Enzymatic Synthesis of Diadenosine 5', 5'''-P^1, P^4-Tetraphosphate (Ap_4A) Analogues by Stress Protein LysU

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Preparative synthesis of analogues of the biologically important molecule diadenosine 5', 5'''-P^1,P^4-tetraphosphate (Ap_4A) is achieved in good yield by enzyme catalysis using the stress protein LysU.

All cells of all organisms synthesise small families of highly conserved proteins known as stress proteins,[1] which protect cells against adverse affects of the environment. We are currently studying[2] the structure, function and chemistry of a number of stress proteins from *Escherichia coli*. LysU is one such protein. Whilst many stress proteins are now known to be molecular chaperones, which assist protein folding and unfolding *in vivo*, LysU is a heat inducible enzyme.[3] This enzyme is an isozyme of the constitutively expressed *E. coli* Lysyl tRNA synthetase enzyme (LysS).[4] Although the usual function of lysyl tRNA synthetase is to catalyse the synthesis of lysyl-tRNA, LysU is also an efficient catalyst for the formation of dinucleotide diadenosine 5',5'''-P^1, P^4-tetraphosphate (Ap_4A) **1** from adenosine 5'-triphosphate (ATP).[5] Ap_4A and other closely related dinucleoside polyphosphates are ubiquitous in Nature if somewhat elusive in biological function. However, there is good evidence that Ap_4A is involved in the control of cell proliferation[6] as well as DNA replication and repair.[6] In addition Ap_4A has been proposed to promote cell survival under environmental stress by acting as an alarmone[7] to signal the onset of environmental stress and promote the synthesis of stress proteins.[6,8] Ap_4A analogues are potentially useful therapeutic compounds.[9] Accordingly, the synthesis of a number of Ap_4A analogues has already been reported using both chemical[10] and enzymatic means.[11,12] However, chemical procedures are multistep and often result in only moderate yields owing to byproduct formation. In cases where enzymatic synthesis has been used, the reactions have usually been performed on a very smallscale and Ap_4A analogues were either not purified to homogeneity and/or were inadequately characterised.[11]

Here, we report the novel use of stress protein LysU for the first preparative enzymatic syntheses of Ap_4A analogues. Initially, LysU was obtained by purification of the protein from a recombinant strain of *E. coli* TG2 which expressed the plasmid pXLys5.[5b] LysU was purified to homogeneity with a yield of *ca.* 250 mg of protein per litre of cell culture and found to be stable to long term storage (several months) at −20 °C. Using this purified protein, two general synthetic procedures were then developed to prepare Ap_4A analogues. In the first competitive procedure, LysU (150 μl, 4 mg ml^{-1}, 5 nmol homodimer) and inorganic pyrophosphatase (300 μl, 200 units mg^{-1}) were added to an aqueous buffered solution of 20 mmol dm^{-3} Tris–HCl, pH 8, (7.9 ml) containing L-lysine (3 mg, 20 μmol), zinc chloride (160 μmol dm^{-3}), magnesium chloride (10 mmol dm^{-3}), potassium chloride (150 mmol dm^{-3}), and the first nucleotide substrate ATP (14 mg, 25 μmol) together with a second nucleotide substrate (*i.e.* a nucleoside 5'-triphosphate, nucleoside 5'-thiotriphosphate, nucleoside 5'-methylenetriphosphate or nucleoside 5'-imidotriphosphate) (50 μmol). After stirring for between 3–24 h at 37 °C, the reaction was terminated by cooling to 4 °C. The Ap_4A analogue was then recovered by reversed-phase HPLC.† In the second sequential procedure, enzyme catalysis was initiated without the presence of the second nucleotide substrate and zinc chloride which were added sequentially after 2.5 h stirring at 37 °C. The reaction mixture was then stirred for a further 1–2 h at 37 °C before termination. Purification of the Ap_4A analogue followed otherwise the same procedure as above. Both reaction procedures were followed by TLC.‡

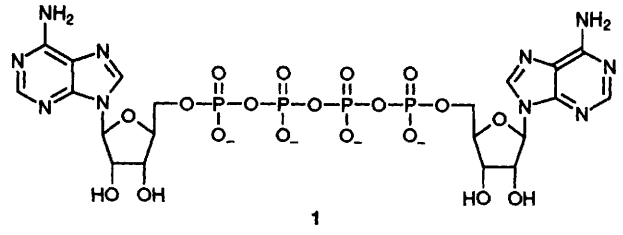

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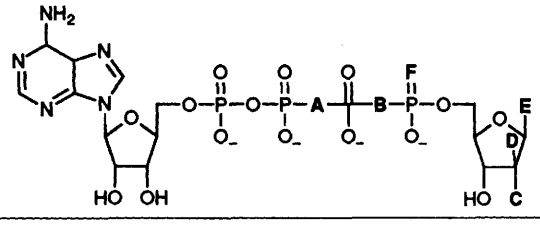
Table 1 shows a representative selection of analogues (some of which are novel and most of which have not previously been synthesised enzymatically) prepared using either the competitive or sequential procedures. All the Ap_4A analogues listed in Table 1 were purified to homogeneity and fully charac-

Table 1 Preparative enzymatic synthesis of Ap_4A analogues by LysU



Compound	Method	Yield (%)	Reported yields (%)
$AppCH_2ppA$ **A** = CH_2; **B** = O; **C** = OH; **D** = H; **E** = adenine; **F** = O	Competitive	59	35[a]
AppNHppA **A** = NH; **B** = O; **C** = OH; **D** = H; **E** = adenine; **F** = O	Competitive	62	n/a[b]
$ApppCH_2pA$ **A** = O; **B** = CH_2; **C** = OH; **D** = H; E = adenine; **F** = O	Competitive	59	22[c]
(αS)Appppp$_s$A **A**, **B** = O; **C** = OH; **D** = H; **E** = adenine; **F** = S	Competitive	46	n/a[b]
Ap_4X **A**, **B** = O; **C** = OH; **D** = H; **E** = xanthine; **F** = O	Competitive Sequential	66 74	n/a[b]
Ap_4I **A**, **B** = O; **C** = OH; **D** = H; **E** = inosine; **F** = O	Sequential	70	n/a[b]
Ap_4(arabino-A) **A**, **B** = O; **C** = H; **D** = OH; **E** = Adenine; **F** = O	Sequential	52	n/a[d]
Ap_4d(5-fluoro-U) **A**, **B** = O; **C** = H; **D** = H; **E** = 5-fluorouridine; **F** = O	Sequential	52	n/a[d]
Ap_4 **A**, **B** = O; **F** = O	Sequential	52	n/a[b]

[a] Ref. 10. [b] Data not available (n/a) because reported purification/characterisations are incomplete. [c] Ref. 18. [d] Data not available (n/a) because the compound is novel.

terised by TLC,‡ ^1H and ^{31}P NMR spectroscopy and MS (negative-ion mode FAB MS). To obtain reproducible mass spectra, special conditions were developed.§ In most cases the appropriate second nucleotide substrate was usually commercially available. However, adeninearabinofuranoside 5′-triphosphate and 5-fluoro-2′-deoxyuridine 5′-triphosphate, used with ATP to form Ap_4(arabino-A) and Ap_4d(5-fluoro-U) respectively (see Table 1), were both synthesised from the corresponding nucleoside by a multistep phosphorylation procedure.[13,14] Also, adenosine (*S*) 5′-α-thiotriphosphate, used with ATP to synthesise (α*S*) Apppp$_s$A (see Table 1), was prepared by converting adenosine into adenosine 5′-thiomonophosphate followed by an enzyme-catalysed diphosphorylation.[15]

The competitive procedure for Ap_4A analogue synthesis was developed from studies on the biochemistry of LysS.[16] Sometimes this procedure was found to produce significant amounts of Ap_4A as a byproduct so reducing the overall yield of the Ap_4A analogue. Also, a number of second nucleotide substrates (*e.g.* inosine 5′-triphosphate and cytosine 5′-triphosphate) were found to act sometimes as mixed-competitive inhibitors of LysU thereby halting Ap_4A analogue synthesis altogether. The sequential procedure was discovered to overcome both these limitations and is now being used more generally to synthesise Ap_4A analogues. Both competitive and sequential procedures rely upon reversed-phase HPLC for purification of the Ap_4A analogues. Owing to the close similarity between the structural and physical properties of the Ap_4A analogues, Ap_4A and unreacted nucleotides, purification of the analogues from reaction mixtures was found to be difficult. However, the final reversed-phase conditions† have proved to be of general utility for the purification of all the Ap_4A analogues we have made so far.

There are limitations on the range of Ap_4A analogues which can be synthesised by LysU. Since LysU has a requirement for ATP, as the first nucleotide substrate, then Ap_4A analogues synthesised usually contain at least one adenosine moiety. Recently we have found that 2′-deoxyadenosine 5′-triphosphate (dATP) may be used in place of ATP as the first nucleotide substrate but thus far no other acceptable nucleoside 5′-triphosphates have been found. By contrast there are very few restrictions on the second nucleotide substrate. Therefore most of the synthetic variation depends on this second substrate which may contain a modification to either the base (purine or pyrimidine), the sugar ring or the triphosphate chain (Table 1).

Ap_4A analogues are probably synthesised by a mechanism similar to that proposed for Ap_4A synthesis by Zamecnik *et al.*[17] (Scheme 1). ATP forms an activated-intermediate with lysine (lysyl adenylate) in step (1) which reacts with the second nucleotide as in step (2). When Ap_4A is formed, ATP takes the place of the second nucleotide. An intermediate (presumably the lysyl adenylate) has been observed by TLC during the sequential procedure, prior to the addition of the second nucleotide substrate and zinc chloride. Whilst the enzyme active-site is very specific for lysyl adenylate formation, the lack of specificity for the second nucleotide suggests that LysU recognises only a small part of the nucleotide structure such as the phosphate chain. Evidence for this suggestion comes from the ready synthesis of adenosine 5′-tetraphosphate (Ap_4) using the sequential enzymatic method (Table 1) where tripolyphosphate (p_3) was used in place of the second nucleotide substrate.

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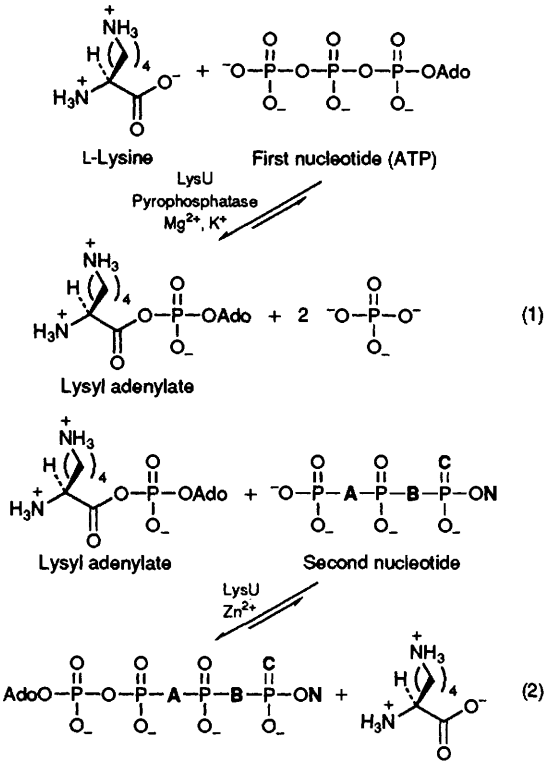

Scheme 1 Proposed mechanism of Ap_4A analogue formation catalysed by LysU: Ado adenosine. The second nucleotide contains structural changes relative to ATP. Changes involve either a nucleoside **N** different to adenosine or changes to the phosphate chain at positions **A**, **B** or **C**. (**A** could be either CH_2, NH or O; **B** could be either CH_2 or O and **C** could be either S or O). See Table 1 for details of analogues synthesised.

Footnotes

† HPLC was performed on a Pharmacia FPLC system using a PepRPC HR 10/10 column pre-equilibrated with 15 mmol dm^{-3}-triethylammonium bicarbonate buffer and eluted with a shallow gradient of MeCN (0–7%) in the same buffer.

‡ TLC was performed using dioxan–H_2O–NH_3 (v/v, 6:3:1) and silica gel 60 F254 precoated (0.2 mm thick) on aluminium-sheets.

§ Negative ion mode FAB MS was performed as follows: Ap_4A analogues (2–3 mg), purified as their triethylammonium salts, were dissolved in a minimum volume of methanol after which NaI solution (1 mol dm^{-3} in acetone) was added until the analogue precipitated out as a sodium salt. The precipitate was pelleted by centrifugation (3000g, 10 min) then washed twice with acetone before being freeze-dried. Analogues were redissolved in a little water and then dispersed in glycerol (10 μl), containing DBU (0.16 mol dm^{-3}). Afterwards, an aliquot of this solution (5 μl) was mixed on a FAB MS probe with additional glycerol (1–2 μl) containing DBU (0.16M). Negative-ion FAB MS were obtained on a KRATOS MS890 mass spectrometer connected to a KRATOS DS90 data system. For all spectra the accelerating voltage was 4 kV, resolution was set to 1000 and the target was bombarded with Xenon atoms at 7–8 kV. (DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.)

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