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Synthesis and reactivity of novel γ-phosphate modified ATP analogues

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ABSTRACT

We hereby present a simple yet novel chemical synthesis of a family of γ-modified ATPs bearing functional groups on the γ-phosphate that are amenable to further derivatization by highly selective chemical manipulations (e.g., click chemistry, Staudinger ligations). A preliminary screen of these compounds as phosphate donors with a typical wild type protein kinase (cdk2) and one of its known substrates $p27^{kip1}$ is also presented.

The kinase-catalysed phosphorylation of protein substrates is a critical regulatory step in many biological processes and this post-translational modification is often deregulated in human diseases such as cancer.[1] One of the major challenges remaining in this field is to fully elucidate complex kinase pathways and unambiguously correlate a substrate-specific phosphorylation event with a given kinase. To address this problem, powerful methods have been developed for phosphoprotein detection and enrichment,[2] including phospho-dependent tagging strategies (Scheme 1).[3]

In many single kinase-based assays for substrate identification, the underlying principle consists of using a γ-phosphate modified ATP analogue that is bound by the kinase to act as the phosphate donor, thereby allowing for the transfer of the γ-modified phosphate group onto the protein substrate to be identified. To date, only a limited number of tags at the γ-phosphate have been used to facilitate protein substrate identification with [γ^{32}P]-ATP and ATPγS being by far the most common.[3] ATP-biotin has been used with kinases to catalyse the transfer of biotin through the phosphate group to peptides but ATP-biotin tends to be a poor phosphate donor with native proteins, indicating that certain γ-modifications may not be tolerated.[4]

For the identification of kinase substrates in more complex systems such as cell lysates or in vivo, an additional complication arises from the fact that the mechanism of ATP binding and phosphate transfer is highly conserved amongst the more than 500 distinct members of the protein kinase super family.[5] Thus the use of a γ-phosphate modified ATP will not be restricted to the kinase of interest. This problem has been elegantly circumvented by the use of chemical genetics (pioneered by Shokat[6]) whereby an unnatural base-modified ATP analogue is used in conjunction with a mutationally enlarged kinase ATP binding site to create a unique kinase/analogue pairing. The conserved nature of the ATP binding site in kinases has made this approach widely applicable to both tyrosine and serine/threonine kinases.[7] However, the number of phosphate modifications that have been exploited in this context still remains limited, [γ^{32}P]-ATP being the most commonly used. More recently, N^6-modified-ATPγS has been used in combination with analogue sensitive mutant kinases to phosphorylate substrates in cell extracts. A good recovery of known substrates was generally observed, although some low abundance substrates were not detected in these initial studies.[8]

This powerful technique has fuelled our interest for the preparation of ATP analogues that are modified at the γ-phosphate.[9] This area of research may indeed benefit from the availability of novel ATP analogues that are γ-modified with tags featuring functional groups amenable to further derivatization. With the long-term aim to prepare new unnatural ATP analogues modified at the γ-phosphate, we initially focused on native ATP derivatives, not those modified at the adenine site. In this Letter, we report the synthesis of ATP-analogues with γ-phosphate tags incorporating azides, alkynes and alkenes, three functional groups commonly used for click-type chemistry or Staudinger ligation. This choice

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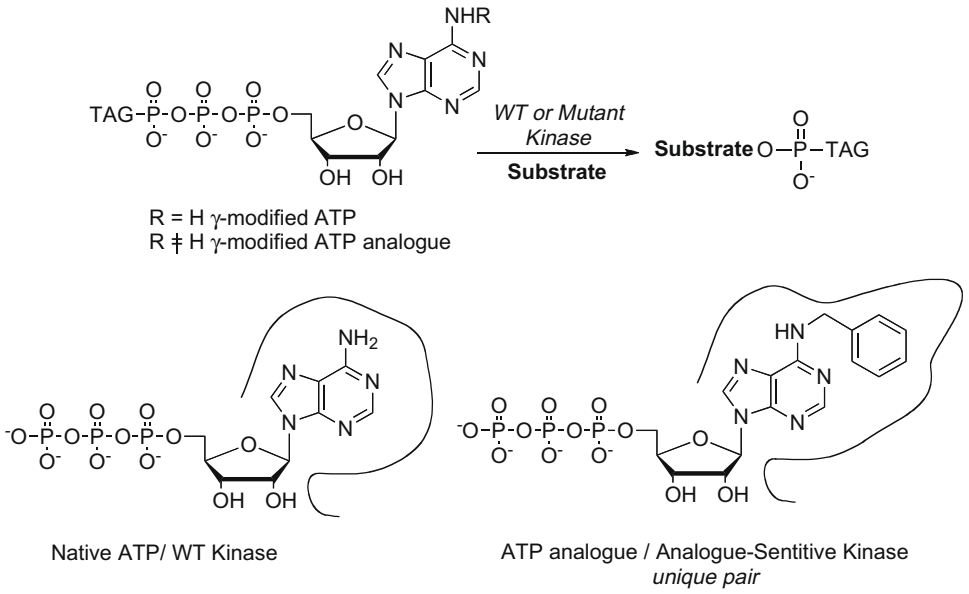


Scheme 1. Phospho-dependent tagging of kinase protein substrates with wild type or analogue sensitive mutant kinase.

was driven by the well-documented value of these transformations in the context of biological systems.[10] We also report the preliminary results of a screen of these compounds as phosphate donors with a typical protein kinase (cdk2) and one of its known substrates $p27^{kip1}$.[11]

We selected the ATP analogues **1–9** as our targets. Compounds **1–4** possess an alkyne functionality attached to the γ-phosphorus atom via a P–O or P–N bond forming reaction. These amines or alcohols would allow us to probe the impact of the chain length and the reactive site on the efficiency of the phosphorylation event. The ATP analogue **5**, derived from allylamine, was prepared since the alkene may be used for subsequent photoclick or Diels–Alder chemistry. The ATP analogues **6–9** were selected based on the ability of the azido group to act as a 1,3-dipole with click partners or to undergo Staudinger ligation with phosphanes. Alkyl-, alkoxy- and aryl-type linkages were considered for this sub-family of analogues (Fig. 1).

Two procedures (A or B) were followed for the preparation of **1–9**, both beginning with the conversion of commercially available ATP (for A) or ADP (for B) into the corresponding tributylammonium salt in order to confer solubility in organic solvents (Scheme 2).[12]

The first procedure (A)[13] was inspired by the chemistry of Hoard and Ott.[14] ATP was dissolved in DMF and treated with 5 equiv of carbonyl diimidazole overnight. The resulting intermediate was reacted, without prior isolation, at room temperature with 5 equiv of the primary amine to be attached. The resulting γ-modified ATP analogues were purified using standard ATP purification methods[12] consisting of DEAE Sephadex resin ion exchange chromatography followed by reverse phase HPLC. Yields of the purified products ranged from 36% to 86% (Scheme 2, Procedure A). When this protocol was applied to pent-4-yn-1-ol or 2-bromoethanol (the precursor of the azido derivative **8**), no coupling took place, ATP being the only crude product observed by ^{31}P NMR after workup. For the preparation of the O-linked ATP analogues **4** and **8**, an alternative procedure (B)[15] was applied. We initially prepared the tagged phosphoric acid derivative by reacting the alcohol with phosphoryl chloride followed by an aqueous quench at pH 7.5 (TEAB buffer). This modified phosphoric acid (5 equiv) was then coupled to activated ADP, prepared using carbonyl diimidazole, to give the corresponding γ-modified O-linked ATP analogues **4** and **10**.[16] A subsequent nucleophilic substitution of **10** with sodium azide was necessary to access compound **8**. The overall yields for this synthetic sequence averaged 45%. Arylated ATP analogue **9** was prepared as described previously.[9a] All compounds were characterised by ^{1}H, ^{13}C and ^{31}P NMR. The ^{31}P NMR spectra all showed the characteristic pattern expected for triphosphate derivatives. Two doublets in the region of ~−1 ppm and −10 ppm and one triplet at ~−21 ppm were observed for the N-linked analogues. For O-linked ATP analogues, the γ-P doublets shifted to around −10 ppm (Table 1). Full characterisations for all compounds are provided in the electronic supporting content.

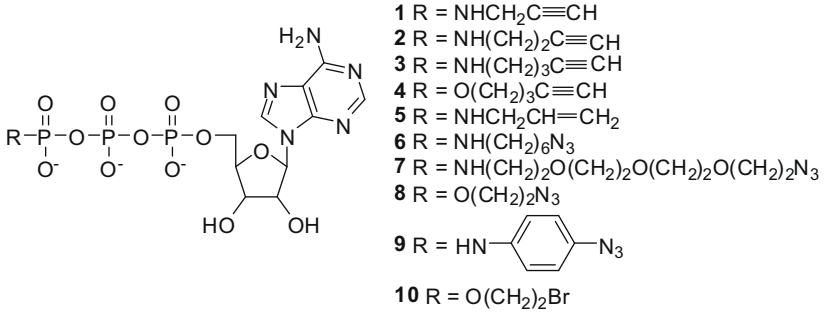
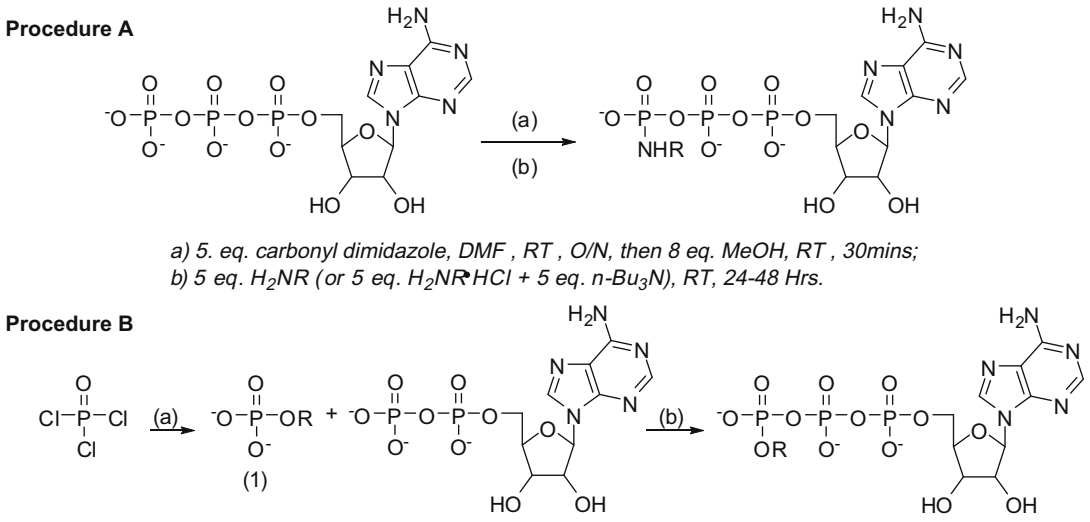


Figure 1. γ-Phosphate modified ATP analogues **1–9**.



Scheme 2. Synthesis of γ-phosphate modified ATP analogues **1–10**.

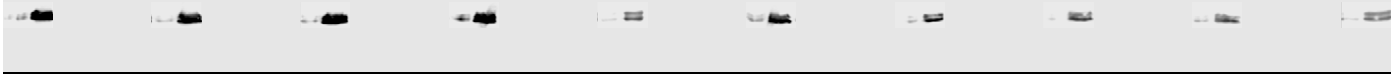
Table 1

γ-Modified ATP	1	2	3	4	5	6	7	8	9
Procedure	A	A	A	B	A	A	A	B	9a
Yield[a] (%)	78	78	49	42[b]	86	36	33	52[c]	61
^{31}P NMR	−1.3	−0.7	−0.1	−9.8	−0.4	−0.3	0.4	−10.3	−9.5
	−10.1	−10.4	−10.4	−10.4	−10.4	−10.4	−10.2	−10.5	−10.5
	−21	−21.8	−21.8	−22.2	−21.8	−21.8	−21.6	−22.2	−21.9

[a] Isolated yield after HPLC purification.
[b] This compound showed substantial degradation under HPLC conditions resulting in an isolated yield of 6%. 42% relates to the isolated yield after ion exchange.[17]
[c] Given the hazardous nature of azidoethanol, **8** was prepared as the bromide **10** followed by treatment with sodium azide.

Table 2
Phosphorylation of p27^{kip1} with ATP analogues **1–9**

ATP	1	2	3	4	5	6	7	8	9
− +	− +	− +	− +	− +	− +	− +	− +	− +	− +



Recombinant p27^{kip1} and cyclin E1/cdk2 were incubated at 30 °C for 1 h in the presence (+) or absence (−) of ATP or compounds **1–9**. Samples were then resolved by SDS–PAGE and immunoblotted for phosphorylated p27^{kip1}.

These novel ATP analogues were then reacted with protein kinase cyclin E1/cdk2 and the known cdk2 substrate p27^{kip1} to verify that phosphate transfer occurred.[16] Kinase assays were performed using recombinant GST purified proteins and phosphorylation was detected by SDS gel electrophoresis and immunoblotting using an anti-phospho p27^{kip1} antibody (Table 2).

Phosphorylation of the p27^{kip1} acceptor substrate was detected with ATP and with each analogue tested albeit with varying degrees of efficiency. These data clearly indicate that phosphotransfer does take place with each of the compounds **1–9.**

In conclusion, we have prepared nine novel γ-phosphate modified ATP analogues and have tested their ability to phosphorylate p27^{kip1} with wild type protein kinase cdk 2. The data shows that phosphate transfer has occurred. The unambiguous verification that the transferred phosphate has retained the desired modification is ongoing. This work lays the foundations to utilize novel chemistry to study protein phosphorylation and may be useful in the identification and isolation of novel kinase substrates.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.028.

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13. *General synthesis of γ-N-modified ATPs*: 0.1 mM ATP [(nBu$_3$NH$^+$) salt] as a 0.1 M solution in DMF was dried over freshly activated molecular sieves overnight. The solution was then decanted off the sieves and 5 equiv carbonyl diimidazole was added. The resulting mixture was then stirred overnight at room temperature under argon. Eight equivalents of MeOH were added and the resulting mixture stirred for 30 min. Five equivalents of H_2NR (or a mixture of 5 equiv H_2NR·HCl and 5 equiv nBu$_3$N) were then added and the reaction stirred for a further 24–48 h under argon at room temperature. The resulting mixture was then diluted with 10 ml 0.05 M triethylammonium bicarbonate buffer (TEAB) and applied to a column of DEAE Sephadex resin that had been pre-swelled in 0.05 M TEAB overnight. The column was then eluted with a gradient from 0.05–0.8 M TEAB, and the desired product eluted between 0.25 and 0.5 M TEAB, whereas unreacted ATP eluted between 0.45 and 0.6 M TEAB. The major UV active components were pooled and concentrated and further purified by RP HPLC.
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15. *General synthesis of γ-O-modified ATPs*: The relevant phosphoric acid was prepared by treating a 0.5 M solution of phosphoryl chloride in diethyl ether at −78 °C with 1 equiv ROH and 1 equiv Et$_3$N. The resulting mixture was then allowed to warm to rt overnight, and quenched by the addition of 10 ml 0.1 M TEAB. The resulting solution was then concentrated to dryness, and redissolved in DMF to generate a 1 M solution which was dried over molecular sieves overnight and used crude in the next step. 0.1 mM ADP [(nBu$_3$NH$^+$) salt] as a 0.1 M solution in DMF was dried over freshly activated molecular sieves overnight. The solution was then decanted and 5 equiv carbonyl diimidazole was added. The reaction mixture was stirred under argon overnight at room temperature. Eight equivalents of MeOH were then added, and the resulting mixture stirred for 30 min. Five equivalents of modified phosphoric acid were added as a 1 M solution in DMF and the reaction stirred for a further 60 h under argon at room temperature. The resulting mixture was then diluted with 10 ml 0.05 M triethylammonium bicarbonate buffer (TEAB) and applied to a column of DEAE Sephadex resin that had been pre-swelled in 0.05 M TEAB overnight. The column was then eluted with a gradient from 0.05–0.8 M TEAB, and the desired product eluted between 0.3 and 0.55 M TEAB, whereas any ATP eluted between 0.45 and 0.6 M TEAB. The major UV active components were pooled and concentrated and further purified by RP HPLC.
16. Up to ~1 μg of GST purified substrate (p27^{kip1} (aa 105–198)) was incubated with cyclin E1/cdk2 purified from Sf9 cells infected with recombinant baculoviruses and 1 mM ATP (or ATP derivative) and K buffer (25 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA) and incubated for 1 hour at 30 °C. Reactions were terminated by adding 5× SDS–PAGE loading buffer. The protein samples were separated by SDS–PAGE and visualized by immunoblotting and ECL detection (Millipore). Purified p27^{kip1} protein is thought to appear as a doublet because of some truncated expression in *Escherichia coli*; however, both versions phosphorylate as detected by anti-phospho p27^{kip1} antibody. Analysis of blots was performed using Aida software. Residual detection of p27^{kip1} present in '-' lanes is due to a low level of non-specificity of the phosphospecific antibody.
17. Following separation by ion exchange chromatography on DEAE Sephadex resin, two compounds were isolated, 42% of **4** and 39% ADP. Following further HPLC purification, **4** was isolated analytically pure in only 6% yield. ADP was also isolated.