nature portfolio

Peer Review File

An ATPγS recycling strategy for practical biocatalytic thiophosphorylation

Corresponding Author: Professor Hans Renata

Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
The manuscript by Renata and coworkers describes a versatile biocatalytic strategy for the selective thiophosphorylation of a range of biologically relevant substrates, including nucleosides, sugars, peptides/proteins, and oligonucleotides. The approach utilizes ATPγS as a thiophosphate donor, coupled with a recycling system based on thiophosphorylated creatine and creatine kinase. This method is successfully applied across multiple enzyme cascades, enabling the synthesis of diverse thiophosphorylated biomolecules. Overall, the methodology is operationally straightforward and is likely to have a broad range of applications. Some sections of the paper require more detailed discussion, however provided the comments below are addressed, I am supportive of publication.
1. The stereocontrolled synthesis of NTPαS is interesting but should be explored in more detail. Can the authors comment on the selectivity of the NMPK and AcKs used in the cascade. Is the TmAcK (S)-selective and the EcAck (R)-selective? Do the NMPKs used generate single stereoisomers? Evidence should be provided.
2. The authors should comment on/compare the efficiency of the different phosphorylation reactions performed. For example, under optimized conditions nucleoside phosphorylation required only 0.25 mol% CK, 5 mol% ADP and 1.2 equivalents of the LiXCrPS, while peptide phosphorylation required 8 mol% CK, 0.4 mM ADP and 40 equivalents LiXCrPS. Why did the peptide phosphorylation require such a large excess of LiXCrPS? The authors should also check the units for consistency – 0.4 mM ADP is used for one of the peptide examples, and 40 mol% for the other peptide making comparisons difficult.
3. Figure 1A: The colour coding used for 2'-OMe and 2'-F is not clear and should be modified. The GalNAc conjugate is drawn in the scheme but not labelled. Phosphatases produce Pi and not PPi. It is not true that there is no reaction when phosphatases are incubated with thiophosphates. The reaction rate is potentially compromised but many phosphatases accept thiophosphate substrates.
4. Page 5, line 111: 'We hypothesized that the low formation of ATP S was due to its instability and unfavorable equilibrium under the reaction conditions'. Do the authors mean that the product is chemically unstable or that the product is converted back to starting material?
5. Table 1: What was the conversion under the reaction conditions written in the scheme – this entry is missing from the table. What was the rationale for selecting the enzyme concentrations used. The absolute concentrations and ratio of NK:CK doesn't appear to have been explored.
6. Page 6 line121: The authors state that NKs from different organisms led to lower reactivity but they are only comparing the final conversions which are not so different. To give a fair comparison of reactivity the authors should provide a time course for the reaction and compare the initial rate.
7. Page 7, line 145: 'During this exploration, we also uncovered the catalytic utility of several enzymes that have never been tested as biocatalysts previously and identified their preferred substrates. Three nucleoside kinases, namely Ag-dNK, Dm-dNK and Tm-TK, were found to be especially useful as they display broad promiscuity and tolerance toward a range of structural variations on their substrates.' It wasn't clear which enzymes had not been previously tested and what the preferred substrates were? Dm-dNK and Tm-TK have been previously exploited as biocatalysts (J. Biol. Chem. 2023, 299, 104746; Adv. Synth. Catal. 2014, 356, 563).
8. Page 9, line 178: 'In many cases, thiophosphate transfer catalyzed by diphosphate kinases was found to be reversible,

which resulted in stalled reaction conversion after the first two hours. This observation was attributed to the intrinsic phosphatase reactivity of these kinases.' The kinase reverse reaction is phosphoryl transfer not hydrolysis.
9. Page 10, line 194: There is no mention of nucleoside monophosphate kinases in the text which makes it confusing. The text reads 'in the second step of these reactions, acetate kinase performs two roles: (1) delivering the second phosphate motif and (2) regenerating ATP'. However, the second phosphate is delivered by the NMPK, the AcK provides the gamma phosphate and regenerates the ATP used by the NMPK.
10. Interestingly, we found that the diastereoselectivity in this sequence was significantly improved during the removal of residual ATP (see SI for details), which might be attributed to selective consumption of one diastereoisomer by hexokinase. This should be tested.
11. Figure 3B: The term 'enzymatic purification' is misleading. The enzyme was used to degrade the residual ATP prior to chromatographic purification.
12. Figure 4C: The names of the enzymes used should be provided for clarity.
13. The reaction conditions are missing from 5B.
14. Table S5: what does pdt mean?

Referee #2

(Remarks to the Author)
A. Summary of key results
Renata & colleagues present a novel thiophosphorylating reagent and a thorough screen of enzymes for establishing a novel biocatalytic reaction manifold for site-selective thiophosphorylation. The work is thorough and comprehensive in its treatment of both the benefits and limitations of the method. The work is reminiscent of Baran's work on novel reagents for synthetic chemistry applications as well as several of Whitesides's classic works on novel enzyme catalyzed reactions useful in synthesis, and in that regard represents a milestone for the field of biocatalysis as it combines advances in synthetic chemistry and enzymology to synthesize previously inaccessible compounds—particularly interesting in this regard are the thiophosphorylated proteins. The supplementary material is comprehensive and thorough.
B: Originality and significance
The reagent described and overall approach to the synthesis of ATP S is clever and novel. The authors show clearly that the method has broad applicability to a variety of useful molecules.
C. Data and methodology
The SI is thorough in its treatment of synthetic methods. One point regarding the SI is that although the authors state that kinases were Ni-NTA purified, I note that the coding sequences provided in the SI lack his-tags excepting SRC and STK3. Can the authors provide the actual coding sequences for the constructs used including tags? They state elsewhere that genes were cloned into pET28, so it may be presumed that the final constructs have the expected pET28 N-terminal his-tag, though that raises some ambiguity for SRC and STK3 which have different N-terminal his-tag sequences than I would expect for pET28.
D. Appropriate use of statistics and treatment of uncertainties
No concerns here—manuscript does not lean on statistical methods for conclusions
E. Conclusions: robustness, validity, reliability
Synthesis of compounds is well-documented, methods are thoroughly described.
F. Suggested improvements
The authors mention in passing that they were unable to prepare ATP S from ADP and presume this to be due to instability and unfavorable equilibrium. However, their control reactions with ADP and phosphocreatine are also low-yielding as in Table S2. In view of the reported favorable Keq for the phosphorylation of ADP using phosphocreatine (e.g. JBC Volume 254, Issue 14p6528-6537July 1979), this result does not make sense. I would recommend that the authors collect more thorough and convincing data on this point, including kinetic assays of phosphorylation to better understand the relative rates of phosphorylation versus thiophosphorylation. Alternatively, obtaining commercial ATP S and subjecting it to the reaction conditions could shed light on the hypothesized instability. Providing at least some further details as to the specific issue (instability or thermodynamics) would be helpful even if this point is ultimately difficult to resolve entirely. This aspect could be relevant for later investigators in that understanding the likely steady-state level of ATP S that can be formed may be relevant when considering the Km of kinases employed.

Do the authors know if the creatine thiophosphate reagent can be taken up into cells (bacterial or mammalian) and used to thiophosphorylate substrates in cells? This could be a valuable thing to investigate in regards to potential future applications, though it may be better left to follow-up reports.
G. References
References are appropriate
H. Clarity and context
The introduction feels a bit lengthy. If the authors could reduce the details of the various drugs and investigational compounds described this might economize for length—I think the general point that phosphorothioates are an important class of compounds can be made more succinctly.

Referee #3

(Remarks to the Author)
Please see attached review

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have thoroughly addressed all my comments and their additional experiments and analyses in the revised manuscript have strengthened the work. The manuscript describes a useful methodology that will be of interest to a broad range of researchers across multiple fields. I am highly supportive of publication in its current form. Congratulation to the team on a great piece of work.

Referee #2

(Remarks to the Author)
In a previous review, I raised concerns about reporting of DNA constructs as well as the stability of the key thiophopshorylating intermediate ATP gamma S. Renata and colleagues have revised the manuscript to address my concerns in convincing fashion and demonstrate that ATP gammaS is unstable under the reaction conditions, which explains why this compound could not be produced in high yield. The response to concerns raised by other reviewers appears equally thorough and persuasive. I have no further concerns about the work that should prevent publication.

Referee #3

(Remarks to the Author)
This revised MS has been improved. The authors have done a good job in answering many of the questions posed by the three referees, including conducting additional experiments in several cases. They have also improved the procedural details and the figures to make clear exactly which enzymes and both thiophosphoryl and phosphoryl donors are used for each step.

As stated in the R3 review, we find the discussion of thermodynamic considerations in selecting and developing the thiophosphocreatine thiophosphoryl donor to be of paramount importance to this paper, particularly for a high level Nature audience that is attuned to the fundamental underlying principles of this work.

The thermodynamic table that the authors have added is helpful in this regard, but it should not be "buried in the SI" along with its references. Rather, in our view, this table and discussion should be presented front-and-center in the manuscript itself as this is conceptually one of the most central issues one must consider in thinking about S-ATP biocatalytic regeneration.

This serves three important purposes:
(1) It clearly gives the reader the very real big picture of which thiophosphoryl donor candidates can thermodynamically come under consideration for the grand challenge of enzymatic thiophosphorylation with catalytic gamma-S-thio-ATP;
2) It shows how the authors chose the very successful course that they did to move forward, but also shows the world that other solutions may well be possible in the future, as synthetic approaches continue to be developed and enzyme availability and handling/immobilization/compartmentalization/flow techniques improve, etc.
3) It also properly gives attribution at the outset to important complementary approaches to this problem over the past several decades going back to the very early first synthesis of gamma-S-ATP in 1971 by Goody and Eckstein (not cited or mentioned in the "point-by-point" response) and several other good references suggested.

In this submission, Xiangyu Wu, Yu Fu, and Hans Renata describe a practical biocatalytic strategy for thiophosphorylation by designing a novel sacrificial thiophosphate donor, creatine thiophosphate (Li_xCrPS), that works in concert with creatine kinase to enable catalytic turnover of ATPγS. When partnered with a large set of kinase enzymes, this system allows for the biocatalytic synthesis of a wide range of phosphorothioate analogues of (i) both natural and unnatural nucleoside monophosphates, (ii) nucleoside (β-thio)diphosphates, (iii) nucleoside (α-thio)triphosphates, (iv) phosphosugars; (v) phospho-inositols and (vi) nicotinamide cofactors (NAD^+ and $NADP^+$) with control of phosphorothioate placement.

Moreover, the Rice team also showcase the methodology for the thiophosphorylations of intermediate-sized peptides and of proteins and nucleic acids, albeit in an as yet limited fashion. The peptide studies are quite well-behaved. On the other hand, in studies on the autophosphorylation domain for the STK3 (serine/threonine kinase 3), the authors uncover the challenge of seeking to achieve autothiophosphorylation in competition with the presumably kinetically faster simple autophoshorylation reaction. The authors are commended for the detailed mass spectrometric analysis of the modified STK3 samples to provide a clear description of the nature of the challenge here, and to show that one can effectively analyze the microheterogeneity of such mixed thiophosphorylated/phosphorylated proteins. By going beyond the challenging, but still much simpler domain of thiophosphorylated nucleotides, sugars and cofactors into the complex domain of protein thiophosphorylation, the Rice team has appropriately taken quite a leap to show the relevance/applicability of the methodology for studies in biomacromolecular systems! Much more needs to be done here, but addressing the challenge and raising some of the issues for the field going forward is entirely appropriate for such a piece for a *Nature* audience.

The core concept put forward here draws direct inspiration from the well-established creatine phosphate/creatine kinase system for ATP regeneration in enzymatic synthesis (P.G. Wang group – ref. 39) and successfully adapts this system for S-ATP analogue synthesis for the first time. It is also worth noting that Baran and coworkers have previously reported the chemical synthesis of thiophosphate-containing nucleoside di- and triphosphate donors using their P(V) platform (ref. 20), that employs carefully designed chiral thiophosphorylation agents with appropriate protecting groups. The present enzymatic approach offers a complementary and more operationally simple route to access similar thiophosphate-containing molecules under mild, protecting-group-free conditions.

Most importantly, the present work marks a real milestone in establishing the first viable catalytic S-ATP regeneration scheme, and this will capture the attention of members of the biocatalytic, chemical biology and biomedical research communities, given the importance of phosphorothioates in studying signal transduction, enzyme mechanism, and in developing chiral phosphorothioate drug candidates such as MK-1454, and phosphorothioate-based anti-sense nucleic acids. The implications of this work are significant. The authors achieved this breakthrough by developing an efficient and economical synthesis of the thiocreatine phosphate donor (Li_xCrPS) and employing this reagent to achieve thiophosphorylation, across a wide range of biomolecular scaffolds from nucleoside phosphates, to sugar phosphates, NAD(P)-cofactors to thiophosphorylated peptides, proteins and nucleic acids. To achieve this, the authors have demonstrated broad compatibility with a wide range of kinases. The optimization studies presented

in both the manuscript and SI are thorough, clearly establishing entry 4 as optimal with as little as 0.16 mol% Hb creatine kinase loading and 1.2 equivalents of Li_xCrPS.

All that said, the manuscript as written is quite dense and difficult to read. Here are some areas in which the manuscript could be significantly improved, both to discuss the most important general concepts that underlie this work, and to make the paper more understandable/readable.

(1) Thermodynamics of S-ATP Regeneration

At the end of the day, the key to S-ATP regeneration is to begin with a thermodynamically higher energy thiophosphoryl donor than S-ATP itself (ΔG'(hydrolysis) ~ -7.7 kcal/mol for ATP itself), and then to be able to synthesize and handle that S-phosphoryl donor and show that donor is compatible with the enzyme that normally handles the corresponding phosphoryl donor. **The authors should articulate these key points right out of the gate to properly frame the problem for a *Nature* audience.** IMO, the best way to do this would be to present an early figure/table that clearly depicts the candidates available based S-ATP based upon the thermodynamic considerations.

Good examples of manuscripts that do this include a nice paper on ATP regeneration [Zhang, J. *et al. Organic Letters.* **2003,** *5*, 2583–2586 (see Table 2 in this paper)] and another on NADH regeneration; See Broussy, S. *et al. Organic Letters* **2009**, *11*, 305-308 (See Figure 1 in this paper). Consider citing these papers when discussing such an essential thermodynamic analysis of the problem at hand.

Here is the sort of table that might be included in this *Nature*-directed manuscript *(all values are given for the actual phosphoryl system, relative to a ΔG'(hydrolysis) ~ -7.7 kcal/mol for ATP itself)*. If the authors, can find numbers for the thiophosphoryl system, they can include. If not, this thermodynamic hierarchy serves to give the viewer an overview of what enzymatic systems and donors might be considered to solve this problem. The authors only discuss the final two systems in the paper, not mentioning the other four presented here even though the Whitesides team has already demonstrated proof of principle for the use of a thermodynamically better thiophosphoryl donor, S-1,3-diphosphoglycerate, even if it is generated in situ:

(Thio)Phosphoryl Donor	~ΔG'(Hydr)	Enzyme	Reference	Donor Cost
Phosphoenol Pyuvate	-13.8 kcal/mol	PEP Kinase		
1,3-Diphosphoglycerate	-12 kcal/mol	1,3-Di-P-Glycerate Kinase	*Whitesides (S-P)*	
Carbamoyl Phosphate	-12 kcal/mol	Carbamate Kinase		
Arginine Phosphate	-10 kcal/mol	Arginine Kinase	*Whitesides*	
Creatine Phosphate	-10 kcal/mol	Creatine Kinase	*Wang/this paper (S-P)*	
Acetyl Phosphate	-10 kcal/mol	Acetate Kinase	*multiple, incl. this paper*	

Whitesides ref (S-1,3-diphosphoglycerate for S-ATP): Abril, O., Crans, D. C. & Whitesides, G. M. *J. Org. Chem.* **1984**, *49,* 1360–1364;
Wang ref (phosphocreatine for ATP): Zhang, J. *et al. Organic Letters.* **2003,** *5*, 2583–2586
Whitesides ref (arginine phosphate for ATP): Bolte, J.; Whitesides, G. M. *Bioorganic Chemistry*, **1984**, *12*, 170-175.

Whitesides ref (acetyl phosphate for ATP): Crans, D. Whitesides, G. M. *J. Org.Chem.* **1983**, *48*, 3130-3132.

Please also cite the first ever synthesis of S-ATP, classical work by Roger Goody and Fritz Eckstein:
Goody, R. S.; Eckstein, F. "Thiophosphate Analogs of Nucleoside Di- and Triphosphates," *J. Am. Chem. Soc.* **1971**, *93*, 6252-6257.

(2) Biocatalysis Schemes

In describing the sequential biocatalytic thiophosphorylation/phosphorylation events in the figures in the paper, particularly **Figures 3 and 4**, the reader is faced with a rather abbreviated description of the enzymes and conditions used to form each bond. What is needed is a clear indication in each scheme of which enzyme is used to form each bond. Color-coding (such as the blue, maroon and teal below for the α-, β- and γ-P positions, for example) would be very helpful in this regard, as would including each enzyme used in the manuscript figures so the reader can follow the biocatalytic synthesis.

In this regard, the Supporting Information is much more helpful, particularly **Methods A-H** (pages 46-52 of the SI). But even here there is some confusion. A good example is **Method C**. Here the procedure for the biocatalytic synthesis of α-S-nucleoside triphosphates is described. **(i)** The initial α-thiophosphorylation is performed with nucleoside kinase, Hb creatine kinase, stoichometric Li_xCrPS and catalytic ADP; **(ii)** the β-phosphorylation is then performed with what one might expect to be a "nucleoside monophosphate kinase." The scheme itself identifies this enzyme as "monophosphate kinase" above the arrow, whereas the text below describes the enzyme as "nucleoside diphosphate kinase." In Figure 3 in manuscript, this enzyme is not even mentioned as far as I can tell. *This needs to clarified so that this work can be repeated. Please give the enzyme and source for each step unambiguously, in both the SI and in* ***Figures 3 and 4****.*
(iii) the γ-phosphate is apparently installed through the action of acetate kinase with acetyl phosphate as stoichiometric phosphoryl donor. Is this correct? If so, please state this explicitly in the text and the SI.

(3) Stereochemistry at Phosphorus

In cases where a bridging phosphorothioate is generated, two diastereomers are possible at the phosphorus stereocenter. In nearly all cases, the authors demonstrate experimentally a high diastereopreference for one stereochemistry at phosphorus. The sole exception appears to be the NAD^+ β-S-analogue, **74**, where a 1:1 ratio of diastereomers is obtained. More discussion is needed. Here are some of the questions:

1. Is the generally high diastereoselectivity at P expected, given the enzyme employed and what is known in the literature?
2. Why is the NAD^+ β-S-analogue case different?

3. How is the absolute stereochemistry at P assigned? The authors show beautiful C18-RP HPLC traces in the SI and seem to claim that the R_p diastereomer is always the slower-eluting diastereomer. How rigorous is this assumption? Please clarify.
4. The authors show a comparison with "standards" in many cases. Where were these standards obtained and how was their absolute stereochemistry assigned?

Additional Corrections/Clarifications

1. The authors should clearly distinguish among conversion, NMR yield, and isolated yield throughout the manuscript.
2. In **Figure 2**, sections A & B are not marked
3. In **Figure 5B**, fluorescence detection in lane 2 lists (-) for Li_xCrPS which seems incorrect
4. Check the manuscript for the use of "adenine diphosphate" vs. "adenosine diphosphate" (more correct) and related designations.

Supporting Information

The extensive SI adds much to the paper and in many ways is more clearly written than the manuscript itself. The SI provides comprehensive experimental details, nice ^{1}H, ^{13}C and ^{31}P NMR spectral data and C18-reverse phase HPLC traces, in support of the biocatalysis-enabled syntheses reported in the main manuscript.

However, there are still a few corrections needed:

1. Look carefully at the use of "nucleoside monophosphate kinase" vs. "nucleoside diphosphate kinase" throughout (see discussion of Biocatalysis Schemes above).
2. There seems a typographical error in the HRMS of compound **56**, instead of 398, it shows 498.xx.
3. In some cases, there seems to be a discrepancy in yield between the manuscript and the SI (e.g. **entry 15**, two different yields). Please clarify and correct, as needed.
4. The HPLC data for compounds **60** & **62** appear to be missing.
5. The authors comment that the reagent Li_xCrPS is highly hygroscopic. As such, it would be helpful to practitioners if the authors provided guidelines/best practices on handling and storing the reagent (dessicator? P_2O_5 side arm? etc.)

Wiess School of Natural Sciences
Hans Renata, Ph.D.
Department of Chemistry

[redacted]

[redacted]

[redacted]

Referee #1:

The manuscript by Renata and coworkers describes a versatile biocatalytic strategy for the selective thiophosphorylation of a range of biologically relevant substrates, including nucleosides, sugars, peptides/proteins, and oligonucleotides. The approach utilizes ATPγS as a thiophosphate donor, coupled with a recycling system based on thiophosphorylated creatine and creatine kinase. This method is successfully applied across multiple enzyme cascades, enabling the synthesis of diverse thiophosphorylated biomolecules. Overall, the methodology is operationally straightforward and is likely to have a broad range of applications. Some sections of the paper require more detailed discussion, however provided the comments below are addressed, I am supportive of publication.

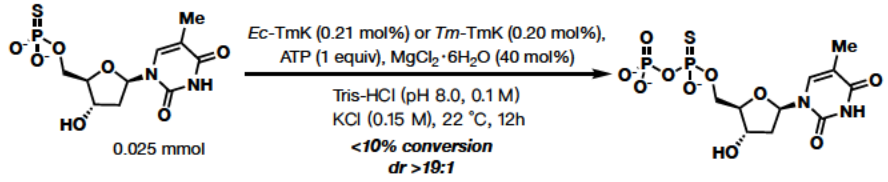
Response:
We thank the reviewer for their assessment of the manuscript.

Question 1: The stereocontrolled synthesis of NTPαS is interesting but should be explored in more detail. Can the authors comment on the selectivity of the NMPK and AcKs used in the cascade. Is the TmAcK (S)-selective and the EcAck (R)-selective? Do the NMPKs used generate single stereoisomers?

Response:
We thank the reviewer for the insightful question. Firstly, two literature precedents (*J. Biol. Chem.***1977**, *252*, 4445–4448; *J. Org. Chem.* **1984**, *49*, 704–706) suggest that monophosphate kinase can stereoselectively phosphorylate adenosine 5'-monothiophosphate. Additionally, we investigated the origin of the diastereoselectivity in our two-enzyme cascade by analyzing the NDPαS intermediate, focusing on two enzymes that afforded high and low diastereoselectivity, respectively (see Section 8.3 in the revised

Email hrenata@rice.edu | Office 713-348-8878 | Fax TBD | Rice University Department of Chemistry - MS 602 |
BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

Supporting Information, with key data and tables reproduced below). In the phosphorylation of the following thymidine mono-thiophosphate with stoichiometric ATP, both *Ec*-TmK and *Tm*-TmK afforded high selectivity, albeit with low conversion. Only one diastereoisomer was observed after purification.



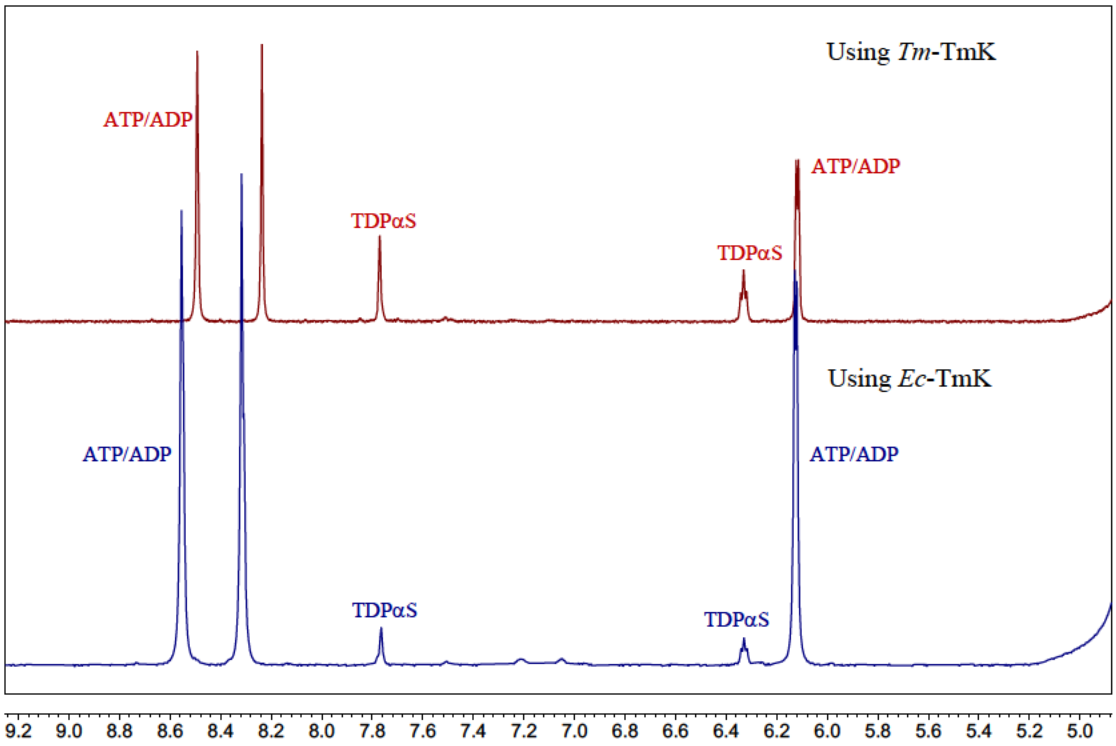


Figure S7. NMR of isolated TDPαS using different monophosphate kinases. High diastereoselectivity was observed for each enzyme.

On the other hand, we also investigated the diastereoselectivity of two cytidine monophosphate kinases. In contrast, low diastereoselectivity ratio (dr) was observed. This ratio is close to the final diastereoselectivity observed in the synthesized CTPαS (around 2:1), and the small discrepancy is attributed to the stereospecificity of acetate kinase.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

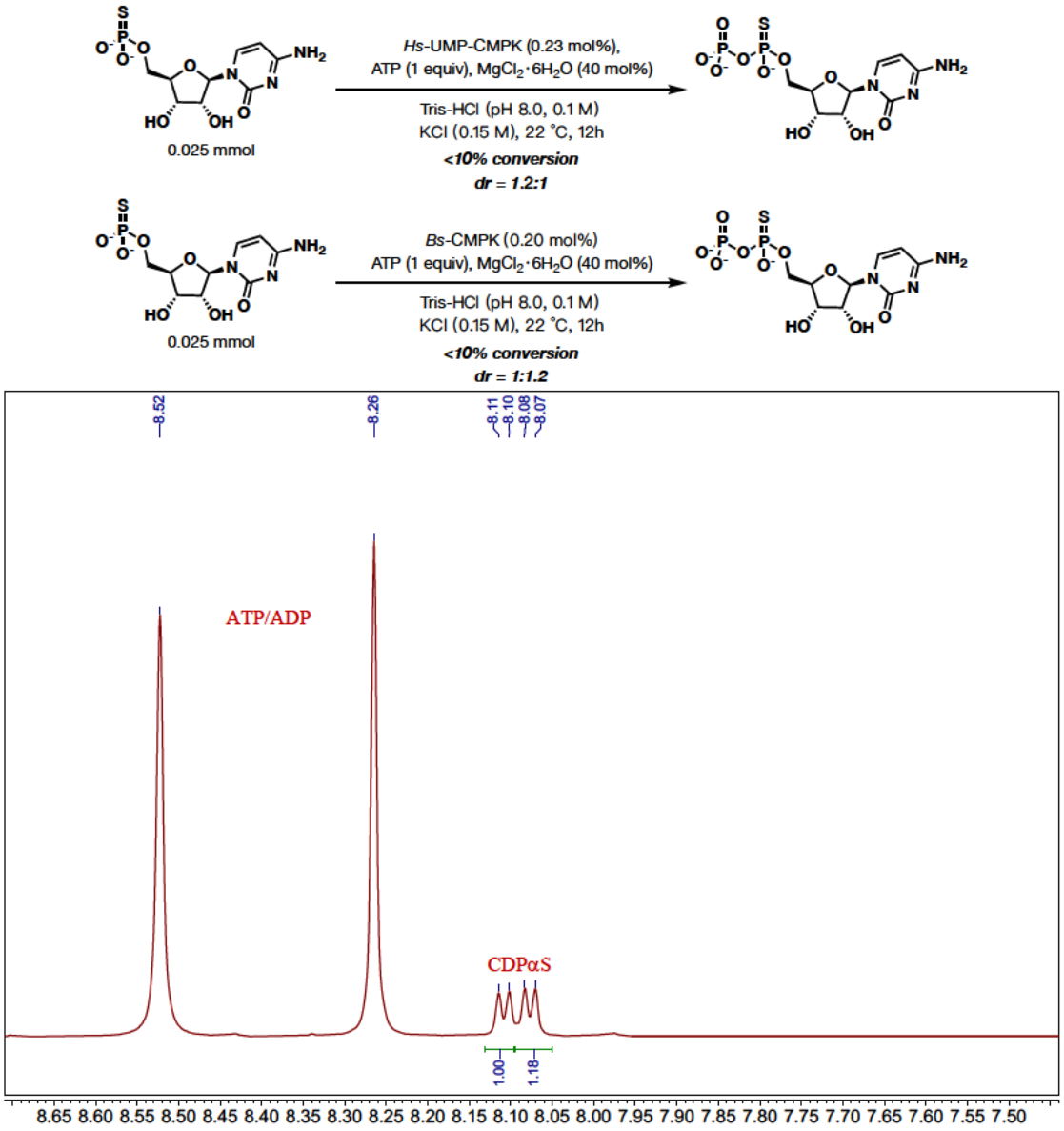


Figure S8. NMR of isolated CDPαS using *Hs*-UMP-CMPK.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

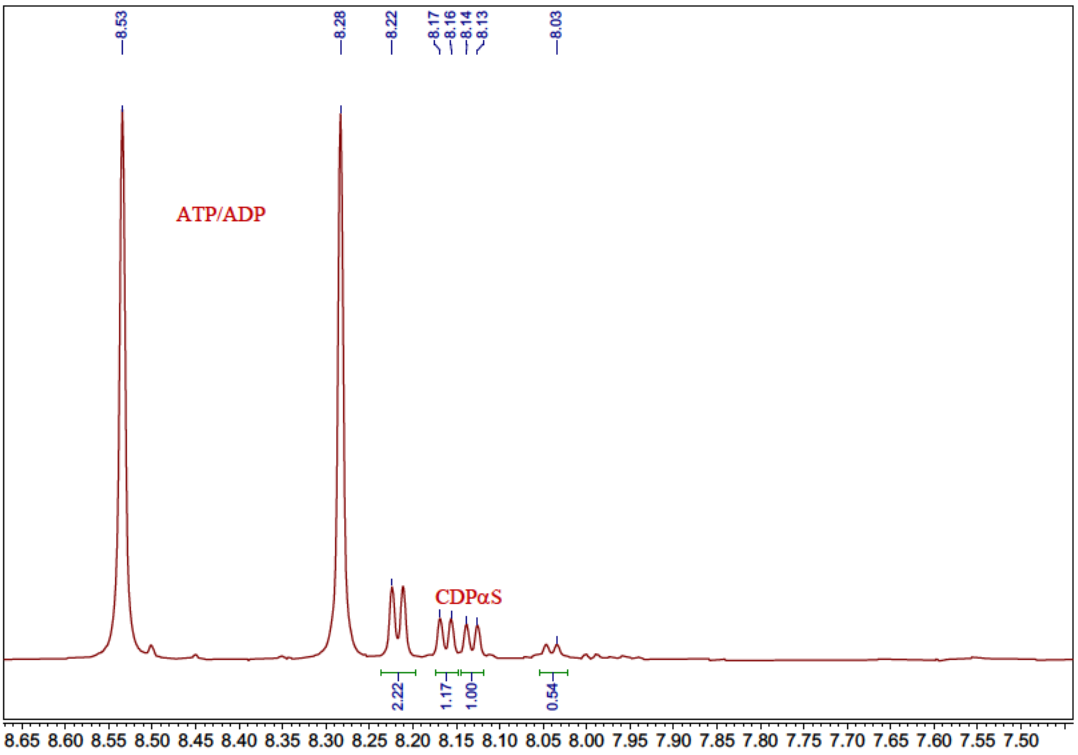


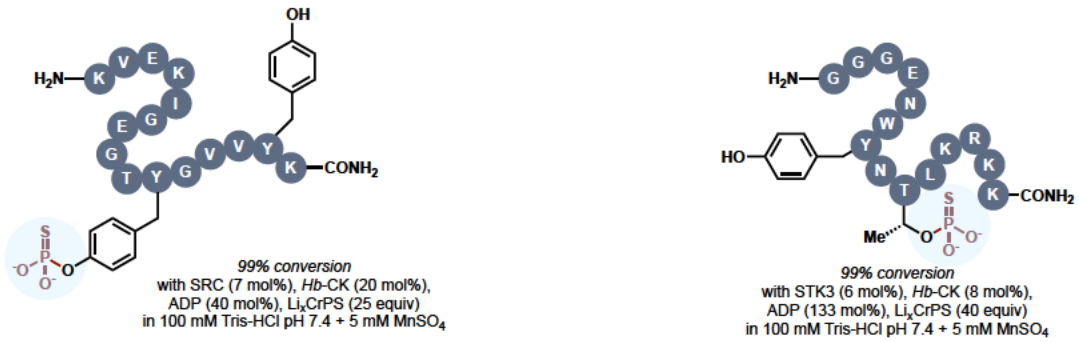
Figure S9. NMR of isolated CDPαS using *Bs*-CMPK. CMPS and CTPαS were also observed.

Overall, we believe the P-stereocenter is mainly determined by the monophosphate kinase (*Ec*-TmK, *Bs*-CMPK, etc.), but acetate kinase might also have minor effects on the final diastereoselectivity ratios observed in the NTPαS products.

Question 2: The authors should comment on/compare the efficiency of the different phosphorylation reactions performed. For example, under optimized conditions nucleoside phosphorylation required only 0.25 mol% CK, 5 mol% ADP and 1.2 equivalents of the LiXCrPS, while peptide phosphorylation required 8 mol% CK, 0.4 mM ADP and 40 equivalents LiXCrPS. Why did the peptide phosphorylation require such a large excess of LiXCrPS? The authors should also check the units for consistency – 0.4 mM ADP is used for one of the peptide examples, and 40 mol% for the other peptide making comparisons difficult.

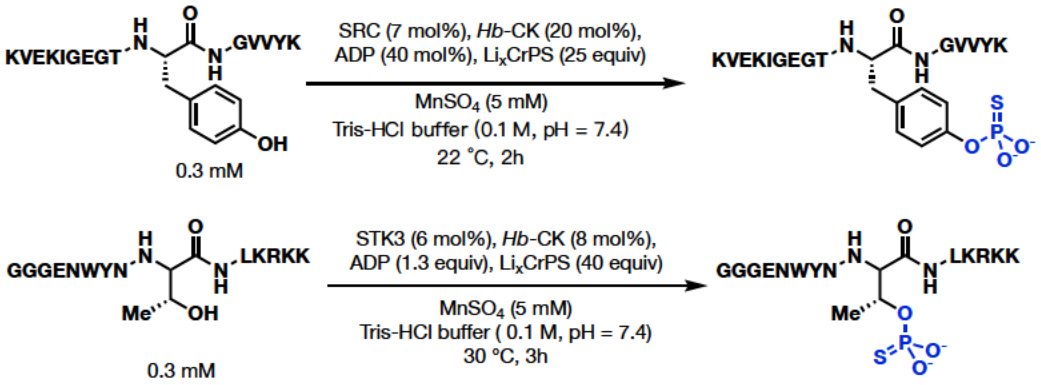
Response:

We have revised the units in Figure 5 for consistency. The revised figure is as follows:



Email hrenata@rice.edu | Office 713-348-8878 | Fax TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

The Supporting Information figures have also been revised to the following:



We also conducted additional experiments to address the question on the need for large excess of Li_xCrPS in peptide thiophosphorylation (see Figures S19–S22, which have been reproduced below). In contrast to the reactions with small molecules like nucleosides, the kinetics of protein kinase reactions are typically slower (see Table II in *Proteins: Struct., Funct., Bioinf.* **2013**, *81*, 568–582. for nucleoside kinase and Table1 in *Biochemistry* **1996**, *35*, 1533–1539. for tyrosine kinase with peptide substrates). Two conditions with lower enzyme/reagent loading were tested for peptide thiophosphorylation using GK-14 as the substrate.

Condition B (lower loading of enzymes): loading of STK3 was reduced to 0.25 mol%, *Hb*-CK was reduced to 0.35 mol%, other conditions remained unchanged.

Condition C (lower loading of reagents and enzymes): loading of STK3 was reduced to 0.25 mol%, *Hb*-CK was reduced to 0.35 mol%, ADP was reduced to 5 mol%, Li_xCrPS was reduced to 1.2 equiv, the concentration of Mn(II) and Tris-HCl buffer remained unchanged.

Both condition B and condition C showed that incomplete conversion of the peptide substrate. The results suggest the original higher loadings of enzymes and reagents are necessary, presumably due to the slower kinetics of these protein kinases compared to other kinases that act on small molecules.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

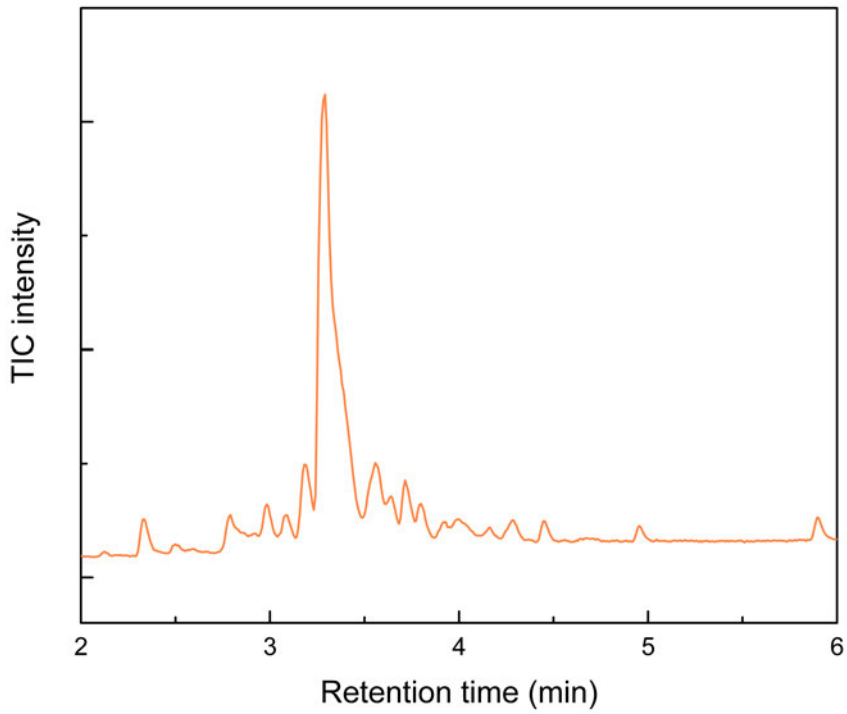


Figure S19. Chromatogram of modified peptide of GK-14 using condition B.

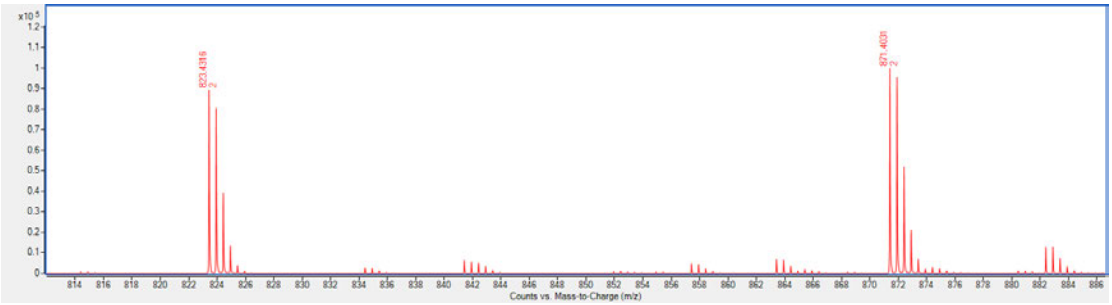


Figure S20. Mass extraction of GK-14 using condition B. Only doubly charged peptide was shown because the singly charged species has weak signals. Thiophosphorylated peptide (m/z =871.4031, two negative charges) and unreacted peptide (m/z =823.4316, two negative charges) can be observed.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

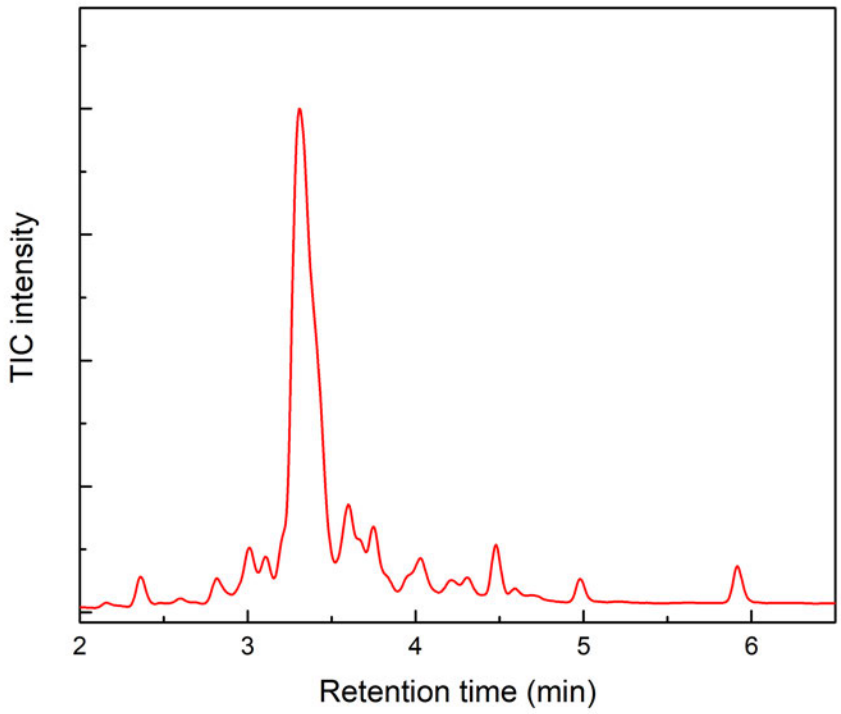


Figure S21. Chromatogram of modified peptide of GK-14 using condition C.

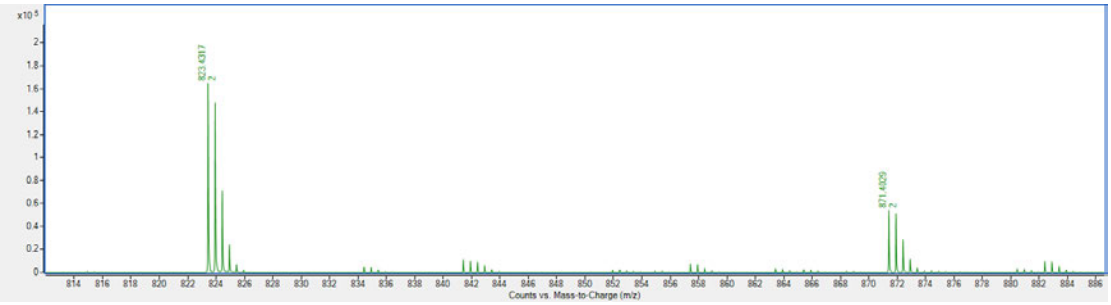


Figure S22. Mass extraction of GK-14 using condition C. Only doubly charged peptide was shown because the singly charged species has weak signals. Thiophosphorylated peptide (m/z =871.4029, two negative charges) and unreacted peptide (m/z =823.4317, two negative charges) can be observed.

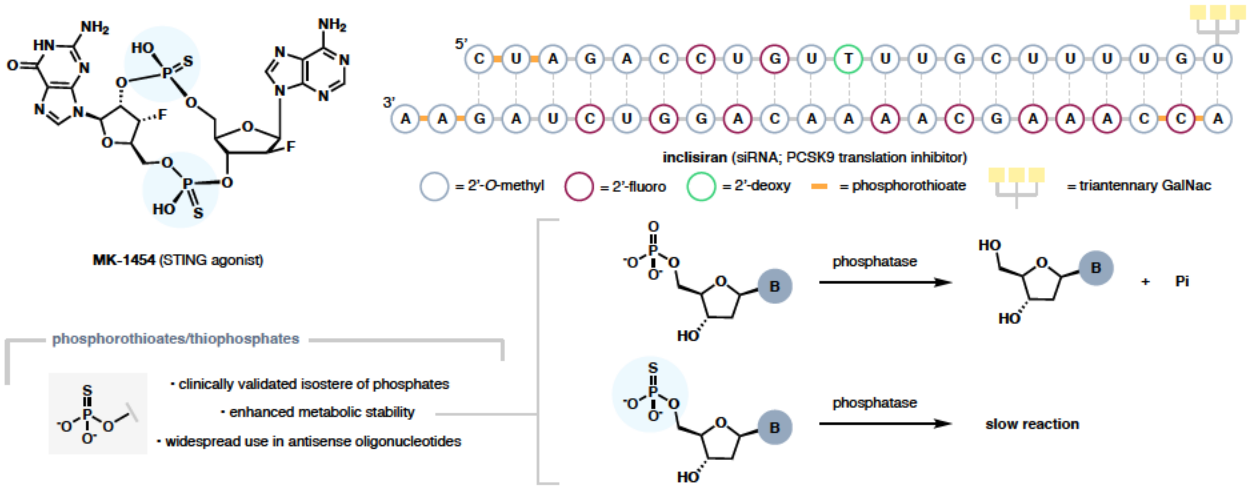
Note: due to the limited amounts of peptide materials, we were not able to run the SRC and STK3 reactions under identical molar equivalents. However, the point stands that higher enzyme loadings and reagent equivalents are needed to drive the reaction to completion.

Question 3: Figure 1A: The colour coding used for 2'-OMe and 2'-F is not clear and should be modified. The GalNAc conjugate is drawn in the scheme but not labelled. Phosphatases produce Pi and not PPi. It is not true that there is no reaction when phosphatases are incubated with thiophosphates. The reaction rate is potentially compromised but many phosphatases accept thiophosphate substrates.

Response:

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

We thank the reviewer for the suggestions. We have revised the figure and the text in Figure 1A to the following:



Question 4: Page 5, line 111: 'We hypothesized that the low formation of ATPγS was due to its instability and unfavorable equilibrium under the reaction conditions'. Do the authors mean that the product is chemically unstable or that the product is converted back to starting material?

Response:

We have performed additional experiments to provide more clarity to this issue (see Section 8.2 in the revised Supporting Information, which has been reproduced below).

We first investigated the stability of ADP, Li_xCrPS and ATPγS. In a 1.5 mL Eppendorf tube was charged:

1. ATPγS (Li^+ salt, 1.0 mg, 1.8 µmol), reaction buffer (200 µL, 0.1 M pH =8.0 Tris-HCl, 0.15 M KCl, 4 mM $MgCl_2$).

2. ATPγS (Li^+ salt, 1.0 mg, 1.8 µmol), Li_xCrPS (2.6 µmol, 5 µL from 0.52 M stock solution), reaction buffer (195 µL, 0.1 M pH =8.0 Tris-HCl, 0.15 M KCl, 4 mM $MgCl_2$).

3. ADP (K^+ salt, 0.8 mg, 1.8 µmol), reaction buffer (200 µL, 0.1 M pH =8.0 Tris-HCl, 0.15 M KCl, 4 mM $MgCl_2$).

4. ATPγS (Li^+ salt, 1.0 mg, 1.8 µmol), reaction buffer (200 µL, 0.1 M pH =8.0 Tris-HCl, 0.15 M KCl, 4 mM $MgCl_2$).

5. ATPγS (Li^+ salt, 1.0 mg, 1.8 µmol), D_2O (500 µL)

Tube 1 and 2 were incubated at 37 °C and 500 rpm for 2 hours, tube 3-5 were left at 22 °C for 2 hours. Internal standard (1.8 mmol, methyl phosphonic acid), 300 µL D_2O was added to each tube (except tube 5). The sample was then analyzed by NMR.

Results:

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

1. We observed that the signals of β- and γ-phosphorus atom of ATPγS become broad when NMR is taken in buffer solution containing Mg^{2+}.(**Figure S3**)

2. In the buffer containing Mg^{2+}, ATPγS was rapidly transformed into ADP and lost its terminal thiophosphate (10% ATPγS left at 37 °C, 30% ATPγS left at 22 °C).

3. ATPγS is sensitive to pH change; loss of thiophosphate was observed after addition of methyl phosphonic acid (tube 5). Addition of Li_xCrPS, which is stored in basic solution containing LiOH, also leads to significant change of chemical shifts (tube 3).

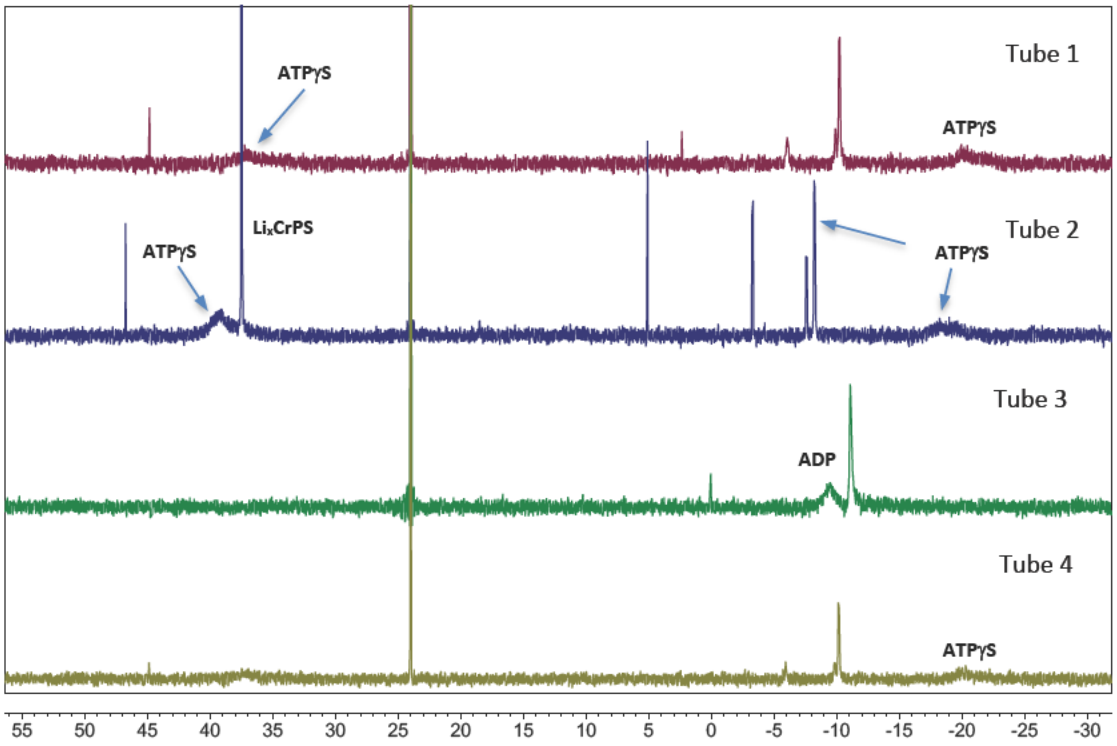


Figure S3. Stability studies of ATPγS. Methyl phosphonic acid is used as internal standard.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

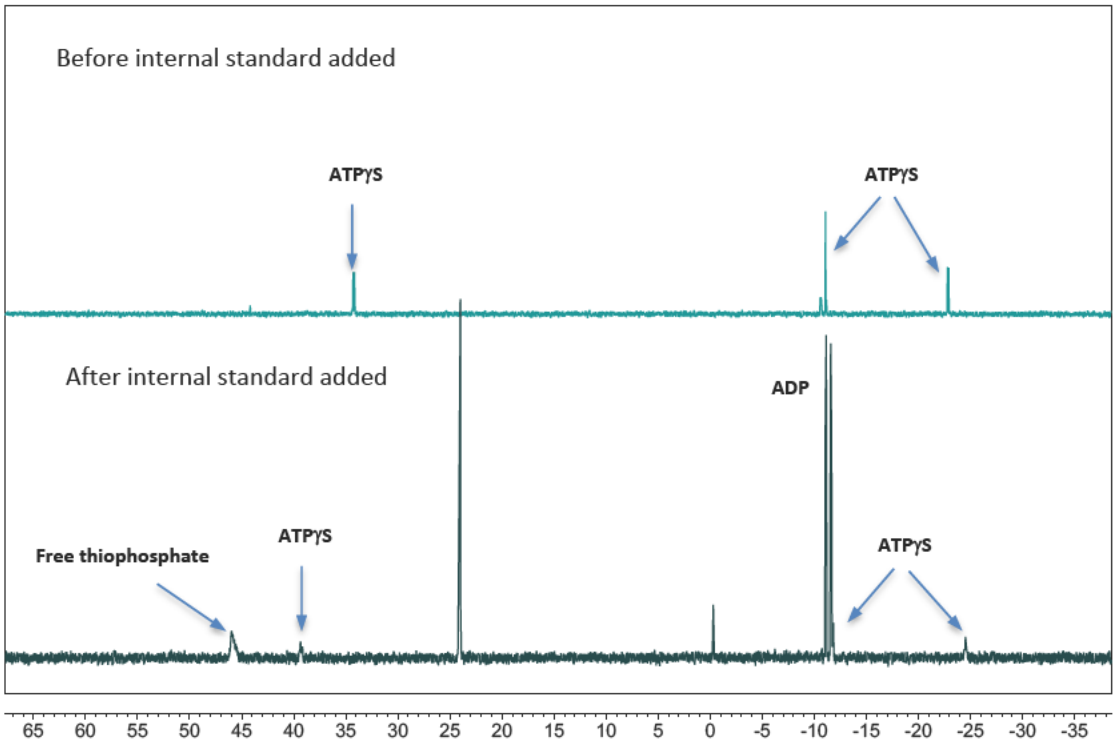


Figure S4. Comparison of ATPγS before and after addition of methyl phosphonic acid.

We then investigated the reactivity of *Hb*-CK with Li_xCrPS and ATPgS. In a 1.5 mL Eppendorf tube was charged:

6. ADP (K^+ salt, 0.8 mg, 1.8 µmol), Li_xCrPS (2.6 µmol, 5 µL from 0.52 M stock solution), *Hb*-CK (0.012 µmol, 10 µL from 50 mg/mL stock solution), reaction buffer (185 µL, 0.1 M pH =8.0 Tris-HCl, 0.15 M KCl, 4 mM $MgCl_2$).

7. ATPgS (Li^+ salt, 1.0 mg, 1.8 µmol), creatine (2.6 µmol, 50 µL from 52 mM stock solution), *Hb*-CK (0.012 µmol, 10 µL from 50 mg/mL stock solution), reaction buffer (140 µL, 0.1 M pH =8.0 Tris-HCl, 0.15 M KCl, 4 mM $MgCl_2$).

The mixture was incubated at 37 °C and 500 rpm for 2 hours, 300 µL D_2O was added to each tube. The sample was then analyzed by NMR.

Results:

The interconversion among ADP + Li_xCrPS and ATPγS + creatine in the presence of creatine kinase is reversible. The enzymatic reaction can reach similar equilibrium regardless whether it was started from the forward or reverse reaction.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

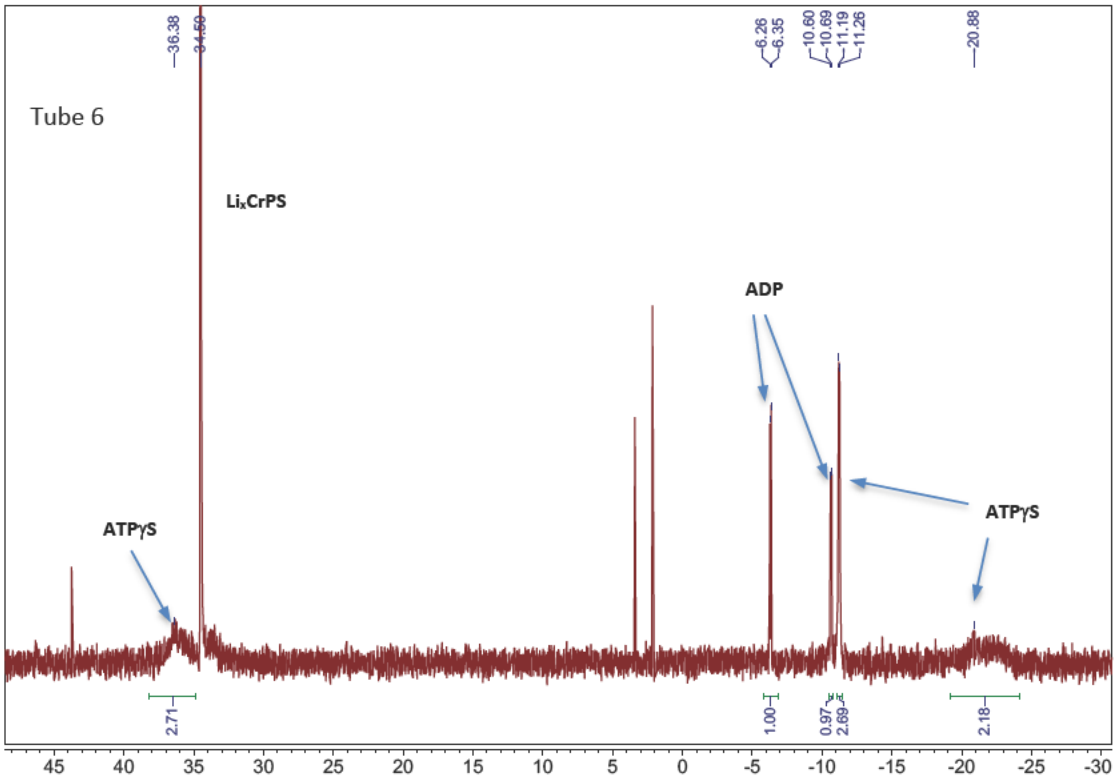
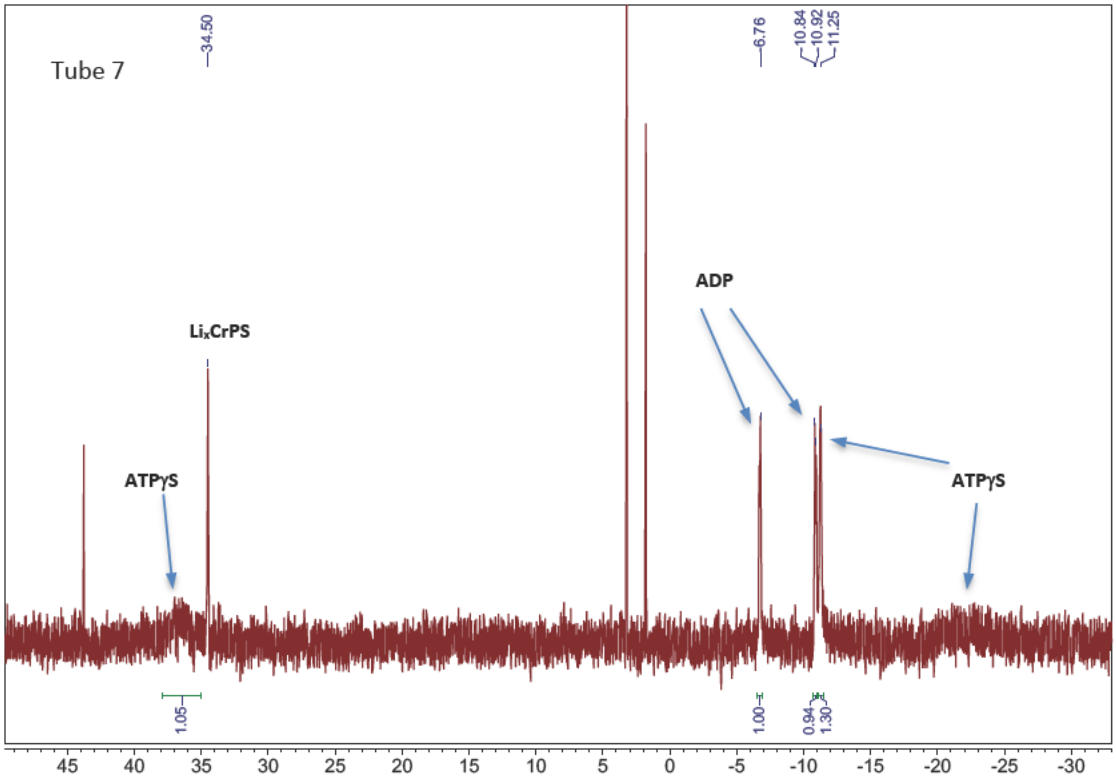


Figure S5. Reversibility studies of ATPγS regeneration system starting from ADP+ Li_xCrPS.



Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

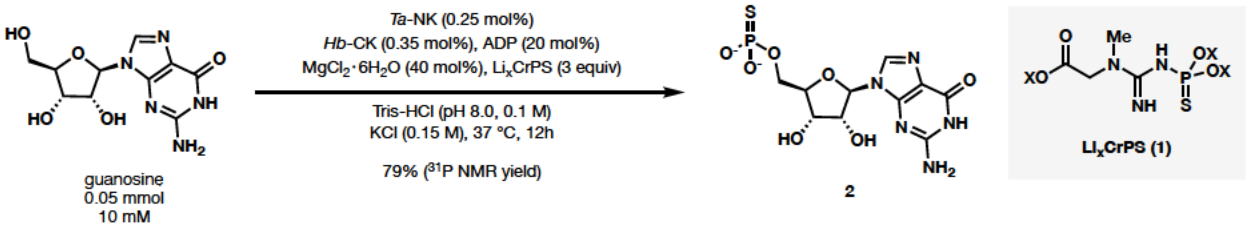
Figure S6. Reversibility studies of ATPγS regeneration system starting from ATPγS + creatine.

Taken together, these additional experiments support our hypothesis on the instability of ATPγS under the reaction and analytical conditions and on the reversible nature of the creatine kinase reaction with Li_xCrPS.

Question 5: What was the conversion under the reaction conditions written in the scheme – this entry is missing from the table. What was the rationale for selecting the enzyme concentrations used.

Response:

The conversion of guanosine is >95% (except for entry 11) based on crude NMR. Table 1 and Table S4 have been revised to include this information. The initial concentration of enzyme was chosen based on experience—roughly 0.2–0.4 mol%, which is also common in many biocatalysis papers, including ours.

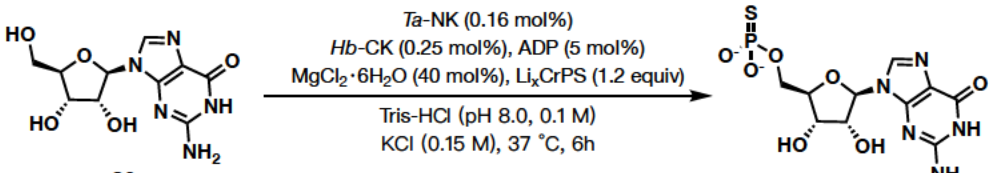


entry	variation from standard conditions	^{31}P NMR yield	conversion
1	10 mol% ADP	83%	>95%
2	2 mol% ADP + 1.2 eq. Li_xCrPS	80%	>95%
3	0.16 mol% *Ta*-NK + 0.25 mol% *Hb*-CK	82%	>95%
4	entry 3 + 5 mol% ADP + 1.2 eq Li_XCrPS	81%	>95%
5	6 h instead of 12 h	80%	>95%
6	0.04 mol% *Ta*-NK + 0.063 mol% *Hb*-CK	74%	>95%
7	*Hm*-CK instead of *Hb*-CK	77%	>95%
8	*Rm*-CK instead of *Hb*-CK	74%	>95%
9	*Mt*-NK instead of *Ta*-NK	72%	>95%
10	*Mj*-NK instead of *Ta*-NK	57%	>95%
11	no enzymes	<2%	<2%

NMR yield was determined by quantitative ^{31}P NMR using methyl phosphonic acid as internal standard.
Conversion was determined by ^{1}H NMR using methyl phosphonic acid as internal standard.

To fully address the question, we also carried out additional control experiments.

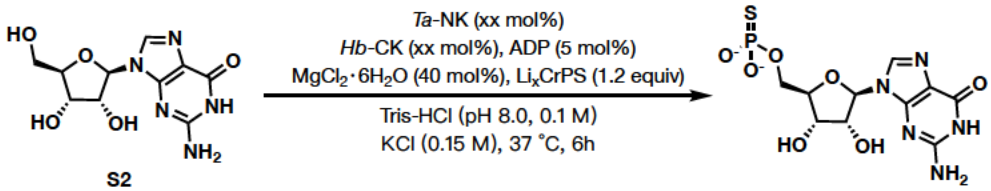
Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu



S2
0.05 mmol

entry	Concentration of S2	^{31}P NMR yield
1	10 mM	79%
2	20 mM	78%
3	5 mM	79%

Concentration of the substrate does not affect yield significantly when other reaction conditions remained identical.



S2
0.05 mmol
20 mM

entry	enzymes	^{31}P NMR yield
1	*Ta*-NK (0.08 mol%)+*Hb*-CK (0.125 mol%)	79%
2	*Ta*-NK (0.04 mol%)+*Hb*-CK (0.0625 mol%)	39%

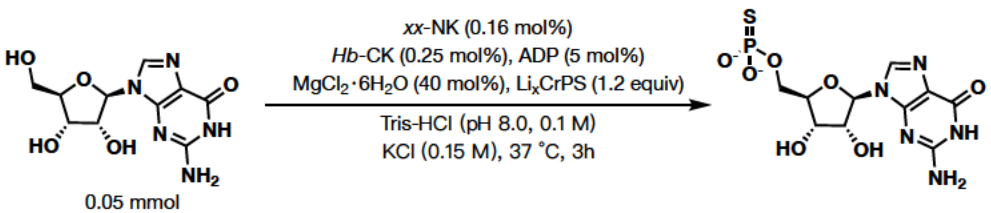
At a higher concentration, the loading of the enzyme could be reduced to half of the original loading without significant drop of yield, but further reducing the enzyme loading led to inefficient reaction. While these additional experiments suggested that the kinase loadings could be lowered for reaction with **S2**, we believe that the final concentrations used in the substrate scope study represent an ideal middle ground to account for substrates that have poor reactivity.

Question 6: The authors state that NKs from different organisms led to lower reactivity but they are only comparing the final conversions which are not so different. To give a fair comparison of reactivity the authors should provide a time course for the reaction and compare the initial rate.

Response:

We thank the reviewer for the suggestion. In response to this suggestion, we carried out a time course study of three different NKs (see Section 8.1, Table S14 and Figure S2 in the revised Supporting Information, which has been reproduced below). The results suggested *Ta*-NK and *Mt*-NK have similar kinetic, while *Mj*-NK has the highest initial reaction rate. All three reached similar conversion and yield after 1 hour, though *Ta*-NK is slightly better than the other two at the end of the 3 h reaction. The lower yield of *Mj*-NK observed in the initial screening (entry 10 in Table1) might be because of experimental error or some unexpected decomposition during a longer reaction time.

Table S14. Time course study of thiophosphorylation of guanosine using different NKs.



Time (min)	*Ta*-NK(%)	*Mj*-NK(%)	*Mt*-NK(%)
0	0	0	0
5	3	43	7
10	11	70	13
15	24	70	21
20	32	70	33
30	44	70	44
60	69	70	70
120	73	71	73
180	80	73	77

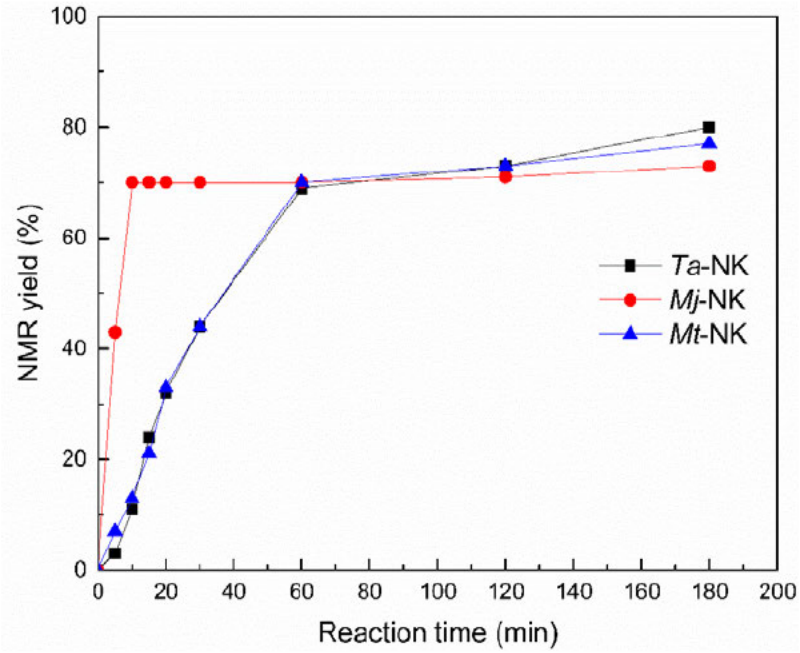


Figure S2. Time course study of thiophosphorylation of guanosine with different nucleoside kinases.

Based on these results, we believe under current enzyme concentration, there is only minor reactivity difference among three NKs. We have revised our original description to the following:

"Minor reactivity differences were observed with nucleoside kinases from other organisms (entry 7–10)."

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

Question 7: 'During this exploration, we also uncovered the catalytic utility of several enzymes that have never been tested as biocatalysts previously and identified their preferred substrates. Three nucleoside kinases, namely Ag-dNK, Dm-dNK and Tm-TK, were found to be especially useful as they display broad promiscuity and tolerance toward a range of structural variations on their substrates.' It wasn't clear which enzymes had not been previously tested and what the preferred substrates were? Dm-dNK and Tm-TK have been previously exploited as biocatalysts (J. Biol. Chem. 2023, 299, 104746; Adv. Synth. Catal. 2014, 356, 563).

Response:

To prevent any ambiguity, we have revised the above sentences to the following:

"During this exploration, three nucleoside kinases, namely *Ag*-dNK, *Dm*-dNK and *Tm*-TK, were found to be especially useful as they display broad promiscuity and tolerance toward a range of structural variations on their substrates."

Question 8: 'In many cases, thiophosphate transfer catalyzed by diphosphate kinases was found to be reversible, which resulted in stalled reaction conversion after the first two hours. This observation was attributed to the intrinsic phosphatase reactivity of these kinases.' The kinase reverse reaction is phosphoryl transfer not hydrolysis.

Response:

We thank the reviewer for pointing out this oversight. The last sentence has been removed and the section now reads:

"In many cases, thiophosphate transfer catalyzed by monophosphate kinases was found to be reversible, which resulted in stalled reaction conversion after the first two hours."

Question 9. There is no mention of nucleoside monophosphate kinases in the text which makes it confusing. The text reads 'in the second step of these reactions, acetate kinase performs two roles: (1) delivering the second phosphate motif and (2) regenerating ATP'. However, the second phosphate is delivered by the NMPK, the AcK provides the gamma phosphate and regenerates the ATP used by the NMPK.

Response:

We thank the reviewer for pointing this issue out. The following sentence has been added to the revised manuscript:

"The second step of these reactions involves a cascade with two enzymes, namely monophosphate kinase to install the β-phosphate and acetate kinase to deliver the γ-phosphate in the presence of acetyl phosphate while also regenerating ATP for the monophosphate kinase."

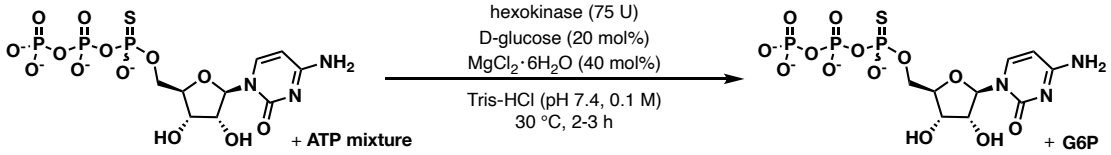
Question 10: Interestingly, we found that the diastereoselectivity in this sequence was significantly improved during the removal of residual ATP (see SI for details), which might be attributed to selective consumption of one diastereoisomer by hexokinase. This should be tested.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

Response:

The suggested experiment has been conducted (see Table S15 and Figures S10 and S11 in the revised Supporting Information, which have been reproduced below). Time course study of hexokinase reaction with CTPαS diastereomers and ATP suggested that ATP is first consumed and the minor isomer of CTPαS can be slowly consumed after all ATP has been consumed. The gradual increase of dr support our original hypothesis.

Table S15. Time course study of dr and removal of ATP in CTPαS using hexokinase.



Time (min)	CTPαS (major)	CTPαS (minor)	dr	ATP
0	62%	23%	2.5:1	8%
5	58%	14%	4.5:1	<2%
10	58%	12%	4.8:1	<2%
15	61%	12%	5.1:1	<2%
20	60%	11%	5.5:1	<2%
30	56%	10%	5.5:1	<2%
40	59%	9%	6.5:1	<2%
50	60%	9%	6.7:1	<2%
60	58%	8%	7.3:1	<2%
180	60%	6%	10:1	<2%

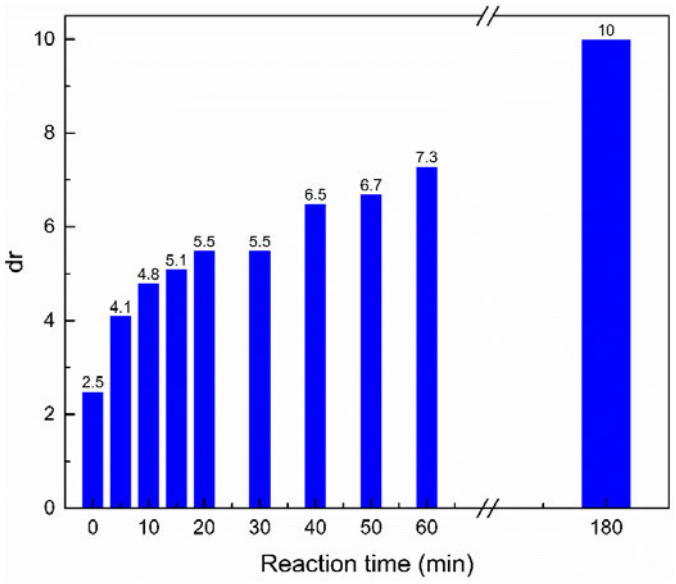


Figure S10. Time course study of dr change for CTPaS using hexokinase.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

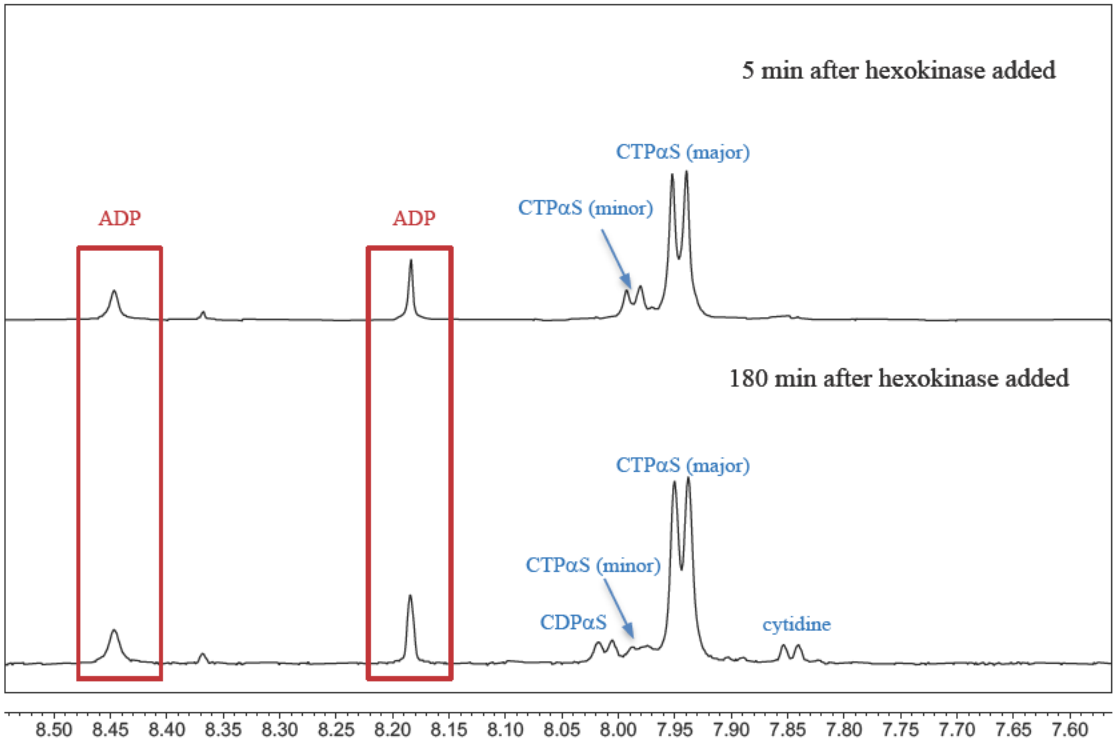
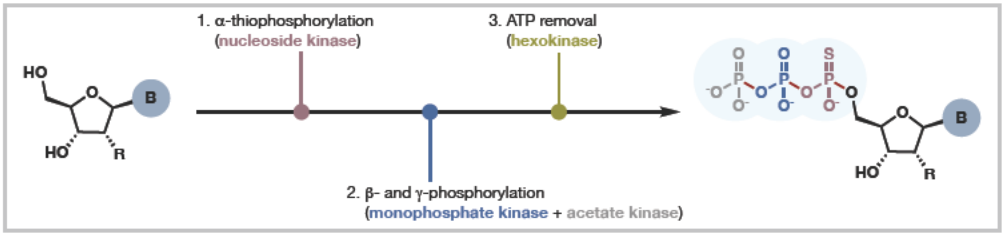


Figure S11. NMR of dr change of CTPαS after hexokinase was added t = 5 min (top) and t =180 min (bottom).

Question 11: Figure 3B: The term 'enzymatic purification' is misleading. The enzyme was used to degrade the residual ATP prior to chromatographic purification.

Response:

We changed the term to "ATP removal". The revised figure is as follows:

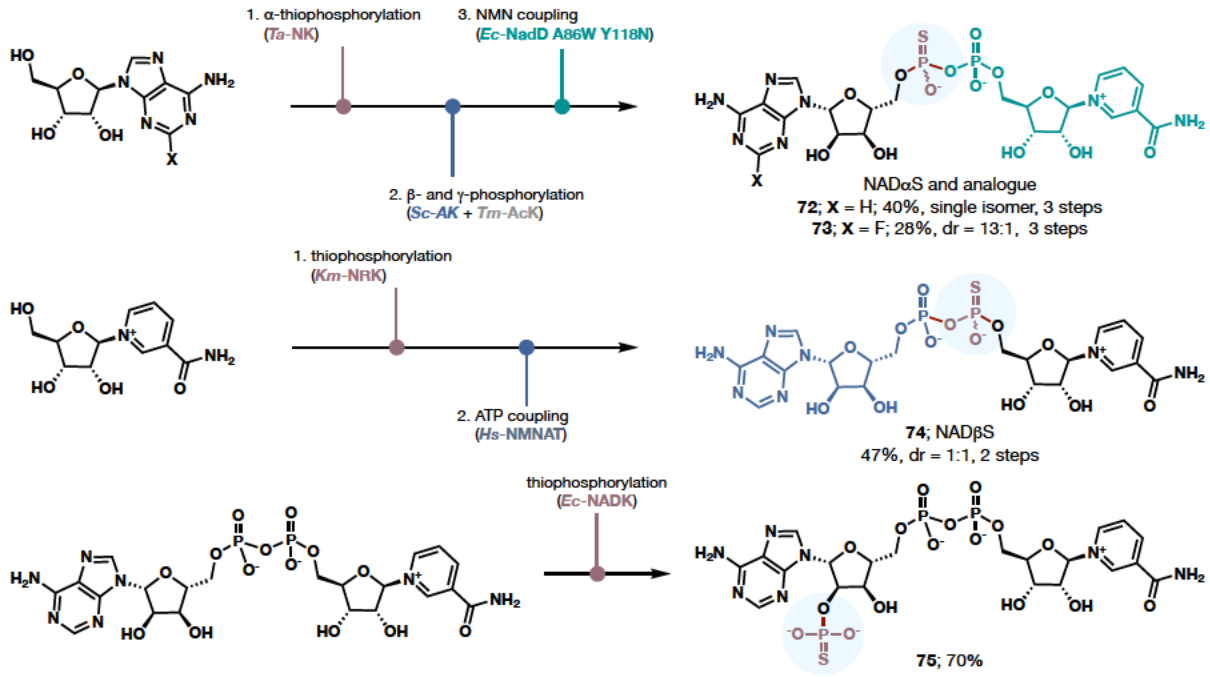


Question 12. Figure 4C: The names of the enzymes used should be provided for clarity.

Response:

Figure 4C has been revised to the following:

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu



13. The reaction conditions are missing from 5B.

Response:

We thank the reviewer for pointing this out. Unfortunately, it is not possible to provide the exact stoichiometry of this reaction, which was adapted from the recommended protocol in the commercial T4 PNK kit (NEB #M0201), as the exact molarity of T4 PNK was not provided by the vendor (the concentration was provided as 10,000 units/mL where one unit is defined as catalyzing the phosphorylation of 20 pmol of oligo in 30 min at 37 ºC).

14. Table S5: what does pdt mean?

Response:

The term "pdt" has been revised to "products" to avoid ambiguity and confusion.

Referee #2

A. Summary of key results

Renata & colleagues present a novel thiophosphorylating reagent and a thorough screen of enzymes for establishing a novel biocatalytic reaction manifold for site-selective thiophosphorylation. The work is thorough and comprehensive in its treatment of both the benefits and limitations of the method. The work is reminiscent of Baran's work on novel reagents for synthetic chemistry applications as well as several of Whitesides's classic works on novel enzyme catalyzed reactions useful in synthesis, and in that regard

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

represents a milestone for the field of biocatalysis as it combines advances in synthetic chemistry and enzymology to synthesize previously inaccessible compounds—particularly interesting in this regard are the thiophosphorylated proteins. The supplementary material is comprehensive and thorough.

B: Originality and significance

The reagent described and overall approach to the synthesis of ATPγS is clever and novel. The authors show clearly that the method has broad applicability to a variety of useful molecules.

Response:

We thank the reviewer for their strong support of our work and insightful suggestions.

Question 1: One point regarding the SI is that although the authors state that kinases were Ni-NTA purified, I note that the coding sequences provided in the SI lack his-tags excepting SRC and STK3. Can the authors provide the actual coding sequences for the constructs used including tags? They state elsewhere that genes were cloned into pET28, so it may be presumed that the final constructs have the expected pET28 N-terminal his-tag, though that raises some ambiguity for SRC and STK3 which have different N-terminal his-tag sequences than I would expect for pET28.

Response:

With the exception of SRC and STK3, all of our kinase plasmids were obtained via DNA synthesis by Twist Bioscience wherein the encoding genes were inserted between the NdeI and XhoI restriction sites within the commercial pET28a(+) vector. On the other hand, the expression plasmids for SRC and STK3 were obtained from Addgene. This information has now been made clearer in the revised Supporting Information. We decided to provide the include the His10 tag and the TEV site for SRC and STK3 because of the non-standard plasmids used and the omission of several N-term residues in the final constructs. To address any ambiguity for these two plasmids, we have also provided their full plasmid sequences.

Question 2: The authors mention in passing that they were unable to prepare ATPγS from ADP and presume this to be due to instability and unfavorable equilibrium. However, their control reactions with ADP and phosphocreatine are also low-yielding as in Table S2. In view of the reported favorable Keq for the phosphorylation of ADP using phosphocreatine (e.g. JBC Volume 254, Issue 14p6528-6537July 1979), this result does not make sense. I would recommend that the authors collect more thorough and convincing data on this point, including kinetic assays of phosphorylation to better understand the relative rates of phosphorylation versus thiophosphorylation. Alternatively, obtaining commercial ATPγS and subjecting it to the reaction conditions could shed light on the hypothesized instability. Providing at least some further details as to the specific issue (instability or thermodynamics) would be helpful even if this point is ultimately difficult to resolve entirely. This aspect could be relevant for later investigators in that understanding the likely steady-state level of ATPγS that can be formed may be relevant when considering the Km of kinases employed.

Response:

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

We thank the reviewer for the questions and suggestions. Additional experiments have been performed to provide some preliminary evidence for the instability of ATPγS and the reaction equilibrium. Please see our response to Reviewer 1's Question 4 and Figure S3–S6.

Question 3: Do the authors know if the creatine thiophosphate reagent can be taken up into cells (bacterial or mammalian) and used to thiophosphorylate substrates in cells? This could be a valuable thing to investigate in regards to potential future applications, though it may be better left to follow-up reports.

Response:

We thank the reviewer for the suggestion. At present, our creatine thiophosphate reagent is stored in basic solution. Based on our experiments, the stability of this reagent is not ideal for use in physiological conditions. One of the future directions of this work is to develop strategies to address this incompatibility, for example through further derivatization in a pro-drug-like approach.

Question 4: The introduction feels a bit lengthy. If the authors could reduce the details of the various drugs and investigational compounds described this might economize for length—I think the general point that phosphorothioates are an important class of compounds can be made more succinctly.

Response:

We thank the reviewer for the suggestion. We have streamlined the sections in question to make the manuscript more concise.

Reviewer #3

In this submission, Xiangyu Wu, Yu Fu, and Hans Renata describe a practical biocatalytic strategy for thiophosphorylation by designing a novel sacrificial thiophosphate donor, creatine thiophosphate (LixCrPS), that works in concert with creatine kinase to enable catalytic turnover of ATPγS. When partnered with a large set of kinase enzymes, this system allows for the biocatalytic synthesis of a wide range of phosphorothioate analogues of (i) both natural and unnatural nucleoside monophosphates, (ii) nucleoside (β-thio)diphosphates, (iii) nucleoside (α-thio)triphosphates, (iv) phosphosugars; (v) phospho-inositols and (vi) nicotinamide cofactors (NAD+ and NADP+) with control of phosphorothioate placement.

Moreover, the Rice team also showcase the methodology for the thiophosphorylations of intermediate-sized peptides and of proteins and nucleic acids, albeit in an as yet limited fashion. The peptide studies are quite well-behaved. On the other hand, in studies on the autophosphorylation domain for the STK3 (serine/threonine kinase 3), the authors uncover the challenge of seeking to achieve autothiophosphorylation in competition with the presumably kinetically faster simple autophoshorylation reaction. The authors are commended for the detailed mass spectrometric analysis of the modified STK3 samples to provide a clear description of the nature of the challenge here, and to show that one can effectively analyze the microheterogeneity of such mixed thiophosphorylated/phosphorylated proteins. By going beyond the challenging, but still much simpler domain of thiophosphorylated nucleotides, sugars and

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

cofactors into the complex domain of protein thiophosphorylation, the Rice team has appropriately taken quite a leap to show the relevance/applicability of the methodology for studies in biomacromolecular systems! Much more needs to be done here, but addressing the challenge and raising some of the issues for the field going forward is entirely appropriate for such a piece for a Nature audience.
The core concept put forward here draws direct inspiration from the well-established creatine phosphate/creatine kinase system for ATP regeneration in enzymatic synthesis (P.G. Wang group – ref. 39) and successfully adapts this system for S-ATP analogue synthesis for the first time. It is also worth noting that Baran and coworkers have previously reported the chemical synthesis of thiophosphate-containing nucleoside di- and triphosphate donors using their P(V) platform (ref. 20), that employs carefully designed chiral thiophosphorylation agents with appropriate protecting groups. The present enzymatic approach offers a complementary and more operationally simple route to access similar thiophosphate-containing molecules under mild, protecting-group-free conditions.

Most importantly, the present work marks a real milestone in establishing the first viable catalytic S-ATP regeneration scheme, and this will capture the attention of members of the biocatalytic, chemical biology and biomedical research communities, given the importance of phosphorothioates in studying signal transduction, enzyme mechanism, and in developing chiral phosphorothioate drug candidates such as MK-1454, and phosphorothioate-based anti-sense nucleic acids. The implications of this work are significant. The authors achieved this breakthrough by developing an efficient and economical synthesis of the thiocreatine phosphate donor (LixCrPS) and employing this reagent to achieve thiophosphorylation, across a wide range of biomolecular scaffolds from nucleoside phosphates, to sugar phosphates, NAD(P)-cofactors to thiophosphorylated peptides, proteins and nucleic acids. To achieve this, the authors have demonstrated broad compatibility with a wide range of kinases. The optimization studies presentedin both the manuscript and SI are thorough, clearly establishing entry 4 as optimal with as little as 0.16 mol% Hb creatine kinase loading and 1.2 equivalents of LixCrPS.

Response:

We thank reviewer 3 for their strong support of our work. All the analyses and suggestions are intriguing and insightful, and have significantly improved the quality of our work.

All that said, the manuscript as written is quite dense and difficult to read. Here are some areas in which the manuscript could be significantly improved, both to discuss the most important general concepts that underlie this work, and to make the paper more understandable/readable.

*Question 1: …Good examples of manuscripts that do this include a nice paper on ATP regeneration [Zhang, J. et al. Organic Letters. **2003,** 5, 2583–2586 (see Table 2 in this paper)] and another on NADH regeneration; See Broussy, S. et al. Organic Letters **2009**, 11, 305-308 (See Figure 1 in this paper). Consider citing these papers when discussing such an essential thermodynamic analysis of the problem at hand.*

Here is the sort of table that might be included in this Nature-directed manuscript (all values are given for the actual phosphoryl system, relative to a ΔG'(hydrolysis) ~ -7.7 kcal/mol for ATP itself). If the authors, can find numbers for the thiophosphoryl system, they can include. If not, this thermodynamic hierarchy

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

serves to give the viewer an overview of what enzymatic systems and donors might be considered to solve this problem. The authors only discuss the final two systems in the paper, not mentioning the other four presented here even though the Whitesides team has already demonstrated proof of principle for the use of a thermodynamically better thiophosphoryl donor, S-1,3-diphosphoglycerate, even if it is generated in situ:

(Thio)Phosphoryl Donor	~ΔG'(Hydr)	Enzyme	Reference	Donor Cost
Phosphoenol Pyuvate	-13.8 kcal/mol	PEP Kinase		
1,3-Diphosphoglycerate	-12 kcal/mol	1,3-Di-P-Glycerate Kinase	*Whitesides (S-P)*	
Carbamoyl Phosphate	-12 kcal/mol	Carbamate Kinase		
Arginine Phosphate	-10 kcal/mol	Arginine Kinase	*Whitesides*	
Creatine Phosphate	-10 kcal/mol	Creatine Kinase	*Wang/this paper (S-P)*	
Acetyl Phosphate	-10 kcal/mol	Acetate Kinase	*multiple, incl. this paper*	

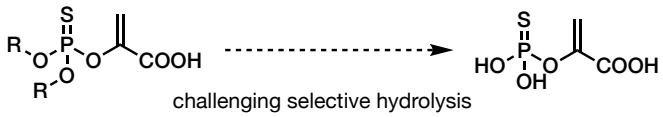
Response:
We thank the reviewer for providing the above table, which has been added to the Supporting Information with the appropriate adjustments as Table S1 and reproduced below.

Table S1. Comparison of different phosphate donors in biological system.

Phosphoryl donor	ΔG (kcal/mol)	Related kinase	Donor Cost	Reference
Phosphoenol pyruvate	-14.8	PEP kinase	$82/mmol	[2]
1,3-bisphosphoglycerate	-11.8	BPG kinase	>$500/mmol	[3]
Carbamoyl phosphate	-12.3	Carbamate kinase	$85/mmol	[4]
Arginine phosphate	-7.7	Arginine kinase	>$500/mmol	[5]
Acetyl phosphate	-10.3	Acetate kinase	$35/mmol	[2,6]
Creatine phosphate	-10.3	Creatine kinase	$15/mmol	[2]

We actually considered the thiophosphate counterparts of several known phosphate donors in biological systems. The choice was made based on thermodynamic properties, availability of corresponding kinases, complexity of regeneration system and prices of donors.

1.Thiophosphoenol pyruvate (thio-PEP): the preparation of thio-PEP is synthetically challenging as the selective hydrolysis of protecting group while keeping the pyruvate motif is not a trivial issue. We were also concerned by the stability of this reagent due to its enol ether motif.

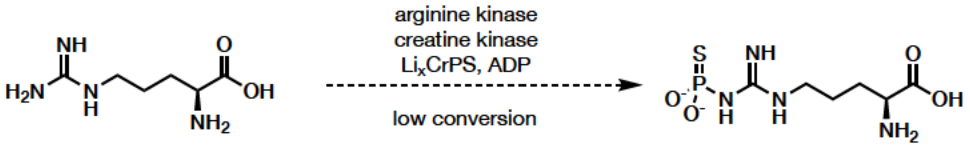


2. Thio-1,3-diphosphoglycerate ((thio)-1,3-BPG): the enzymatic synthesis of (thio)-1,3-BPG was considered at the beginning. However, based on Whitesides' system[6], several expensive enzymes are required, and immobilization of these enzymes are beyond our expertise.

3. Carbamoyl thiophosphate: The synthesis could be challenging because of the good leaving group ability of the carbamoyl motif.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

4. Arginine thiophosphate: We think selective functionalization of arginine's guanidine to install only one thiophosphate group would require multi-step synthesis and careful reverse phase chromatography. This would affect the practicality of the overall transformation. The free energy of arginine phosphate hydrolysis is slightly better than ATP, as such, the overall equilibrium of the thiophosphate transfer might be favored. We later found the arginine thiophosphate cannot be generated when arginine was reacted with creatine thiophosphate and ADP in the presence of creatine kinase and arginine kinase, suggesting arginine thiophosphate might be a thiophosphoryl donor with more favorable thermodynamic properties but also more challenging to use.



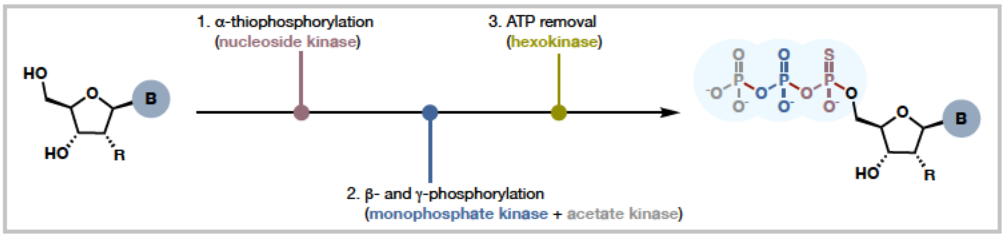
Considering the above, we think acetyl thiophosphate and creatine thiophosphate are the most practical donors and we focused on their synthesis.

We have provided the above discussions in our updated supporting information as Section 4.1

Question 2: In describing the sequential biocatalytic thiophosphorylation/phosphorylation events in the figures in the paper, particularly ***Figures 3 and 4****, the reader is faced with a rather abbreviated description of the enzymes and conditions used to form each bond. What is needed is a clear indication in each scheme of which enzyme is used to form each bond. Color-coding (such as the blue, maroon and teal below for the α-, β- and γ-P positions, for example) would be very helpful in this regard, as would including each enzyme used in the manuscript figures so the reader can follow the biocatalytic synthesis.*

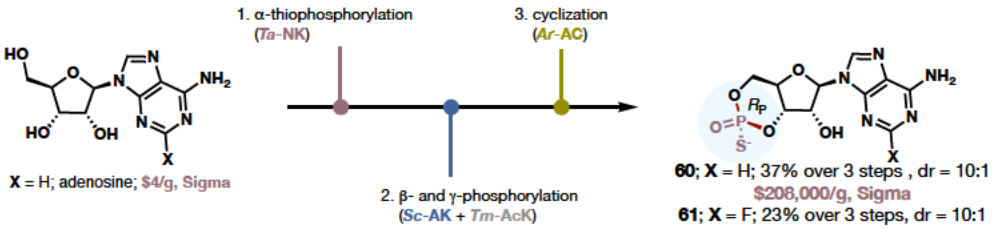
Response:

Per the reviewer's suggestions, we have revised the corresponding figures to make the presentation clearer. The heading of Figure 3B has been revised to the following:



Revised Figure 3C:

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu



Finally, please refer to our response to Reviewer #2 for the new version of Figure 4C.

In this regard, the Supporting Information is much more helpful, particularly ***Methods A-H*** *(pages 46-52 of the SI). But even here there is some confusion. A good example is* ***Method C****. Here the procedure for the biocatalytic synthesis of α-S-nucleoside triphosphates is described.* ***(i)*** *The initial α-thiophosphorylation is performed with nucleoside kinase, Hb creatine kinase, stoichometric LixCrPS and catalytic ADP;* ***(ii)*** *the β-phosphorylation is then performed with what one might expect to be a "nucleoside monophosphate kinase." The scheme itself identifies this enzyme as "monophosphate kinase" above the arrow, whereas the text below describes the enzyme as "nucleoside diphosphate kinase." In Figure 3 in manuscript, this enzyme is not even mentioned as far as I can tell. This needs to clarified so that this work can be repeated. Please give the enzyme and source for each step unambiguously, in both the SI and in* ***Figures 3 and 4****.*

Response:

We have revised the Supporting Information and the Figures accordingly.

(iii) *the γ-phosphate is apparently installed through the action of acetate kinase with acetyl phosphate as stoichiometric phosphoryl donor. Is this correct? If so, please state this explicitly in the text and the SI.*

Response:

The requested change has been made to the manuscript. The revised text reads:

"The second step of these reactions involves a cascade with two enzymes, namely monophosphate kinase to install the β-phosphate and acetate kinase to deliver the γ-phosphate in the presence of acetyl phosphate while also regenerating ATP for the monophosphate kinase."

Question 3: In cases where a bridging phosphorothioate is generated, two diastereomers are possible at the phosphorus stereocenter. In nearly all cases, the authors demonstrate experimentally a high diastereopreference for one stereochemistry at phosphorus. The sole exception appears to be the NAD$^+$ β-S-analogue, ***74****, where a 1:1 ratio of diastereomers is obtained. More discussion is needed. Here are some of the questions:*

Is the generally high diastereoselectivity at P expected, given the enzyme employed and what is known in the literature?

Why is the NAD$^+$ β-S-analogue case different?

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

Response:

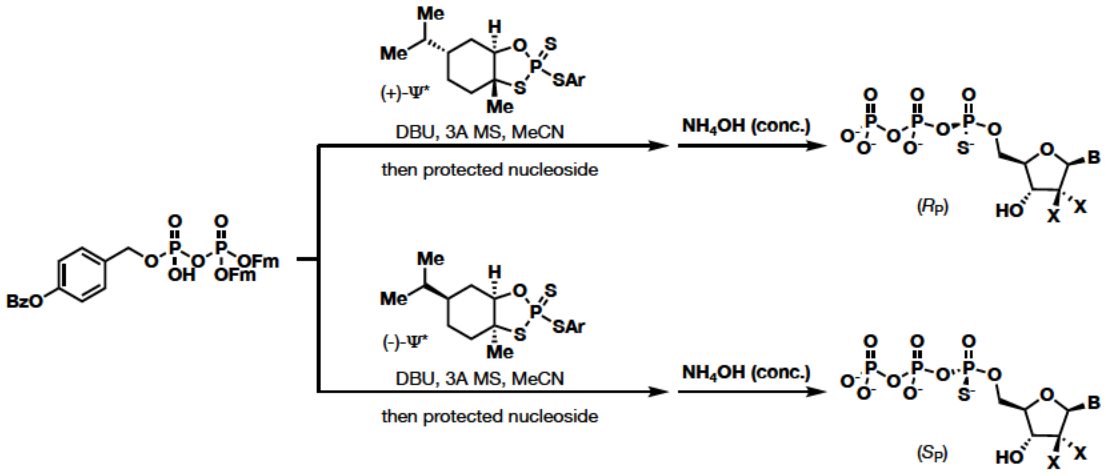
Merck's biocatalytic cascade for the synthesis of MK-1454 (*Nature* **2022**, *603*, 439) serves as an initial reference point to suggest that achieving diastereoselectivity at phosphorus is feasible. This study also cites two literature precedents (*J. Biol. Chem.***1977**, *252*, 4445–4448; *J. Org. Chem.* **1984**, *49*, 704–706) that suggest that monophosphate kinase can stereoselectively phosphorylate adenosine 5'-monothiophosphate. However, to the best of our knowledge, this feature has not been systematically studied with a wide array of substrates and no one has provided any molecular basis for the stereospecificity observed. Conversely, we are uncertain about the origin of stereoselectivity for thio-NAD^+ derivatives and nothing is known about this topic in the literature. At this point, we can only speculate that some transferases/kinases are not as stereospecific as others due to a lack of evolutionary pressure to achieve this feature.

How is the absolute stereochemistry at P assigned? The authors show beautiful C18-RP HPLC traces in the SI and seem to claim that the R_P diastereomer is always the slower-eluting diastereomer. How rigorous is this assumption? Please clarify.

The authors show a comparison with "standards" in many cases. Where were these standards obtained and how was their absolute stereochemistry assigned?

Response:

We synthesized the NTPαS standards (both R_P and S_P form) using Baran's method (*Nat. Chem.* **2024**, *16*, 249–258) wherein the absolute configuration of the P-center is controlled by the absolute configuration of limonene-derived thiophosphorylation reagent. The enzymatically-synthesized NTPαS products were compared with these standards using LCMS or NMR.



Additional Corrections/Clarifications

1. The authors should clearly distinguish among conversion, NMR yield, and isolated yield throughout the manuscript.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry - MS 602 | BioScience Research Collaborative - Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

Response:

We have made the requested distinctions throughout the manuscript.

2. In Figure 2, sections A & B are not marked

Response:

This was a mistake on our end. Figure 2 was not supposed to have panels A and B. This mistake has been corrected in the revised manuscript.

3. In Figure 5B, fluorescence detection in lane 2 lists (-) for LixCrPS which seems incorrect

4. Check the manuscript for the use of "adenine diphosphate" vs. "adenosine diphosphate" (more correct) and related designations.

Response:

These mistakes have been corrected.

Supporting Information

The extensive SI adds much to the paper and in many ways is more clearly written than the manuscript itself. The SI provides comprehensive experimental details, nice ^{1}H, ^{13}C and ^{31}P NMR spectral data and C18-reverse phase HPLC traces, in support of the biocatalysis-enabled syntheses reported in the main manuscript.

However, there are still a few corrections needed:

1. Look carefully at the use of "nucleoside monophosphate kinase" vs. "nucleoside diphosphate kinase" throughout (see discussion of Biocatalysis Schemes above).

Response:

The term "nucleoside diphosphate kinase" has been changed to "nucleoside monophosphate kinase" throughout the manuscript.

2. There seems a typographical error in the HRMS of compound 56, instead of 398, it shows 498.xx.

Response:

This mistake has been corrected.

3.In some cases, there seems to be a discrepancy in yield between the manuscript and the SI (e.g. entry 15, two different yields). Please clarify and correct, as needed.

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu

Response: Thanks for your suggestions. We actually think the yield data for Figure 2 entry 15 is consistent with our SI data (67%). But we carefully checked data for other entries to ensure the consistency.

4. The HPLC data for compounds 60 & 62 appear to be missing.

Response:

We did not obtain HPLC data for compound **60** and **62** because their stereochemistry was assigned based on the accepted mechanism of adenylate cyclase, in which the α-P stereocenter was inverted to R_P from the (S_P)-ATPαS starting material. Unfortunately, the two stereoisomers of the product standards are prohibitively expensive to obtain for data comparison.

5. The authors comment that the reagent Li_xCrPS is highly hygroscopic. As such, it would be helpful to practitioners if the authors provided guidelines/best practices on handling and storing the reagent (dessicator? P_2O_5 side arm? etc.)

Response:

We thank the reviewer for raising this question. To avoid storage issues, we dissolved the reagent in aqueous solution and determine its concentration before use via quantitative ^{31}P NMR. The solution of Li_xCrPS can be stored at -20 °C without decomposition for at least a year.

We hope that the revisions have been made to your satisfaction and we thank you for taking the time to evaluate this work.

Sincerely,

Hans Renata, Ph.D.

Professor, Department of Chemistry

Rice University

Email hrenata@rice.edu | **Office** 713-348-8878 | **Fax** TBD | Rice University Department of Chemistry – MS 602 | BioScience Research Collaborative – Ste.369 | 6500 Main St. | Houston, TX 77030 | chemistry.rice.edu