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# Synthesis of a Biologically Important Adenosine Triphosphate Analogue, ApppD

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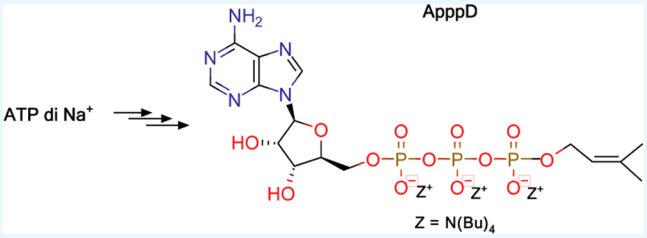
Supporting Information

**ABSTRACT:** The chemical synthesis of a adenosine triphosphate analogue, 1-adenosin-5′-yl 3-(3-methylbut-2-enyl) triphosphoric acid diester (ApppD), is described. ApppD is known to be an active metabolite of the mevalonate pathway in the human body like its structural isomer isopentenyl ester of ATP (ApppI). Very recently, ApppI has been found to possess novel function(s); now it will also be possible to examine the effects of ApppD more precisely because it can be synthesized in reasonable amounts. 1-Adenosin-5′-yl 3-(3-methylbut-2-enyl) diphosphoric acid diester (AppD; a adenosine diphosphate analogue) was also isolated from the synthesis mixture. Both ApppD and AppD were characterized by $^{1}H$, $^{13}C$, $^{31}P$ NMR and mass spectrometry methods.




## INTRODUCTION

It is clear that osteoporosis is one of the diseases becoming increasingly prevalent in the developed countries; that is, it is associated with advanced age and nowadays global lifespans are expanding. Bisphosphonates (BPs), which are stable analogues of the pyrophosphate naturally found in cells, have been used for decades in the treatment of osteoporosis and many other bone-related diseases, such as hypercalcemia and Paget's disease.[1,2] In 1969, first-generation BPs were found to have therapeutic properties; these were "simple" non-nitrogen-containing BPs (non-NBPs) such as etidronate and clodronate (see Figure 1). It has been shown that pyrophosphate influences the formation and dissolution of hydroxyapatite, which is a major component of bone mineral.[3] Earlier, the mechanism of action of these first-generation BPs (non-NBPs) was proposed to rely on their binding to the surface of bone; however, in 1992 non-NBPs such as clodronate were shown to form adenosine triphosphate (ATP) analogues, which could induce the apoptosis of osteoclasts (bone-absorbing cells).[4,5] After the second-generation nitrogen-containing BPs (NBPs), such as pamidronate, alendronate, and risedronate (see Figure 1), were synthesized, their mechanism of action was found to be different from that of the non-NBPs; these compounds were shown to prevent protein isoprenylation by inhibiting farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway.[6,7] FPPS catalyzes the condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate to form first geranyl pyrophosphate and then FPP (see structures in Figure 2), a metabolite which is vital for protein prenylation and cell signaling.[8] In 2006, Mönkkönen et al. reported that administration of NBPs led to the formation of an ATP analogue, the isopentenyl ester of ATP (ApppI, Figure 2), which induced cell death.[9] There is convincing evidence that NBPs also possess anticancer effects; regarding this property, the mechanism of action has been proposed to be attributable to the metabolites described above.[10]

1-Adenosin-5′-yl 3-(3-methylbut-2-enyl) triphosphoric acid diester (ApppD) is a structural isomer of ApppI, and it is also one of the metabolites induced by NBPs; actually, it has been reported that the cellular ApppD concentration is over double that of ApppI after treatment with NBPs.[11] Because ApppI has become rather widely available during recent years,[12−14] more studies related to this have been performed, leading to the publication of some interesting and unexpected results. For example, very recently, Ishchenko et al. showed that ApppI could evoke a calcium-dependent inhibition of pain sensation.[15] In contrast, little is known about the effects of ApppD in biological systems, as this compound has been hardly available to bioscientists.

## RESULTS AND DISCUSSION

The methods for the preparation of ATP γ-O-alkyl esters have been reported.[16−18] According to SciFinder search, the chemical synthesis of ApppD has not been reported earlier; there is only one paper describing the preparation of ApppD by ligases.[19] In principal, there are two possible ways to prepare ATP derivatives such as ApppI: (1) using an activated ADP (adenosine diphosphate) derivative with the appropriate monophosphate derivative as starting materials or (2) using the tetrabutylammonium (TBA) salt of ATP and an appropriate tosylated (mesylated) alcohol or alkylhalide.[20,12] One intriguing method for the future would also be the possibility to synthesize ApppD from ApppI, either chemically or enzymatically. Both of the above-mentioned approaches

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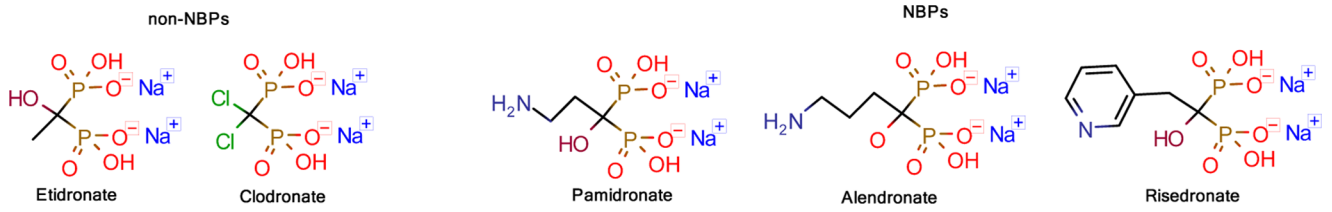



**Figure 1.** Examples of non-NBPs and NBPs.

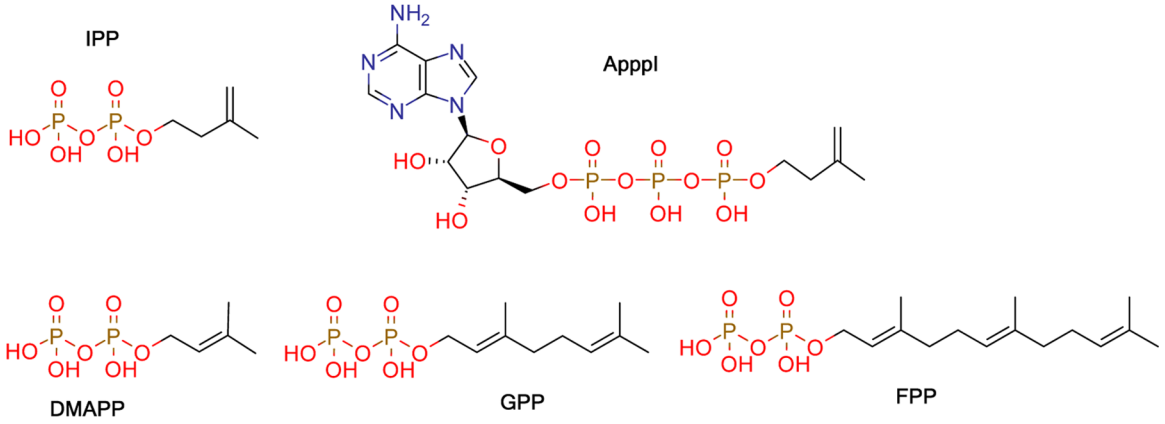
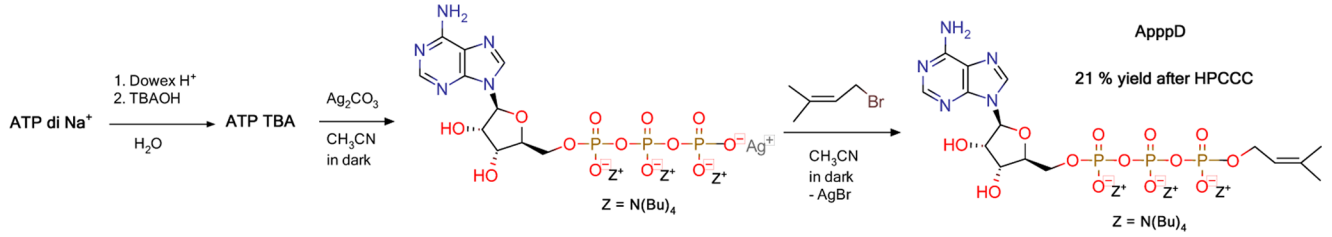



**Figure 2.** Important metabolites in the mevalonate pathway.

**Scheme 1. Synthesis of ApppD**




were tested. First, the preparation of tosylated 3,3-dimethylallyl alcohol [$(H_3C)_2C{=}CCH_2OTs$] was tested, a compound which would be an excellent starting material along with an ATP TBA salt from which to prepare ApppD; this has been the approach used when ApppI is synthesized with an appropriate tosylated alcohol.[12] Unfortunately, this was not possible because according to $^1$H NMR spectrum, there had been degradation of the desired product during the purification by column chromatography. Furthermore, an extensive literature search (with SciFinder) failed to locate any mentions of tosylated 3,3-dimethylallyl alcohol. The situation was identical when striving to isolate the mesylated derivative. Next, an ATP TBA salt was prepared and a reaction was attempted with the commercially available 3,3-dimethylallyl bromide [$(H_3C)_2C{=}CCH_2Br$] in anhydrous acetonitrile at 45 °C, but again without success, even though this kind of method has been reported to produce an isoprenoid conjugate of ADP.[21] Then, the preparation of an appropriate monophosphate starting material [$(H_3C)_2C{=}CCH_2OP(O)(MeO)_2$] was tried, a compound which could then react with the ADP TBA salt via a few more synthesis steps, according to a procedure reported elsewhere.[12] Dimethyl phosphate (commercially available) was first converted to its TBA salt and dissolved in anhydrous acetonitrile, 3,3-dimethylallyl bromide was added, and the reaction mixture was stirred for overnight at 50 °C, but according to $^1$H and $^{31}$P NMR spectra, no reaction occurred. In addition, $Ag_2CO_3$ was tested with dimethyl phosphate and 3,3-dimethylallyl bromide under the same conditions mentioned above because of our earlier positive experience with $Ag_2CO_3$ for the preparation of etidronate prodrugs,[22] however, again without success. Finally, it was tested what would happen with the ATP TBA salt, $Ag_2CO_3$, and 3,3-dimethylallyl bromide in anhydrous acetonitrile at 45 °C, and this approach was successful (see Scheme 1). The synthesis was repeated several times to be sure that it was not some kind of "accident"; this was not the case, the synthesis worked rather well in all runs. The overall yield for ApppD was only about 21%; this is reasonable for this kind of compound and comparable to the results reported elsewhere for ApppI.[12−14]

It can be proposed that the driving force for the synthesis of ApppD in the method reported here is the formation of an extremely stable and (water) insoluble AgBr. The crude reaction mixture was successfully purified by high-performance countercurrent chromatography (HPCCC) using exactly the same method described elsewhere.[14] Interestingly, the method reported here for the synthesis of ApppD also resulted in the formation of 1-adenosin-5′-yl 3-(3-methylbut-2-enyl) diphosphoric acid diester (AppD; see the structure in Figure 3); this compound could be isolated by HPCCC purification of the crude reaction mixture as a side-product (see HPCCC chromatogram in the Supporting Information). The observed ratio of TBA/ApppD was higher than the ratio of TBA/ATP



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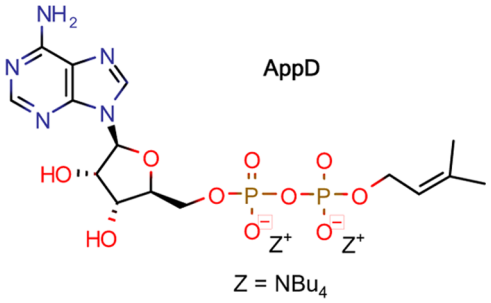


**Figure 3.** Structure of AppD.

used in the synthesis, and it can be different in the different HPCCC runs. The TBA content in the final product also exceeded the maximum number of anionic sites in ApppD (which is 3). This suggests that TBA is not solely acting as a counterion, but it is also exerting some of its generally recognized solvation effects in the purification process. The same phenomenon was observed previously in the purification of ApppI by HPCCC.[14]

## CONCLUSIONS

The chemical synthesis of ApppD has been reported for the first time with a reasonable, 21%, overall yield. ApppD is the active metabolite of the mevalonate pathway with high biological importance. Ongoing biological studies with ApppD and ApppI will clarify their importance and physiological actions more precisely. The newly isolated side-product, AppD, may also have importance in some biological systems.

## EXPERIMENTAL SECTION

$^{1}$H, $^{31}$P, and $^{13}$C NMR spectra were recorded on a 600 MHz spectrometer operating at 600.2, 243.0, and 150.9 MHz, respectively. The solvent ($D_2O$) residual peak was used as a standard for $^{1}$H measurement, which could be referred to as 4.79 ppm. $CD_3OD$ was added as a standard (49.00 ppm)[23] for $^{13}$C measurement with 85% $H_3PO_4$ being used as an external standard in the $^{31}$P measurements. The $^{n}J_{HP}$ couplings were calculated from proton spectra, and all $J$ values are given in hertz. The $^{n}J_{CP}$ couplings were calculated from carbon spectra with the coupling constants given in parenthesis as hertz. An HPCCC method reported elsewhere was used in the purification of ApppD and AppD.[14] Mass spectra were recorded on a quadrupole time-of-flight mass spectrometer (qTOF-MS) using electrospray ionization (ESI) in the negative ionization mode. The purity of the products was determined from $^{1}$H and $^{31}$P NMR spectra and was ≥95% unless stated otherwise.

**Synthesis and Purification of the ApppD TBA Salt.** A commercially available ATP disodium salt (200 mg, 0.36 mmol) was dissolved in distilled water (4 mL), and then freshly washed Dowex $H^+$ resin (1.0 g) was added and the reaction mixture was stirred for 5−6 min before rapid filtration of Dowex, which was washed with water (3−4 mL). The commercially available 40% TBA hydroxide in water (705 μL, 3.0 equiv) was added to the combined water fractions, and the reaction mixture was stirred for a few minutes before evaporation to dryness under high vacuum. The residue was dried carefully for a few days under high vacuum with warming before it was dissolved in anhydrous DMF (2−3 mL); $Ag_2CO_3$ (51 mg, 0.18 mmol, 0.5 equiv) and finally 3,3-dimethylallyl bromide (84 mg, 65 μL, 0.56 mmol, ca. 1.5 equiv) were added, and the reaction mixture was stirred for 24 h at 45 °C *in the dark*. The reaction mixture was filtered and the filtrate was evaporated to dryness in vacuo. The residue was dissolved in MeOH (3−4 mL), water (ca. 0.5−1 mL) was added [after the water addition and vortexing, fine slightly yellow solids were formed (most probably AgBr)], and the mixture was centrifuged to remove all solids and subsequently evaporated to dryness in vacuo. A crude product (505 mg) was obtained as a white "foamy" solid (highly hygroscopic!). The crude product was purified by *exactly* the same HPCCC method as utilized in the purification of ApppI.[14] Appropriate fractions were collected and evaporated to dryness in vacuo (see HPCCC chromatogram in the Supporting Information as an example). ApppD × 4.1 TBA salt (117 mg, 21%) was obtained as a colorless solid (highly hygroscopic!).

**ApppD 4.1 × TBA Salt.** White "foamy" solid (very hygroscopic), 117 mg (21%). $^{1}$H NMR ($D_2O$) $\delta$ 8.52 (s, 1H), 8.25 (s, 1H), 6.12 (d, $J$ = 6.1, 1H), 5.24−5.19 (m, 1H), 4.57−4.54 (m, 1H), 4.40−4.36 (m, 1H), 4.36−4.29 (m, 2H), 4.28−4.20 (m, 2H), 3.22−3.15 (m, 33H, from TBA salt), 1.68−1.60 (m, 33H, from TBA salt), 1.57 (s, 3H), 1.54 (s, 3H), 1.40−1.30 (m, 33H, from TBA salt), 0.94 (t, $J$ = 7.4, 50H, from TBA salt), one "sugar" proton signal partly under the HDO-line at 4.8. $^{13}$C NMR ($D_2O$ + $CD_3OD$ set as a reference at 49.00 ppm) $\delta$ 156.6, 153.8, 150.1, 140.90, 140.87, 120.2 (d, $^{3}J_{CP}$ = 8.2), 119.6, 87.6, 85.1 (d, $^{2}J_{CP}$ = 9.3), 75.4, 71.4, 66.2 (d, $^{3}J_{CP}$ = 5.6), 64.0 (d, $^{2}J_{CP}$ = 5.7), 59.1 (virtual t, from TBA salt), 25.7, 24.1 (from TBA salt), 20.1 (virtual t, from TBA salt), 18.0, 13.8 (from TBA salt); $^{31}$P NMR ($D_2O$) $\delta$ −11.11 (d, $^{2}J_{PP}$ = 19.4), −11.57 (d, $^{2}J_{PP}$ = 19.4), 23.34 (t, $^{2}J_{PP}$ = 19.4). MS (ESI-qTOF) calcd. for $C_{15}H_{23}N_5O_{13}P_3$ $[M\text{-}H]^-$ 574.0505, found: 574.0520.

**AppD 2.8 × TBA Salt.** White "foamy" solid (very hygroscopic), separated as a side-product (28 mg, 6.5%). $^{1}$H NMR ($D_2O$) $\delta$ 8.50 (s, 1H), 8.25 (s, 1H), 6.12 (d, $J$ = 6.0, 1H), 5.21−5.16 (m, 1H), 4.54−4.52 (m, 1H), 4.39−4.36 (m, 1H), 4.32−4.26 (m, 2H), 4.22−4.17 (m, 2H), 3.22−3.15 (m, 22H, from TBA salt), 1.68−1.59 (m, 22H, from TBA salt), 1.56 (s, 3H), 1.52 (s, 3H), 1.39−1.30 (m, 22H, from TBA salt), 0.93 (t, $J$ = 7.4, 33H, from TBA salt), one "sugar" proton signal partly under the HDO-line at 4.8. $^{13}$C NMR ($D_2O$ + $CD_3OD$ set as reference at 49.00 ppm) $\delta$ 156.5, 153.7, 150.1, 140.9, 140.6, 120.2 (d, $^{3}J_{CP}$ = 8.2), 119.6, 87.8, 85.0 (d, $^{2}J_{CP}$ = 9.4), 75.2, 71.4, 66.1 (d, $^{3}J_{CP}$ = 5.4), 63.9 (d, $^{2}J_{CP}$ = 5.6), 59.1 (virtual t, from TBA salt), 25.7, 24.1 (from TBA salt), 20.1 (virtual t, from TBA salt), 18.0, 13.8 (from TBA salt); $^{31}$P NMR ($D_2O$) $\delta$ −11.03 (d, $^{2}J_{PP}$ = 21.8), −11.58 (d, $^{2}J_{PP}$ = 21.8). MS (ESI-qTOF) calcd. for $C_{15}H_{22}N_5O_{10}P_2$ $[M\text{-}H]^-$ 494.0842, found: 494.0851.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00531.

$^{1}$H, $^{13}$C, and $^{31}$P NMR spectra and HPCCC chromatogram for ApppD (PDF)

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### Notes

The author declares no competing financial interest.

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