels of Ap4A may be related to cell proliferation and environmental stress in prokaryotes and lower eukaryotes, as well as to have a function in extracellular signaling in higher eukaryotes. In addition, there is considerable current interest in an extracellular rôle for Ap4A and Ap3A in blood platelet physiology. Both molecules are stored in high concentration in the metabolically inactive, dense granules of platelets[5] and are released when platelets are stimulated to undergo aggregation.[6] Indeed, Ap3A has been shown gradually to induce platelet aggregation, most likely through its hydrolysis in plasma to ADP. While Ap4A strongly inhibits platelet aggregation induced by ADP, it too can be degraded to ADP, which is a known agonist of platelet aggregation. In response to this situation, we have evaluated[7] Ap4A analogues as antithrombotic agents that cannot be cleaved to ADP.

†Dedicated to the memory of Professor Tsujiaki Hata, friend and associate for many years, who himself held a close interest in diadenosine and diguanosine polyphosphates.

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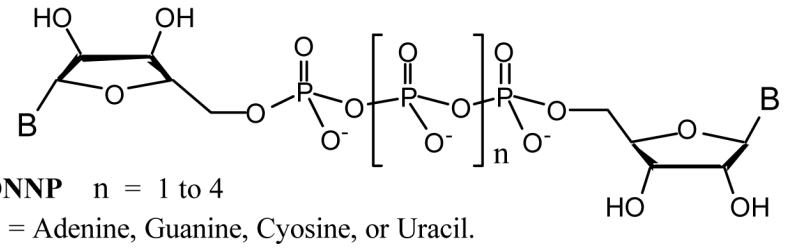


DNNP n = 1 to 4

B = Adenine, Guanine, Cyosine, or Uracil.

The major problem frustrating accurate definition of the cellular function of DNPPs, especially of the Ap_nAs, is their rapid degradation by both specific and non-specific hydrolases and phosphorylases[8] which leads to the maintenance of DNPPs at submicromolar levels in resting cells, notwithstanding their continuous biosynthesis. Chemical synthesis of DNPP analogues that are stable to enzymatic cleavage has provided useful tools which have been deployed alike in the elucidation of some of their biological functions and in the mechanism of action of some specific DNPP pyrophosphohydrolases.[9] Much excitement has attached to the recent identification of the human Fhit (Fragile histidine triad) protein, encoded by a gene located on the short arm of chromosome 3, which spans the fragile site FRA3B and is disrupted in many human tumours.[10] This putative tumour suppressor is a DNPP hydrolase dependent on Mn^{2+} that prefers Ap3A ($k_{cat}/K_M = 2 \times 10^6$ s^{-1} M^{-1}) to Ap4A (6.7×10^3 s^{-1} M^{-1}).[11] These results strongly suggest that Ap3A or similar dinucleotide polyphosphates may be factors in tumorigenesis.[10] Moreover, the high degree of protein homology between the sequence of Fhit and that of the Ap4A hydrolase from *S. pombe*, a known *asymmetric* Ap4A hydrolase,[12] suggests that Fhit may be related in activity to the eukaryotic Ap3A hydrolase from lupin whose mechanism of action we have already characterised.[9]

We have previously described syntheses of a range of phosphonate analogues[13,14] of Ap3A and Ap4A as part of a programme to investigate the mechanism of action the corresponding, specific dinucleoside polyphosphate hydrolases and to uncover the biological function of such dinucleoside polyphosphates. This paper describes the design and synthesis of two novel analogues of Ap3A, programmed to be resistant to hydrolysis by Fhit, and generated for use in X-ray crystal structure and NMR solution structure determinations of binary complexes with that protein.

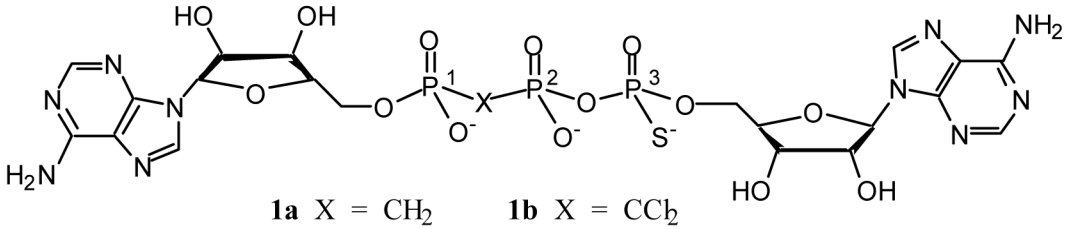
Results and Discussion

The pattern of cleavage of Ap3A by the eukaryotic specific Ap3A hydrolase involves attack of water at P^1 with cleavage of the P^1,P^2 bridge (FIG. 1).[9] As we have shown earlier,[15] such cleavage can be blocked either by the use of an isosteric carbon function in the P^1,P^2-bridge locus or by the introduction of sulfur with *R* configuration at P^1. Because of the C2 symmetry of the Ap3A substrate, the 1-R_P,3-R_P isomer of diadenosine 5′,5‴-P^1,P^3-dithiotriphosphate, APsPPsA, should be an effective inhibitor for Fhit. However, the assumption that Fhit operates by water attack at P^1 is insecure, not least because the lack of sequence homology between lupin and human Ap4A hydrolases and the Ap3A hydrolase[16] from *S. pombe* makes it imperative to generate analogues of Ap3A that are able to resist water attack at *either* P^1(P^3) *or* P^2. Moreover, bis-thiation at P^1,P^4 in Ap4A has been observed to drive the *asymmetrical* Ap4A hydrolase from the brine shrimp, *Artemia*, actually to cleave APsPPPsA *symmetrically* to ADPαS, albeit at 3.4 % of the regular V_{max} rate.[17]

There are two acceptable design solutions to this problem. The first seeks to incorporate a bisphosphonomethylphosphinate, PCPCP, linker into an analogue of Ap3A.[13] The second combines thiation at one end of the analogue with an isosteric carbon bridge at the other. The former design retains the simplicity of a C2-symmetry analogue, which may well prove to be

important for either x-ray crystallographic studies or for NMR investigations. However, the introduction of two methylene bridges may affect the binding of the APCPCPA analogue too adversely for effective agonist/antagonist activity. By contrast, the dissymmetry of the analogues generated in the second approach has the potential benefit of offering *alternative* modes of binding for such APsPXPA analogues to the Fhit protein, and thereby optimising affinity and agonist/antagonist effects.

While our studies on the synthesis of species such as $APCF_2PCF_2PA$ and $APCCl_2PCCl_2PA$ will be described elsewhere, we here report the synthesis of the first two analogues of Ap3A of the second type, specifically diadenosine 5′,5‴-(P^1,P^2-methylene-P^3-thio)-P^1,P^3-triphosphate **1a** and diadenosine 5′,5‴-(P^1,P^2-dichloro-methylene-P^3-thio)-P^1,P^3-triphosphate **1b**.



1a X = CH_2 **1b** X = CCl_2

Syntheses were achieved by condensation of adenosine 5′-thiophosphate activated by diphenyl phosphorochloridate[18] with an excess of adenosine 5′-P^1,P^2-methylenebisphosphonate, AMPPCP, or adenosine 5′-P^1,P^2-dichloromethylenebisphosphonate, $AMPPCCl_2P$, respectively (FIG. 2). While other phosphorylating agents have been used for the large scale synthesis of Ap4A itself,[19] diphenyl phosphorochloridate remains the most effective condensing agent in our hands for these thiated isosteric analogues with methylene bridges. Analogue **1b** was obtained in a yield of 45% and **1a** in 18% yield (with recovery of 61% starting material, AMPPCP). Both of the required P^1,P^2-methylene analogues of ADP, AMPPCP and $AMPPCCl_2P$, were conveniently prepared by the method of Poulter.[20,21]

These condensation process necessarily generates a mixture of two diastereoisomers for **1a** and **1b** at the thiophosphate centre. Their existence is clearly manifest in the ^{31}P NMR resonance for both species, most strongly in the signals for the thiophosphate residue. Compound **1a** showed an AMX NMR spectrum with doublets for each of the two diastereoisomers at δ_P 43.0 ppm (J = 31.4) and 43.3 ppm (J = 31.4) while **1b** showed two doublets for P^3 at 43.77 ppm (J = 31.4), and 43.53 ppm (J = 34.5). Unfortunately, neither of these diastereoisomeric mixtures could be separated by anion exchange HPLC: **1a** gave a single peak while **1b** gave partial resolution with a shoulder peak. It may thus be necessary to seek partial resolution of these isomers by the use of stereoselective enzymatic hydrolysis. Positive ion FAB-MS spectra of the products showed a homologous series of peaks of mass increment 22 based on the protonated parent molecule: **1a** ionises with 0-3 sodium ions and **1b** with 0-2 sodium ions.

We have completed preliminary studies using cloned Fhit protein which show that the protein binds well to both of these analogues without cleaving them. Moreover, Fhit forms a crystalline complex with **1a**, details of which will be published elsewhere.[22]

Experimental

Proton NMR were recorded on a Bruker AC-250 spectrometer at 250 MHz, unless otherwise stated. Chemical shifts (δ_H) are accurate to ± 0.01 ppm. Phosphorus nmr were recorded on a Bruker AM-250 spectrometer at 101.3 MHz. Chemical shifts were quoted in ppm downfield

from 85% H_3PO_4 as the external reference. Fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS80RF mass spectrometer. HPLC was performed using MA7Q anion exchange column (BIO-RAD) with eluent of a linear 0.05 M / 0.7 M aqueous NH_4HCO_3 gradient: from 100% 0.05 M NH_4HCO_3 to a mixture of 0.05 M aqueous NH_4HCO_3 and 0.7 M NH_4HCO_3 (4:1, v/v) in 10 min and then isocratic elution for another 2 min. The flow was 4 ml min-1 and the eluent was monitored at 254 nm. Ion-exchange medium pressure column chromatography (mplc) was performed using a DEAE A25 Sephadex (Aldrich) column (3 cm × 20 cm) and linear gradient of triethylammonium bicarbonate buffer (TEAB), pH 7.5. A flow of 6 ml min-1 was used and the eluent was monitored at 254 nm. TEAB (2 M) was prepared by adding triethylamine (557 ml, 4 mol) to distilled water (1.2 L) in a glass vessel cooled by ice, then CO_2 was bubbled through until a pH of 7.5 was achieved. The buffer was diluted to a final volume of 2 L.

Diadenosine 5′,5‴-(*P*^{1},*P*^{2}-dichloromethylene-*P*^{3}-thio)-*P*^{1},*P*^{3}-triphos-phate 1b

Adenosine 5′-thiophosphate (180 mg, 0.308 mmol) as the bis-(triethyl-ammonium salt) and tri-*n*-octylamine (0.141 ml, 0.323 mmol) were shaken in methanol (7 ml) until dissolution was achieved. The solution was evaporated under reduced pressure. The residue was then coevaporated with pyridine (3 × 10 ml) and further dried under vacuum over P_2O_5 for 12 h. The oily residue was then dissolved in dry dioxane (3 ml). Diphenyl phosphorochloridate (0.098 ml, 0.471 mmol) and tri-*n*-butylamine (0.176 ml, 0.739 mmol) was added. The mixture was stirred at rt. and the initial cloudy solution became clear gradually. After 3.5 h, the solvent was evaporated and oily residue was washed with dry diethyl ether (3 × 10 ml) and then coevaporated with dry pyridine (2 × 10 ml). Adenosine 5′-(*P*^{1},*P*^{2}-dichloromethylene) diphosphate (165 mg, 0.237 mmol) as its tris-triethylammonium salt and tri-*n*-butylamine (0.113 ml, 0.474 mmol) were shaken in dry methanol (5 ml) until the dissolution was achieved, then the solution was evaporated under reduced pressure. The residue was coevaporated with pyridine (3 × 10 ml) and further dried over P_2O_5 overnight. The resulting oil was dissolved in dry pyridine (3.6 ml) and this solution was added to the activated nucleoside above. The reaction mixture was stirred at rt overnight and then evaporated under reduced pressure. The oily residue was partitioned between dichloromethane (2 × 15 ml) and water (50 ml), the aqueous layer evaporated under reduced pressure, and the residue chromatographied on a DEAE A-25 Sephadex column with a gradient eluent of aqueous (TEAB) from 0.05 M to 0.5 M in 4 L. The title compound as triethylammonium salt was eluted at a concentration of 0.37 M TEAB. The product-containing fractions were pooled and evaporated under reduced pressure. The residue was coevaporated with methanol (3 × 15 ml) and the title compound was obtained as its triethylammonium salt. To convert this salt into the sodium salt, the product was dissolved in 2 ml methanol and added dropwise to a stirred solution of NaI (1 M) in acetone (50 ml). The precipitate was collected by centrifugation and washed with acetone (4 × 50 ml). Yield 97 mg (45% as trisodium salt).

δ_P (D_2O): 43.8 (d, J = 34.5) and 43.5 (d, J = 34.5) (P^3, two diastereoisomers), 8.5 (d, J = 20.0, P^1), -1.4 (dd, J = 20.0 and 34.5) and -1.5 (dd, J = 20.0 and 34.5) (P^2, two diastereoisomers). δ_H (D_2O): 8.45 (s), 8.42 (s), 8.40 (s) and 8.36 (s) [2H in total], 8.04 (m, 2H), 5.97 (m, 2H) and 4.56-4.25 (m, 10H). FAB-MS(positive): *m/z* 839 ($M+H^+$), 861 ($M+Na^+$) and 883 ($M+2Na^+-H^+$).

Diadenosine 5′,5‴-(*P*^{1},*P*^{2}-methylene-*P*^{3}-thio)-*P*^{1},*P*^{3}-triphosphate 1a

This compound was prepared similarly to **1b**. Starting from 221 mg adenosine 5′-(*P*^{1},*P*^{2}-methylene)-diphosphate as its tris-triethylammonium salt, the sodium salt of the title compound was obtained as a white powder (58 mg, 18%) with recovery of 61% of starting material, adenosine 5′-(*P*^{1},*P*^{2}-methylene)-diphosphate as its tris-triethyl-ammonium salt (136 mg, 0.25 mmol).

δ_p (D_2O): 43.0 (d, J = 31.4) and 43.3 (d, J = 31.4) (P^3, two diastereoisomers), 17.9 (d J = 8.4) and 18.0 (d, J = 7.8) (P^1, two diastereoisomers), 7.6 (dd, J = 31.2 and 7.9, P^2). δ_H (D_2O): 8.50 (s), 8.43 (s), 8.35 (s) and 8.31 (s) [2H in total], 8.02 (m, 2H), 5.91-6.01 (m, 2H), 4.05-4.74 (m, 10H), 3.28 (m, 2H, PCH_2P). FAB-MS (positive): *m/z* 771 ($M+H^+$), 793 ($M+Na^+$), 815 ($M+2Na^+-H^+$), 837 ($M+3Na^+-2H^+$).

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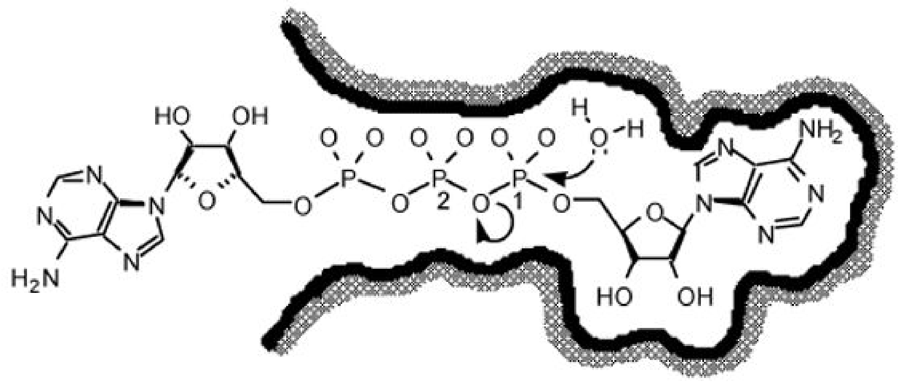
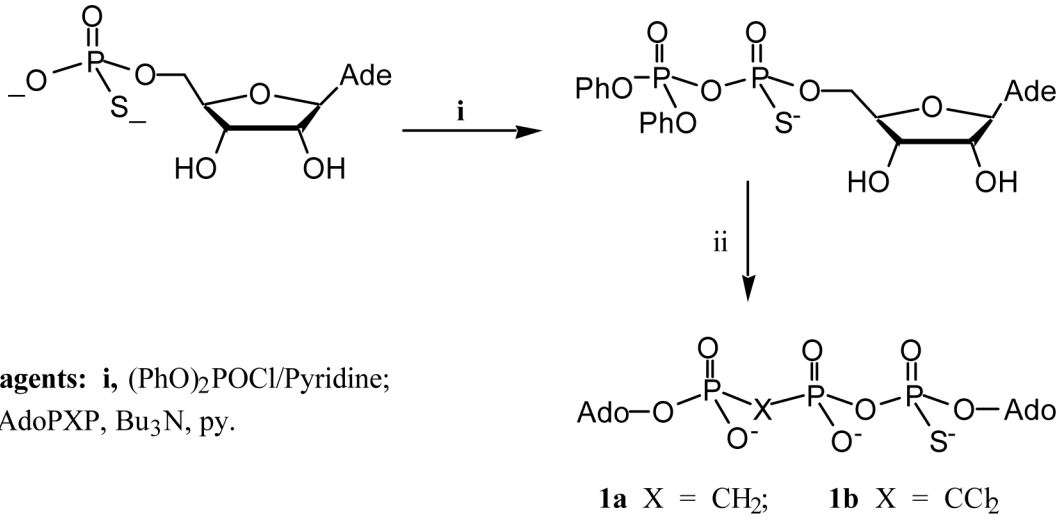


Fig. 1.



Reagents: i, $(PhO)_2POCl$/Pyridine; ii, AdoPXP, Bu_3N, py.

1a X = CH_2; **1b** X = CCl_2

Fig. 2.