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CONJUGATION OF NUCLEOSIDE TRIPHOSPHATES TO AN AMINO LINKER

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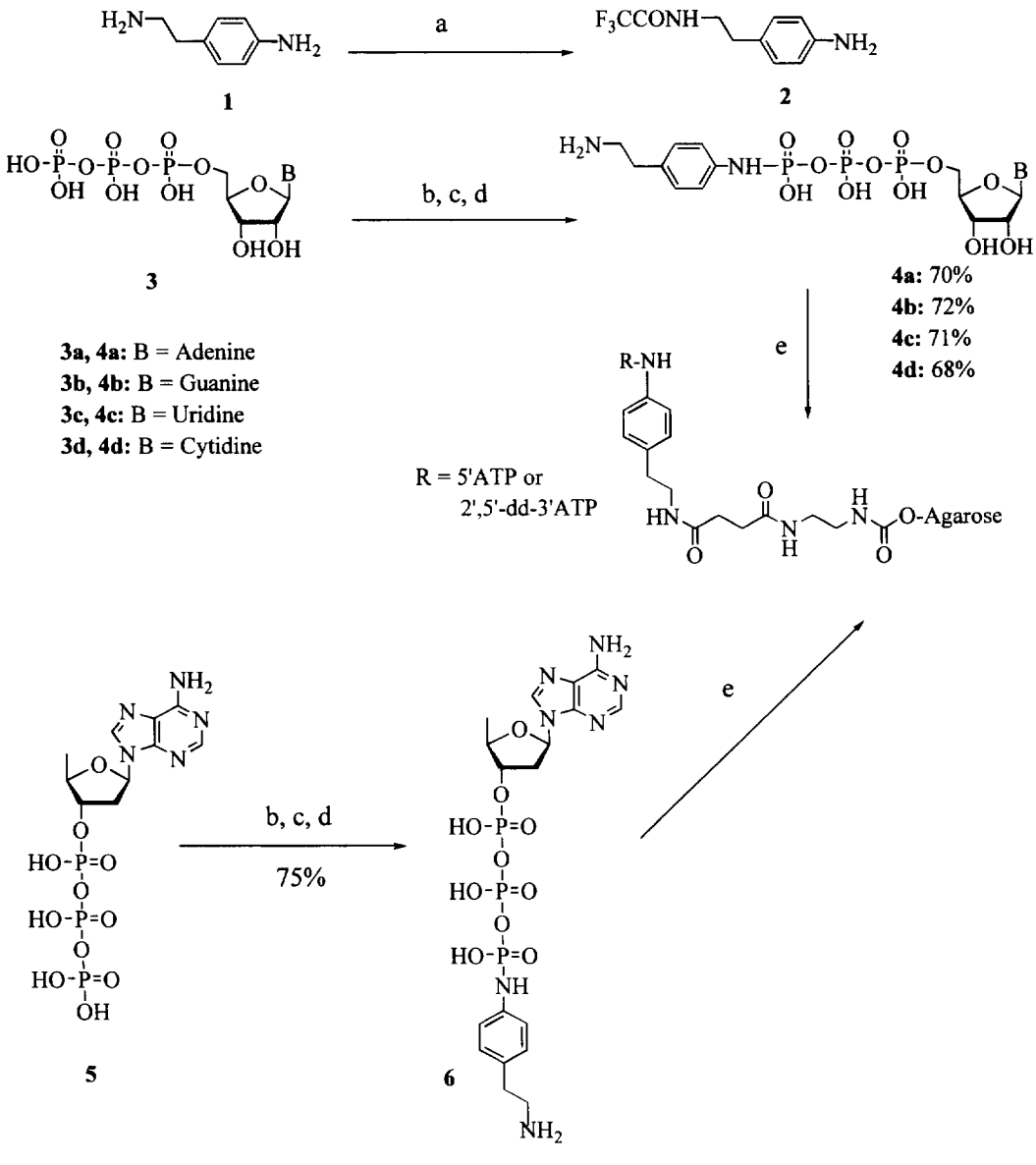
Abstract:A number of nucleoside triphosphates have been linked to a para-aminophenethylamino moiety providing an improved method for the immobilization of nucleotides. © 1997, Elsevier Science Ltd. All rights reserved.

Adenylyl cyclases are a family of enzymes that catalyze the formation of cAMP from adenosine-5'-triphosphate (5'ATP).[1] The isolation of adenylyl cyclases is very difficult since these enzymes are present in extremely low abundance and very labile.[1] A hexahistidine tagged enzyme has been purified by chelation affinity chromatography.[2] Native adenylyl cyclases have been purified by affinity chromatography using immobilized forskolin[3] and immobilized calmodulin[4]. However, neither of these ligands are specific for adenylyl cyclases,[5] and moreover only four of the ten known isoforms of adenylyl cyclases binds to calmodulin. Recently, we showed that 2',5'-dideoxyadenosine-3'-triphosphate (2',5'-dd-3'ATP) was a potent noncompetitive inhibitor of adenylyl cyclases (IC_{50} ~40 nM), suggesting that this family of nucleotides may constitute a new class of regulators that affect cell function.[6] This paper describes the preparation of *p*-aminoethylanilides of nucleoside triphosphates and their coupling to an Affi-Gel 10 matrix. Although several nucleotides have been conjugated to a matrix for affinity chromatography and are commercially or readily available, none are targeted to binding domains for nucleoside-3'-polyphosphates.[7] During the course of our investigation concerning the inhibitory site of adenylyl cyclases we wanted to immobilize 2',5'-dd-3'ATP to obtain a new method to purify adenylyl cyclases and possibly some other proteins that would recognize this class of nucleoside 3'-polyphosphates. For the preparation of this column, various attachments on 2',5'-dd-3'ATP were considered. Substitution on the adenine moiety or on the 2' and 5' positions of the ribose impair the capacity to inhibit adenylyl cyclases.[8] We therefore choose to couple the matrix to the terminal phosphate of the nucleotide. We tried first to couple it to a phenylenediamine according to the procedure developed by Pfeuffer to immobilize 5'GTP for the purification of G-proteins (guanine-nucleotide dependent regulatory proteins).[9] Although this method has proved useful for immobilizing nucleoside 5'-triphosphates, we were not successful in preparing conjugate with 2',5'-dd-3'ATP. The nucleotide was totally decomposed in the reaction mixture. As this nucleotide is much more unstable than 5'GTP for example, and also has to be prepared in several steps from 2'-deoxyadenosine,[10] we developed an alternative method for coupling



Reagents: (a) CF_3COOMe, $CHCl_3$, 1 h; (b) EDCI, pH 5.6-5.9, H_2O, 3 min; (c) **2**, pH 6.5, dioxane, 90 min; (d) NH_3, 6 h; (e) Affi-Gel 10, HEPES, $MgCl_2$, pH 7.5, H_2O, 12 h, 4 °C.

nucleoside triphosphates to a linker by their gamma phosphate more efficiently. For that purpose we choose to use a 4-aminophenylethylamino unit as a linker. The anilino junction gives a stable bond with phosphate and the primary alkylamine moiety can react efficiently with the NHS-ester functions of Affigel 10. The alkylamino part of the linker was protected as a trifluoroacetamide. The aminoamide **2** was prepared quantitatively by treatment of the diamine **1** with methyltrifluoroacetate. This amine was coupled to nucleoside triphosphates activated by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) via a trimetaphosphate.[11, 12] The conditions of the coupling we found to be optimal were similar to those described by Pollock and Auld.[13] The nucleotides **4a** and **6** were conjugated to an Affi-Gel 10 matrix. We found that the presence of $MgCl_2$ increased the yield of the coupling to 50 %.[14]

Preliminary results showed that adenylyl cyclases bind to, and can released from, this 2',5'-dd-3'ATP affinity column. Optimization of the conditions for chromatography are in progress.

In conclusion, the results obtained in this study indicate that the adducts **4** and **6**, readily obtained in a one-pot reaction, provide a considerable improvement for the immobilization of nucleotides on a matrix, especially when conjugation to phenylenediamine is not possible. Furthermore, these tetherable ligands could be used to prepare several other bioconjugates for the study of nucleotide binding proteins in general. More specifically, the 2',5'-dd-3'ATP affinity column is a promising tool for purifying 2',5'-dd-3'ATP-binding proteins and unraveling signalling pathways that lead to the inhibition of adenylyl cyclases physiologically.

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12. Procedure for the synthesis of the adducts **4** and **6**: 0.5 g of EDCI·HCl was added over 3 min to a solution of 0.22 mmol of nucleoside triphosphate in 10 mL of water while the pH was maintained between 5.6 and 5.9 by the dropwise addition of a 0.2 N HCl solution. After 10 min 6 mL of a solution of 0.46 g (2 mmol) of the amine **2** in dioxane was added. The pH was adjusted to 6.5. The reaction was left at room temperature for 90 min and then it was cooled to 5 °C in an ice-water bath. Ammonia was bubbled through the mixture for 20 min and it was left at room temperature for 6 h. The deprotected adduct was purified by anion exchange chromatography on QAE Sephadex, eluting the product with a gradient of $Et_3N{\cdot}H_2CO_3$.

Compound **4a**, trisodium salt: ^{1}H NMR (250 MHz, D_2O) δ 2.39-2.51 (m, 4H, CH_2-CH_2), 3.89-4.06 (m, 3H, H-5', H-5" and H-4'), 4.09 (t, 1H, H-3'), 4.42 (t, 1H, J = 5.3 Hz, H-2'), 5.79 (d, 1H, J = 5.4 Hz, H-1'), 6.81 (s, 4H, H-aryl), 8.17 (s, 1H, H-2), 8.31 (s, 1H, H-8); ^{31}P NMR (100 MHz, D_2O) δ -18.31 (dd, J = 18.7 Hz, P-2), -7.05 (dt, J = 18 Hz, P-1), -5.46 (d, J = 19.8 Hz, P-3).

Compound **4b**, trisodium salt:^{1}H NMR (250 MHz, D_2O) δ 2.38-2.52 (m, 4H, CH_2-CH_2), 3.83-3.93 (m, 3H, H-5' , H-5" and H-4'), 4.00-4.03 (m, 1H, H-3'), 4.35 (t, 1H, J = 5.1 Hz, H-2'), 5.63 (d, 1H, J = 5.7 Hz, H-1'), 6.90 (s, 4H, H-aryl), 7.91 (s, 1H, H-8); ^{31}P NMR (100 MHz, D_2O) δ -18.35 (dd, $J_{P\text{-}P}$ = 17.9 Hz, $J_{P\text{-}P}$= 19.7 Hz, P-2), -7.09 (dt, $J_{P\text{-}H}$ = 5.4 Hz, $J_{P\text{-}P}$ = 17.8 Hz, P-1), -5.59 (d, $J_{P\text{-}P}$ = 19.8 Hz, P-3).

Compound **4c**, trisodium salt: ^{1}H NMR (250 MHz, D_2O) δ 2.61 (t, 2H, J = 6.6 Hz, CH_2), 2.78 (t, 2H, J = 7.6 Hz, CH_2), 4.00-4.09 (m, 4H, H-3', H-4', H-5' and H-5"), 5.84 (t, 1H, J = 7.6, H-1'), 5.87 (d, 1H, J = 7.6 Hz, H-5), 7.06 (dd, 4H, J = 8.5 Hz, J = 19.2 Hz, H-aryl), 7.65 (d, J = 7.6 Hz, H-6); ^{31}P NMR (100 MHz, D_2O) δ -18.69 (dd, J = 19.1 Hz, P-2), -7.26 (dt, J = 18.2 Hz, P-1), -5.79 (d, J = 20.0 Hz, P-3).

Compound **4d**, trisodium salt: ^{1}H NMR (250 MHz, D_2O) δ 2.58 (t, 2H, J = 6.7 Hz, CH_2), 2.73 (t, 2H, J = 6.7 Hz), 3.94-4.14 (m, 5H, H-3', H-4', H-5' and H-5"), 5.76 (d, 1H, J = 2.5 Hz, H-1'), 5.96 (d, 2H, J = 7.5 Hz, H-5), 6.99 (dd, 4H, J = 8.5 Hz, J = 15.8 Hz, H-aryl), 7.75 (d, 1H, J = 7.5 Hz, H-6); ^{31}P NMR (100 MHz, D_2O) δ -18.47 (dd, J = 17.3 Hz, J = 19.7, P-2), -6.95 (dt, $J_{P\text{-}H}$ = 2.3 Hz, $J_{P\text{-}P}$ = 17.3 Hz, P-1), -5.68 (d, J = 19.7, P-3).

Compound **6**, trisodium salt: ^{1}H NMR (250 MHz, D_2O) δ 1.13 (d, 3H, J = 6.6 Hz, 3H-5'), 2.11 (t, 2H, J = 7.2 Hz, CH_2), 2.37-2.48 (m, 4H, H-2', H-2" and CH_2), 4.14-4.18 (m, 1H, H-4'), 4.55-4.60 (m, 1H, H-3'), 5.99 (t, 1H, J = 7.2 Hz), 6.70 (dd, 4H, J = 8.5 Hz, J = 31 Hz, H-aryl), 8.08 (s, 1H, H-2), 8.14 (s, 1H, H-8); ^{31}P NMR (100 MHz, D_2O) δ -18.26 (dd, J = 18.6 Hz, P-2), -8.05 (dd, $J_{P\text{-}H}$ = 7.9 Hz, $J_{P\text{-}P}$ = 8.4 Hz, P-1), -5.64 (d, J = 18.8 Hz, P-3).

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14. Procedure for the synthesis of the 2',5'-dd-3'ATP affinity matrix: Affi-Gel 10 (Bio-Rad) resin (25 mL) was washed with a cold aqueous solution of 20 mM HEPES and 50 mM $MgCl_2$ and mixed with a precooled solution of the amine **3** (100 μmol) in 15 mL of the same buffer (pH 7.5). The medium was gently agitated overnight at 4 °C. A phosphate assay of an aliquot of the resin following ashing showed that 50% of the ligand bound to the resin.

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