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Synthesis of novel fluorescent-labelled dinucleoside polyphosphates

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Abstract—A novel tandem synthetic–biosynthetic procedure is described for the synthesis of four new fluorescent dinucleoside polyphosphates: mant–Ap_4A, mant–$AppCH_2ppA$, TNP–Ap_4A and TNP–$AppCH_2ppA$. These compounds are expected to supplement the existing etheno (ε) and 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) labelled derivatives, being the fluorescent probes of choice to investigate polyphosphate/enzyme binding behaviour.
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5′,5‴-P^1,P^4-Dinucleoside polyphosphates (Ap_nAs) are an important family of nucleotides with diverse intracellular and extracellular biological roles.[1,2] Many questions about these compounds' metabolic behaviour in vivo remain to be answered, hence there is a clear need for increased numbers of fluorescent probes suitable for molecular recognition and binding studies in vivo. Fluorescent etheno (ε) labelled analogues have had limited success in such studies.[3] These compounds fluoresce at 410 nm (violet) when excited at 307 nm but have a fluorescence quantum yield less than 1/10th that of ε-AMP. Hence in vivo hydrolysis of ε-Ap_nA derivatives results in a significant increase in fluorescence output without change in the emission profile.[4] Furthermore, there is realistic concern that the ε-derivatisation of an adenine ring inhibits molecular recognition more substantially than modification of other nucleotide functional groups. Fluorescent BODIPY labelled analogues by contrast have proved to be excellent Ap_4A hydrolyse substrates, but have more the appearance of an Ap_n analogue than an Ap_nA analogue suggesting limited utility in molecular recognition and binding studies.[5] In an attempt to improve upon this situation, we report here a novel tandem synthesis–biosynthesis of new fluorescent Ap_nA analogues that are expected to be significantly more biocompatible.

The central members of the Ap_nA class of compounds are Ap_4A (diadenosine-5′,5‴-P^1,P^4-tetraphosphate) and Ap_3A (diadenosine-5′,5‴-P^1,P^3-triphosphate). Our main interest has been in Ap_4A therefore we have focussed primarily on the preparation of novel fluorescent Ap_4A analogues. For alternative fluorophores, we were attracted to the *N*-methylanthraniloyl (mant) and trinitrophenyl (TNP) moieties.[6,7] Both attach to the ribose and not the purine ring of adenosine nucleotides, and both have a proven track record as fluorescent labels of adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) that do not appear to impair the molecular recognition behaviour of respective fluorescent analogues.[8–13] The utility of mant- and TNP- moieties is also enhanced by their mutually complementary UV–vis absorption and fluorescence properties. Mant–ATP **1** shows intense fluorescence in water (I_{max} 448 nm (light blue); A_{max} 356 nm). The I_{max} becomes characteristically blue-shifted with an increase in fluorescence quantum yield (4–5-fold in 50% ethanol), upon contact with a more hydrophobic environment (i.e., as a result of mant–ATP binding to a protein or due to the presence of nonpolar solvents). TNP–ATP **2** shows minimal fluorescence in water. This is because the TNP-moiety equilibrates between an aromatic and semi-quinoid tautomer. In water the aromatic tautomer dominates the equilibrium and has no intrinsic fluorescence, but in contact with a more hydrophobic environment the semi-quinoid tautomer will dominate and this does possess intrinsic fluorescence properties (I_{max} 540 nm (green); A_{max} 410 nm).[7]

Initially, we attempted to synthesise mant and TNP fluorescent derivatives of Ap_4A and the nonhydrolysable analogue $AppCH_2ppA$ (diadenosine 5′,5‴-P^1,P^4-[β,γ-methylene] tetraphosphate) direct from their respective

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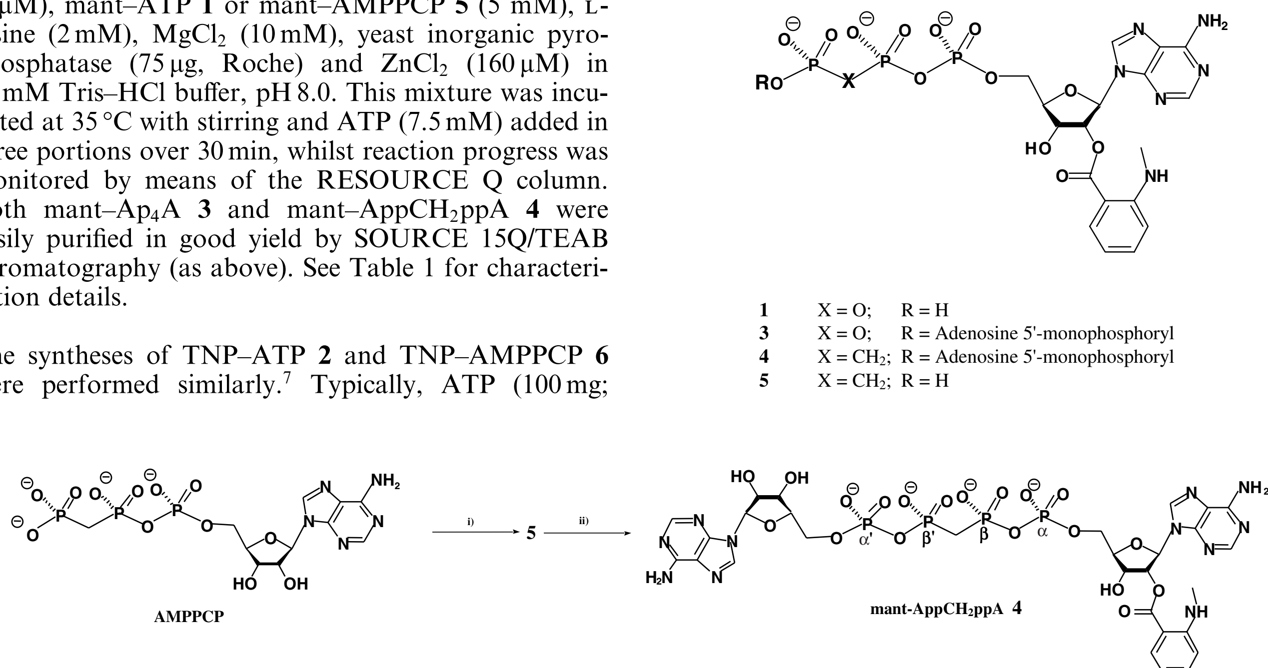
parent nucleotide polyphosphates. However, yields were poor (<40%) owing to hydrolysis, double substitution and purification problems. Therefore, we elected to combine chemical modification with enzymic synthesis in order to derive a high yielding and reproducible approach to the synthesis of potentially useful fluorescent Ap_4A analogues starting with mant–Ap_4A **3** and mant–$AppCH_2ppA$ **4**, respectively. The resulting tandem synthesis–biosynthesis procedure is illustrated for the preparation of mant–$AppCH_2ppA$ **4** (Scheme 1). ATP and AMPPCP (Sigma) were converted through to their mant-derivatives by reaction of the parent compound with *N*-methylisatoic anhydride (MIA) in aqueous solution. In a typical procedure, ATP (50 mg, 0.09 mmol) in deionised water (2 mL, adjusted to pH 9.6 with NaOH) was heated to 40 °C in a water bath. MIA (50 mg, 0.28 mmol) was added in four portions over 1 h and kept in suspension by rapid stirring, whilst pH 9.6 was maintained by titration with 1 M NaOH. The reaction was monitored by RESOURCE Q anion exchange chromatography as previously described.[14] After 3 h the reaction was terminated by reducing the pH to 7.0 with the addition of 1 M HCl and then the resulting solution was chilled on ice to precipitate excess MIA. Supernatant was centrifuged then applied to a Sephadex LH 20 column (1.6 × 70 cm; Amersham Biosciences) packed and eluted in water (0.5 mL/min). Mant–ATP fractions (eluting between 80–120 mL) were identified by light blue fluorescence under a UV lamp (365 nm). These were then further applied in one portion to a SOURCE 15Q column (35 mL; Amersham Biosciences), and the column eluted by a 0–100% 2M TEAB gradient (200 mL; 4 mL/min) with mant–ATP **1** eluting pure at approximately 55% gradient. Mant–AMPPCP **5** was prepared in a similar way. Ap_4A analogues were then generated using our own LysU biosynthesis procedure.[14–16] Typically, a reaction mixture (4 mL) was prepared consisting of LysU (9 μM), mant–ATP **1** or mant–AMPPCP **5** (5 mM), L-lysine (2 mM), $MgCl_2$ (10 mM), yeast inorganic pyrophosphatase (75 μg, Roche) and $ZnCl_2$ (160 μM) in 50 mM Tris–HCl buffer, pH 8.0. This mixture was incubated at 35 °C with stirring and ATP (7.5 mM) added in three portions over 30 min, whilst reaction progress was monitored by means of the RESOURCE Q column. Both mant–Ap_4A **3** and mant–$AppCH_2ppA$ **4** were easily purified in good yield by SOURCE 15Q/TEAB chromatography (as above). See Table 1 for characterisation details.

The syntheses of TNP–ATP **2** and TNP–AMPPCP **6** were performed similarly.[7] Typically, ATP (100 mg; 0.18 mmol) in deionised water (2 mL) was vigorously stirred at 40 °C. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) (150 mg; 0.51 mmol) was then added whilst pH was maintained at 9.6 with additions of 1 M NaOH. After 16 h, the reaction mixture was freeze-dried, washed with ice-cold acetone (300 mL) to remove excess TNBS, dried and then dissolved again in deionised water (1.5 mL). This solution was centrifuged, and then applied to the Sephadex LH 20 column packed and eluted in water (0.5 mL/min) as above. TNP–ATP **2** was eluted cleanly (monitoring at 255 and 409 nm) and characterised directly; TNP–AMPPCP **6** was prepared in a similar manner. Going forward, conversion of **2** and **6** into their respective TNP–Ap_4A **7** and TNP–$AppCH_2ppA$ **8** analogues was performed once again by means of the LysU biosynthetic methodology using 2 mM TNP–nucleotide with 6 mM ATP and elution through the Sephadex LH 20 column to achieve product purification (as above). See Table 1 for characterisation details.

The preparations and purifications of mant–Ap_4A **3**, mant–$AppCH_2ppA$ **4**, TNP–Ap_4A **7** and TNP–$AppCH_2ppA$ **8** analogues were achieved in excellent yields and represent an important step forward for research into the biology of Ap_nAs. Our recent efforts to obtain a reproducible preparative method for Ap_4A analogue biosynthesis[14] have now been applied successfully in a novel tandem synthesis–biosynthesis to generate new fluorescent analogues with high potential utility as probes for Ap_4A functions in vitro and in vivo. The nonhydrolysable analogues mant–$AppCH_2ppA$ **4**, and TNP–$AppCH_2ppA$ **8** are expected to be of particular utility in conjunction with mant–ATP **1** and TNP–ATP **2** for the spatial localisation of purine P2 and polyphosphate-selective receptors in neurological tissue not



Scheme 1. (i) MIA (3 equiv), pH 9.6, 40 °C, 3 h; (ii) ATP (1.5 equiv) with LysU (9 μM), pyrophosphatase (75 μg), L-lysine (2 mM), $MgCl_2$ (10 mM), $ZnCl_2$ (160 μM) in 50 mM Tris–HCl buffer, pH 8.0, 35 °C, 1 h.

Table 1. Characterisation details of fluorescent Ap_4A analogues

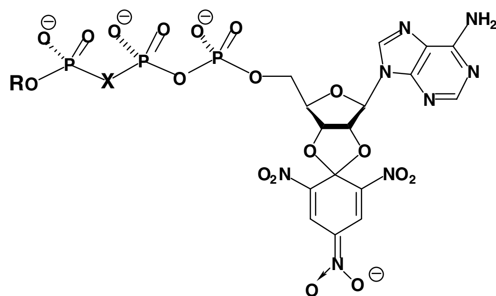
Derivative	(ES) $[M-H]^-$ (m/z)	Overall yield (%)	A_{max} (nm)	I_{max} (nm)	^1H and ^{31}P (δ ppm)
mant–Ap_4A	968.1	85	357	446	^1H: 8.5 (1H, s, 8-H-Ad), 8.4 (1H, s, 8-H-Ad [mant]), 8.1 (1H, s, 2-H-Ad), 8.0 (1H, s, 2-H-Ad [mant]), 7.9–7.8 (1H, d m, 1-H-mant), 7.4–7.3 (1H, m, 3-H-mant), 6.7–6.5 (1H, m, 2-H-mant), 6.2–6.1 (1H, d m, 4-H-mant)[a], 5.9 (2H, m, 1′-H-rib) ^{31}P: −11.2 (2P, m, α-P), −23.0 (2P, m, β-P)
mant–$AppCH_2ppA$	966.1	85	357	446	^1H: 8.6 (1H, s, 8-H-Ad), 8.5 (1H, s, 8-H-Ad[mant]), 8.2 (1H, s, 2-H-Ad), 8.1 (1H, s, 2-H-Ad[mant]), 8.0–7.8 (1H, d m, 1-H-mant), 7.5–7.4 (1H, m, 3-H-mant), 6.8–6.6 (1H, m, 2-H-mant), 6.3–6.1 (1H, d m, 4-H-mant), 5.9 (2H, m, 1′-H-rib), 2.6–2.4 (2H, t, O-CH_2-O, J_{H-P} 20.8 Hz) ^{31}P: −10.6 (2P, m, α-P), 8.0 (2P, m, β-P)
TNP–Ap_4A	1046.0	80	412	546[b]	^1H: 9.1 (~1H, s, H-TNP), 8.4 (1H, s, 8-H-Ad), 8.35 (1H, s, 8-H-Ad[TNP]), 8.1 (1H, s, 2-H-Ad), 8.05 (1H, s, 2-H-Ad[TNP]), 6.2 (1H, m, 1′-H-rib[TNP]), 5.85 (1H, m, 1′-H-rib) ^{31}P: −11.2 (2P, m, α-P), −22.7 (2P, m, β-P)
TNP–$AppCH_2ppA$	1043.8	80	412	546	^1H: 8.6 (~1H, s, H-TNP), 8.4 (1H, s, 8-H-Ad), 8.35 (1H, s, 8-H-Ad[TNP]), 8.1 (1H, s, 2-H-Ad), 8.05 (1H, s, 2-H-Ad[TNP]), 6.4 (1H, m, 1′-H-rib[TNP]), 5.95 (1H, m, 1′-H-rib), 2.6–2.4 (2H, t, O–CH_2–O, J_{H-P} 21 Hz) ^{31}P: −10.4 (2P, m, α-P), 9.3 (2P, m, β-P)

Mass spectrometry was carried out using an electrospray Bruker Esquire 3000 machine set to 100% fragmentation strength. Samples were applied in 1:1 acetonitrile/water containing 0.1% acetic acid. UV–vis and fluorescence spectra were obtained on an Ultraspec 4000 UV–vis spectrometer and a Shimadzu RF-5301PC fluorescence spectrophotometer, respectively. Proton and phosphorous NMR spectra were recorded on a 400 MHz Bruker Ultrashield with samples in D_2O.

[a] Mant protons numbered anticlockwise with respect to Scheme 1.

[b] Emission maxima are for aqueous solution, TNP derivatives show a shift to 535 nm in 50% ethanol/water.

to mention the identification of polyphosphate-selective receptors/binding proteins located inside cells.



2	X = O; R = H
6	X = CH_2; R = H
7	X = O; R = Adenosine 5'-monophosphoryl
8	X = CH_2; R = Adenosine 5'-monophosphoryl

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