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Isolation and identification of diadenosine 5′,5‴-P^1,P^4-tetraphosphate binding proteins using magnetic bio-panning

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ABSTRACT

We report the development of a synthetic, biotin-conjugated diadenosine tetraphosphate (Ap_4A)-'molecular hook' attached to magnetic beads enabling the isolation of Ap_4A-binding proteins from bacterial cells or mammalian tissue lysates. Characterisation and identification of isolated binding proteins is performed sequentially by mass spectrometry. The observation of positive controls suggests that these newly observed proteins are putative Ap_4A-binding partners, and we have expectations that others can be found with further technical improvements in our methods.

Dinucleoside polyphosphates (Np_nNs) are a family of ubiquitous, naturally occurring molecules consisting of two nucleosides bridged by 2–7 phosphate groups. Members of this family can be extracted from a wide range of prokaryotic or eukaryotic cells or tissues and have been suggested to possess a diverse range of intracellular and extracellular biological roles.[1,2] These include activities in bacterial cell division,[3] in the invasion of mammalian cells,[4] the regulation of apoptosis,[5] plus roles in the cardio-vascular system,[6,7] neurotransmission,[8,9] analgesia,[10] and tissue protection.[11] Most especially Np_nNs may have a key role in cell stress signalling and in management of the heat shock response.[12] Finally, two dinucleoside polyphosphates, (Diquafosol and Denufosol) have even undergone clinical trials as treatments for dry eye and cystic fibrosis.[13]

Diadenosine tetraphosphate (Ap_4A) is one of the most studied members of the Np_nN family. Even so, the in vivo biology of Ap_4A remains poorly understood, and only a very limited numbers of protein binding-partners are known.[13–15] Given that known Np_nN activities are potentially quite significant, there is now an imperative to develop up-to-date methodologies to enhance our understanding of the specific roles of Ap_4A in biology in the first instance with an eye thereafter to more general roles of the Np_nN family. As a first step towards this, we have developed new isolation methods that were intended to enable the subsequent identification of novel, putative Ap_4A binding proteins in diverse cellular lysates by means of mass spectrometry.

Our approach to isolation was based on the use of superparamagnetic magnetic particles. Such particles have been used in a wide range of applications, including molecular imaging[16] and magnetic thermotherapy.[17] Larger 'beads' have also been used as a solid matrix for the separation of proteins, peptides, DNA/RNA and small-molecule drugs[18] and even for targeted delivery.[19–21] Earlier in the 1990s, streptavidin-coated magnetic iron oxide beads were used to enrich intracellular binding partners of proteins or peptides for characterisation from whole-cell lysates.[22,23] Here we report on how this concept can be adapted further for the magnetic bio-panning, isolation and characterisation of new Ap_4A binding proteins. Central to our approach was the preparation of a synthetic, biotin-conjugated Ap_4A-'molecular fish-hook' with the capacity to bind to *bona fide* Ap_4A binding proteins on the one hand and simultaneously to streptavidin-coated superparamagnetic particles on the other hand. It is our expectation that specific protein interactions with Ap_4A are most likely by recognition

Abbreviations: Ap_4A, diadenosine tetraphosphate; ATP, adenosine 5′-triphosphate; ESI-MS, electrospray ionisation mass spectroscopy; IMP, inosine 5′-monophosphate; IMPDH, IMP dehydrogenase; NAD, nicotinamide adenine dinucleotide; Np_nN, dinucleoside polyphosphate.

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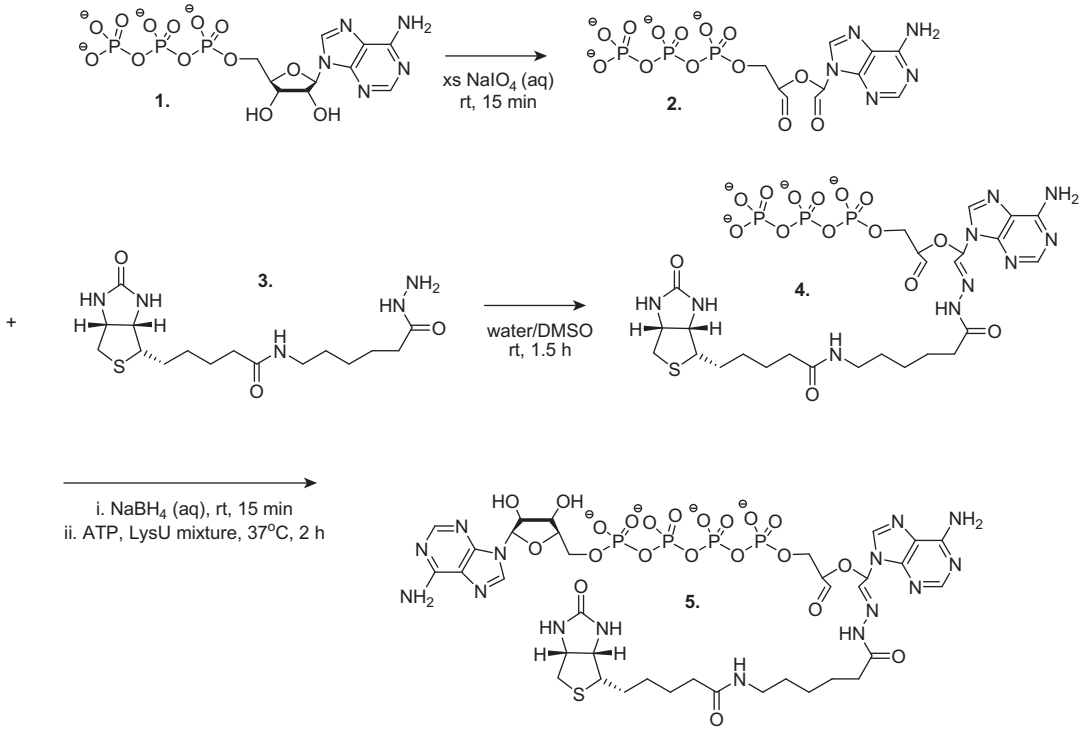

Scheme 1. Tandom chemical/enzymatic synthesis of biotin-LC-Ap_4A.

of the adenines or the polyphosphate chain. Our conjugate (**5**) was designed via modification of the ribose to reduce the risk of binding interference. Given the attachment to a relatively large magnetic bead, we also chose a long linker to reduce steric hindrance.

We have previously reported on the use of tandem chemical/enzymatic synthetic procedures for the production of fluorescent Ap_4A analogues for use in affinity binding studies.[24] In particular, the ability of LysU – a heat inducible lysyl-tRNA synthetase from *Escherichia coli* (*E. coli*) – to produce a variety of Ap_4A-like analogues from mononucleotides has proved to be very valuable. Forming phosphate–phosphate bonds via chemical synthetic approaches is often difficult or hard to control (with resulting purification problems), although there have been some interesting recent developments.[25] LysU-catalysed biosynthesis is more limited in term of acceptable substrates but is fast and highly specific – often giving 90–95% yields with optimised conditions.

A number of alternate synthetic routes were investigated but the simplest was a one-pot biotinylation involving pre-oxidation of adenosine triphosphate (ATP) (**1**) with sodium periodate to yield dial (**2**) that was conjugated in situ to biotin-LC-hydrazide (**3**) (Sigma–Aldrich) (see Scheme 1).[26] The resulting biotin-LC-ATP (**4**) was then coupled enzymatically to a second ATP, giving biotin-LC-Ap_4A (**5**) in a reaction that could be easily monitored by ion-exchange HPLC.[24] The overall yield was 30% – that was suboptimal due to only partial biotinylation in step II. However, the advantage of this one-pot process was found to be the ease of purification (any excess Ap_4A produced is degraded to Ap_3A during the long LysU incubation,[27] allowing product (**5**) to elute from purification column in especially pure form.

Attachment of the biotin-LC-Ap_4A 'molecular fish-hook' (**5**) to streptavidin-linked magnetic beads (M-270 Dynabeads™, Invitrogen) was carried out with stirring at room temperature, followed by a series of wash steps.[28] Gratifyingly, positive control LysU (in 50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$ pH 8.0) was found to bind to the resulting Ap_4A-labelled beads but not to unlabelled (control) beads. Following this confirmation, initial studies were then carried out with an *E. coli* lysate prepared as described.[29] Magnetic bio-banning of the lysate with Ap_4A-labelled and control beads was then carried out according to Figure 1.[30] All washes, eluted fractions and the beads themselves were analysed by means of SDS–PAGE electrophoresis.

Interesting results from this initial phase were then repeated and after Coomassie Brilliant Blue staining of the gels, appropriate bands were excised, trypsinised, then subject to analysis by mass spectrometry.[31] Proteins identified from the lysate (using the Escherichia_coli_08 protein bank on the NCBL website) were found to include the important positive control GroEL protein, a previously identified and well known Ap_4A-binding protein.[12–14] The most dominant putative new Ap_4A binding protein identified was inosine 5′-monophosphate (IMP) dehydrogenase (IMPDH) but others were also identified (see Supplementary data, Table 1).

LysU (another positive control) was not observed in our screen but this is not surprising since the bacterial cultures were grown at 37 °C, normal growth temperature, and the gene for LysU is only expressed under heat shock conditions. Otherwise, we were also unable to observe several other known *E. coli* Ap_4A-binding proteins. However, these are either membrane bound proteins (not investigated here) or else active Ap_4A-hydrolases which would be able to consume the biotin-LC-Ap_4A 'molecular fish-hook' enzymatically during the magnetic bio-panning process after binding. Accordingly, studies are now underway to develop Ap_4A-'molecular fish-hooks' that are resistant to hydrolysis but retain binding efficacy.

Our magnetic molecular bio-panning approach was also extended to the investigation of murine brain lysates[32] using the same procedures as above (see Fig. 2). In this case, several hsp70 family proteins were identified (Hspa8, Hspa5 and Hspa1b). While these have not been previously reported to bind Ap_4A, the prokaryotic hsp70 family equivalent 'DnaK' has.[14] This suggests that these hsp70 proteins may also be considered an encouraging positive control. Otherwise, as with the *E. coli* lysate, several other novel and interesting putative Ap_4A-binding proteins were identified (see Supplementary data, Table 2).

Although our studies are still at an early stage, we have still succeeded in identifying several new, potentially interesting putative

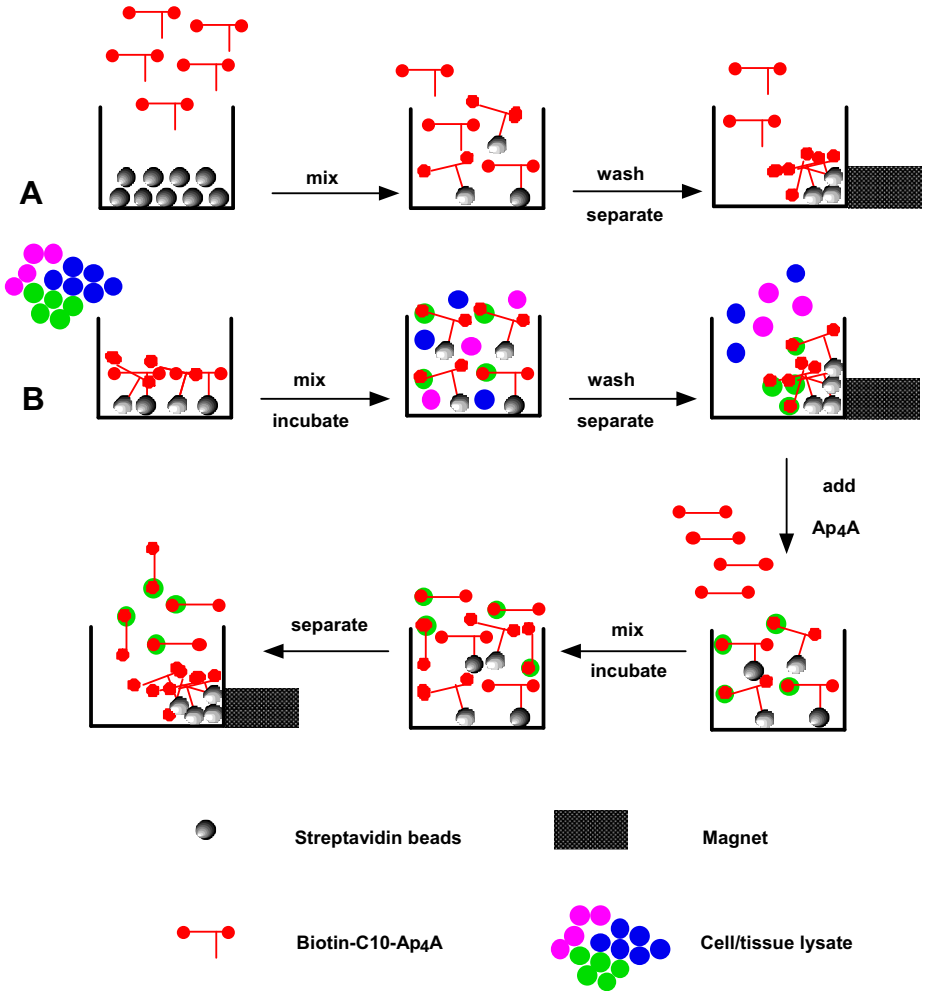

Figure 1. Flow-diagram of target screening of Ap_4A from cell or tissue lysate using a magnetic bio-panning technique. Biotin-conjugated Ap_4A-'molecular fish-hooks' (biotin-LC-Ap_4A, **5**) (shown in red) are mixed with streptavidin-associated superparamagnetic particles (grey spheres): unbound material is removed by wash and separation (A); (B) lysate is added for the binding of putative Ap_4A-binding proteins (green spheres); unbound material is removed by wash and separation: Ap_4A-binding proteins (green spheres) are steadily eluted and separated from the particles after Ap_4A and other wash steps (see Fig. 2).

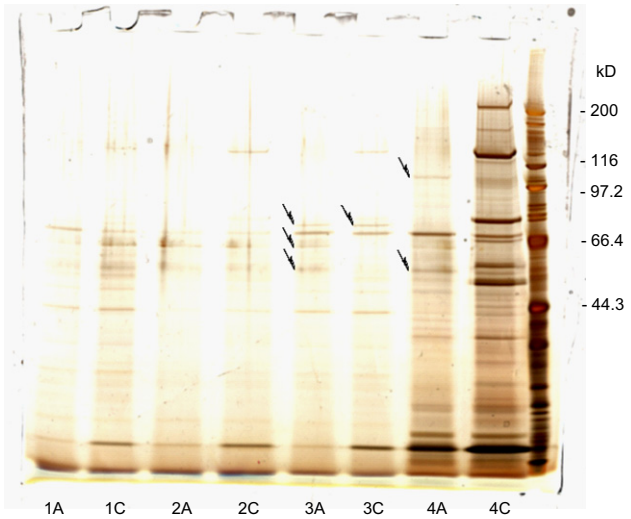

Figure 2. Silver stained SDS-PAGE of putative protein-binding partners to biotin-LC-Ap_4A from murine brain lysate. Columns labelled with 'A' are from Ap_4A-bearing beads, 'C' are negative controls. Samples are from: **1**, third buffer wash; **2**, non-specific NaCl elution; **3**, specific Ap_4A elution; **4**, remaining beads.

Ap_4A-binding partners. From the *E. coli* list, IMPDH (also known as guanosine 5′-monophosphate oxidoreductase) is responsible for catalysing the rate-limiting step of de novo guanosine 5′-triphosphate biosynthesis and the nicotinamide adenine dinucleotide (NAD)-dependent reduction of IMP into xanthosine 5′-monophosphate.[33] Indeed, several adenine dinucleotides and dinucleotide-like compounds, for example, NAD, thiazole-4-carboxamide- and selenazole-4-carboxamide-adenine dinucleotides,[34] and mycophenolic adenine dinucleotide,[35] are reported to act as potent inhibitors of IMPDH function. Given the structural similarities, Ap_4A could certainly bind into the IMP/NAD pocket of IMPDH and exhibit putative biological effects. These putative effects will need to be investigated by means of biological and biophysical studies.[12]

The chaperone/peptidyl-prolyl *cis-trans* isomerase SurA is known to promote protein folding by increasing the rate of prolyl residue *cis-trans* isomerisation. Once again, the reason why this protein might be an Ap_4A binding protein will require some detailed study. Given the apparent modulatory behaviour of Ap_4A seen in most recent studies,[9,12] there may be a very significant role for Ap_4A-binding to SurA protein given that inactivation of SurA typically results in a reduction of *E. coli* growth rate, viability and increased susceptibility to certain antibiotics.[36] On another tack, our discovery that a LysR family transcriptional regulator is a

putative Ap_4A-binding protein is also very interesting given that the LysRS isozyme – LysU – appears to be the primary source of Ap_4A biosynthesis in *E. coli*.

Turning to the murine brain, carbamoyl-phosphate synthase 1 (Cps1) is identified here as a putative Ap_4A-binding protein. This is also intriguing. Cps1 is involved in the urea cycle, where the enzyme plays an important role in removing excess ammonia from the cell. The known ligands of Cps1 include ATP and other nucleotides.[37] Indeed Cps1 requires ATP to transform excess ammonia into carbamoyl phosphate, and Ap_5A that has been found previously to act as a CpsI inhibitor.[38] Could Ap_4A have similar properties and act as a modulator of urea cycle to prevent the over accumulation of extracellular ammonia that might otherwise cause neurological damage problems? Once more detailed biological and biophysical studies are now required. Finally, the identification of three Hsp70 family proteins (namely Hspa8, Hspa5 and Hspa1b) as putative Ap_4A binding proteins also appears very significant. Previous studies have concluded that Ap_4A-binding to molecular chaperone proteins is associated with the modulation of cellular stress responses and recovery from stress injury.[13,39] Therefore, Ap_4A binding with these Hsp70 family proteins could indeed conceivably follow the same pattern.

In conclusion, we report that a first proof of concept study has been concluded for the identification of putative Ap_4A binding proteins from two different biological sources by a magnetic bio-panning procedure. Given the structural similarities between Ap_4A and adenine mononucleotides (e.g. ADP/ATP) there will always be concern as to the specificity of the identified partners. The use of a high [Ap_4A] elution wash should increase the probability of isolating specific binders however this study must be followed up with other biophysical, NMR or probe-based studies before they could be definitively declared as such.[40] On balance, however, the method appears to work. We predict that other interesting binding protein partners will be identified as our methodology is refined and as the search is extended to include other Np_nN family members and other lysate sources. The results will then guide future investigations into the role of Ap_4A and other Np_nN family members in biology and medicine.

Acknowledgments

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The mice were housed in the specific pathogen-free Animal Centre of Shanghai Jiao Tong University and all the experimental operations were done according to the ethic review from The Animal Care & Welfare Committee of that institute.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.070.

References and notes

1. McLennan, A. G. *Pharmacol. Ther.* **2000**, *87*, 73.
2. Guranowski, A. *Front. Biosci.* **2004**, *9*, 1398.
3. Nishimura, A.; Moriya, S.; Ukai, H.; Nagai, K.; Wachi, M.; Yamada, Y. *Genes Cells* **1997**, *2*, 401.
4. Ismail, T. M.; Hart, C. A.; McLennan, A. G. *J. Biol. Chem.* **2003**, *278*, 32602.
5. Vartanian, A. A.; Suzuki, H.; Poletaev, A. I. *Biochem. Pharmacol.* **2003**, *65*, 227.
6. Stavrou, B. M. *Curr. Med. Chem. Cardiovasc. Hematol. Agents* **2003**, *1*, 151.
7. Bobbert, P.; Schluter, H.; Schultheiss, H. P.; Reusch, H. P. *Biochem. Pharmacol.* **2008**, *75*, 1966.
8. Gomez-Villafuertes, R.; Pintor, J.; Gualix, J.; Miras-Portugal, M. T. *J. Pharmacol. Exp. Ther.* **2004**, *308*, 1148.
9. Melnik, S.; Wright, M.; Tanner, J. A.; Tsintsadze, T.; Tsintsadze, V.; Miller, A. D.; Lozovaya, N. *J. Pharmacol. Exp. Ther.* **2006**, *318*, 579.
10. Giraldez, L.; Diaz-Hernandez, M.; Gomez-Villafuertes, R.; Pintor, J.; Castro, E.; Miras-Portugal, M. T. *J. Neurosci. Res.* **2001**, *64*, 174.
11. Wang, Y.; Chang, C. F.; Morales, M.; Chiang, Y. H.; Harvey, B. K.; Su, T. P.; Tsao, L. I.; Chen, S.; Thiemermann, C. *J. Neurosci.* **2003**, *23*, 7958.
12. Tanner, J. A.; Wright, M.; Christie, E. M.; Preuss, M. K.; Miller, A. D. *Biochemistry* **2006**, *45*, 3095.
13. Gendaszewska-Darmach, E.; Kucharska, M. *Purinergic Signal.* **2011**, *7*, 193.
14. Johnstone, D. B.; Farr, S. B. *EMBO J.* **1991**, *10*, 3897.
15. Fuge, E. K.; Farr, S. B. *J. Bacteriol.* **1993**, *175*, 2321.
16. Bjornerud, A.; Johansson, L. *NMR Biomed.* **2004**, *17*, 465.
17. Hilger, I.; Hergt, R.; Kaiser, W. A. *IEE Proc. Nanobiotechnol.* **2005**, *152*, 33.
18. Salaklang, J.; Steitz, B.; Finka, A.; O'Neil, C. P.; Moniatte, M.; van der Vlies, A. J.; Giorgio, T. D.; Hofmann, H.; Hubbell, J. A.; Petri-Fink, A. *Angew. Chem., Int. Ed.* **2008**, *47*, 7857.
19. Li, W.; Ma, N.; Ong, L. L.; Kaminski, A.; Skrabal, C.; Ugurlucan, M.; Lorenz, P.; Gatzen, H. H.; Lutzow, K.; Lendlein, A.; Putzer, B. M.; Li, R. K.; Steinhoff, G. J. *Gene Med.* **2008**, *10*, 897.
20. Neuberger, T.; Schöpf, B.; Hofmann, H.; Hofmann, M.; von Rechenberg, B. *J. Mag. Mag. Mat.* **2005**, *293*, 483.
21. Arruebo, M.; Fernández-Pacheco, R.; Ibarra, M. R.; Santamaría, J. *Nano Today* **2007**, *2*, 22.
22. Kausch, A. P.; Owen, T. P., Jr.; Narayanswami, S.; Bruce, B. D. *Biotechniques* **1999**, *26*, 336.
23. Luers, G. H.; Hartig, R.; Mohr, H.; Hausmann, M.; Fahimi, H. D.; Cremer, C.; Volkl, A. *Electrophoresis* **1998**, *19*, 1205.
24. Wright, M.; Tanner, J. A.; Miller, A. D. *Anal. Biochem.* **2003**, *316*, 135.
25. Mohamady, S.; Taylor, S. D. *J. Org. Chem.* **2011**, *76*, 6344.
26. Carried out as follows: (i) 5 mM aqueous ATP ((**1**) retention time 3.93 min) was stirred with excess sodium periodate to give ribose-oxidised dialdehyde-ATP ((**2**) 4.06 min); (ii) The pH of the mixture was adjusted to 4.5 and a concentrated solution of biotin-LC-hydrazide (**3**) in DMSO was slowly added over 30 min, with vigorous stirring. HPLC analysis after a further hour showed the presence of biotin-LC-ATP (**4**) at 3.69 min; (iii) the periodate was quenched with sodium borohydride (3.87 min) and the Tris-HCl added to 50 mM, pH 8.0; (iv) finally, L-lysine (to 2 mM), $MgCl_2$ (10 mM), $ZnCl_2$ (160 μM), LysU (10 μM), inorganic pyrophosphatase (Sigma–Aldrich; 6 U/mL) was added in sequence and the mixture warmed to 37 °C. Aqueous ATP (to 6 mM) was then added in four portions over the period of 1 h and resulting mixture left for a further hour to give biotin-LC-Ap_4A (**6**) as the majority product (4.24 min); (v) purification was easily carried out by SOURCE-15Q/TEAB ion exchange HPLC as previously described[24] and combined product fractions lyophilised to a highly hydroscopic, white crystalline solid, before storage at −20 °C. Major side products from this reaction included Ap_3A, ADP and **4**. Compound **5** was identified by ESI-MS: $[M-H]^-$ 1185.7 *m*/*z*, expected ($C_{36}H_{52}N_{15}O_{21}P_4S^-$) 1186.2 *m*/*z* and ^{31}P NMR (202 MHz, D_2O, 21500x) δ −11.63 (2P, m, $J_{\alpha\beta/\delta\gamma}$ 22.2, Pα,δ), −23.16 (1P, d, $J_{\gamma\delta}$ 18.2, Pγ), −23.28 (1P, d, $J_{\beta\alpha}$ 24.2, Pβ), purity confirmed as >95% by HPLC.
27. Wright, M.; Boonyalai, N.; Tanner, J. A.; Hindley, A. D.; Miller, A. D. *FEBS J.* **2006**, *273*, 3534.
28. Beads and **5** were stirred in coupling buffer (5 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl) at rt for 30 min, before settling of the beads with the aid of a neodymium ceramic magnet, removal of the supernatant and washing (3×) with coupling buffer. Beads were then resuspension in phosphate buffer (20 mM sodium phosphate, pH 8.0) before being stored at 4 °C (<3 days).
29. *E. coli* BL21 (DE3), pLysS cells were grown at 37 °C in LB medium supplemented with carbenicillin (50 μg/ml) until OD_{600} = 1.5, then harvested by centrifugation. Cell pellets were lysed by probe sonication in lysis buffer (50 mM Tris–HCl, 2 mM BME, 0.1% Triton X-100, 0.6 mM benzamidine, 3 mg DNAase, 20 mM $MgCl_2$, protease inhibitor tablet; pH 8.0). After addition of streptomycin sulphate, the lysate was centrifuged at 12,000*g* to remove cell debris and precipitated nucleotides. It was then fractionated with 20% and 60% ammonium sulfate, with the precipitated protein fractions being redissolved in HEPES buffer (50 mM, 100 mM NaCl, protease inhibitor tablet, pH 8.0) before storage with 30% glycerol, in small portions at −20 °C.
30. As follows: (i) 30 μL of beads were washed (3×) with 200 μL Tris–HCl buffer (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, pH 8.0); (ii) incubated at 4 °C for 40 min with 30 μL of each lysate fraction in 200 μL Tris–HCl buffer; (iii) beads washed (3×) with Tris–HCl; (iv) initial elution for 10 min (4 °C) in Tris–HCl with NaCl concentration increased to 1 M; (v) competition elution with 4 mM Ap_4A in Tris–HCl buffer.
31. LTQ, Thermo Finnigan, positive charge testing mode, micro spray injecting way, 170 °C capillary temperature, 0.15 mm and 15 cm column, 470-1800 DAL scanning scope.
32. Prepared as follows: after injection with a lethal dose of ketamine, two healthy C57BL/6 mice (16 weeks old) were perfused intracardially with 25 mL of normal saline. The brains were rapidly removed and put in ice-cold RIPA lysis buffer (25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; 3 mL/g tissue), including a protease inhibitor cocktail, phenylmethylsulfonyl fluoride and sodium orthonavate. After being homogenised, the tissue lysate was centrifuged at 12,000*g* and the supernatant aliquots were stored at −40 °C.

33. Collart, F. R.; Huberman, E. *J. Biol. Chem.* **1988**, *263*, 15769.
34. Franchetti, P.; Cappellacci, L.; Pasqualini, M.; Petrelli, R.; Jayaprakasan, V.; Jayaram, H. N.; Boyd, D. B.; Jain, M. D.; Grifantini, M. *Bioorg. Med. Chem.* **2005**, *13*, 2045.
35. Chen, L.; Petrelli, R.; Olesiak, M.; Wilson, D. J.; Labello, N. P.; Pankiewicz, K. W. *Bioorg. Med. Chem.* **2008**, *16*, 7462.
36. Justice, S. S.; Hunstad, D. A.; Harper, J. R.; Duguay, A. R.; Pinkner, J. S.; Bann, J.; Frieden, C.; Silhavy, T. J.; Hultgren, S. J. *J. Bacteriol.* **2005**, *187*, 7680.
37. Maddocks, S. E.; Oyston, P. C. *Microbiology* **2008**, *154*, 3609.
38. Powers, S. G.; Griffith, O. W.; Meister, A. *J. Biol. Chem.* **1977**, *252*, 3558.
39. McLennan, A. G.; Barnes, L. D.; Blackburn, G. M.; Brenner, C.; Guranowski, A.; Miller, A. D.; Rovira, J. M.; Rotllan, P.; Soria, B.; Tanner, J. A.; Sillero, A. *Drug Dev. Res.* **2001**, *52*, 249.
40. Wright, M.; Miller, A. D. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 943.