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Syntheses of stable, synthetic diadenosine polyphosphate analogues using recombinant histidine-tagged lysyl tRNA synthetase (LysU)

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ABSTRACT

Recombinant *Escherichia coli* lysyl-tRNA synthase (LysU) has been previously utilised in the production of stabile, synthetic diadenosine polyphosphate (Ap_nA) analogues. Here we report on the extended use of a new recombinant histidine residue-tagged LysU as a tool for highly controlled phosphate—phosphate bond formation between nucleotides, avoiding the need for complex protecting group chemistries. Resulting high yielding tandem LysU-based biosynthetic–synthetic/synthetic–biosynthetic strategies emerge for the preparation of varieties of Ap_nA analogues directly from inexpensive natural nucleotides and nucleosides. Analogues so formed make a useful small library with which to probe Ap_nA activities in vitro and in vivo leading to the discovery of new, potentially potent biopharmaceuticals active against chronic pain and other chronic, high-burden disease states.

Dinucleoside polyphosphates (Np_nNs; where N is a nucleoside, p is phosphate and *n* is 2–7) are a class of ubiquitous, naturally occurring molecules with apparent involvement in a variety of distinct inter- and intracellular signalling activities.[1–3] Their existence has been reported for decades but their in vivo activities and functions remain unclear. Although previously described as both stress 'alarmones' or even as just metabolic side products, they are now thought to be involved potentially in a variety of distinct inter- and intracellular signalling activities associated with cellular stress and damage responses in particular.[4–6] Recent reports describe Np_nNs potentially active in: the cardio-vascular system,[7–9] tissue protection/ischemia,[10–13] nucleoside receptor interactions,[13–15] neurochemistry,[13,16] insulin secretory activity,[17] eye,[18] and in HIV maturation.[19–21] In mechanistic terms, interactions with a number of target proteins have also been identified,[22] and these interactions have been extensively characterized and understood in at least one instance involving GroEL of the heat shock protein 60 (Hsp 60) family.[23] Over the years, attempts to study the biological function of Np_nNs have been hampered by their chemical and metabolic lability in vivo. Whilst being generally more stable than mononucleotides in vitro, they share rapid turnover in vivo and natural dinucleosides are rapidly degraded in both the intra- and extra-cellular localities by families of specific and non-specific phosphatases.[24] As a result, both naturally released and exogenously added Np_nNs are likely to have unpredictable (and probably brief) life spans. Accordingly, there have been attempts to address this problem of chemical and metabolic lability in vivo through the synthesis of Np_nN analogues with selected oxo-bridges in polyphosphate chains substituted by methylene or imido-bridges.[25] In at least one instance, the use of such an analogue led to a substantial reappraisal of Np_nN biology,[13] suggesting that such analogues should be of real value in the future exploration of Np_nN biology and even its therapeutic benefits. A number of chemical and enzymatic methods have been reported for the synthesis of Np_nN analogues.[13,25–28] Of particular relevance here has been the use of heat-shock protein (heat-inducible) *Escherichia coli* lysyl-tRNA synthetase (*E. coli* LysU) to catalyse the synthesis of diadenosine polyphosphate (Ap_nA) analogues from a primary ATP substrate and a variety of possible second nucleotide substrates.[27,28] Since these primary reports, LysU has been shown to be an enzyme with multiple catalytic activities,[29] and also a multifunctional protein with functions extending beyond biocatalysis.[30] Moreover, the biosynthetic capabilites of LysU have been increasingly linked to other up-stream synthetic steps leading to the development of tandem synthetic–biosynthetic procedures for the preparation of bespoke soluble and solid-phase immobilized Ap_nA binding protein probes for use with pure proteins and also complex biological samples.[22,23,31–33]

Here we report on the realization of new LysU-based biosynthetic–synthetic/synthetic–biosynthetic strategies for the prepara-

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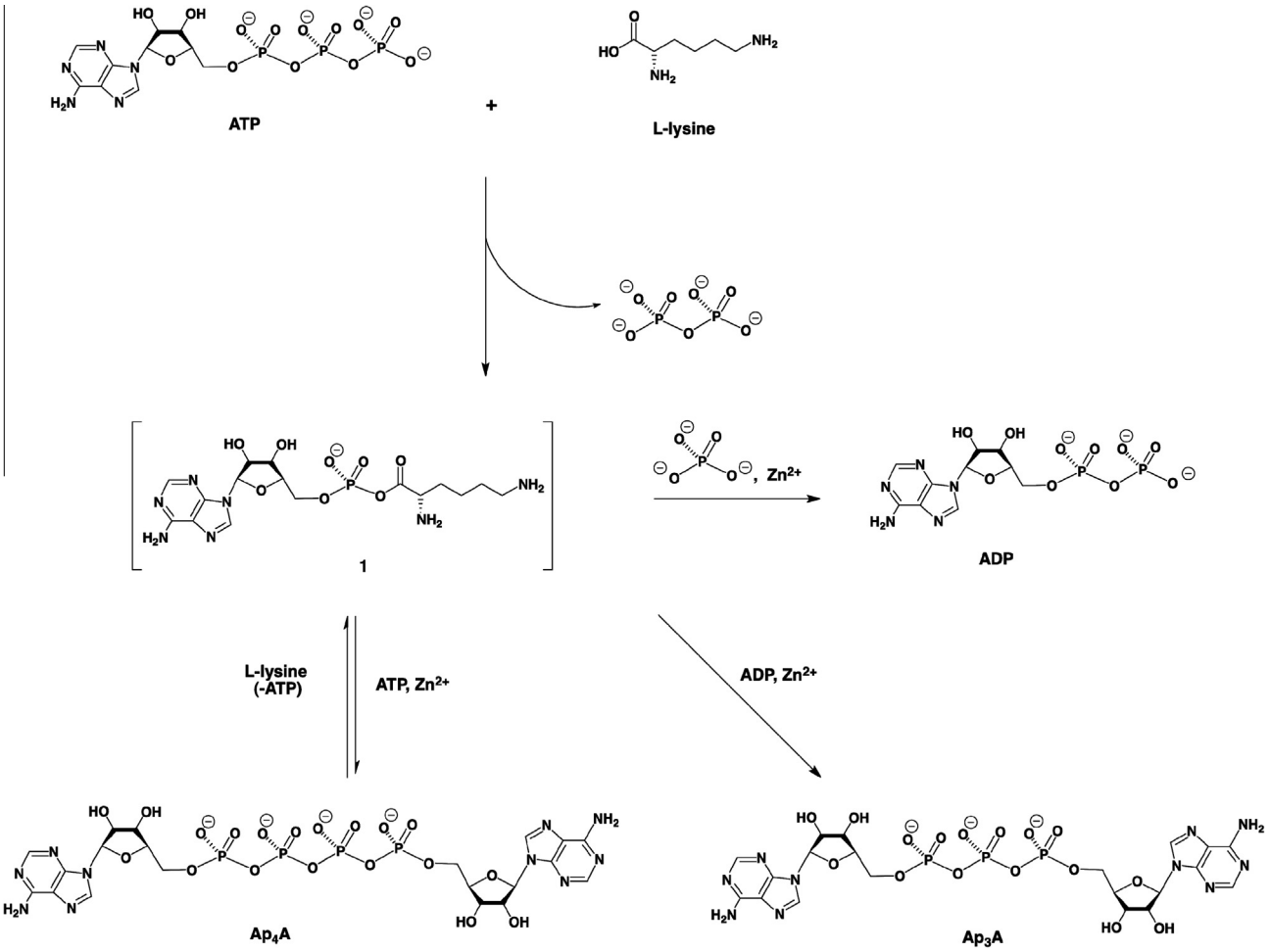
tion of stable, synthetic Ap_nA analogues in high yield and purity directly from natural, inexpensive nucleotides/nucleosides, These strategies extend the synthetic range of LysU-based procedures for the preparation of potentially important Ap_nA analogues for biological and even therapeutic studies. Recent mechanistic studies demonstrated that dimeric LysU has dual diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) and diadenosine-5′,5‴-P^1,P^3-triphosphate (Ap_3A) synthase activities.[34] Syntheses of both take place through the formation of a lysyl-adenylate **1** intermediate from ATP and L-lysine (see Scheme 1).[29,34] Thereafter, the terminal phosphate of a second nucleotide substrate combines with the enzyme-bound lysyl-adenylate, thereby liberating free L-lysine and generating either Ap_4A or Ap_3A depending upon the identity of the second nucleotide substrate. The first step involving lysyl-adenylate **1** intermediate formation is highly specific but reversible. Therefore inorganic pyrophosphatase-mediated controlled hydrolysis of pyrophosphate is required in order to prevent the back-reaction taking place, and thereby essentially rendering this first step committed. Fortunately, the second product formation step is highly promiscuous and a wide variety of nucleotide di-, tri-, and tetraphosphates are acceptable as second nucleotide substrates. This promiscuity was also found to extend to inorganic phosphate and to tripolyphosphate.[28,34] Hence for the purposes of further extending the synthetic reach of LysU, we considered the possibility that pyrophosphate and pyrophosphate analogues might also act as *bona fide* second substrates. This being the case, we surmised that the capabilities of LysU to act as a biosynthetic tool for the high yielding syntheses of highly purified, stable, synthetic Ap_nA analogues could be considerably enhanced. In pursuing this line of enquiry, we were also greatly assisted by over-expression of recombinant histidine-tagged LysU and its highly efficient purification from cell lyzates using nickel-affinity chromatography, as very recently reported.[30] Resulting samples of recombinant LysU could be produced readily with minimal difficulty, enhanced purity and corresponding enzyme activity.

The first applications of our new LysU protocols are summarized (see Scheme 2). Initially ATP derived lysyl-adenylate **1** intermediate was coupled to methylene-diphosphonic acid yielding β,γ-methylene-ATP ($AppCH_2p$) **2**, and **1** was also coupled to imido-diphosphonic acid giving rise to β,γ-imido-ATP (AppNHp) **3**.[35] Thereafter, ATP derived lysyl adenylate **1** intermediate was then coupled to **2** in a fresh enzymatic reaction giving β,β′-methylene-diadenosine 5′,5‴-P^1,P^4-tetraphosphate ($AppCH_2ppA$) **4**. Similarly, **1** was coupled cleanly to **3** thereby yielding β,β′-imido-diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AppNHppA) **5** instead.[35] The use of consecutive enzymatic reactions to prepare **4** from **1** via **2**, and also **5** from **1** via **3**, was necessary in order to optimize overall recovered product yields and minimize losses due to the fact that LysU can catalyze a range of different reactions giving rise to a number of different products at different times (see Scheme 1).[29,34]

Following on from this, we then observed that $AppCH_2ppA$ **4** could be converted by a two stage process into a double open-ring diol **6** involving first 2′,3′-*vic*-diol cleavage and open ring bis-aldehyde formation using sodium periodate, followed second by



Scheme 1. Surface mechanism of LysU catalyzed Ap_4A and Ap_3A synthase activities determined previously.[29,34]