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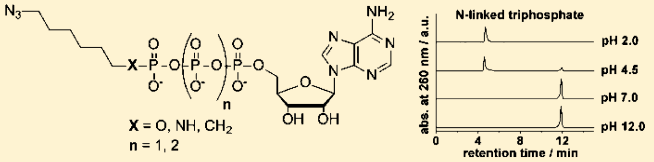
# Synthesis and Stability of Phosphate Modified ATP Analogues

Stephan M. Hacker, Martin Mex, and Andreas Marx*

Department of Chemistry and Konstanz Research School of Chemical Biology, Universität Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany

Supporting Information

**ABSTRACT:** Nucleotides modified at the phosphate have numerous applications. Nevertheless, the number of attachment modes is limited and little is known about their stability. Here, we present results on the elaboration of the synthesis of five classes of ATP analogues and studies concerning their stability. We show that the nitrogen-linked ATP analogue is less stable, whereas the oxygen- and novel carbon-linked adenosine tri- and tetraphosphate analogues are stable from pH 3 to 12 rendering them interesting for further applications and designs.

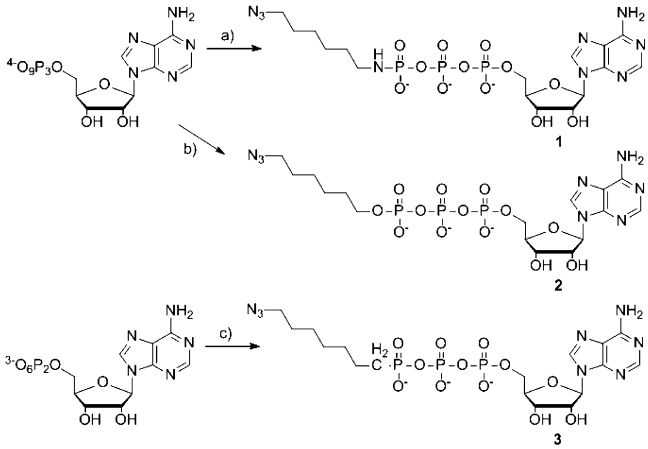



Modified nucleotides are valuable tools to understand and modify biological processes.[1−3] Recently, nucleotide analogues modified at the terminal phosphate chain have gained significant interest. One of their applications is the study of DNA polymerase catalyzed DNA synthesis. Different dNTP analogues have already been used for monitoring the activity of these enzymes in real time.[4,5] In this context, these analogues can be efficiently used for continuous single molecule DNA sequencing.[6−8] Furthermore, phosphate-modified ATP analogues have been widely used for studying protein kinases. In this approach peptides or proteins can be modified with different fluorescent,[9] cross-linking,[10] electrochemical,[11,12] and affinity[13] labels for subsequent detection or manipulation. Additionally, many other processes can be studied using phosphate-modified nucleotide probes. These include, e.g., modification of nucleic acids by polynucleotide kinases,[14] detection of GTP binding to GTPases,[15] and affinity purification of nucleotide binding proteins.[16]

Nevertheless, up to now only limited possibilities exist to modify triphosphates at the γ-phosphate and only little is known about the stability of different kinds of phosphate-modified nucleotides. To address these shortcomings we synthesized different phosphate-modified ATP analogues and studied their stability. On the one hand we set out to synthesize one member of the commonly used nitrogen- and oxygen-linked triphosphates, respectively (Scheme 1). On the other hand, we synthesized a, to date, unexplored carbon-linked ATP analogue that would add to the variety of terminally modified ATP analogues that can be used in biological studies. We chose to introduce a 6-azidohexyl residue at every ATP analogue due to the ease of the synthesis. We decided to introduce an azide at the linker, as this functional group may be further functionalized using copper-catalyzed azide−alkyne cycloaddition ("click chemistry") or reduced to the corresponding amine opening the possibilities for subsequent amide chemistry.[17−20]

First, we set out to synthesize the nitrogen-linked nucleotide, γ-*N*-(6-azidohexyl) adenosine triphosphate (**1**)[21] (Scheme 1). The synthesis of nitrogen-linked triphosphate analogues is very well established.[4,9−13,16,21] We performed the synthesis using EDC as the activator in water at pH 6.0. Under these conditions the commercially available disodium salt of ATP can be used without the need for cation exchange. Using 6-azidohexylamine[22] as a reagent we obtained nucleotide **1** in 12% yield.

**Scheme 1. Synthesis of Modified Triphosphate Analogues**[a]




[a](a) 6-Azidohexylamine, EDC-HCl, water, pH 6.0, 12 h, RT, 12%; (b) 6-azido-1-bromohexane, DMF, 12 h, RT, 22%; (c) (i) CDI, DMF, 24 h, RT, (ii) MeOH, 3 h, RT, (iii) 7-azidoheptyl phosphonate, $Et_3N$, 24 h, 40 °C, 11%.

Oxygen-linked nucleoside triphosphates can be synthesized by coupling of a nucleoside diphosphate with the monophosphate of the molecule to be attached.[21] This methodology requires the synthesis of two phosphorylated building blocks. In one case, it has been stated that triphosphates may alternatively be alkylated at the γ-phosphate using an alkyl bromide in water.[23] This procedure failed to produce oxygen-linked

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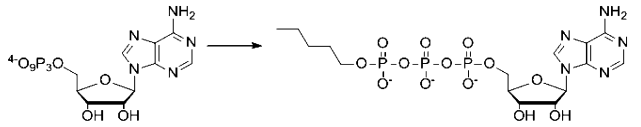
nucleotide γ-*O*-(6-azidohexyl) adenosine triphosphate (**2**) in our hands.

Therefore, we set out to optimize the conditions for alkylation. We used 1-bromopentane as a model alkylating reagent. First, we tried to perform the coupling with disodium ATP in a two-phase system using water and diethyl ether or water and chloroform as the solvents to allow dissolution of ATP as well as the alkylating reagent (Table 1). This failed to produce the alkylated product. To be able to work under anhydrous conditions we transformed ATP into its tetrabutylammonium salt by passing it through an ion exchange column preequilibrated with tetrabutylammonium bromide. Using tetrabutylammonium ATP in anhydrous DMSO we were able to produce the alkylated ATP analogue at room temperature within 12 h in an isolated yield of 20%. Changing the solvent to DMF gave almost the same yield. Using this optimized protocol we also alkylated ATP with 6-bromo-1-azido-hexane[24] in DMF under strictly anhydrous conditions at room temperature (Scheme 1). This gave compound **2** in 22% yield. Attachment of the linker to the γ-phosphate could be proven by 2D-$^{31}$P−$^{1}$H-HMBC NMR experiments (data not shown).

**Table 1. Optimization of the Reaction Conditions for Alkylating the γ-Phosphate of ATP**[a]



| entry | solvent | counterion of ATP | yield |
|---|---|---|---|
| 1 | water/diethyl ether | $Na^+$ | –[b] |
| 2 | water/chloroform | $Na^+$ | –[b] |
| 3 | DMSO | $Bu_4N^+$ | 20%[c] |
| 4 | DMF | $Bu_4N^+$ | 17%[c] |

[a]All reactions were stirred for 12 h at room temperature and then analyzed by RP-HPLC. [b]No product detected by HPLC. [c]Isolated yield after ion-exchange chromatography and RP-HPLC.

ATP analogues such as carbon-linked nucleotide γ-*C*-(7-azidoheptyl) adenosine triphosphate (**3**) have not been reported. First, we synthesized 7-azido-heptyl-phosphonate. Starting from known diethyl 7-bromoheptylphosphonate[25] we performed a substitution reaction of the bromine with sodium azide followed by saponification of the phosphonic acid esters using TMS-Br.[26] 7-Azido-heptylphosphonic acid was purified by a very simple procedure. An alkaline aqueous solution of the reaction product was washed with diethyl ether, acidified, and extracted with diethyl ether to give the product in 50% yield. To synthesize compound **3** we coupled this phosphonic acid to ADP. We tested the coupling reaction of tetrabutylammonium ADP in DMF/DMSO using CDI or EDC as an activator, respectively. Whereas activation with EDC gave traces of the product, using CDI 11% of compound **3** was obtained.

In order to study the stability of the synthesized analogues toward hydrolysis at different pH conditions, we set up an assay using reversed-phase HPLC (RP-HPLC) detection. All isolated compounds were dissolved in buffered solutions of varying pH at a concentration of 100 μM and incubated at 37 °C for 2 h. The pH values ranged from pH 2.0 to 12.0. We chose to use a citrate, acetate, phosphate, or carbonate buffer at a concentration of 50 mM. In this manner, differences between the chosen pH and p$K_a$ of the corresponding buffer salt could be kept below 1.5 to ensure constant pH during the whole experiment. RP-HPLC analysis (Figure 1) revealed that oxygen-linked compound **2** is stable over the applied pH range from 2.0 to 12.0. The novel carbon-linked compound **3** is almost comparably stable and showed only slight degradation at pH 2.0. In contrast, nitrogen-linked compound **1** completely decomposes at pH 2.0 and shows the first signs of degradation at pH 5.5. HR-ESI-MS analysis revealed that this lack of stability is due to hydrolysis of the P−N bond to give unmodified ATP. This hydrolysis of the P−N bond of these triphosphate analogues has been reported using strongly acidic conditions[13] but has not been studied in detail.

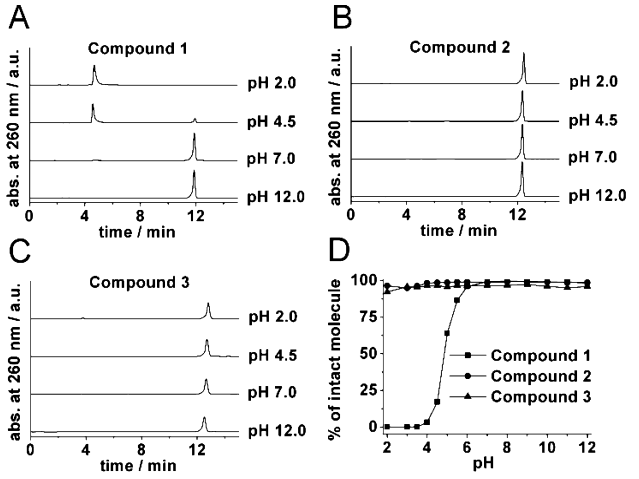



**Figure 1.** (A–C) RP-HPLC traces after incubation of compounds **1**, **2**, and **3** at indicated pH values for 2 h at 37 °C monitored by the absorption at 260 nm; abs.: absorbance; a.u.: arbitrary units. (D) Evaluation of the amount of intact probe after incubation. % of intact molecule was calculated as the ratio of the integral of the peak corresponding to the starting material to the sum of the integrals of all peaks.

As the use of longer phosphate chains has been shown to be useful for certain biochemical applications[4−8,27] we also synthesized an O-linked tetraphosphate, δ-*O*-(6-azidohexyl) adenosine tetraphosphate (**4**), and a C-linked tetraphosphate, δ-*C*-(7-azidoheptyl) adenosine tetraphosphate (**5**) (Figure 2A). Again, we used tetrabutylammonium ATP in DMF and chose to couple it to 6-azido-hexylphosphate and 7-azido-heptyl-phosphonate, respectively. Screening CDI and EDC as an activator, we found that in both cases using EDC gave higher yields of the desired products. Oxygen-linked tetraphosphate **4** was synthesized in 24% yield, and carbon-linked tetraphosphate **5** was synthesized in 28%. The latter molecule comprises the first adenosine tetraphosphate analogue modified with a linker via a carbon atom. By applying the same stability assay as described above, the tetraphosphate analogues showed the same high stability as the triphosphate analogues (Figure 2B, 2C). This indicates that stability is mainly determined by the type of attachment.

In conclusion, we successfully synthesized five different terminally modified adenosine polyphosphates with different attachment modes to the phosphate chain. Nitrogen-linked ATP analogue **1** could be obtained in a straightforward manner by activation of ATP with EDC and subsequent coupling to 6-azidohexylamine. To obtain oxygen-linked triphosphate **2** we optimized the conditions for alkylation of the γ-phosphate of ATP. Using tetrabutylammonium ATP and 6-azido-1-bromo-hexane in DMF under strictly anhydrous conditions gave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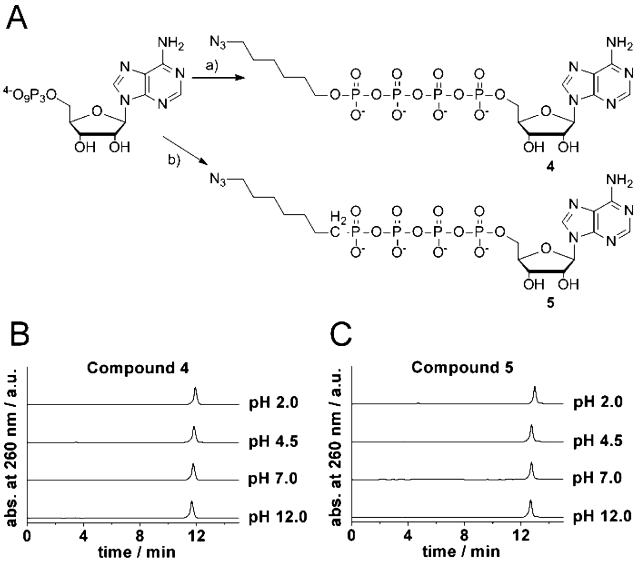


**Figure 2.** (A) Synthesis of modified tetraphosphate analogues. (a) (i) EDC−HCl, 2.5 h, RT, (ii) MeOH, 3 h, RT, (iii) 6-azidohexyl phosphate, $Et_3N$, 24 h, 40 °C, 24%; (b) (i) EDC−HCl, 2.5 h, RT, (ii) MeOH, 3 h, RT, (iii) 7-azidoheptyl phosphonate, $Et_3N$, 24 h, 40 °C, 28%; (B and C) RP-HPLC traces after incubation of compounds **4** and **5** at indicated pH values for 2 h at 37 °C monitored by the absorption at 260 nm; abs.: absorbance; a.u.: arbitrary units.

compound **2**. The novel carbon-linked triphosphate **3** was obtained by coupling tetrabutylammonium ADP with 7-azidoheptyl-phosphonic acid. Both the oxygen- (**4**) and the carbon-linked (**5**) adenosine tetraphosphate analogues were obtained by activation of tetrabutylammonium ATP with EDC and subsequent coupling to 6-azidohexyl-phosphate and 7-azidoheptyl-phosphonate, respectively. The presented carbon-linked adenosine tri- and tetraphosphate each comprise the first entity of these types of molecules and thus add to the diversity of terminally linker-modified ATP analogues.

Having all these molecules in hand we tested their stability toward different pH conditions. The nitrogen-linked triphosphate **1** readily decomposes at slightly acidic pH. This limits the use of this kind of probes in biological studies. In contrast, oxygen-linked triphosphate **2** is very stable over a broad range of pH values. Furthermore, the so far unexplored carbon-linked analogue **3** and **5** are stable from pH 3.0 to 12.0. They can serve as a new alternative to the oxygen-linked counterpart. Interestingly, for both the oxygen- and carbon-linked analogues a change from triphosphate to tetraphosphate does not alter the stability, showing that tetraphosphate analogues are also stable alternatives.

The results presented herein provide valuable information for the design of future nucleotide probes that bear modifications at the γ-phosphate, a class of nucleotides that are increasingly applied, e.g., for elucidating the complexity of kinase regulation[9−13] or the advent of single molecule sequencing.[6−8]

## EXPERIMENTAL SECTION

**General Procedures.** ATP and ADP were purchased as their sodium salts and converted to their tetrabutylammonium salts by passing through a column of CHELEX 100 cation-exchange resin preequilibrated with tetrabutylammonium bromide. HR-ESI-MS spectra were recorded in the negative mode. Preparative RP-HPLC was performed using a 21 mm × 250 mm C18 column and an isocratic gradient of 5% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.0, TEAA) over 10 min followed by a linear gradient from 5% to 40% acetonitrile in 50 mM TEAA over 30 min at a flow rate of 15 mL/min. Anion exchange chromatography was performed using a column of DEAE sepharose and an isocratic gradient of 0.1 M triethylammonium bicarbonate buffer (pH 7.5, TEAB) over 100 mL followed by a linear gradient from 0.1 to 1 M TEAB within 900 mL at a flow rate of 2.5 mL/min. Representative chromatograms of anion-exchange chromatography and RP-HPLC for compound **1** can be found in the Supporting Information.

***γ-N*-(6-Azidohexyl) Adenosine Triphosphate Tris-(triethylammonium) Salt 1.** ATP (disodium salt, 27 mg, 49 μmol) and 6-azido-hexylamine (70 mg, 490 μmol, 10 equiv) were dissolved in 10 mL of water, and the pH was adjusted to 6.0. EDC hydrochloride (199.4 mg, 1.04 mmol, 21 equiv) was added, and the solution was kept at room temperature for 12 h. The pH was adjusted to 8.5, and the solvent was evaporated. Purification by anion-exchange chromatography (eluting after 320 min at 0.8 M TEAB) and RP-HPLC (eluting after 25 min at 23% acetonitrile) gave 5.7 μmol (5.3 mg, 12%) of colorless oil. $^1$H NMR ($D_2O$, 400 MHz): δ 8.59 (s, 1H, H8), 8.29 (s, 1H, H2), 6.15 (d, $J$ = 5.9 Hz, 1H, H1′), 4.80 (m, 1H, overlapping with HOD, H2′), 4.56 (m, 1H, H3′), 4.40 (m, 1H, H4′), 4.25 (m, 2H, H5′a, H5′b), 3.22 (m, 2H, overlapping with $Et_3N-CH_2$, $N_3-CH_2$), 2.79 (q, $J$ = 8.0 Hz, 2H, $CH_2-NH-P$), 1.45−1.28 (m, 8H, overlapping with $Et_3N-CH_3$, 4 × $CH_2$-linker); $^{31}$P NMR ($D_2O$, 162 MHz): δ −0.9 (d, $J$ = 20.6 Hz, 1P), −11.6 (d, $J$ = 18.6 Hz, 1P), −23.0 (t, $J$ = 20.1 Hz, 1P); HR-ESI-MS: found, 630.1017; calculated, 630.0987 ($M-H^+$, $C_{16}H_{27}N_9O_{12}P_3^-$); deviation: 4.8 ppm.

***γ-O*-(6-Azidohexyl) Adenosine Triphosphate Tris-(triethylammonium) Salt 2.** Tetrabutylammonium ATP (285 μmol, 1 equiv) and 6-azido-1-bromohexane (293 mg, 1.43 mmol, 5 equiv) were separately dissolved in 5 mL of DMF each and stored over molecular sieves for 12 h. The solutions were combined and kept at room temperature for 12 h. Purification by anion-exchange chromatography (eluting after 240 min at 0.6 M TEAB) and RP-HPLC (eluting after 27 min at 25% acetonitrile) gave 64 μmol (60 mg, 22%) of a colorless oil. $^1$H NMR (MeOD, 400 MHz): δ 8.61 (s, 1H, H8), 8.22 (s, 1H, H2), 6.11 (d, $J$ = 5.4 Hz, 1H, H1′), 4.73 (t, $J$ = 5.2 Hz, 1H, H2′), 4.57 (m, 1H, H3′), 4.28 (m, 3H, H4′, H5′a, H5′b), 4.00 (pq, $J$ = 6.5 Hz, 2H, $P-O-CH_2$), 3.25 (t, $J$ = 6.9 Hz, 2H, $N_3-CH_2$), 1.61 (m, 2H, $CH_2$-linker), 1.54 (m, 2H, $CH_2$-linker), 1.36 (m, 4H, 2 × $CH_2$-linker); $^{13}$C NMR (MeOD, 100 MHz): δ 157.1 (C6), 153.7 (C2), 150.9 (C4), 141.0 (C8), 120.1 (C5), 88.8 (C1′), 85.6 (d, $J$ = 9.3 Hz, C4′), 76.1 (C2′), 71.8 (C3′), 66.9 (d, $J$ = 6.1 Hz, $CH_2-O-P$), 66.5 (d, $J$ = 5.1 Hz, C5′), 52.3 ($N_3-CH_2$), 31.7 (d, $J$ = 8.4 Hz, $P-O-CH_2-CH_2$), 29.8 ($CH_2$-linker), 27.5 ($CH_2$-linker), 26.4 ($CH_2$-linker); $^{31}$P NMR (MeOD, 162 MHz): δ −11.2 (d, $J$ = 18.3 Hz, 1P), −11.7 (d, $J$ = 18.6 Hz, 1P), −23.2 (t, $J$ = 18.5 Hz, 1P); HR-ESI-MS: found, 631.0839; calculated, 631.0827 ($M-H^+$, $C_{16}H_{26}N_8O_{13}P_3^-$); deviation: 1.9 ppm.

**7-Azidoheptyl-phosphonate.** Diethyl 7-bromoheptyl-phosphonate (3.3 g, 6.72 mmol) and sodium azide (1.4 g, 20.8 mmol) were dissolved in 15 mL of acetone and refluxed overnight. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under reduced pressure. The residue was dissolved in 27 mL of acetonitrile and cooled to −5 °C. Trimethylsilyl bromide (5.14 g, 33.6 mmol) was added dropwise, and the solution was warmed to room temperature and stirred overnight. 45 mL of methanol and 15 mL of water were added, and the solution was stirred for 40 h. The solvents were evaporated, and the residue was dissolved in 1 M NaOH solution. The solution was washed with ether, acidified, and extracted with ether. The latter ether phase was dried over magnesium sulfate and concentrated to give 747 mg (3.38 mmol, 50%) of the product as a colorless oil. $^1$H NMR (MeOD, 400 MHz) δ 5.63 (s, 2H, $H_2PO_3$); 3.28 (t, $J$ = 6.8 Hz, 2H, $N_3-CH_2$); 1.74−1.6 (m, 6H, $H_2PO_3-(CH_2)_2$ and $N_3-CH_2-CH_2$); 1.46−1.32 (m, 6H, $N_3-(CH_2)_2-(CH_2)_3$); $^{13}$C NMR (MeOD, 100 MHz): δ 52.4, 31.5 (d, $J$ = 16.5 Hz), 29.8, 29.8, 27.9 (d, $J$ = 138.1 Hz), 27.6, 23.7 (d, $J$ = 5.1 Hz); $^{31}$P NMR (MeOD, 162 MHz) δ 30.5 (s, 1P); HR-ESI-MS: found: 220,0841 calculated: 220,0846 ($M-H^+$, $C_7H_{15}N_3O_3P$); deviation: 2.3 ppm.


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**γ-C-(7-Azidoheptyl) Adenosine Triphosphate Tris-(triethylammonium) Salt 3.** Tetrabutylammonium ADP (232 μmol, 1 equiv) and 7-azido-heptyl-phosphonate (480 mg, 2.32 mmol, 10 equiv) were separately dissolved in 3 mL of DMF/DMSO 4:1 (v/v) each and stored over molecular sieves for 24 h. CDI (188 mg, 1.16 mmol, 5 equiv) was added to ADP, and the solution was kept at room temperature for 12 h. Methanol (75 μL) was added, and the solution was kept at room temperature for 3 h. Triethylamine (650 μL, 470 mg, 4.64 mmol, 20 equiv) was added. This solution was combined with the solution of 7-azido-heptyl-phosphonate and kept at 40 °C for 24 h. Purification by anion-exchange chromatography (eluting after 240 min at 0.6 M TEAB) and RP-HPLC (eluting after 25 min at 23% acetonitrile) gave 26 μmol (24 mg, 11%) of a colorless oil. $^1$H NMR (MeOD, 400 MHz): δ 8.59 (s, 1H, H8), 8.20 (s, 1H, H2), 6.09 (d, $J$ = 5.6 Hz, 1H, H1′), 4.73 (t, $J$ = 5.3 Hz, 1H, H2′), 4.57 (m, 1H, H3′), 4.31 (m, 1H, H5′a), 4.24 (m, 2H, H4′, H5′b), 3.25 (t, $J$ = 6.8 Hz, 2H, $N_3$−C**H**$_2$), 1.82 (m, 2H, C**H**$_2$−P), 1.67 (m, 2H, C**H**$_2$-linker), 1.55 (m, 2H, C**H**$_2$-linker), 1.34 (m, 6H, 3 × C**H**$_2$-linker); $^{13}$C NMR (MeOD, 100 MHz): δ 157.4 (C6), 154.0 (C2), 151.1 (C4), 141.4 (C8), 120.3 (C5), 89.0 (C1′), 85.9 (d, $J$ = 9.1 Hz, C4′), 76.3 (C2′), 72.0 (C3′), 66.6 (d, $J$ = 5.8 Hz, C5′), 52.6 ($N_3$−**C**H$_2$), 32.4 (d, $J$ = 18.3 Hz, P−CH$_2$−**C**H$_2$), 30.3 (d, $J$ = 138.0 Hz, **C**H$_2$−P), 30.2 (CH$_2$-linker), 30.0 (CH$_2$-linker), 27.9 (CH$_2$-linker), 24.9 (d, $J$ = 4.8 Hz, CH$_2$-linker); $^{31}$P NMR (MeOD, 162 MHz): δ 17.6 (d, $J$ = 22.5 Hz, 1P), −11.6 (d, $J$ = 17.7 Hz, 1P), −23.0 (t, $J$ = 20.6 Hz, 1P); HR-ESI-MS: found, 629.1056; calculated, 629.1034 (M−H$^+$, $C_{17}H_{28}N_8O_{12}P_3^-$); deviation: 3.5 ppm.

**δ-O-(6-Azidohexyl) Adenosine Tetraphosphate Tetrakis-(triethylammonium) Salt 4.** Tetrabutylammonium ATP (217 μmol, 1 equiv) and 6-azido-hexyl-phosphate (484 mg, 2.17 mmol, 10 equiv) were separately dissolved in 3 mL of DMF each and stored over molecular sieves for 12 h. EDC hydrochloride (125 mg, 651 μmol, 3 equiv) was added to ATP, and the solution was kept at room temperature for 2.5 h. Methanol (70 μL) was added, and the solution was kept at room temperature for 3 h. Triethylamine (600 μL, 438 mg, 4.34 mmol, 20 equiv) was added. This solution was combined with the solution of 6-azido-hexyl-phosphate and kept at 40 °C for 24 h. Purification by anion-exchange chromatography (eluting after 280 min at 0.7 M TEAB) and RP-HPLC (eluting after 21 min at 18% acetonitrile) gave 52 μmol (58 mg, 24%) of a colorless oil. $^1$H NMR (MeOD, 400 MHz): δ 8.64 (s, 1H, H8), 8.23 (s, 1H, H2), 6.10 (d, $J$ = 6.1 Hz, 1H, H1′), 4.77 (t, $J$ = 5.6 Hz, 1H, H2′), 4.65 (m, 1H, H3′), 4.35 (m, 1H, H5′a), 4.24 (m, 2H, H4′, H5′b), 4.02 (pq, $J$ = 6.4 Hz, 2H, P−O−C**H**$_2$), 3.26 (t, $J$ = 6.9 Hz, 2H, $N_3$−C**H**$_2$), 1.68−1.50 (m, 4H, 2 × C**H**$_2$-linker), 1.46−1.36 (m, 4H, 2 × C**H**$_2$-linker); $^{13}$C NMR (MeOD, 100 MHz): δ 156.6 (C6), 153.0 (C2), 150.9 (C4), 141.6 (C8), 120.0 (C5), 88.7 (C1′), 86.1 (C4′), 76.3 (C2′), 72.0 (C3′), 67.0 (**C**H$_2$−O−P), 66.7 (C5′), 52.4 ($N_3$−**C**H$_2$), 31.7 (CH$_2$-linker), 29.8 (CH$_2$-linker), 27.6 (CH$_2$-linker), 26.5 (CH$_2$-linker); $^{31}$P NMR (MeOD, 162 MHz): δ −11.0 (m, 1P), −11.6 (m, 1P), −23.3 (m, 2P); HR-ESI-MS: found, 711.0508; calculated, 711.0490 (M−H$^+$, $C_{16}H_{27}N_8O_{16}P_4^-$); deviation: 2.5 ppm.

**δ-C-(7-Azidoheptyl) Adenosine Tetraphosphate Tetrakis-(triethylammonium) Salt 5.** Tetrabutylammonium ATP (100 μmol, 1 equiv) and 7-azido-heptyl-phosphonate (207 mg, 1.0 mmol, 10 equiv) were separately dissolved in 3 mL of DMF each and stored over molecular sieves for 12 h. EDC hydrochloride (57.5 mg, 300 μmol, 3 equiv) was added to ATP, and the solution was kept at room temperature for 2.5 h. Methanol (33 μL) was added, and the solution was kept at room temperature for 3 h. Triethylamine (279 μL, 202 mg, 2.0 mmol, 20 equiv) was added. This solution was combined with the solution of 7-azido-heptyl-phosphonate and kept at 40 °C for 24 h. Purification by anion-exchange chromatography (eluting after 280 min at 0.7 M TEAB) and RP-HPLC (eluting after 25 min at 23% acetonitrile) gave 28 μmol (31 mg, 28%) of a colorless oil. $^1$H NMR (MeOD, 400 MHz): δ 8.63 (s, 1H, H8), 8.20 (s, 1H, H2), 6.11 (d, $J$ = 6.2 Hz, 1H, H1′), 4.79 (t, $J$ = 5.7 Hz, 1H, H2′), 4.66 (m, 1H, H3′), 4.34 (m, 1H, H5′a), 4.24 (m, 2H, H4′, H5′b), 3.26 (t, $J$ = 6.9 Hz, 2H, $N_3$−C**H**$_2$), 1.83 (m, 2H, C**H**$_2$−P), 1.67 (m, 2H, C**H**$_2$-linker), 1.56 (m, 2H, C**H**$_2$-linker), 1.35 (m, 6H, 3 × C**H**$_2$-linker); $^{13}$C NMR (MeOD, 100 MHz): δ 156.1 (C6), 152.4 (C2), 150.8 (C4), 141.8 (C8), 119.9 (C5), 88.7 (C1′), 86.0 (d, $J$ = 7.8 Hz, C4′), 76.4 (C2′), 72.0 (C3′), 66.7 (C5′), 52.4 ($N_3$−**C**H$_2$), 32.1 (d, $J$ = 17.9 Hz, P−CH$_2$−**C**H$_2$), 30.0 (CH$_2$-linker), 29.9 (CH$_2$-linker), 29.9 (d, $J$ = 138.8 Hz, **C**H$_2$−P), 27.7 (CH$_2$-linker), 24.5 (CH$_2$-linker); $^{31}$P NMR (MeOD, 162 MHz): δ 17.7 (d, $J$ = 19.6 Hz, 1P), −11.6 (m, 1P), −23.3 (m, 2P); HR-ESI-MS: found, 709.0729; calculated, 709.0697 (M−H$^+$, $C_{17}H_{29}N_8O_{15}P_4^-$); deviation: 4.5 ppm.

**pH Stability Assay.** Compounds were dissolved at a concentration of 100 μM in 50 μL of 20 mM citrate buffer (pH 2.0, 3.0, 3.5), acetate buffer (pH 4.0, 4.5, 5.0, 5.5), phosphate buffer (pH 6.0, 7.0, 8.0), or carbonate buffer (pH 9.0, 10.0, 11.0, 12.0), respectively, and incubated for 2 h at 37 °C. The mixtures were neutralized by addition of 50 μL of 100 mM phosphate buffer (pH 7). The samples were analyzed by analytical RP-HPLC using a 4 mm × 250 mm C18 column and a gradient from 5% to 18% acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.0) over 15 min. The product formed by incubation of compound **1** at pH 2.0 was subjected to HR-ESI-MS: found, 505.9881; calcd, 505.9874 (ATP−H$^+$, $C_{10}H_{15}N_5O_{13}P_3^-$); deviation: 1.4 ppm.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Copies of NMR spectra of novel compounds and representative chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


### Corresponding Author

*E-mail: andreas.marx@uni-konstanz.de.


### Notes

The authors declare no competing financial interest.

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