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New Fluorescent and Photoactivable Analogs Acting on Nucleotide Binding Enzymes

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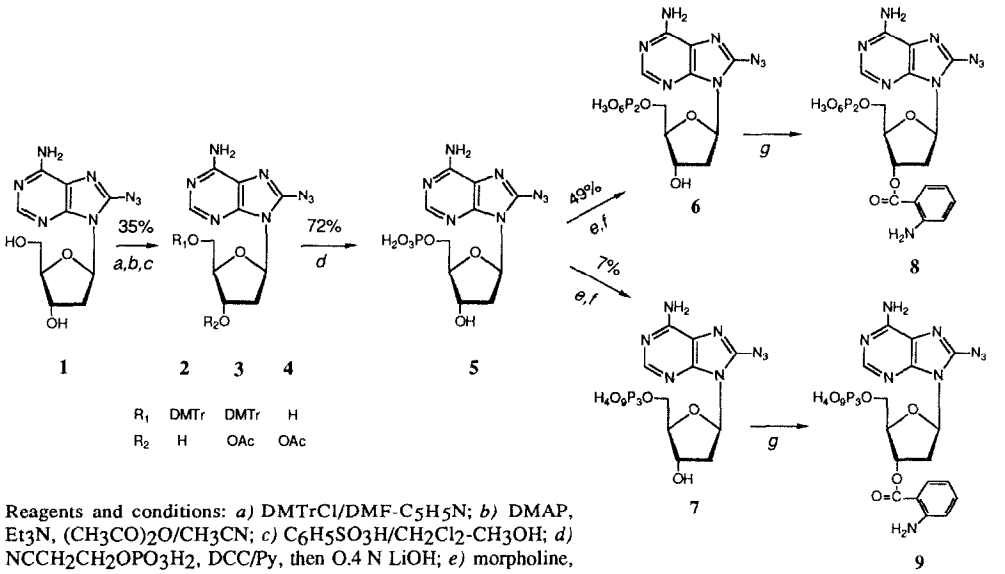
Abstract: *We describe a seven-step synthesis of 8-azido-3'-O-anthraniloyl-2'dADP and 2'dATP from 8-azido-2'deoxyadenosine. These compounds can be used as fluorescent and photoactivable probes for the nucleotide-binding site of kinases or cyclases.*

Nucleotide analogs with functional reactive groups have been increasingly used to characterize substrate and/or effector sites of ATP-dependent enzymes[1-3]. The most widely employed compounds are the azido-derivatives of ATP or ADP. Under weak ultraviolet light, the azido group changes rapidly to the corresponding nitrene which can then react with the side chain of several amino acid residues[4-9].

Recently, we have shown that the fluorescent 3'-O-anthraniloyl derivative of 2'dATP (Ant-dATP) is a strong competitive inhibitor (K_i 10 μM) of two calmodulin (CaM)-activated bacterial adenylate cyclases[10]. Binding of this analog to *Bordetella pertussis* or *Bacillus anthracis* enzyme is specific and is accompanied by a several-fold increase in its quantum yield. As 8-azido-ATP showed no or little decrease in its affinity for the active site of adenylate cyclase[11,12], we predicted that 8-azido-3'-O-anthraniloyl-2'dATP might act as a useful fluorescent and photoactivable probe for the nucleotide-binding site of bacterial adenylate cyclase(s) with approximately ten times higher affinity than that of the natural substrate.

Attempts to prepare 8-azido-2'dATP by the same procedure employed to obtain 8-azido-ATP (i.e. bromination of AMP[13], treatment of 8-bromo-AMP with NaN_3[14,15] and conversion of 8-azido-AMP to 8-azido-ATP) failed. In our hands 2'dAMP was not stable enough to treatment with bromine in buffered aqueous solutions which led to cleavage of the glycosidic bond. For this reason we have selected 8-azido-2'deoxyadenosine (**1**) as a starting material to prepare the corresponding mono-, di- and triphosphate (**5**, **6** and **7**).



Reagents and conditions: *a)* DMTrCl/DMF-C_5H_5N; *b)* DMAP, Et_3N, $(CH_3CO)_2O/CH_3CN$; *c)* $C_6H_5SO_3H/CH_2Cl_2$-CH_3OH; *d)* $NCCH_2CH_2OPO_3H_2$, DCC/Py, then 0.4 N LiOH; *e)* morpholine, DCC/tBuOH-H_2O; *f)* $O[P(O)(OH)_2]_2$ tetrabutylammonium salt/DMSO; *g)* isatoic anhydride/H_2O (pH 10).

Selective protection of 5'OH of **1** with dimethoxytrityl chloride in a mixture of DMF/pyridine gave **2**. Selective acylation[16] of 3'OH of **2** with acetic anhydride/triethylamine in the presence of a catalytic amount of DMAP at room temperature led to **3**. Removal of the DMTr group by treatment with 2% benzenesulfonic acid gave **4**. The free 5'OH group of **4** was phosphorylated by condensation with cyanoethyl phosphate[17] in presence of DCC in anhydrous pyridine. Treatment with 0.4 N LiOH for 1 h, yielded, upon simultaneous decyanoethylation and deacetylation, the phosphomonoester **5**. The phosphomonoester **5** was converted into 8-azido-2'-deoxyadenosine 5'-phosphomorpholidate in quantitative yield by refluxing with morpholine in the presence of DCC using a mixture of t-butanol and water as solvent[18]. Treatment with tetrabutylammonium pyrophosphate in dry DMSO for two days yielded di- and triphosphate derivatives **6** and **7** which were purified by ion-exchange chromatography on a DEAE-cellulose column (bicarbonate form) using a linear gradient of triethylammonium bicarbonate (0 to 0.35 M) as eluant. The free 3'OH group of **6** and **7** was anthranoylated by reaction with isatoic anhydryde to give **8** and **9**. Compounds **8** and **9** were purified by chromatography on Lichroprep RP-18 (25-40 μm) using triethylammonium acetate as eluant, followed by reverse phase HPLC on a Nucleosil 5C18 column. The di- and triphosphate derivatives have been characterized by IR, UV, FAB-mass[19], and NMR spectroscopy[19].

Compound **9** inhibited competitively *B. pertussis* adenylate cyclase. The K_i value of 74 μM was close to that predicted from the apparent K_i values of Ant-dATP and 8-azido-ATP determined previously, which seems to indicate the "cumulative" effects of the two substitutions operated on the natural nucleotide. As expected, the affinity of **9** for bacterial adenylate cyclase was 8 times higher than that of the natural substrate[20], due to the presence of azidoadenine and anthraniloyl moieties, respectively. Compounds **8** and **9** exhibited characteristic UV spectra with maxima at 280 (ε_{mM} = 12.5) and 333 nm (ε_{mM} = 4.7). Photolysis of these compounds under weak UV-light (radiation below 300 nm was cut off with a glass filter) was accompanied by a decrease in absorbance at 280 nm, whereas the maximum at 333 nm was unaffected. Photolysis of adenylate cyclase alone had no effect on enzyme activity but with a four-fold excess of compound **9** ower its K_i value, the bacterial enzyme was inactivated in a time-dependent manner. Two mM 3'dATP protected almost completely adenylate cyclase against inactivation by **9**. Labeling of bacterial enzymes upon photolysis with **9** was easily visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the reaction mixture and exposure of the wet gel to UV-light. Other ATP-utilizing enzymes such as adenylate kinase, phosphoglycerate kinase or creatine kinase were equally well labeled by the compound **9**.

In conclusion, the photoreactive ATP analog 8-azido-Ant-dATP, with its environment-sensitive fluorescent anthraniloyl group, is a new and useful tool for studying ATP-binding proteins with particular relevance on the as yet poorly characterized class of ATP-cyclizing enzymes. The covalently attached fluorescent nucleotide can be used to identify the amino acid residue(s) of the active site or as an *in situ* probe for monitoring conformation changes at or near the active site.

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19. In the ^{1}H-NMR spectrum of compounds **8** and **9** the protons of the anthraniloyl moiety are labeled as H-2A, H-3A, H-4A, H-5A.
Compound **8**: IR: υ_{max} 2158 cm^{-1} (N_3). M.S. (FAB$^-$): 570 (M-H)$^-$. ^{1}H-NMR 300 MHz: (D_2O): δ = 2.47 (m, 1H, H-2"); 3.26 (m, 1H, H-2'); 4.15 (m, 2H, H-5' and H-5"); 4.42 (m, 1H, H-4'); 5.56 (m, 1H, H-3'); 6.35 (dd, 1H, H-1'); 6.64 (t, 1H, H-3A); 6.72 (d, 1H, H-5A); 7.28 (t, 1H, H-4A); 7.83 (d, 1H, H-2A); 8.09 (s, 1H, H-2).
^{31}P-NMR 121.5 MHz: (D_2O): δ (PO_4H_3 as reference) = -9.8 (d, 1P, P_α, $J_{P_\alpha\text{-}P_\beta}$ = 18.21 Hz); -10.75 (d, 1P, P_β, $J_{P_\beta\text{-}P_\alpha}$ = 18.21Hz).
Compound **9**: IR: υ_{max} 2158 cm^{-1} (N_3). M.S. (FAB$^-$): 650 (M-H)$^-$; 624 (M-H-26). ^{1}H-NMR 300 MHz: (D_2O): δ = 2.40 (m, 1H, H-2"); 3.17 (m, 1H, H-2'); 4.14 (m, 2H, H-5' and H-5"); 4.34 (m, 1H, H-4'); 5.52 (m, 1H, H-3'); 6.27 (dd, 1H, H-1'); 6.53 (t, 1H, H-3A); 6.64 (d, 1H, H-5A); 7.16 (t, 1H, H-4A); 7.70 (d, 1H, H-2A); 7.97 (s, 1H, H-2).
^{31}P-NMR 121.5 MHz: (D_2O): δ (PO_4H_3 as reference) = -9.83 (d, 1P, P_γ, $J_{P_\gamma\text{-}P_\beta}$ = 20.6 Hz; -10.80 (d, 1P, P_α, $J_{P_\alpha\text{-}P_\beta}$ = 20.6 Hz); -22.42 (t, 1P, P_β, $J_{P_\beta\text{-}P_\alpha}$ = $J_{P_\beta\text{-}P_\gamma}$ = 20.6 Hz).
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