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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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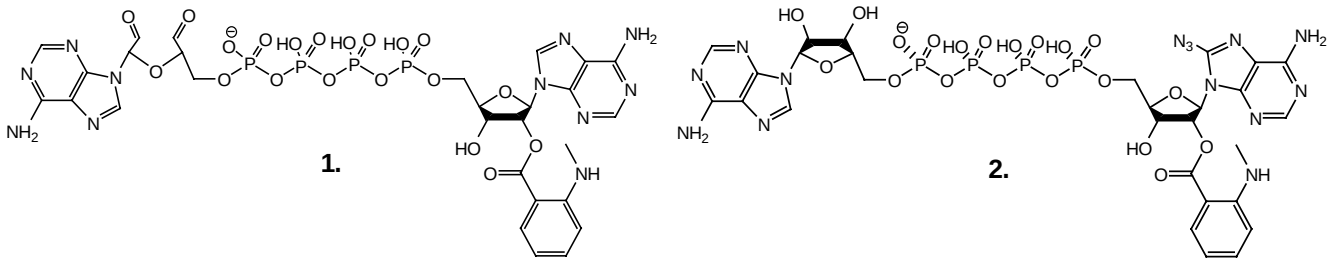
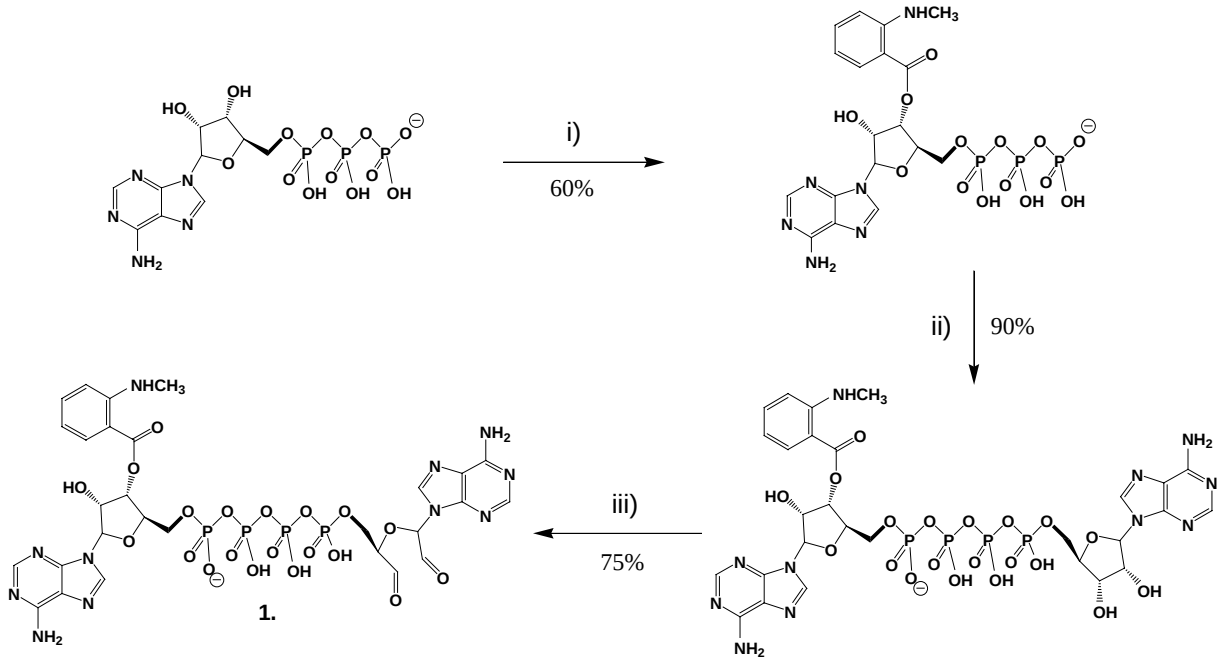


Figure 1. Structures of target fluorescent labelled Ap_4A analogue affinity probes.

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.