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Novel fluorescent labelled affinity probes for diadenosine-5′,5‴-P^1,P^4-tetraphosphate (Ap_4A)-binding studies

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Abstract—Tandem synthetic–biosynthetic procedures were used to prepare two novel fluorescent labelled affinity probes for diadenosine-5′,5‴-P^1,P^4-tetraphosphate (Ap_4A)-binding studies. These compounds (dial-mant-Ap_4A and azido-mant-Ap_4A) are shown to clearly distinguish known Ap_4A-binding proteins from *Escherichia coli* (LysU and GroEL) and a variety of other control proteins. Successful labelling of chaperonin GroEL appears to be allosteric with respect to the well-characterized adenosine 5′-triphosphate (ATP)-binding site, suggesting that GroEL possesses a distinct Ap_4A-binding site.

Dinucleoside polyphosphates (Ap_nAs, $n = 2–6$) are an important family of nucleotides with potentially diverse intracellular and extracellular biological roles.[1,2] Many questions about the behaviour of these compounds in vivo (intracellular and extracellular) remain to be answered and confirmed examples of Ap_nA-binding proteins remain few. Therefore, there is a clear need for appropriate probes for further extensive Ap_nA-molecular recognition and binding studies.[3] Previously, we reported the use of a tandem synthetic–biosynthetic procedure to synthesise a variety of novel fluorescent labelled diadenosine-5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) analogues.[4] Here, we report how this procedure has been extended for the synthesis and initial testing of two fluorescent labelled Ap_4A analogue affinity probes, 2′,3′-dial-adenosine(2′/3′-*O*-[*N*-methylanthraniloyl]adenosine)-5′,5‴-P^1,P^4-tetraphosphate (dial-mant-Ap_4A **1**) and adenosine(8-azido-2′/3′-*O*-[*N*-methylanthraniloyl]-adenosine)-5′,5‴-P^1,P^4-tetraphosphate (azido-mant-Ap_4A **2**) (see Fig. 1).

In 1991, Johnstone and Farr reported the synthesis of radiolabelled azido-Ap_4A (β- or δ-[^{32}P]8-N_3AppppA) via 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) assisted coupling of 8-azido-adenosine monophosphate (azido-AMP) to either α- or γ-labelled [^{32}P]ATP. Using these radio-labelled Ap_4A analogue affinity probes, Johnstone and Farr were able to identify an initial set of Ap_4A-binding proteins within *Escherichia coli* cell lysates after extensive resolution by 2D-PAGE.[5] Following on from this and our previous work on fluorescent Ap_4A analogues, we wished to develop alternative fluorescent labelled Ap_4A analogue affinity probes in order to do two things:

1. to extend the original observations of Johnstone and Farr for the detection of other intracellular Ap_4A-binding proteins within *E. coli*.
2. to detect and identify intracellular Ap_4A-binding proteins within other micro-organisms and eukaryotic cells.

We anticipated that any such new fluorescent labelled affinity probes should retain sufficient Ap_4A structural elements to be readily recognised by Ap_4A-binding proteins but not control proteins and be equipped with an affinity tag appropriate for efficient covalent labelling of corresponding binding proteins. Labelled proteins would then be subjected to chromatography, separation, characterisation and final identification by mass spectrometry, making use of up-to-date proteomics techniques and approaches.

After some consideration, we selected *N*-methylanthraniloyl (mant) as an appropriate fluorophore for

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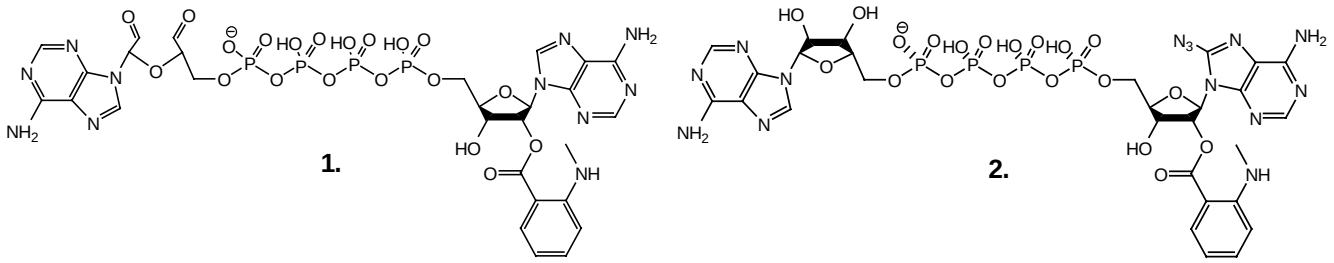
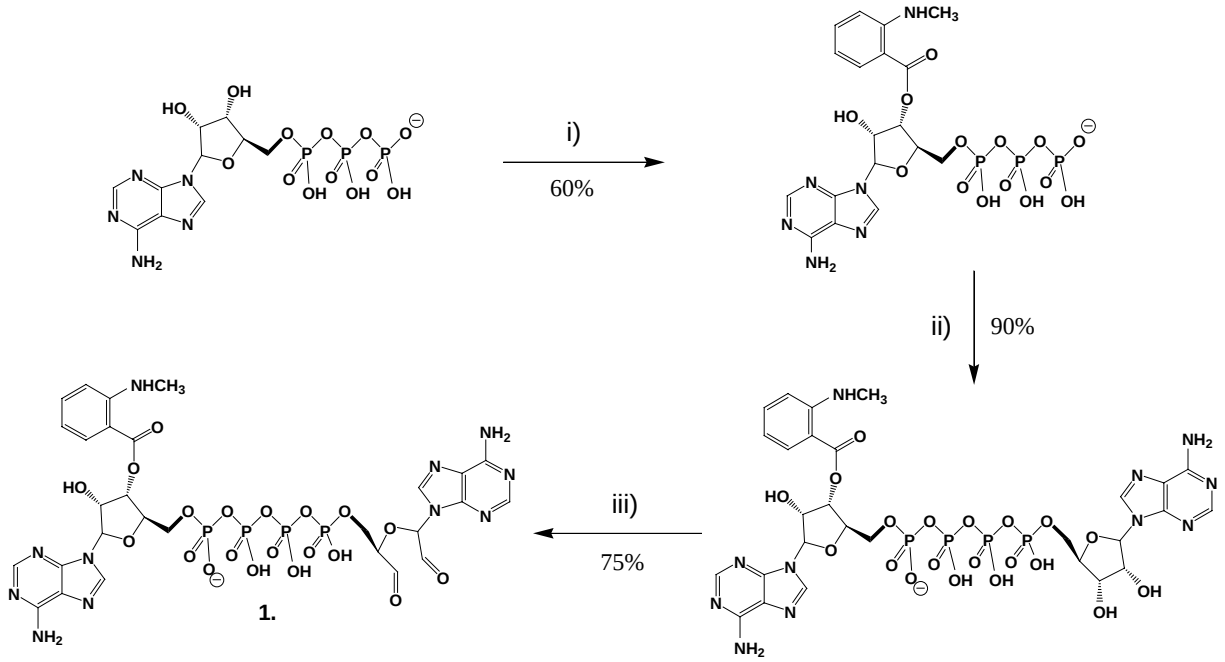


Figure 1. Structures of target fluorescent labelled Ap_4A analogue affinity probes.

fluorescent labelled Ap_4A analogue affinity probes.[5,6] The mant group is attached by the 2′,3′-hydroxyl groups of ribose and has a proven track record as a fluorescent label of adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP), whose presence does not appear to impair molecular recognition and binding behaviour.[7–13] Mant labelled-nucleotides show an intense fluorescence in water (I_{max} 448 nm [light blue]; A_{max} 356 nm), with an I_{max} that becomes characteristically blue-shifted with a concurrent increase in fluorescence quantum yield (4- to 5-fold in 50% ethanol) upon contact with a more hydrophobic environment. As to the affinity tag we elected to use either the azido functional group, which is stable in dark but photolyses to a highly reactive N·-adenine radical on exposure to UV[14–16] or a ring-opened ribose-dialdehyde, which has been shown to spontaneously form stable Schiff base-like moieties with primary amines in proteins.[17–19]

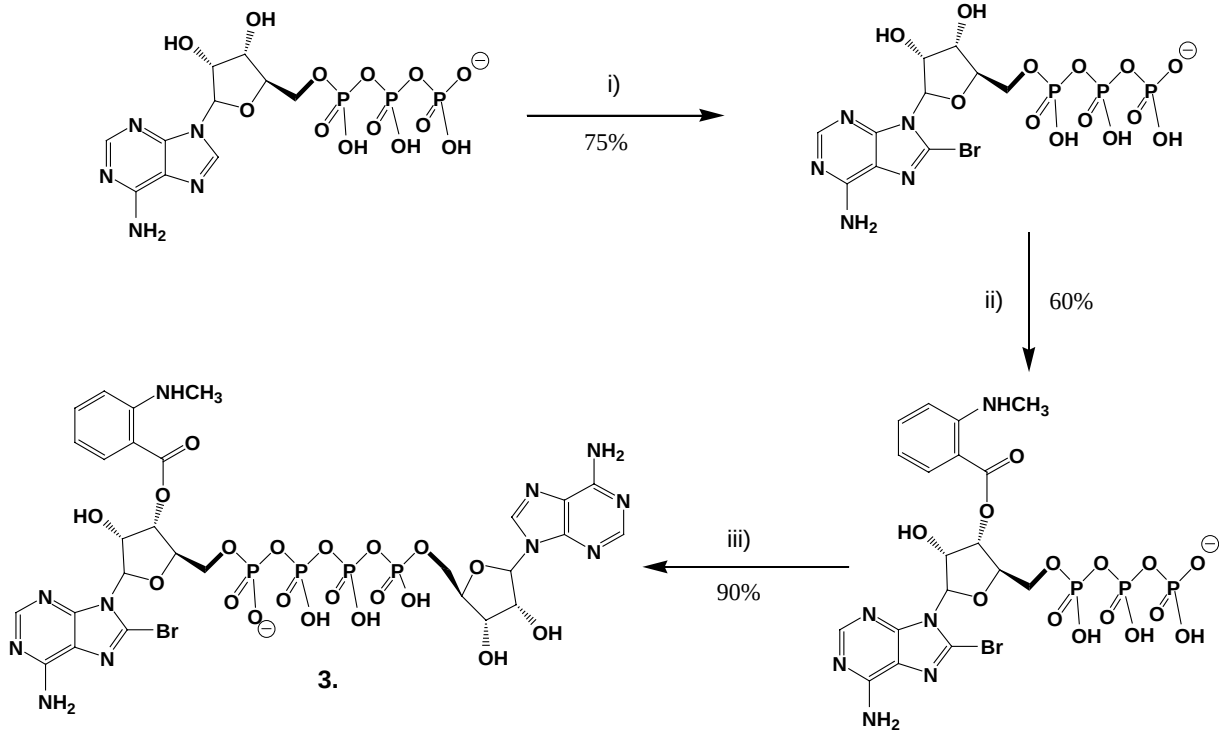
The route to dial affinity probe **1** was relatively simple (see Scheme 1);[20] the synthesis of mant-Ap_4A has been reported previously[4] and conversion to the dialdehyde was effected by the means of sodium periodate oxidation.[17] The main obstacles were the purification and handling of the final product owing to the ribose-dialdehyde reactivity. Post formation, the dial affinity probe **1** needed to be isolated from amine-containing reagents and buffers (e.g., **1** [100 μM] in 20 mM Tris–HCl, pH 8.0, at rt was found by anion-exchange HPLC[4] to have completely disappeared after 45 min). Indeed, the dial affinity probe **1** was even found to react (albeit slowly) with the triethylammonium hydrogen carbonate buffer (TEAB) used in our standard anion-exchange chromatography-based purification protocol.[21] Therefore, an alternate ethanol extraction purification technique had to be used despite its poorer efficiency. The extracted dial affinity probe **1** appeared to be unstable to lyophilisation so was stored as an aqueous ethanol solution at −20 °C until required. HPLC analysis identified several minor impurities (<16% total molarity) that were judged to be polyphosphate chain hydrolysis products by comparison of their retention times with known standards (mant-AMP, mant-ADP and dial-ADP).



Scheme 1. Tandem synthetic–biosynthetic procedure for the preparation of dial-mant-Ap_4A **1** from 2 equiv of ATP. Reagents and conditions: (i) MIA (4 equiv) pH maintained at 9.6, 50 °C, 1.5 h; (ii) ATP (1.5 equiv) with LysU (9 μM), pyrophosphatase (75 μg), L-lysine (2 mM) and $MgCl_2$ (10 mM) and $ZnCl_2$ (160 μM) in 50 mM Tris–HCl buffer, pH 8.0, 37 °C, 1 h; (iii) $NaIO_4$ (1 equiv), rt, 30 min.

Synthesis of azido affinity probe **2** was comparatively more complex (see Scheme 2). Bromination of ATP was the first stage of a three-step tandem synthetic–biosynthetic procedure to give Br-mant-Ap_4A **3**.[4,21,22] Conditions for *E. coli* lysyl t-RNA synthase (LysU) catalyzed synthesis of **3** were as for mant-Ap_4A, with some adjustments. Br-mant-ATP precipitates in the presence of Mg^{2+} ions, therefore a reaction mixture containing a reduced $MgCl_2$ content (3.5 mM) was required even though the rate of enzyme catalysis was retarded. The produced Br-mant-Ap_4A **3** was then onwards converted to the azido affinity probe **2** by prolonged heating with triethylammonium azide freshly prepared using the ion-exchange procedure described by Haley.[23,24] Further characterisation details for all three compounds are given in Table 1.

The binding selectivities of both affinity probes were analyzed by experiments with a range of 'candidate' Ap_4A-binding proteins. The list included two proteins known to bind Ap_4A, namely LysU[25,26] and the *E. coli* chaperonin GroEL.[5,27] The remaining proteins chosen were trypsin (porcine pancreas), L-lactic dehydrogenase (L-LDH, rabbit muscle) and kallikrein (porcine pancreas), none of which are expected to be Ap_4A-binding proteins. Labelling assays[28] were performed by incubating



Scheme 2. Tandem synthetic–biosynthetic procedure for the preparation of Br-mant-Ap_4A **3** from 2 equiv of ATP. Reagents and conditions: (i) Br (3 equiv), 1 M NaOAc pH 3.8, 37 °C, 1.5 h; (ii) MIA (5 equiv) pH maintained at 9.6, 50 °C, 3 h; (iii) ATP (2 equiv in two portions) with LysU (9 μM), pyrophosphatase (75 μg), L-lysine (2 mM), $MgCl_2$ (3.5 mM), $ZnCl_2$ (160 μM) in 10 mM Tris–HCl buffer, pH 8.0, 37 °C, 21 h.

Table 1. Characterisation details of fluorescent labelled Ap_4A analogue affinity probes[30]

Derivative	Retention time (min)	(ES) $[M-H]^-$ (*m/z*)	Overall yield (% from ATP)	^1H and ^{31}P (δ ppm)
Dial-mant-Ap_4A **1**	8.12	965.7	46	^1H: 9.1 (2H, d, H-aldehyde), 8.3–8.5 (2H, m, 8-H-Ad), 7.8–8.3 (2H, m, 2-H-Ad), 7.7–7.8 (1H, d m, 1-H-mant), 7.4–7.5 (1H, m, 3-H-mant), 6.6–6.8 (1H, m, 2-H-mant), 6.0–6.4 (1H, m, 4-H-mant) ^{31}P: −11.2 (1P, m, α-P), −22.7 (1P, m, β-P)
Br-mant-Ap_4A **3**	8.75	1046.2, 1048.0	29	^1H: 8.3 (1H, s, 8-H-Ad), 8.0–8.2 (2H, m, 2-H-Ad), 7.8 (1H, d, 1-H-mant), 7.4 (1H, m, 3-H-mant), 6.7 (1H, m, 2-H-mant), 6.1–6.2 (1H, d m, 4-H-mant), 5.9 (2H, m, 1′-H-rib) ^{31}P: −11.2 (1P, m, α-P), −21.7 (1P, m, β-P)
Azido-mant-Ap_4A **2**	8.80	1009.1	16	^1H: 8.3 (1H, s, 8-H-Ad), 8.0–8.1 (2H, m, 2-H-Ad), 7.8 (1H, d, 1-H-mant), 7.4 (1H, m, 3-H-mant), 6.7 (1H, m, 2-H-mant), 6.1–6.2 (1H, d, 4-H-mant), 5.9 (2H, m, 1′-H-rib) ^{31}P: −11.1 (1P, m, α-P), −21.7 (1P, m, β-P)

equal quantities of each probe and protein for 1 h in the dark, before exposing the mixtures containing **2** to UV light to initiate photocoupling. Excess probe was removed by the use of 5 or 10 kDa (as appropriate) molecular weight cutoff centrifugal concentrators (Viva Science) and the proteins were repeatedly washed in fresh buffer before measuring their fluorescence in a spectrofluorophotometer. The increase in fluorescence ΔI_{438} above background was then determined for each 'candidate' Ap_4A-binding protein-containing mixture. Only mixtures containing bone fide Ap_4A-binding proteins were expected to exhibit a significant increase in fluorescence ΔI_{438} above background, owing to affinity probe binding and covalent attachment.[14–19]

Fluorescence emission spectra from each protein-containing mixture are shown (Fig. 2a). As expected, only mixtures containing GroEL and LysU exhibited a significant increase in fluorescence emission intensity above background presumably due to specific protein labelling by either affinity probe as stated above. In the case of other protein 'candidates,' the dial affinity probe **1** was observed not to introduce any substantial fluorescence emission intensity increase above background consistent with minimal protein labelling of non-Ap_4A-binding proteins. By contrast, the azido affinity probe **2** was found to be somewhat less selective and measurable increases in fluorescence emission intensity were observed above background consistent with some non-specific labelling of these non-Ap_4A-binding proteins, especially trypsin. The differences between the various 'candidate' proteins in the presence of either affinity probe can be seen more clearly when increase in fluorescence intensity ΔI_{438} above background is plotted alone as a function of 'candidate' protein (Fig. 2b). These data clearly show that LysU and GroEL are the only Ap_4A-binding proteins as expected (see above) but in addition they illustrate that the dial affinity probe **1** is much more selective than the azido affinity probe **2**. The addition of 1 M NaCl to the incubation buffer retarded the absolute degree of labelling (to about 35%) but did not significantly affect the demonstrated specificity (data not shown) thereby confirming the probes' specific affinity for LysU and GroEL.

The binding assay above was also able to reveal additional information regarding differences in the Ap_4A-binding sites of LysU and GroEL. Assays were carried out with LysU and GroEL in 20 mM HEPES buffer, pH 8.0, containing 10 mM $MgCl_2$, 10 mM KCl and 4 mM ADP or ATP (in the case of GroEL, ATP was exchanged with non-hydrolysable β,γ-methylene adenosine triphosphate [AMPPCP] since GroEL is a potent ATPase, while LysU is not in the absence of L-lysine). The results are striking (Fig. 2c). In the case of LysU, there was an almost 90% drop in fluorescence emission intensity consistent with ADP and ATP competition with the affinity label for binding to protein. In the case of GroEL, there was no drop in fluorescence emission intensity at all consistent with a complete absence of ADP or AMPPCP competition with the affinity label for binding to protein, hence suggesting the existence of an alternative Ap_4A-binding site independent of the well-known ADP/ATP site located in each monomer of this homo-oligomeric protein. Of course these results could be interpreted as demonstrating that these well-known ADP/ATP sites are binding the affinity probes several orders of

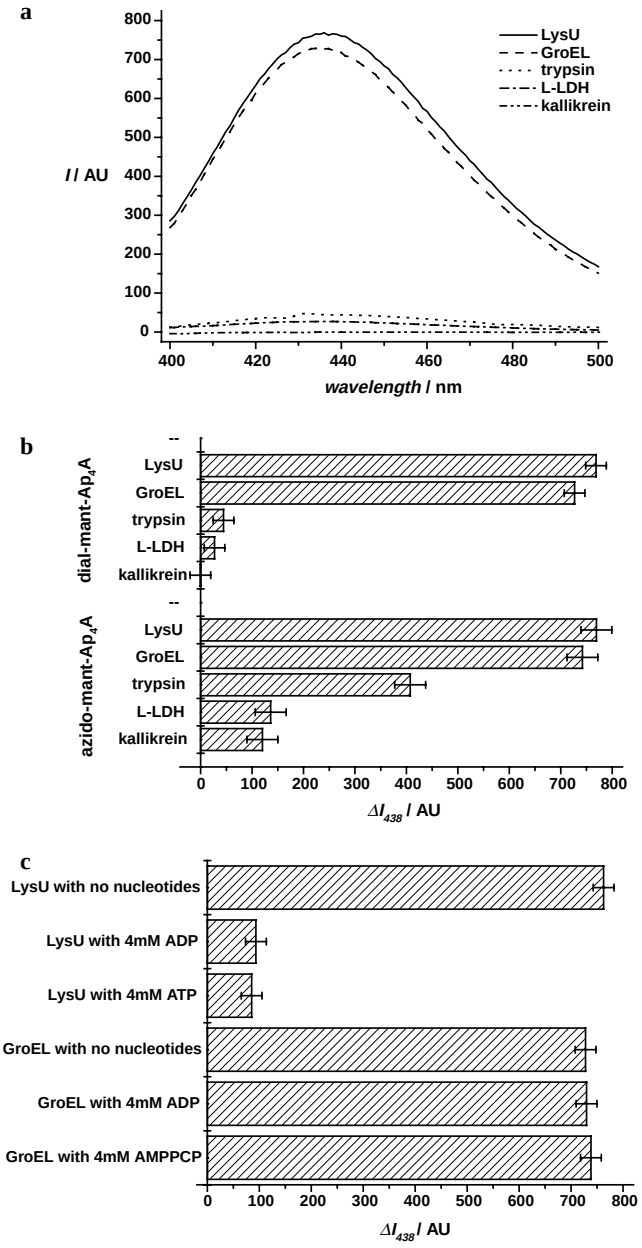


Figure 2. Detecting labelled proteins by change in fluorescence spectra (excitation at 356 nm). (a) Fluorescence spectra of proteins after 1 h incubation at rt with dial-mant-Ap_4A **1**. LysU and GroEL are clearly labelled by dial affinity probe **1**. (b) ΔI_{438} values for all 'candidate' proteins after 1 h incubation at rt with either dial-mant-Ap_4A **1** or azido-mant-Ap_4A **2**. (c) Effect of ADP or ATP/AMPPCP on dial-mant-Ap_4A **1** labelling of LysU or GroEL. Mono-nucleotides affect only LysU fluorescence emission intensity consistent with the same site competition between ADP or ATP and the dial affinity probe **1** for binding to LysU. All spectral data were recorded on a Shimadzu RF-5301PC spectrofluorophotometer; excitation 356 nm, high-sensitivity setting, slits 3 nm for excitation and 5 nm for emission, employing a quartz cuvette (1 cm pathlength).

magnitudes more efficiently than either ADP or AMP-PCP, but this is very unlikely in view of other data that we have also acquired which support the concept of two different binding sites (paper under preparation).

In conclusion, the preparations of dial-mant-Ap_4A **1**, azido-mant-Ap_4A **2** and Br-mant-Ap_4A **3** are reported. Initial tests with both show that **1** and **2** are successful fluorescent labelled Ap_4A analogue affinity probes for Ap_4A-binding proteins but that dial-mant-Ap_4A **1** in particular shows excellent specificity and differentiation between bone fide Ap_4A-binding proteins and control proteins. We are also able to report initial evidence confirming binding of an Ap_4A analogue to GroEL at a site (as yet unidentified) distinct from the well-known ADP/ATP binding site found in each GroEL monomer.

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20. In a typical procedure, mant-Ap_4A (100 mg, 0.103 mmol, >95% pure by HPLC) was freeze-dried from water and then methanol to remove any trace of TEAB from earlier synthesis steps. This lyophilisate was then dissolved in deionised water (4× 1 ml) and aqueous 0.3 M $NaIO_4$ (85 μl) was added to each batch (1 ml) over 30 min. Each reaction was monitored by anion-exchange HPLC until the oxidation was judged complete. Combined product mixtures were freeze-dried and then extracted with aqueous 96% ethanol (3× 1 ml) and the residue (containing only trace polyphosphate) discarded. The yield from this last step was estimated from the mass of lyophilised mant-Ap_4A, reduced by a purity factor (84 ± 3%) calculated from HPLC peak areas.[4,29]
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22. In a typical procedure, ATP (400 mg, 0.72 mmol) was dissolved with 1 M sodium acetate, pH 3.8 (8 ml). Liquid bromine (120 μl) was added and the reaction was monitored to completion by anion-exchange HPLC. Excess bromine was neutralised by dropwise addition of concentrated aqueous ammonia until the solution turned from brown to red, prior to initial product purification using a Sephadex LH 20 column (1.6 × 70 cm; Amersham Biosciences) packed and eluted in water (0.5 ml/min). Bromo-ATP (Br-ATP) eluted impure between 60 and 75 ml. Further purification was achieved using a SOURCE 15Q column (2× 35 ml; Amersham Biosciences) that was eluted with a 0–40% gradient of 2 M TEAB (200 ml; 7 ml/min). Br-ATP-containing fractions were combined and freeze-dried. Thereafter, pure Br-ATP (300 mg, 0.51 mmol) was dissolved in deionised water (8 ml) and heated with stirring to 50 °C. The pH was maintained at 9.6 by dropwise addition of 1 M NaOH aq, while *N*-methylisatoic anhydride (MIA, 500 mg, 2.82 mmol) was added in portions over 1.5 h. Br-mant-ATP was purified as previously described for mant-ATP.[4]
23. In this procedure, lyophilised LiN_3 (1 g, 20.4 mmol) was dissolved in dry DMF (50 ml) and applied to triethylammonium Dowex gel (40 ml) in a glass chromatography column. The column eluant was collected directly into a flask (50 ml) containing **3** (60 mg, 0.058 mmol) previously dried under vacuum from methanol solution. After 16h at 65 °C in the dark, azide exchange was judged to be complete and solvent with impurities was removed by SOURCE 15Q column that was eluted with a 0–50% gradient of 2 M TEAB (6 ml/min). Fractions containing the azido affinity probe **2** were combined and freeze-dried, after which the probe was stored in aqueous solution at −20 °C.
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28. Typical experimental conditions were as follows: samples of each 'candidate' Ap_4A-binding protein (50 μM final concentration) were combined with the appropriate affinity probe (50 μM final concentration) in 20 mM HEPES, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM KCl (200 μl final volume) (in the dark in the case of the azido affinity probe **2**), and the protein-containing mixtures subjected to vortex mixing. After 1 h incubation at rt, the protein-containing mixtures were subjected to vortex mixing again and those containing azido affinity probe **2** exposed to a 254 nm UV lamp for 5 min. Thereafter, each mixture was then introduced to a centrifugal concentrator (4 ml) and

concentrated and diluted three times with 20 mM HEPES, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM KCl to remove any free, unreacted affinity probe (each round being a dilution to 3 ml and a reconcentration to 100 μl). Fluorescence emission spectra (400–500 nm) of each protein-containing mixture were then recorded.

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30. Purity and retention times were measured with SOURCE 15Q anion-exchange media (2 ml) packed into a 0.5×10 cm glass Tricorn column (0.5×10 cm) (both from Amersham Biosciences), mounted on an Aglient 1100 series HPLC equipped with an auto loader and thermostatic sample chamber. The column was loaded in degassed 5 mM Tris–HCl, pH 8.0, before elution with a programmed gradient of 1 M NaCl in the same buffer. Fractions were detected by absorbance at A_{256} and A_{280}, injections were 2 μl for reaction monitoring and 5-15 μl for fraction analysis, flow rate was 2 ml/min and maximum pressure was set at 200 bar. Standard gradient program (% of 1 M NaCl) was, 1 min 0%, 1.5 min 20%, 6 min 70%, 8 min 90%, 10 min 100%, 11.5 min 0% and 13.5 min 0%. Mass spectrometry was carried out using an electrospray Bruker Esquire 3000 machine set to 100%, 75% or 50% fragmentation strength for dial-mant-Ap_4A **1**, Br-mant-Ap_4A **3** and azido-mant-Ap_4A **2**, respectively. Samples were applied in 1:1 acetonitrile/water containing 0.1% acetic acid. ^{1}H and ^{31}P NMR spectra were recorded on a 400 MHz Bruker Ultrashield with samples in D_2O. Coupling constants could not be reliably resolved.