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Simple Synthesis of P^1P^2-Diadenosine 5′-Pyrophosphate

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Abstract: Pyrophosphate-linked coenzymes play essential roles in several biochemical systems. Symmetrical diadenosine-5′-pyrophosphate (Ap2A) has been synthesized from adenosine-5′-phosphate in virtually quantitative yield. The simple procedure is carried out in anhydrous pyridine using adenosine phosphoromorpholidate and adenosine monophosphate *bis*-(tri-n-butylammonium salt) as coupling reagents.

Keywords: Coenzymes, diadenosine, morpholidate, pyrophosphate

Diadenosine polyphosphates are a family of endogenous bioactive compounds increasingly recognized as important mediators of functional responses in several mammalian cell types. The general structure of these compounds is ApnA, where n is a variable (2–6) number of phosphates. The members of this large family of compounds containing at least three phosphate groups are generally agonists of purinergic receptors of excitable cells (neuronal or muscle cells),[1,2] whereas the dinucleoside diphosphates Ap2A, Ap2G, and Gp2G represent a new class of growth-promoting extracellular mediators, which are released from platelets after activation.[3]

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Most recently, ADP-ribosyl cyclases (ADPRC) from lower and higher metazoa have been shown to synthesise Ap2A and two isomers thereof (P18 and P24), containing an unusual N-glycosidic bond between one of the adenine moieties and the ribose. These dinucleotides are present and metabolized in $ADPRC^+$ mammalian cells where they affect intracellular calcium and cell proliferation.[4]

The growing scientific interest regarding Ap2A and the fact that the dinucleotide is not commercially available prompted us to develop a rapid and simple chemical synthesis for this dinucleotide, which may be of interest to scientists in various fields of mammalian physiology.

Synthesis of pyrophosphate bonds has relied on chemical activation of one phosphate derivative and subsequent reaction with the other one. The reaction involves the nucleophilic attack of one phosphate anion on the activated derivative of the other.

The synthesis of this dinucleotide presents some difficulties because of the low nucleophilic reactivity of the phosphate moiety. In the literature, different methods applicable to the synthesis of P^1P^2-diadenosine-5′-pyrophosphate have been reported, the majority of these including the use of an appropriate adenosine-5′-monophosphate salt. The most direct method for preparing diesters of pyrophosphoric acid is through condensation of the pyridium salt of 5′-adenosine-monophosphate (5′-AMP) with dicyclohesylcarbodiimide (DCC),[5] which has the advantage, over other dehydrating agents, of preferentially attacking acidic groups, but its slight excess in the activating solution requires a further step to filter the crystallized dicyclohexylurea.

The same product was obtained by reacting adenosine-5′-monophosphate with trifluoroacetic anhydride overnight and evaporating it to dryness. This method[6] exploits a simple exchange reaction but has the great drawback of a low yield (17% by the same authors) of the purified product. The use of a different approach based on intermediates such phosphoramidates[7] or phosphorimidazolide-activated nucleosides[8] does not allow the development of satisfactory procedures for the synthesis of Ap2A. Phosphorimidazolidates exhibit inherent high polarity and instability, so their purification and preservation is rather difficult. In an attempt to synthesize the product without the use of different steps to activate the phosphate as salt or other forms, Kanavarioti et al., following a particular oxidation–reduction condensation method, discovered a one-pot synthesis of the symmetrical pyrophosphate of 5′-adenosine.[9] The synthesis, although showing a good yield, has great disadvantages: the insolubility of the employed reagents and the need for particular chromatographic steps of purification.

The introduction of water-soluble carbodiimides as activating agents in a variety of reactions that involve the formation of pyrophosphate

bonds[10] led Ng and Orgel to publish a simple synthesis of diadenosine pyrophosphate.[11] This method is based on activation of adenosine monophosphate with water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (CDI) in an aqueous solution also containing phosphate buffer (HEPES), but unfortunately the reaction yielded several by-products chemically similar to the desired product. The unreacted adenosine monophosphate, the carbodiimide adducts, and the high amount of salts present in the reaction mixture requires a suitable chromatographic purification.[12] Thus, although several procedures have been described, none appeared to be convenient. For this reason, we developed an alternative approach involving the use of phosphoromorpholidates derivatives.

The aim of the present communication is to introduce a rapid, simple, and low-cost protocol for the synthesis of Ap2A using commercially available reactives. The use of phosphoromorpholidates[13] is suitable to obtain the corresponding P^1P^2-diadenosine-5′-pyrophosphate in virtually quantitative yields.

Because the resulting phosphoromorpholidate derivatives exhibit high reactivity toward various nucleophilic functions, they can be used as active intermediates for the synthesis of pyrophosphate compounds by the reaction with phosphate nucleophiles. Our approach overcomes the drawbacks of the conventional methodology of pyrophosphate synthesis. The homodimer Ap2A was obtained using a modified version of the phosphoromorpholidate coupling method where the *bis*-(tri-n-butylammonium salt) of 5′-adenosinemonophosphate (**1**) was coupled to the adenosine-5′-phosphoromorpholidate 4-morpholine-N,N′-dicyclohexylcarboxamidine salt (5′-AMP phosphoromorpholidate, **2**), commercially available as Sigma A1127, or carried out according to the procedure of Moffat,[13] without further purification.

A specific salt, *bis*-(tri-n-butylammonium salt) of 5′-adenosinemonophosphate, which is readily soluble in organic solvents, needs to be prepared prior to reaction. It was obtained by modification of our previously published method,[14] where the reaction of 5′-adenosine monophosphate free acid (5′-AMP) with tributylamine in methanol gave the corresponding TBA salt of 5′-AMP(**1**), readily soluble in pyridine.

Mass spectrometry confirmed the structure, and the final product (95% yield) was used without further purification. The attempt of condensation of the phosphoromorpholidate with adenosine monophosphate free acid failed, probably because of the insolubility of AMP free acid in pyridine or some others organic solvents. It needs to use the more reactive ammonium salt.

The P^1P^2-diadenosine-5′-pyrophosphate **(3)** was indeed obtained through the condensation between *bis*-(tri-n-butylammonium salt)

5′-adenosinemonophosphate, dissolved in anhydrous pyridine, and a solution of the same solvent containing the equimolar concentration of 5′-AMP phosphoromorpholidate. The reactants were dried by several evaporations of anhydrous pyridine until the last amounts of water were removed, and finally the solution was gently stirred under nitrogen at room temperature.

The phosphoromorpholidate is strongly activated toward the nucleophilic attack by phosphate groups, and hence the conversion of intermediates to the pyrophosphate is quite easy. Because of the use of the tertiary base salt, a longer reaction period at room temperature is necessary, but the final yield of the desired compound is excellent.

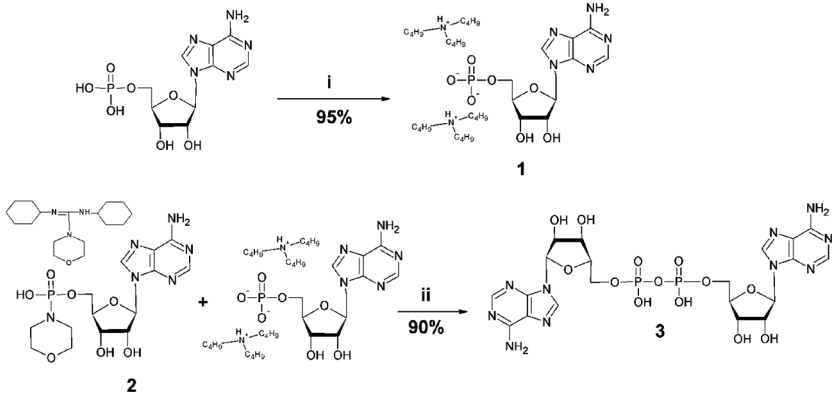
After 72 h, when thin-layer chromatography (TLC) indicated complete consumption of starting materials, the pyridine was removed under vacuum, and the mixture was dissolved in water. No side products were observed, except for low amounts of 5′-adenosine monophosphate.

The aqueous mixture was adjusted to pH 8 with 1N sodium hydroxide to liberate the free guanidine adduct (4-morpholine-N,N′-dicyclohexylcarboxamidine), which was removed by ether extraction (as reported by Rhajbhandary et al.).[15]

The synthetic reactions are summarized in Scheme 1.

The final product was purified by preparative HPLC using a C18 reversed-phase column.

Buffer A was an aqueous solution of 10 mM ammonium formate adjusted to pH 4.0, and buffer B was a solution of 50% buffer A and 50% methanol. An HPLC chromatogram of the purified product, using the same HPLC preparative conditions, is shown in Fig. 1, panel A.



Scheme 1. Reagents and conditions: (i) methanol, TBA, rt, 30 min; (ii) (a) pyridine, rt, 72 h, (b) H_2O, NaOH 1N, pH = 8.

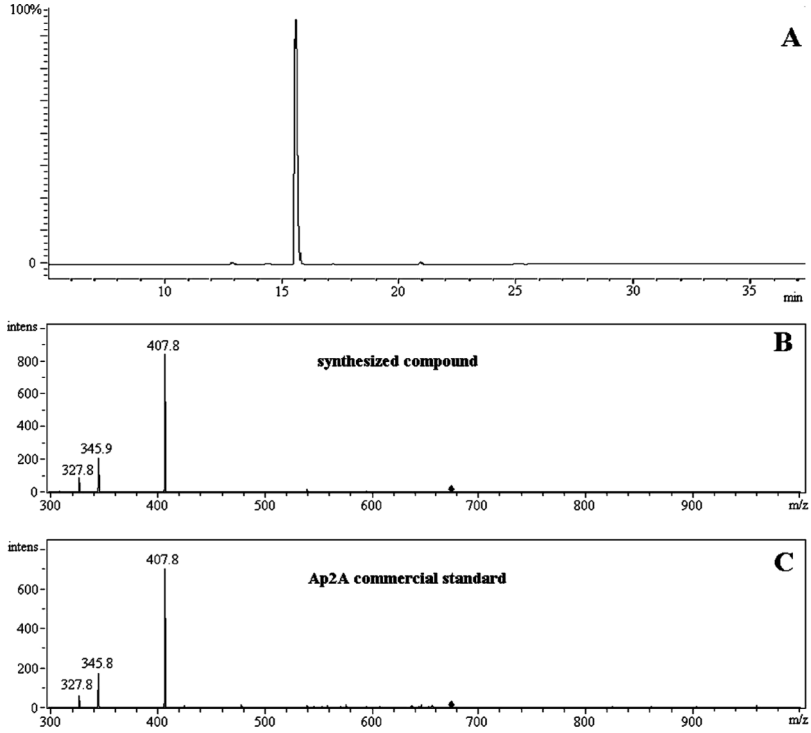


Figure 1. HPLC chromatogram of the purified product Ap2A (panel A). The MS/MS spectrum of the synthesized compound (panel B) is compared to the MS/MS spectrum of the commercial standard Sigma D-7376 (panel C).

The powder (90% yield) was then analyzed by electrospray mass spectrometry (ESI-MS), confirming presence of the expected molecular weight (m/z calculated 676.43; found 676.4). The MS/MS analysis performed on the synthetic molecule and on the commercial standard (Sigma D-7376, now out of production) on the ion-trap instrument showed a high degree of correspondence of fragment ions, as shown in Fig. 1 (panels B and C).

The same synthesis was carried out, in the same experimental conditions, with other solvents such as N,N-dimethylformamide and DMSO, but with yields not higher than 40–50%, showing the evident catalytic role of anhydrous pyridine in the entire procedure.

The presence of the P^1P^2-diadenosine-5′-pyrophosphate bridge was also confirmed enzymatically, by digestion with pyrophosphatase from *Crotalus adamanteus* venom. Approximately 20 nmol of Ap2A was incubated at 37 °C with 40 µl of 30 mM tris-HCl at pH 8, 10 µl of 5 mM $MgCl_2$ and 5 µl of Pyrophosphatase (Sigma-Aldrich P7383). Aliquots

were removed after 60 min and chromatographed directly using the same HPLC preparative conditions. Enzymatic degradation yielded the expected 5′-AMP, as detected and identified by HPLC on the basis of the retention time (identical to that of the commercial standard). This experiment demonstrated that the diphosphate chain binds the adenosine moieties via phosphoester bonds with the 5′-oxygens of the riboses.

The Ap2A molecule, synthesized as described, was indeed tried out in biochemical experiments. The ability of Ap2A to affect $[Ca^{2+}]_i$, when applied extracellularly to intact cells at micromolar concentrations, and to antagonize P24-induced proton gradient dissipation and cytotoxicity was demonstrated with already published methods.[16]

In summary, we propose a convenient strategy for the synthesis of pyrophosphate bonds by the use of readily available phosphoromorpholidate derivatives and demonstrate the usefulness of our pyrophosphorylation strategy on the synthesis of Ap2A and its analogs. The major advantages of this method are (1) the considerably reduced costs and the opportunity of employing commercial reagents compared to that of previous methods, (2) the absence of by-products, allowing a good final yield, and (3) the possibility of application to asymmetric dinucleoside pyrophosphates (e.g., Ap2T, Ap2G, Ap2C).

The chemical synthesis, although straightforward, has great importance to synthesis of various nucleoside and nucleotides not commercially available or hardly achievable with other synthetic protocols but fundamental as extracellular mediators in the biochemical and biomedical fields.

EXPERIMENTAL

The melting points were recorded on a Buchi 535 melting-point apparatus. All mass spectrometric low-resolution analysis were performed on an Agilent MSD 1100 ion trap instrument acquiring spectra in the negative ion mode. All measures were conducted in FIA (flow injection analysis) mode in a range including the expected m/z ratios using water–acetonitrile 50:50 with 0.1% formic acid as carrier flow.

The elemental composition analysis was performed in DIA, in the negative ion mode, on a high-resolution q-TOF geometry mass spectrometer (Qstar XL system, Applied Biosystems/MDS Sciex, Toronto, Canada) using an electrospray ion source. The compound was dissolved in a water–acetonitrile 50:50 solution containing triethylamine at final concentration of 10 mM. All NMR spectra were acquired on a Varian MercuryPlus 300-MHz spectrometer.

Peak assignment in ^{1}HNMR spectra was also made with the aid of correlation spectroscopy (COSY) and heteronuclear single quantum

coherence (HSQC) experiments. Peak assignment in ^{13}CNMR spectra was also obtained using HSQC experiment.

bis-(Tri-n-butylammonium) Salt 5′-Adenosine Monophosphate (1)

A solution of tributylamine (TBA) (0.2 mmol, 47 µl) was added dropwise to a stirred suspension of 5′-adenosine-monophosphate free acid monohydrate (5′-AMP) (0.1 mmol, 36.5 mg) in 10 ml of methanol. The mixture was vigorously stirred at room temperature, and after 30 min, a clear solution was obtained. The solution was evaporated to dryness. The process of dissolving and evaporating was repeated few times in the presence of anhydrous pyridine to obtain the TBA salt of 5′-AMP, readily soluble in pyridine.

Adenosine-5′-phosphoromorpholidate-4-morpholine-N,N′-dicyclohexylcarboxamidine Salt (2)

A solution of dicyclohexylcarbodiimide (1 mmol, 206 mg) in t-butyl alcohol (5 ml) was added dropwise to a refluxing solution of the adenosine-5′-phosphate free acid (0.25 mmol, 91 mg) in a mixture of water (5 ml), t-butyl alcohol (5 ml), and purified morpholine (1 mmol, 85 µl).

The addition was completed in about 3 h, and the mixture was refluxed overnight until TLC (n-butanol–water–acetic acid 5:3:2) showed completion of the reaction. The mixture was then cooled to room temperature, and any crystalline material was removed by filtration and carefully washed with t-butyl alcohol. The filtrate was evaporated until the t-butyl alcohol was largely removed, and the remaining aqueous phase was extracted three times with ether. The clear aqueous solution was then evaporated to dryness in a vacuum.

The residue was then transferred in a centrifuge tube, and a minimum volume of methyl alcohol (1.5 ml) was added. The addition of dry ether (10 ml) precipitated a gummy solid, which on trituration with fresh ether changed to a dry white powder. The adenosine-5′-phosphoromorpholidate was obtained as a salt of 4-morpholine-N,N′-dicyclohexylcarboxamidine (90% yield, 159 mg). ^{1}H NMR (D_2O, 300 MHz): 8.40 (s, 1H), 8.17 (s, 1H), 6.07 (d, $J = 5$ Hz, 1H), 4.76 (t, $J = 5$ Hz, 1H), 4.49 (t, $J = 5$ Hz, 1H), 4.35–4.29 (m, 1H), 4.07–3.94 (m, 2H), 3,75 (t, J = 5 Hz, 4H), 3.57–3.48 (m, 4H), 3.40 (t, J = 5 Hz, 4H), 3.33–3.21 (m, 2H), 2.97–2.82 (m, 4H), 1.94–1.82 (m, 4H), 1.77–1.69 (m, 4H), 1.59–1.55 (m, 2H), 1.36–1.01 (m, 10H).

Electrospray mass spectrometry (ESI-MS) conducted in negative ion mode gave unique significative signal at m/z 415.7[M-H]$^-$. The calculated m/z neutrally charged for $C_{14}H_{21}N_6O_7P$ was 416.78.

P^1P^2-diadenosine-5′-pyrophosphate (3)

4-Morpholine-N,N′-dicyclohexylcarboxamidinium salt of adenosine-5′-phosphoromorpholidate (0.1 mmol, 71 mg) was dried by three evaporation steps of its solution in anhydrous pyridine. Separately the equimolar concentration of *bis*-(tri-n-butylammonium) salt 5′-adenosine monophosphate (0.1 mmol, 72 mg) was dissolved in 2 ml of anhydrous pyridine, and the compound was rendered anhydrous by two evaporations of solutions. The two pyridine solutions were mixed and dried by several evaporation steps of anhydrous pyridine solution until the solution became clear, and finally the reaction was carried out by gently stirring at room temperature under N_2. Silica-gel TLC was used to monitor the course of reaction (TLC: n-butanol–water–acetic acid 5:3:2). After 72 h, time in which the morpholidate completely disappeared, the solvent was evaporated under vacuum and the residual pyridine was removed by the addition and evaporation of water (2–3 ml). The mixture was indeed dissolved in 10 ml of water and the pH was adjusted to 8 with 1 N sodium hydroxide. The basic solution was then washed three times with 15 ml of diethyl ether, and the organic layers were back-extracted once with distilled water and then evaporated to dryness. The final product was purified by preparative HPLC using a Luna C18 reversed-phase column (21.20 × 250 mm, Phenomenex, Torrance CA, USA). The solvent program was a gradient starting at 100% buffer A for 2 min and linearly increasing to 100% B in 38 min.

Buffer A was an aqueous solution of 10 mM of ammonium formate adjusted to pH 4.0, and buffer B was a solution of 50% buffer A and 50% methanol.

The solution was evaporated to dryness under vacuum, and the residue was repeatedly lyophilized to afford colorless and water-soluble crystals of P^1P^2-diadenosine-5′-pyrophosphate (90% yield, 61 mg, mp 187–190 °C, lit.[17] mp 184–189 °C).

^{1}H NMR δ (D_2O, 300 MHz): 8.14 (s, 2H, *H8*), 7.97 (s, 2H, *H2*), 5.89 (d, $J = 5$ Hz, 2H, *H1′*), 4.53 (t, $J = 5$ Hz, 2H, *H2′*), 4.40 (t, $J = 5$ Hz, 2H, *H3′*), 4.35–4.27 (m, 4H, *H4′* + *H5′*), 4.24–4.16 (m, 2H, *H5″*). ^{13}C NMR δ (D_2O, 75 MHz): 154.073 (*C6*), 151.413 (*C2*), 148.097 (*C4*), 139.587 (*C8*), 117.793 (*C5*), 87.284 (*C1′*), 83.508 (*C4′*), 74.835 (*C2′*), 70.058 (*C3′*), 65.285 (*C5′*).

Electrospray mass spectrometry (ESI-MS) conducted in negative ion mode gave unique remarkable signal at m/z 675.4 $[M\text{-}H]^-$. The average calculated m/z, neutrally charged for $C_{20}H_{26}N_{10}O_{13}P_2$, was 676.43.

The elemental composition analysis showed an m/z ratio of 675.1045 amu (calculated for $C_{20}H_{26}N_{10}O_{13}P_2[M\text{-}H]^-$ m/z 675.1083 amu) with an error of −5.62 ppm, in the range of the instrumental factory specification.

To obtain the more stable lithium salt of Ap2A, the aqueous solution of Ap2A pyrophosphate was adjusted to pH 5 with 0.1 M lithium hydroxide, then concentrated to a small volume and finally evaporated to a syrup under vacuum. Crystallization with a mixture of acetone and ethyl alcohol (5:1) at temperature of 0 °C allowed us to obtain the precipitated lithium salt of Ap2A.

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