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Synthesis of a hydrolytically stable, fluorescent-labeled ATP analog as a tool for probing adenylyl cyclases

Thomas Emmrich [a], Ali El-Tayeb [b,†], Hesham Taha [c], Roland Seifert [c], Christa E. Müller [b], Andreas Link [a,*]

[a] Institute of Pharmacy, Ernst-Moritz-Arndt-University, Friedrich-Ludwig-Jahn-Straße 17, D-17487 Greifswald, Germany
[b] PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany
[c] Department of Pharmacology and Toxicology, University of Regensburg, Universitätsstraße 31, 93040 Regensburg/Institute of Pharmacology, Medizinische Hochschule Hannover, Carl-Neuberg-Straße 1, 30625 Hannover, Germany

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ABSTRACT

(2*R*,3*S*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-hydroxy-4-[2-(methylamino)benzamido]tetrahydrofuran-2-yl-methoxy[(hydroxy)phosphoryloxy][(hydroxy)phosphoryl]dichloromethylphosphonic acid was synthesized as a chemically and metabolically stable analog of ATP substituted with a fluorescent methylanthranoyl (MANT) residue. The compound is intended for studying the binding site and function of adenylyl cyclases (ACs), which was exemplified by studying its interaction with *Bacillus anthracis* edema factor (EF) AC exotoxin.

Adenylyl cyclases (ACs) play a key role in signal transduction and various isoforms represent attractive drug targets, for example, for male contraceptives[1] or for therapeutic intervention in cardiovascular diseases and ageing.[2,3] In addition, several AC toxins are involved in the pathogenesis of bacterial diseases. A prominent example is the exotoxin edema factor (EF) of *Bacillus anthracis*, a calmodulin-dependent AC which is part of the bacterium's deadly triad that leads to the clinical symptoms of anthrax.[4]

ACs catalyze the conversion of ATP into the second messenger cAMP and thus, ATP analogs have been used as experimental tools to study the binding sites of these enzymes. Specifically, *N*-methylanthranoyl-(MANT-) substituted nucleotides[5,6] have been extensively studied. The *N*-methylanthranoyl group inserts into a hydrophobic pocket in the catalytic centers of structurally diverse ACs, thereby preventing transition of AC from the catalytically inactive closed to the catalytically active open conformation.[7,8] Moreover, MANT-nucleotides can be used as fluorescent tools to analyze conformational changes in ACs with high sensitivity and temporal resolution.[7–10] However, known MANT-nucleotides prepared via straightforward acylation of ATP with an activated *N*-methylanthranilic acid derivative are associated with problems arising from the ester bond formed. The resulting 2′- and 3′-acylated ATP derivatives are obtained as mixtures of two isomers **1a** and **1b** that cannot be separated due to acyl migration (Fig. 1).[5] However, the use of mixtures of two structurally distinct nucleotide derivatives is not optimal for the evaluation of isoform specificity.[11] Derivatives with two MANT groups attached to the 2′- as well as to the 3′-OH group would circumvent this problem but have shown a strong reduction in inhibitory potency in the case of related inosine derivatives.[10] Another approach includes the use of mono-deoxygenated sugars (vide infra), where intramolecular acyl migration is excluded, albeit with the concomitant loss of a potential hydrogen bond donor. Furthermore, these carboxylic acid esters are not resistant towards hydrolysis which may complicate the interpretation of the obtained results.[12] In addition, nucleoside triphosphates could readily be cleaved by ecto-nucleotidases, including nucleoside triphosphate diphosphohydrolases (NTPDases)[13] and alkaline phosphatases.[14] Consequently, we designed, synthesized and characterized a MANT-ATP analog that is stabilized with respect to hydrolysis in aqueous media and in biological fluids.

The notorious instability of carboxylic acid esters in biological fluids can be overcome by various strategies. An obvious choice based on our preceding work on 2′-amido-substituted adenosine derivatives as antiparasitic drugs[15] was the exchange of the 2′-OH group by an NH_2-group enabling the formation of comparably stable carboxylic acid amides. The amides thus obtained are no longer prone to intramolecular acyl migration or fast hydrolysis by water or esterases present in many in vitro or in vivo assays. Moreover, it can be excluded that the fluorescent MANT-moiety will be transferred to nucleophilic groups of surrounding biomolecules, in analogy to an intermolecular acyl migration. Furthermore,

* Corresponding author.
E-mail address: link@pharmazie.uni-greifswald.de (A. Link).
† A. El-Tayeb is on leave from the University of Al-Azhar, Assiut, Egypt.

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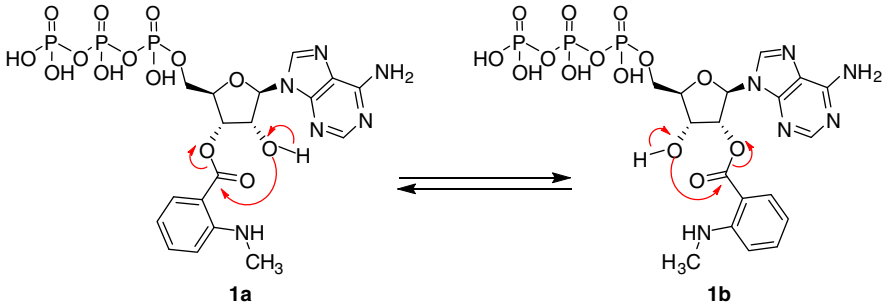


Figure 1. Intramolecular acyl migration of classical MANT-substituted fluorescent ATP derivatives.

the hydrogen bond donor properties of the residual NH of the carboxylic acid amide closely imitate the 2′-OH-group of ATP. In contrast, carboxylic acid esters, such as **1b** only possess an H-bond acceptor at the same position (2′-oxygen atom). The feasibility of such an approach was previously demonstrated by amide **2**, which was synthesized as a stable substrate analog of aminoacyl-tRNA synthetases and contributed to the characterization of the binding site and the elucidation of the function of the enzymes (Fig. 2).[16]

For the parallel preparation of arrays of 2′-amido-2′-deoxyadenosine derivatives as potentially bioactive nucleosides we employed a polymer-assisted acylation protocol making use of Kenner's safety-catch linker. In the present study, where only a single derivative had to be synthesized on a multi-mg scale, we decided to prepare the desired intermediate 2′-MANT-amino-2′-deoxyadenosine (**4**) in solution for practical reasons.

For this purpose we adapted a synthetic approach published by Sauer et al.[17], in which silica-supported ion exchange reagents were employed to facilitate the work-up after hydroxybenzotriazole-catalyzed, $N^{2'}$-selective acylation at the ribose moiety.

Taking advantage of a microwave-assisted heating protocol, **4** was obtained from commercially available 2′-amino-2′-deoxyadenosine **3**, in a moderate yield of 42.6 % (Scheme 1).[18] Care must be taken, as the product is susceptible to degradation by daylight irradiation. For the preparation of nucleoside triphosphates and analogues there are numerous protocols available in the literature.[19] In our hands, the method originally established by Yoshikawa et al.[20] performed best and was adapted with modifications.[21] To further enhance the stability of the targeted nucleotide probe both towards chemical and enzyme-catalyzed hydrolysis we prepared a dichloromethylene analogue (**7**) of the MANT-ATP derivative. In contrast to the corresponding triphosphate this structure cannot be cleaved by NTPDases or phosphatases due to the replacement of the terminal phosphate by a phosphonate. Therefore it is more suitable for both in vitro and especially in vivo assays. The selected bioisosteric replacement of the oxygen bridge promised to be particularly suitable due to its electronic properties, which have an impact on the pK_a value, and therefore the protonation state of the γ-phosphate/phosphonate.

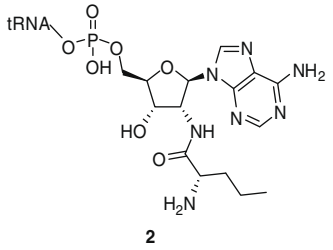


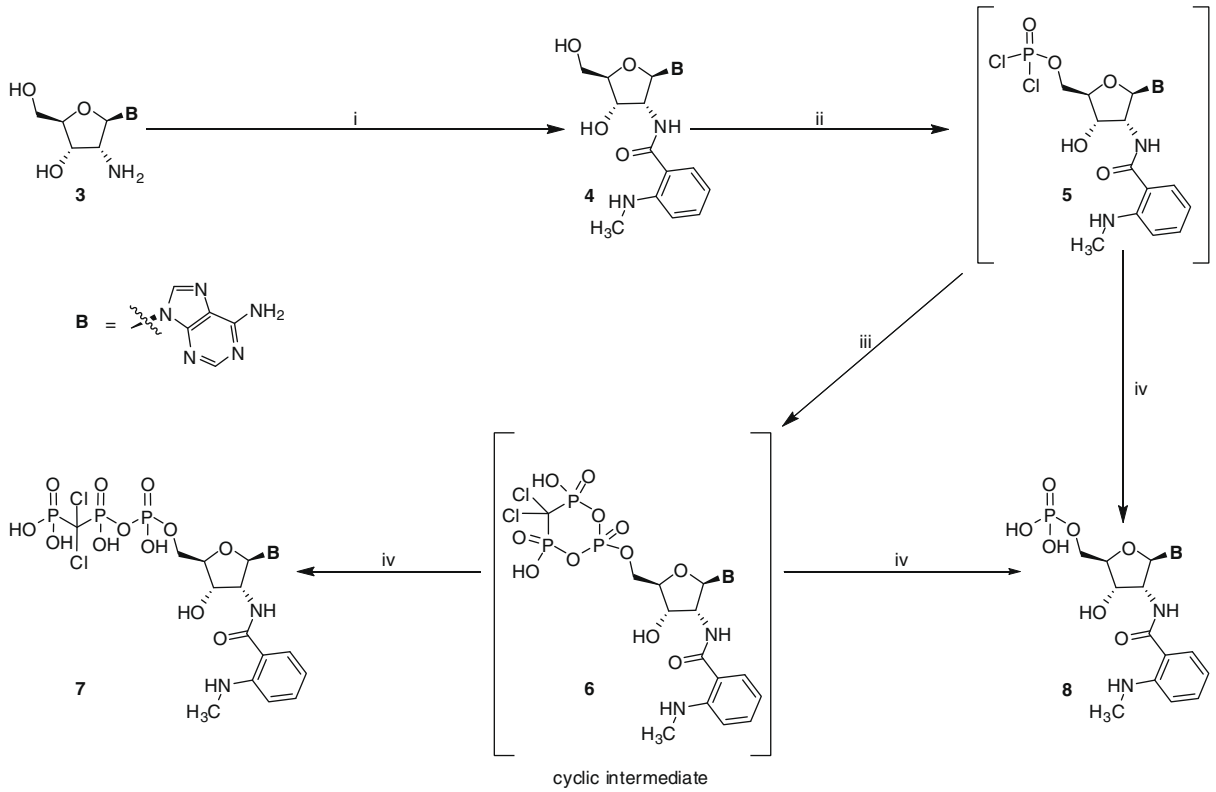
Figure 2. 2′-Amido-2′-deoxyadenosine derivative as a stable substrate analog of aminoacyl-tRNA synthetases.[16]

Reaction of the fluorescent-labeled nucleoside **4** with phosphorus oxychloride in trimethyl phosphate afforded the reactive 5′-dichlorophosphate intermediate **5** which was directly reacted with dichloromethylenediphosphonic acid in *N*,*N*-dimethylformamide (DMF) to form the cyclic intermediate **6**. Hydrolysis of **6** with triethylammonium hydrogen carbonate afforded the corresponding triphosphate analog **7** (Scheme 1).[22–24] The synthesized nucleotide **7** was purified by anion exchange chromatography using FPLC (ÄKTA FPLC, Amersham Biosciences, equipped with an XK 26 mm/20 cm length column filled with Sephadex DEAE A-25 gel) to remove the monophosphate **8** obtained as a side-product. In a second step, the product was further purified by reversed phase HPLC to remove inorganic impurities such as inorganic phosphates and buffer components. The structure of the synthesized nucleotide was confirmed by ^{1}H and ^{31}P NMR spectra, in addition to LC/ESI mass spectra obtained both in positive and negative mode.[22,24]

To demonstrate the suitability of compound **7** as a new tool to probe the active centers of ACs, we initially investigated the interaction of **7** with *B. anthracis* EF. The inhibition of EF3, the isolated catalytical domain of EF, by **7** was determined with a radiometric assay using [α-^{32}P]ATP as substrate, thus yielding a K_i-value of 8.98 ± 0.8 μM.

This is approximately 9–90 times weaker compared to recently assayed congeners of this class of compounds, for example, MANT-ATP (0.98 ± 0.08 μM) or MANT-CTP (0.10 ± 0.008), respectively.[9] Furthermore, we studied the direct interaction of **7** with EF3 in FRET-experiments. At an excitation wavelength of 280 nm, the ubiquitous aromatic amino acids tryptophan and tyrosine are excited, thus emitting light at 350 nm,[7–10] which can then excite the MANT-moiety of **7**, provided a close proximity of donor and acceptor moiety. This radiation-free transfer of energy results in an increased fluorescence of the MANT-residue at 420–450 nm. A detailed description of the assay was recently published by Taha et al.[9]

2′-MANT-AppCCl$_2$p **7** (300 nM) was excited at λ_{ex} 350 nm, and emission was recorded from λ_{em} 380–550 nm. In the presence of Tris buffer and in the absence of the organic solvent DMSO, compound **7** showed only a small fluorescence signal (Fig. 3A) as was the case for the reference nucleotide 2′-MANT-3′-d-ATP (Fig. 3B). The addition of DMSO at increasing concentrations augmented the fluorescence of both nucleotides in a concentration-dependent manner. The maximum increase in fluorescence with 2′-MANT-3′-d-ATP was seen with 80–100% (v/v) DMSO. 2′-MANT-AppCCl$_2$p was more responsive to changes in the hydrophobic environment, that is, the maximum increase in fluorescence was already observed with 40–60% (v/v) DMSO. With both nucleotides, we observed a shift of the emission maximum from λ_{em} 450 nm to λ_{em} 425 nm (blue-shift). Thus, in principle, both nucleotides could be used to monitor conformational changes in EF upon addition of calmodulin.[9] For 2′-MANT-3′-d-ATP we have recently observed large calmodulin-dependent increases in direct nucleotide fluorescence



Scheme 1. Reagents and conditions: (i) Acetonitrile/*N*,*N*-dimethylacetamide (DMA), *N*-methylanthranilic acid, *N*-hydroxybenzotriazole (HOBt), polystyrene-supported *N*,*N*′-dicyclohexylcarbodiimide (PS-DCC), 4-dimethylaminopyridine (DMAP), microwave heating for 6 min; (ii) $(OMe)_3PO$, $POCl_3$, proton sponge, 5 h, 0 °C; (iii) NBu_3, bis(tri-*n*-butylammonium) dichloromethylenediphosphonate in DMF, 5 min, 0 °C; (iv) $Et_3NH^+HCO_3^-$, pH 7.4–7.6, 1 h, rt.

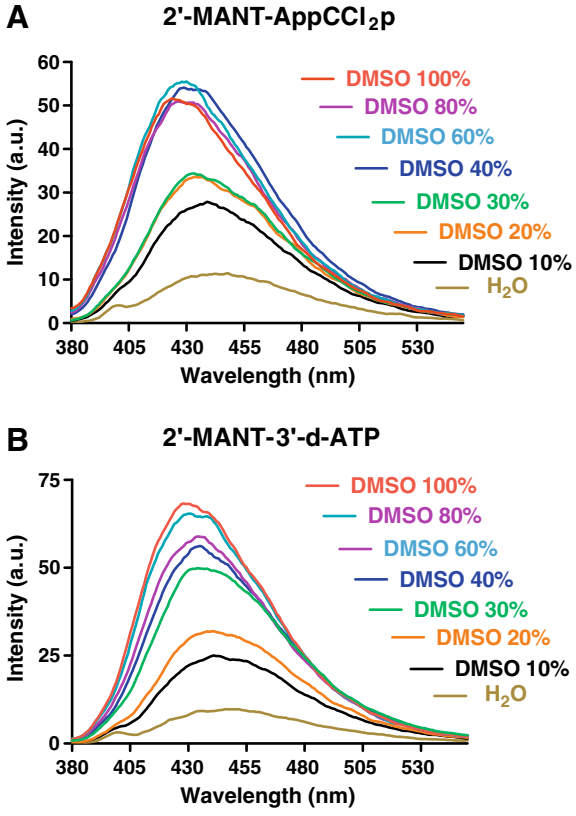


Figure 3. Increasing addition of DMSO to 300 nM solutions of (A) 2′-MANT-AppCCl$_2$p (**7**) resp. (B) 2′-MANT-3′-d-ATP leads to both a hypsochromic shift and a concentration-dependent increase in intensity.

(λ_{em} 350 nm) upon interaction with EF. Moreover, we observed calmodulin-dependent FRET from multiple tryptophan and tyrosine residues in EF to 2′-MANT-3′-d-ATP.[9] In marked contrast, with 2′-MANT-AppCCl$_2$p, no such changes were observed (data not shown). A likely explanation for these negative results is the fact that the affinity of the nucleotide as determined in functional AC activity competition results was too low. Accordingly, the interaction of 2′-MANT-AppCCl$_2$p with EF is not sufficiently tight enough to ensure the development of direct fluorescence and FRET signals. Another hypothesis to explain the observed results is that either the specific charge distribution in the vicinity of the fluorophore leads to a strong attenuation of the signals, or nearby molecules like chlorine ions quench the otherwise expected signals, or a combination of both effects.

Concluding, we have synthesized for the first time an ATP analog, which combines numerous advantages of previously described derivatives in one molecule, that is, a hydrolytically stable triphosphate analog, a fluorescent label that does not undergo intramolecular acyl migration and maintenance of both 2′- and 3′-hydrogen bond donor capabilities.

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18. Synthesis of (2*R*,3*R*,4*S*,5*R*)-*N*-2-(6-amino-9*H*-purin-9-yl)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-3-yl-[2-(methylamino)]benzamide (**4**). In a 8 ml microwave reaction vessel were placed 200 mg of 2′-amino-2′-deoxyadenosine (**3**, 1.0 equiv, 752 μmol), 125 mg of *N*-methylanthranilic acid (1.1 equiv, 830 μmol), 102 mg of anhydrous HOBt (1 equiv, 752 μmol), 800 mg of PS-DCC, loading level 1.9 mmol g^{-1}, 2 equiv) and catalytic amounts of 4-DMAP (1 mol %, 1 mg). Washing the polymer-supported reagent with the solvent system of the reaction is advisable. After addition of a 1:1 solvent mixture of MeCN and DMA (4 mL), the reaction vessel was immediately closed and the mixture was heated in a monomode microwave oven at 100 °C for 6 min, allowing a ramp time of 2 min. Applying constant external cooling by pressurized air, the heating power was around 150 W at the beginning of the reaction and just below 90 W for maintaining the temperature. After cooling to rt, the reaction was stopped by adding the same volume of MeOH and vigorous stirring for 5 min followed by filtering off insoluble impurities. The filtrate was shaken with SiliaBond carbonate (SiliCycle Inc., silica-supported trimethylammonium carbonate, loading level 0.70 mmol g^{-1}) for 1 h at 25 °C; MeOH may be added to allow thorough mixing. After filtration and concentration under reduced pressure a slightly yellowish oil was obtained which was subjected to column chromatography on silica gel ($CHCl_3$/MeOH 19:1). Compound **4** was obtained upon drying in vacuo for 6 h as an off-white solid, yield: 128 mg (42.6%). It was stored under inert gas (argon) and protected from light, as the compound slowly decomposed upon irradiation by daylight, and turned brownish within several weeks. Decomposition came along with a decrease in fluorescence and could be monitored by fluorimetry. ^{1}H NMR (MeOH-d_4, 200 MHz, ppm): δ 2.72 (s, 3H, CH_3), 3.77–3.97 (m, 2H, C(5′)H), 4.26 (s, 1H, C(4′)H), 4.49 (d, 1H, C(3′)H, ^3J = 5.2 Hz), 5.28 (dd, 1H, C(2′)H, $^3J_1$ = 8.3 Hz, $^3J_2$ = 5.4 Hz), 6.14 (d, 1H, C(1′)H, ^3J = 8.4 Hz), 6.54–6.64 (m, 2H, MANT-C(3)H and -C(5)H), 7.28 (dt, 1H, MANT-C(4)H, ^3J = 7.6 Hz, ^4J = 1.6 Hz), 7.48 (dd, 1H, MANT-C(6)H, ^3J = 7.6 Hz, ^4J = 1.4 Hz), 8.18 (s, 1H, C(8)H), 8.29 (s, 1H, C(2)H). ^{13}C NMR (MeOH-d_4, 50 MHz, ppm): δ 29.87 (CH_3), 57.62 (C2′), 63.84 (C5′), 72.67 (C3′), 89.70, 89.77 (C1′ and C4′), 112.04 (MANT-C3), 115.87, 115.90 (MANT-C1 and -C5), 116.46 (C5), 129.25 (MANT-C6), 134.11 (MANT-C4), 141.74 (C8), 150.44 (C4), 151.51 (MANT-C2), 153.58 (C2), 157.62 (C6), 171.95 (CO). Fluorescence data (excitation wavelength is defined as λ_{ex}, fluorescence maximum as λ_{em}): λ_{ex} = 434 nm→λ_{em} = 354.36 nm, λ_{ex} = 354 nm→λ_{em} = 435.63 nm, λ_{ex} = 265 nm→λ_{em} = 433.85 nm. LC/ESI-HRMS (Shimadzu IT-TOF): positive mode 400.171 ($[M+H]^+$), negative mode 398.158 ($[M-H]^-$), calculated: 399.1655.
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24. Synthesis of (2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3-hydroxy-4-[2-(methyl amino)benzamido]tetrahydrofuran-2-ylmethoxy[(hydroxy)phosphoryloxy]-[(hydroxy)phosphoryl]dichloromethylphosphonic acid (**7**). Synthesis of **7** from **4** was performed in analogy to described procedures.[17–19] ^{1}H NMR (D_2O + MeOH-d_4, 500 MHz, ppm): δ 2.67 (s, 3H), 4.36–4.54 (m, 2H), 4.6 (m, 1H), 5.05 (m, 2H), 6.26 (d, 1H, *J* = 8.19 Hz), 6.78 (m, 2H), 7.42 (m, 2H), 8.16 (s, 1H), 8.45 (s, 1H). ^{31}P NMR (D_2O + MeOH-d_4, 202 MHz, ppm): δ 7.37, 0.60, −10.19. LC/ESI-MS: positive mode 706 ($[M+H]^+$), negative mode 704.0 ($[M-H]^-$), calculated: 705.