Diversification of 4’-Methylated Nucleosides by Nucleoside Phosphorylases

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The growing demand for 4’-modified nucleoside analogs in medicinal and biological chemistry is contrasted by the challenging synthetic access to these molecules and the lack of efficient diversification strategies. Herein, we report the development of a biocatalytic diversification approach based on nucleoside phosphorylases, which allows the straightforward installation of a variety of pyrimidine and purine nucleobases on a 4’-alkylated sugar scaffold. Following the identification of a suitable biocatalyst as well as its characterization with kinetic experiments and docking studies, we systematically explored the equilibrium thermodynamics of this reaction system to enable rational yield prediction in transglycosylation reactions via principles of thermodynamic control.
Diversification of 4ʹ-Methylated Nucleosides by Nucleoside Phosphorylases

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Nucleosides are central biomolecules that play key roles in a variety of cellular processes by serving as enzymatic cofactors, building blocks of DNA and RNA and energy transport systems. As such, modified nucleosides mimicking their natural counterparts have a long history in medicinal and biological chemistry.[1–4] Today, modified nucleosides are indispensable pharmaceuticals for the treatment of various types of cancer and viral infections and further represent important tools in chemical biology for a spectrum of imaging applications.[5,6] Despite the great demand for these molecules, the synthesis of nucleosides is still regarded as challenging and inefficient.[7] While nucleosides with ribosyl or 2ʹ-desoxyribosyl moieties can be accessed from naturally occurring nucleosides or carbohydrates,[5–10] the preparation of sugar-modified nucleosides typically suffers from lengthy reaction sequences and low total yields.[11–18] Furthermore, a heavy reliance on protecting groups entails low overall efficiencies[7] and several sugar modifications at the 2ʹ or 4ʹ positions are known to limit diastereoselectivity in glycosylation approaches,[19,20] severely complicating the synthetic access to many target compounds. More importantly, established routes typically exhibit a lack of divergence as they tend to be specific to one nucleoside. As such, the introduction of desired substitutions at the nucleobase often requires complete or partial re-synthesis of the target molecule since a general strategy for the efficient diversification of modified nucleosides has not been reported to date (Scheme 1, top). With the advent of scalable routes for the de novo synthesis of selected 4ʹ-modified nucleoside analogs, as reported recently by Britton,[21] such a diversification strategy would readily provide access to a variety of sought-after nucleosides.

We envisioned that nucleoside phosphorylases could provide a biocatalytic platform for late-stage diversification of 4ʹ-modified nucleosides. These enzymes catalyze the reversible phosphorolysis of nucleosides to the corresponding nucleobases and pentose-1-phosphates via an S_{N}2-like mechanism.[22,23] The reaction sequence involving phosphorolysis of one nucleoside and in situ reverse phosphorolysis to the target nucleoside is generally known as a transglycosylation, and effectively transfers the sugar moiety from one nucleoside to another.[24] While this reactivity is well-established for ribosyl and 2ʹ-desoxyribosyl nucleosides[9] and a few 2ʹ-modified nucleosides (Scheme 1, center), there are no examples in the literature of the enzymatic synthesis of 4ʹ-modified nucleosides, except for Merck’s recent report of a 5-step enzymatic cascade for the synthesis of the 4ʹ-alkynylated nucleoside drug Ilatravir.[25] Therefore, the feasibility of transglycosylation reactions with 4ʹ-modified nucleosides as well as the thermodynamics of such a cascade process are notably underexplored. Herein, we address this gap by reporting on the phosphorolysis and transglycosylation of the simplest 4ʹ-alkylated pyrimidine nucleoside, 4ʹ-methyluridine (1a). Following the identification of a suitable biocatalyst, and a characterization of its reactivity with kinetic experiments and docking studies, we explored the thermodynamics of the phosphorolysis of 1a and leveraged this information in transglycosylation experiments to access a range of 4ʹ-methylated pyrimidine and purine nucleosides.

In the absence of obvious pyrimidine nucleoside phosphorylase (PyNP) candidates for the phosphorolysis of 1a, we began our investigation by screening a small panel of PyNPs with known broad substrate spectra. To our surprise, only the PyNP from *Thermus thermophilus* (TtPyNP)[26,27] showed measurable conversion of 1a under screening conditions (Figure S1). Other broad-spectrum PyNPs, such as those from *Geobacillus thermoglucosidasius* (GtPyNP)[28] or *Bacillus subtilis*,[23] displayed no activity with 1a (Figure 1A). To substantiate the observed conversion of 1a by TtPyNP, we performed a series of control experiments. Reactions either without...
phosphate, without enzyme or with denatured enzyme gave no conversion. Similarly, no conversion was observed under reaction conditions outside of the working space of TtPyNP (pH 3 or pH 12, Figure 1A).[26] NMR analysis of a reaction mixture with TtPyNP and 1a corroborated the proposed reactivity and creation of the pentose-1-phosphate 3, as evident from the rise of an additional HMQC signal at 5.57 ppm showing a strong H,P-HMQC signal (Figure 1B). Consistent with the native reactivity of PyNPs, inversion at the anomeric position was evident by this signal lacking NOE contacts to the 4ʹ-methyl group of 3, while the corresponding anomeric proton in 1a showed clear correlation to the methyl substituent.

Having established the activity of TtPyNP with 1a, we conducted kinetic experiments to provide further insights into this enzymatic transformation. Although TtPyNP is inhibited by pyrimidine nucleobases such as uracil (2a),[26] we could observe Michaelis-Menten behavior of the enzyme with 1a (Figure 1C). Interestingly, the apparent Michaelis-Menten constant $K_M$ of the phosphorolysis of 1a ($K_M = 3.37$ mM) indicated that TtPyNP has a much lower affinity for 1a compared to natural nucleosides like uridine or thymidine ($K_M < 1$ mM),[27] suggesting that productive binding of the modified substrate 1a might present a challenge due to the increased steric bulk. In addition to a lower affinity for 1a, TtPyNP also displayed a lower rate constant compared to uridine (0.59 vs 5.05 s$^{-1}$ for 1 mM substrate at 60 °C and pH 9) which showed a similar temperature-dependence as indicated by phosphorolysis experiments at different temperatures monitored by UV spectroscopy (Figure 1D).[29,30] Collectively, these results demonstrate that, unlike other nucleoside phosphorylases, TtPyNP selectively converts the 4ʹ-methylated nucleoside 1a to the corresponding sugar phosphate 3, albeit with a lower rate constant and substrate affinity compared to the native substrates.

Next, we performed preliminary in silico docking studies to rationalize why 1a is only converted by TtPyNP and not by other closely related and highly promiscuous enzymes such as $Gt$PyNP. We hypothesized that conversion of this substrate would primarily be limited by steric hindrance during substrate binding, since i) uridine and 1a only differ by a single methyl group distant from the anomeric position and ii) TtPyNP displays significantly lower affinity for 1a than for uridine. PyNPs generally exhibit marked flexibility during their catalytic cycle with a transition from an open conformation to a closed state requiring a domain movement of approximately 8 Å.[31] Since all first sphere residues in the closed state are highly conserved and identical between the tested PyNPs, we anticipated that initial binding in the open conformation would be a limiting factor, as TtPyNP offers slightly more space than GtPyNP due to a threonine-serine substitution at the back of the active site, as evident from sequence alignments.[28] To examine this hypothesis, we obtained an X-ray crystal structure of GtPyNP at 1.9 Å resolution (see Supporting Information for details; PDB ID 7m7k) and used AutoDock Vina implemented in YASARA to dock uridine and 1a into the open conformations of this structure and the known X-ray crystal structure of TtPyNP (PDB ID 2dsj).[32] Docking of uridine and 1a into TtPyNP yielded structures in good agreement with the native mode of substrate binding via H-bonding to the nucleobase and positioning of the anomeric carbon near the phosphate binding pocket (Figures 2A and 2B). Likewise, uridine could be docked into GtPyNP in a similar position to the cocystalized substrate (Figures 2C and S8), where the 4ʹ-position of uridine is located in proximity to Thr84 (Ser83 in TtPyNP). However, we were unable to obtain sensible docking results for 1a with GtPyNP as the increased steric bulk at the 4ʹ-position consistently led to a rotation of the sugar scaffold into an unproductive pose (Figure 2D). This suggested that the subtle space-creating mutation to a serine in TtPyNP might be a key factor for conversion of 1a. Consistent with this conclusion, the slightly more sterically congested TtPyNP-S83T mutant significantly lost activity compared to the parent enzyme ($k_{obs} = 0.25$ s$^{-1}$ vs $k_{obs} = 0.59$ s$^{-1}$, Table S2), while the reverse substitution in GtPyNP installed a low but measurable level of activity in this enzyme ($k_{obs} = 0.02$ s$^{-1}$ for GtPyNP-T84S). Moreover, all other enzymes we screened initially, and which were inactive with 1a, also possess a threonine at this position, which likely impedes their ability to bind this substrate productively. Although such subtle but crucial space-creating mutations are rare, there is precedent from other enzymes in the literature.[34] Together, these results indicate that sufficient space in the open conformation of PyNPs is a prerequisite for conversion of sterically more demanding substrates such as 1a. Clearly, there are other factors influencing the rate constant of this
transformation, as evident from the order of magnitude difference between the rate constants of the active enzymes, but these must arise from mutations far from the active site, as all other residues in possible contact with the substrate are identical between the tested enzymes.

Since the phosphorolysis of ribosyl and 2′-desoxyribosyl nucleosides is under tight thermodynamic control,[23] we were then interested in the thermodynamics and reversibility of the phosphorolysis of 1a to enable a diversification of the scaffold via transglycosylation. Time-course experiments with 1a and varying excesses of phosphate revealed incomplete conversion of the substrate, with the equilibrium positions being consistent with an equilibrium constant K of 0.16 (at 60 °C and pH 9, Figure 1D). Further experiments to monitor the equilibrium at 75 °C and 90 °C revealed that the phosphorolysis of 1a has an apparent reaction enthalpy $\Delta_H^\circ$ of 8.9 kJ mol$^{-1}$ and an apparent reaction entropy $\Delta_S^\circ$ of 11.7 J mol$^{-1}$ K$^{-1}$ (Figure S2). Interestingly, these values closely resemble the equilibrium constants and thermodynamic parameters of the phosphorolysis of uridine,[23] indicating that substitutions distant from the anomeric center have little influence on the equilibrium thermodynamics of nucleoside phosphorolysis. These results also pointed to the reversibility of this transformation, opening the door for transglycosylation reactions from the sugar phosphate 3 to yield other nucleosides.

With a solid understanding of the thermodynamics and kinetics of the phosphorolysis of 1a by TtPyNP, we proceeded to diversify this scaffold by subjecting the sugar phosphate 3 to subsequent enzymatic catalysis with different nucleobases in situ. Using this transglycosylation approach (Figure 3A and Scheme 1, center), we aimed to access a variety of 4′-methylated nucleosides from 1a in a one-pot manner. After confirming the stability of 3 through equilibrium shift experiments (Figure S6),[35] we subjected 1a to phosphorolysis using only minimal phosphate in the presence of different pyrimidine nucleobases 2b−2e belonging to a panel of 5-substituted uracil analogs (Figures 3A and 3B). Analysis of the reaction mixtures by HPLC revealed consumption of 1a and the respective uracil analog with concurrent formation of new products (Figure 3B), which HRMS analysis identified as the nucleoside products arising from glycosylation of 2b−2e with 3. Equilibrium state thermodynamic calculations[24] based on transglycosylation experiments with different sugar donor concentrations revealed apparent equilibrium constants of phosphorolysis of 0.12−0.73 for these products 1b−1e (Figure 3B and S3). The trifluoromethylated pyrimidine 2f could also be converted, although the instability of the starting material and product in aqueous solution[36]
Figure 3. Transglycosylation reactions (A) provided access to different pyrimidine (B) and purine nucleosides (C) as well as the equilibrium thermodynamics of the process, which can be exploited for yield prediction (D). [a] 2f is converted, but 1f and 2f hydrolyse to the corresponding carboxylates under the reaction conditions. [b] Reaction mixtures with purines additionally contained the purine nucleoside phosphorylase from Geobacillus thermoglucosidasius (PNP). Please see the externally hosted supplementary information for raw data and calculations.

precluded us from obtaining equilibrium data (Figure S4). A similar elaboration of in situ generated 3 with purine nucleobases proceeded smoothly using the promiscuous purine nucleoside phosphorylase from Geobacillus thermoglucosidasius. Notably, the adenosine analogs 1g–1i were generated in much higher conversions, corresponding to equilibrium constants of phosphorolysis of 0.01–0.02, reflecting the more favorable thermodynamics typically observed for 6-aminopurines. The guanosine and inosine analogs 1j and 1k could also be accessed, although with lower conversions indicative of higher equilibrium constants (Figure 3C). These experiments not only confirmed that nucleoside transglycosylations with the methylated precursor 1a can deliver a range of modified nucleosides in a one-pot manner, but also that the equilibrium thermodynamics of this system largely resemble those of the well-described ribosyl nucleosides. These findings further indicated that these transglycosylations would offer themselves to rational reaction engineering using established principles of thermodynamic reaction control to predict and maximize conversions in these reactions. Indeed, thermodynamic calculations based on the obtained equilibrium constants suggested that 1b could, for instance, be obtained in 84% conversion from 1a using 4 equivalents of nucleobase, which we confirmed experimentally (Figures 3D and S5). Similarly, 1i could be obtained in quantitative conversion with 4 equivalents of 2i, in agreement with our predictions. As a proof of synthetic utility, we subjected 1a to transglycosylation with 5 equivalents of 2e and obtained the iodinated 1e in 68% conversion (61% predicted) and ca. 40% isolated yield.

In conclusion, we identified and characterized TtPyNP as a biocatalyst for the diversification of 4ʹ-methylated nucleosides. Reversible phosphorolysis of a methylated precursor 1a yields stable the pentose-1-phosphate 3 which can be employed as a sugar synthon to access a range of modified nucleosides in one pot. Our investigations revealed that sufficient space near the active site in the open conformation of PyNPs appears crucial for binding and conversion of 1a. Furthermore, the equilibrium thermodynamics of the phosphorolysis of 4ʹ-methylated nucleosides largely resemble those of ribosyl nucleosides, indicating that substitutions distant from the anomeric position have only minor effects on the conversions in these systems. Leveraging principles of thermodynamic reaction control enabled us to access a spectrum of 4ʹ-methylated nucleosides bearing different pyrimidine and purine bases in transglycosylation reactions. Lastly, we expect that other 4ʹ-modified nucleoside analogs can be obtained with such biocatalytic systems in a similar fashion (probably with comparable equilibrium thermodynamics), although bulkier 4ʹ-substitutions will likely require some extent of protein engineering to improve activity.

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Supporting Information

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Author contributions (with definitions as recommended by Brand et al.\textsuperscript{[1]})

Conceptualization, F.K.; Data curation, F.K., P.P.; Formal analysis, F.K., R.S., S.W., P.P. and S.K.; Funding acquisition, P.N., G.B., D.B.W., A.S. and A.K.; Investigation, F.K., R.S., S.W., C.K., A.P.L., P.P. and S.K.; Methodology, F.K., R.S., S.W. and S.K.; Project administration, F.K.; Resources, P.N., G.B., D.B.W., A.S. and A.K.; Software, - ; Supervision, F.K.; Validation, - ; Visualization, F.K. and P.P.; Writing—original draft, F.K.; Writing—review & editing, all authors.

Data availability

All data depicted visually in the items in the main text (Figures 1–3) as well as in the Supplementary Information (Figures S1–S16, see below) are available as tabulated data from the text below and from the externally hosted Supplementary Information at zenodo.org.\textsuperscript{[2]} The data and model of GtPyNP with bound uridine were deposited to the Protein Data Bank (PDB) under accession code 7m7k.

General remarks

All chemicals used in this study were of analytical grade or higher and purchased from Sigma Aldrich (Steinheim, Germany), Carbosynth (Berkshire, UK), Carl Roth (Karlsruhe, Germany), TCI Deutschland (Eschborn, Germany) or VWR (Darmstadt, Germany) and used without prior purification. 4′-Methyluridine (1a) was synthesized as described recently.\textsuperscript{[3]} Water deionized to 18.2 MΩ-cm with a Werner water purification system was used for the preparation of all enzymatic reactions as well as purification and storage buffers. For the preparation of NaOH solutions for quenching, deionized water was used. Analytical HPLC analyses were carried out with an Agilent 1200 series system equipped with an Agilent DAD detector and a Phenomenex (Aschaffenburg, Germany) reversed phase Kinetex EVO C18 column (250 × 4.6 mm). Elution was performed at a flow rate of 1 mL min\textsuperscript{-1} by running 3% MeCN in 20 mM ammonium acetate buffer for 5 min, followed by a linear gradient to 40% MeCN in 20 mM ammonium acetate buffer over 20 min. Eluting compounds were detected at 260 nm. Preparative HPLC was carried out using a Knauer HPLC system equipped with a Smartline Detector 2600 and two AZURA P 2.1 L pumps, equipped with 11 mL ss pump heads. A flow rate of 21.24 mL min\textsuperscript{-1} and a Kinetex® 5 μm EVO C18 250 × 21.2 mm RP column were used with deionized water and chromatography-grade MeCN as eluents. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVIII 500 or AVIII 700 with the deuterated solvent acting as an internal deuterium lock. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra are referenced to sodium trimethylsilylpropansulfonate (DSS) as internal standard. The chemical shift of DSS was set to 0 ppm.\textsuperscript{[4]} \textsuperscript{31}P NMR spectra were calibrated according to the IUPAC recommendation, using a unified chemical shift scale based on the proton resonance of tetramethylsilane as the primary reference.\textsuperscript{[4]} Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant(s) (Hz), and integration. High resolution mass
spectrometry (HRMS) data were collected by MS service of the Technische University Berlin by LC-MS analysis of samples. To this end, chromatography was performed on a Grom-Sil-120-ODS-4-HE (Grace) column (length 50mm, ID 2mm, 3µm) running a linear gradient of 20% MeCN (+ 0.1% formic acid) in H₂O (+ 0.1% formic acid) over 10 min at a flow rate of 0.3 mL min⁻¹, followed by a column wash with 100% MeCN (+ 0.1% formic acid) and HRMS data was collected on a Thermo Fisher Scientific LTQ Orbitrap XL with electrospray ionization. Data handling and routine calculations were carried out in Excel or LibreOffice, NMR data analysis in MestreNova, MS data analysis in Proteowizard and openchrom, fitting and data plotting in OriginLab, metadata writing for spectral unmixing in LibreOffice, spectral unmixing with data_toolbox⁵,⁶ modelling and docking in YASARA and protein viewing in ChimeraX.⁷ Crystallographic software is described below.
Enzymes

*Thermus thermophilus* pyrimidine nucleoside phosphorylase (*TtPyNP*)
Uniprot ID Q5SHF9_THET8.

*Geobacillus thermoglucosidasius* pyrimidine nucleoside phosphorylase (*GtPyNP*)
Uniprot ID A0A369WPE5_PARTM.

*Geobacillus thermoglucosidasius* purine nucleoside phosphorylase (*GtPyNP*)
Uniprot ID A0A178U1V7_PARTM.

*Escherichia coli* thymidine phosphorylase (*EcTP*)
Uniprot ID TYPH_ECODH

*TtPyNP-S83T* was ordered as a synthetic gene from GeneArt (Invitrogen).

MRMVDLIAKRRDGYESKEEIDFIQRGTNGIDPYYQMSAFAFAMAVFFRGMTEETATLMAMVRSGDVLDSKIEG
MKVDKHSGGVGDTPPTTLGPLVAVGSTVMGKSGRGLHTGGTIDKLESVPGFHVEIDNEQFIELVKNKIAIGQ
TVGATLPAKDAKLYARDSVTATVDSENSIPASSIMKKKIAAGADALVDVKTGAGAAMKDFAGAKRALTAMVEIGKVRG
KTMAVISDMSQPLGAYVGNALVEKAPTDLKGKPEDLQELCGLSGMVYLAEKASSLEARALLEASIREGKALET
FKVFLSQAQGDASVDDPTKLPQAKYRWELEAEPEFYAEIADVEGTAAMLLGAGRATKEATIDLSVLVGLVHKV
GDAVKGESLVTIYSNTENIEEVQKLKSLSSPVAKPTLIYETIS
Experimental details and supplementary items

**Wild-type enzymes** were cloned as described in previous reports\(^5,8\) and the available glycerol stocks of the enzymes from previous projects\(^5,9,10\) were used directly for this work. BsPyNP was obtained as a freeze-dried enzyme from Sigma Aldrich and dissolved to 1 g L\(^{-1}\) in 2 mM phosphate buffer (pH 7).

**Cloning of the mutant enzymes** was carried out via BamHI/HindIII sites in the plasmid pGW3 (gift by Matthias Gimpel, unpublished). Codon-optimized genes were obtained (GeneArt Invitrogen/Thermo Fisher Scientific, Massachusetts, USA) and cloned into pGW3 using the recipient strain *Escherichia coli* DH5α. The correct sequence was confirmed with Sanger Sequencing (LGC Genomics, Berlin, Germany). pGW3 is a 2\(^{\text{nd}}\) generation derivative of pCTUT7, which was optimized with respect to tightness of the LacO in comparison to the 1\(^{\text{st}}\) generation derivate used in a previous work.\(^8\)

**Protein expression and purification** was performed as described recently\(^5,8\) in *Escherichia coli* BL21 using the EnPresso protocol for 50mL (Enpresso, Berlin, Germany). Briefly, all enzymes were heterologously expressed in *E. coli* as His\(^6\)-tagged proteins through IPTG-induced overexpression. Purification was achieved through cell disruption, heat treatment of the crude extract (80 °C for TtPyNP, 60 °C for GtPyNP, both for 30 min; except for EcTP where no heat treatment was performed) and Ni-NTA affinity chromatography. Proteins were eluted with buffer containing 250 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl (pH 8) and stored as stock solutions at −20 °C in 50% (v/v) glycerol. Except for TtPyNP, all proteins were stored in elution buffer with 50% (v/v) glycerol. TtPyNP was subjected to gel filtration using a PD-10 desalting column to remove the phosphate from the purification process and stored in 10 mM MOPS buffer with 50% (v/v) glycerol. Typical protein stock concentrations were around 1 g L\(^{-1}\) (calculated with 1 AU cm\(^{-1}\) at 280 nm being equal to a protein concentration of 1 g L\(^{-1}\)). Under these storage conditions, no decay of activity could be detected over the course of more than 1.5 years.

**Enzymatic phosphorolysis reactions** were performed in 200 µL PCR tubes and prepared from stock solutions of nucleoside, potassium phosphate and buffer. Typical reaction volumes were 50–150 µL, depending on the experiment and substrate concentration. Reactions were preheated to the respective temperature for 30 s and initiated by the addition of 2–10 µL suitably diluted enzyme stock solution (predilution in 10 mM MOPS buffer, pH 7). Samples were withdrawn at timely intervals after reaction initiation, as detailed in the metadata files freely available online.\(^2\)
Reaction monitoring of phosphorolysis reactions was achieved via spectral unmixing. From live reactions, samples were withdrawn and quenched in 100 mM aqueous NaOH as described previously.\cite{5,6} Sample dilution factor was adjusted to reach final concentrations of 100–200 µM UV-active reaction components (please note that the exact concentration is not relevant here since spectral unmixing only takes spectral shape and not absolute intensity into account). For instance, from reactions with 1 mM 1a 50 µL of the reaction mixture were pipetted into 250 µL 100 mM NaOH for quenching and dilution. Of the diluted alkaline sample, 200 µL were transferred to UV/Vis-transparent 96-well plates (UV star, GreinerBioOne, Kremsmünster, Austria) for analysis. UV absorption spectra were recorded from 250–350 nm with a BioTek PowerWave HT platereader and subjected to spectral unmixing using analogously obtained reference spectra of 1a and 2a.\cite{2} Reference spectra used in this study are freely available from the externally hosted Supplementary Information.\cite{2} The degree of conversion was determined directly from the spectral fit which considers the UV-active substrate and product in relation to one another.\cite{5} For activity determination, only sampling points showing 3–10% conversion of the nucleoside substrate were considered. This lower bound was set due to the inherent inaccuracy of the UV-based method employed (roughly ±0.3 percentage points, due to the inherent error in spectral acquisition, as described in the original publication)\cite{5} and the upper bound was applied as recommended by Cornish-Bowden\cite{11} for equilibrium reactions. All datapoints outside this window were not included for calculation of activity and marked accordingly in the datasets available in the Supplementary Information.\cite{2} Datapoints that displayed baseline shifts or other spectral anomalies were also excluded from consideration. Background correction was performed as described recently.\cite{6} Experimental spectra were fitted either across the entire spectrum or over one of the information-rich shoulder regions of pyrimidine nucleosides/nucleobases, as appropriate for the analysis. All background corrections and the corresponding datafiles are detailed in the metadata files in the externally hosted supplementary information.\cite{2}

Enzymatic activity was determined by linear approximation of the conversion over time with a forced intercept at the origin. All raw data and the datapoints considered for calculation are freely available online with outliers and excluded datapoints clearly marked.\cite{2} The observed rate constant $k_{obs}$ was obtained by considering the degree of conversion (mol per second) per mol enzyme applied, using the molar extinction coefficient of $Tt$PyNP of 26,930 cm$^{-1}$ M$^{-1}$ as predicted by Protparam\cite{12} (i.e. the stock solution of 1 g L$^{-1}$ had a concentration of 37.1 µM).

Enzyme screening (Figure 1A) was performed using reaction mixtures of 1 mM 1a, 20 mM potassium phosphate and 30 µg mL$^{-1}$ enzyme ($Tt$PyNP, $Gt$PyNP, $Bs$PyNP or $Ec$TP) at 50 °C in 50 mM MOPS buffer pH 7 in a final volume of 50 µL. These reactions were carried out at a neutral pH in this buffer system
to accommodate for the working space of the enzymes used. Later reactions were performed at pH 9 since TtPyNP retains excellent activity and stability under alkaline conditions[9] and pentose-1-phosphates (such as 3) are much more resistant to hydrolysis under alkaline conditions.[13,14] The reactions were quenched by addition of 250 µL 100 mM NaOH to the reaction mixtures after 30 min. The resulting samples were analyzed by UV spectroscopy as described above. For each protein, control reactions with uridine were performed under identical conditions, all of which gave conversion of the nucleoside to or near equilibrium (Figure S1). For both uridine and 1a, control reactions without protein were carried out, which resulted in no conversion of the starting material.

Figure S1. UV spectra obtained from enzyme screening reactions. All enzyme preparations are active with uridine and effected equilibrium conversion of the substrate (A), except for TtPyNP which displays only submaximal activity at 50 °C.[9] Only TtPyNP displayed appreciable activity with 1a (B). The raw data are available online.[2] For illustrative purposes, all UV spectra shown here were background corrected and normalized to the isosbestic point of base cleavage (271 nm for this substrate).[6]

The control reactions for TtPyNP (Figure 1A) were performed with standard reaction mixtures of 1 mM 1a, 20 mM potassium phosphate and 26.9 µg mL⁻¹ TtPyNP (1 µM, 1 mol%) at 60 °C in 50 mM MOPS buffer pH 7 in a final volume of 60 µL. For the reaction without enzyme, the TtPyNP volume was replaced with water. For the reaction without phosphate, the phosphate volume was replaced with water. For the reaction with boiled enzyme, TtPyNP was subjected to 100 °C in the reaction mixture in the PCR tube with lid heating (105 °C) for 20 min to denature the protein (TtPyNP has a half-life of 2.3 min at 100 °C)[9] prior to addition of 1a. The reaction at pH 3 was carried out in a buffer mix consisting of 5 mM citrate, 10 mM MOPS and 20 mM glycine (all final concentration) adjusted to pH 3. The reaction at pH 12 was carried out with 25 mM NaOH instead of MOPS buffer. All reactions were quenched through addition of 200 µL 100 mM NaOH and analyzed as described above.
The NMR spectra of the sugar phosphate 3 (Figure 1B) were recorded directly from a reaction mixture. To this end, a reaction mixture containing 16 mM 1a, 83 mM potassium phosphate (pH 7), 100 µg mL\(^{-1}\) (3.71 µM, 0.02 mol%) TtPyNP, approximately 5 mM imidazole, 5% (v/v) glycerol, 10% D\(_2\)O with DDS was prepared in a total volume of 500 µL. Prior to the reaction, \(^1\)H (with and without water suppression) and \(^31\)P spectra were recorded. The solution was then heated to 70 °C in the NMR tube for 1.5 h before analysis of the reaction products. For the assignment of the signals, the following spectra were used: \(^1\)H, H,H-COSY, H,H-NOESY, \(^1\)H-1D-TOCSY, \(^13\)C{\(^1\)H}, H,C-HSQC, H,C-HMBC, \(^31\)P, H,P-HMQC. Solvent suppression was used to suppress the water signal for the \(^1\)H, H,H-COSY and H,H-NOSY experiments. The following data were obtained for the reaction components and are shown below in Figures S10–S14. All raw spectra are available from the externally hosted Supplementary Information.[2]

1a
\(^1\)H NMR (700 MHz, H\(_2\)O/D\(_2\)O): δ=7.88 (d, \(^3\)J\(_{5,6}\)=8.4 Hz, 1H; H-6), 5.98 (d, \(^3\)J\(_{1',2'}\)=6.3 Hz, 1H; H-1’), 5.92 (d, \(^3\)J\(_{5,6}\)=8.4 Hz, 1H; H-5), 4.51 (t, \(^3\)J\(_{1',2'}\)=6.1 Hz, 1H; H-2’), 4.22 (d, \(^3\)J\(_{2',3'}\)=5.9 Hz, 1H; H-3’), 3.63 (1H, H-5’), 1.28 (s, 3H; Me).

A NOE contact between H-1’ and the methyl group could be observed in the H,H-NOESY.

\(^{13}\)C NMR (175 MHz, H\(_2\)O/D\(_2\)O): δ=169.0 (C-4), 154.8 (C-2), 144.7 (C-6), 105.4 (C-5), 90.8 (C-1’), 90.4 (C-4’), 76.5 (C-2’), 74.3 (C-3’), 69.1 (C-5’), 20.2 (Me).

2a
\(^1\)H NMR (700 MHz, H\(_2\)O/D\(_2\)O): δ=7.54 (d, \(^3\)J\(_{5,6}\)=7.8 Hz, 1H; H-6), 5.81 (d, \(^3\)J\(_{5,6}\)=7.8 Hz, 1H; H-5).

\(^{13}\)C NMR (175 MHz, H\(_2\)O/D\(_2\)O): δ=170.3 (C-4), 156.1 (C-2), 146.5 (C-6), 103.9 (C-5).

3
\(^1\)H NMR (700 MHz, H\(_2\)O/D\(_2\)O): δ=5.57 (dd, \(^3\)J\(_{H,P}\)=6.6 Hz, \(^3\)J\(_{J,2}\)=4.2 Hz, 1H; H-1), 4.23 (dd, \(^3\)J\(_{J,2}\)=6.1 Hz, \(^3\)J\(_{J,2}\)=4.2 Hz, 1H; H-2), 4.00 (d, \(^3\)J\(_{J,2}\)=6.1 Hz, 1H; H-3), 3.46 (m, 1H; H-5), 1.30 (s, 3H; Me).

A NOE contact between H-1 and the methyl group could not be observed in the H,H-NOESY.

\(^{31}\)P NMR (203 MHz, H\(_2\)O/D\(_2\)O): δ=1.89 (determined via H,P-HMQC due to strong overlap with the phosphate buffer).

\(^{13}\)C NMR (175 MHz, H\(_2\)O/D\(_2\)O): δ=100.0 (d, \(^3\)J\(_{C,P}\)=4.1 Hz; C-1), 89.8 (C-4), 75.0 (C-2), 74.8 (C-3), 70.0 (C-5), 21.5 (Me).
The Michaelis-Menten-type kinetics of TtPyNP (Figure 1C) were investigated with reactions containing 50 mM potassium phosphate and 12 µg mL\(^{-1}\) TtPyNP (0.45 µM) in 50 mM glycine/NaOH buffer pH 9 with 0.5–5 mM 1a. Reaction volumes, sampling times and sampling volumes are indicated in Table S1. Raw data and calculations for this experiment are freely available online.\(^2\) The obtained data were fit to the Michaelis-Menten equation according to

\[
k_{obs,max} = \frac{k_{obs} [S]}{K_M + [S]} \tag{S1}
\]

where \(k_{oobs, max}\) is the maximal observed rate constant [s\(^{-1}\)], \(k_{obs}\) is the rate constant [s\(^{-1}\)] observed at the substrate concentration \([S]\) [mM] and \(K_M\) is the Michaelis-Menten constant [mM]. The fit yielded \(k_{oobs, max} = 2.54 \pm 0.35\) mM and \(K_M = 3.37 \pm 0.88\) mM (\(R^2 = 0.96\)).

**Table S1.** Conditions used for kinetics experiments for \(K_M\) determination.

| 1a [mM] | Sampling times [min] | Reaction volume [µL] | Sampling volume [µL] | Volume of quenching NaOH [µL] |
|---------|----------------------|----------------------|----------------------|-----------------------------|
| 0.5     | 1.5, 3, 4.5          | 200                  | 65                   | 150                         |
| 1       | 2, 4, 6              | 150                  | 50                   | 200                         |
| 2       | 4, 8, 12             | 75                   | 25                   | 200                         |
| 5       | 10, 20, 30           | 30                   | 10                   | 200                         |

The temperature-dependence of the activity of TtPyNP with 1a (Figure 1D) was determined using reaction mixtures of 1 mM 1a and 50 mM potassium phosphate in 50 mM glycine/NaOH buffer at pH 9 and the indicated temperature in a total volume of 150 µL. Depending on the temperature (and, therefore, on rate of phosphorolysis), 6–24 µg mL\(^{-1}\) TtPyNP were used (6 µg mL\(^{-1}\) for 70 °C, 12 µg mL\(^{-1}\) for 60 °C and 24 µg mL\(^{-1}\) for 50 °C), to permit sampling of all reactions within the same time domain. From all reactions, samples of 50 µL were withdrawn after 2, 4 and 6 min, quenched in 300 µL 100 mM NaOH and analyzed as described above. Raw data and calculations for this experiment are freely available online.\(^2\) The obtained data were fit to the Eyring equation\(^15\)

\[
k_{obs} = \frac{\kappa k_B T}{h} \exp \left( - \frac{\Delta R H^\ddagger + T \Delta R S^\ddagger}{RT} \right) \tag{S2}
\]

\[
\ln \left( \frac{k_{obs}}{T} \right) = \ln \left( \frac{\kappa k_B}{h} \right) - \left( \frac{\Delta R H^\ddagger}{T} \right) + \left( \frac{\Delta R S^\ddagger}{R} \right) \tag{S3}
\]

where \(k_{obs}\) is the observed rate constant [s\(^{-1}\)], \(\kappa\) is the transmission coefficient (herein assumed to be unity), \(k_B\) is the Boltzmann constant (1.38 \cdot 10^{-23} \text{ J K}^{-1}), T is the temperature [K], \(h\) is the Planck constant (6.626 \cdot 10^{-34} \text{ J s}), \(R\) is the universal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)), \(\Delta R H^\ddagger\) the transition state enthalpy
[J mol⁻¹] and $\Delta_R S^\ddagger$ is the transition state entropy [J mol⁻¹ K⁻¹]. The fit of the experimental data shown in Figure 1D for 1a yielded $\Delta_R H^\ddagger = 99.18 \pm 17.47$ kJ mol⁻¹ and $\Delta_R S^\ddagger = 36.64 \pm 52.49$ J mol⁻¹ K⁻¹ ($R^2 = 0.94$). The data shown for uridine were taken from our earlier report[9] and the fit shown in Figure 1D gave $\Delta_R H^\ddagger = 101.55 \pm 2.53$ kJ mol⁻¹ and $\Delta_R S^\ddagger = 72.18 \pm 7.59$ J mol⁻¹ K⁻¹ ($R^2 = 0.99$).

The thermodynamic control of the phosphorolysis of 1a (Figure 1E) was probed with reaction mixtures of 1 mM 1a and 100 µg mL⁻¹ TtPyNP (3.71 µM, 0.37 mol%) in 50 mM glycine/NaOH buffer at pH 9 and 60 °C in a total volume of 160 µL with either 2, 5, 10 or 20 mM potassium phosphate (equivalent to 2, 5, 10 or 20 equivalents of phosphate over the nucleoside 1a). The reaction mixtures were incubated in a PCR cycler with lid heating (70 °C). Samples of 25 µL were quenched in 225 µL 100 mM NaOH after 2, 8, 30, 60, 111 and 165 min and analyzed by spectral unmixing as described above and in the metadata files available online.[2] Likewise, the raw data for this experiment are freely available in the externally hosted supplementary information.[2] The obtained data were fit to equation (S4) which was derived as detailed below.

\[
c = \frac{-K - Kx + \sqrt{(K + Kx)^2 + 4Kx(1 - K)}}{2(1 - K)} \tag{S4}
\]

where $c$ is the extent of conversion in the equilibrium (in fractions, not %), $K$ is the equilibrium constant of phosphorolysis and $x$ is the excess of phosphate (i.e. equivalents of phosphate over the nucleoside substrate). All these variables are dimensionless. The fit of the experimental data yielded $K = 0.1605 \pm 0.0056$ ($R^2 = 0.99$) for $c = 0.395, 0.55, 0.7$ and 0.79 for 2, 5, 10 or 20 equivalents of phosphate, respectively. For simplicity’s sake, we performed all further calculations building on this equilibrium constant with $K = 0.16$.

Derivation of equation (S4) for the determination of phosphorolysis equilibrium constants from phosphorolysis equilibria with different phosphate excesses:

Nucleoside phosphorolysis is a thermodynamically controlled reaction which closely adheres to the law of mass action.[10]

\[
K = \frac{[B][P1P]}{[N][P]} \tag{S5}
\]

where $[B]$, $[P1P]$, $[N]$ and $[P]$ are the equilibrium concentration of the nucleobase (2a in this case), pentose-1-phosphate (3 in this case), nucleoside (1a in this case) and orthophosphate (all in an arbitrary molar concentration), respectively. Herein, we assume that stoichiometry holds true (S6), as do the mass balances of all components in this system (S7–S9).

\[
[B] = [P1P] \tag{S6}
\]

\[
[N] = [N]_0 - [B] \tag{S7}
\]
\[ [P] = [P]_0 - [B] \]  
\[ [B] = c[N]_0 \]  
(S8)

where \([N]_0\) is the initial nucleoside concentration and \([P]_0\) is the initial phosphate concentration. The initial concentrations of the products (nucleobase and pentose-1-phosphate) are regarded as zero. In a scenario where the phosphate concentration is expressed as a function of the nucleoside concentration, the following equations (S10–S12) can be formulated.

\[ x = \frac{[P]_0}{[N]_0} \]  
(S10)

\[ [N]_0 = \frac{[P]_0}{x} = 1 \]  
(S11)

\[ [P]_0 = x[N]_0 = x \]  
(S12)

Substitution of equations (S6), (S9), (S11) and (S12) in the law of mass action (S5) then gives a simplified equation with variables that are either easily accessible experimentally or known from the experimental setup.

\[ K = \frac{(c[N]_0)^2}{([N]_0 - c[N]_0) ([P]_0 - c[N]_0)} = \frac{c^2}{(1 - c)(x - c)} \]  
(S13)

This quadratic equation can be rewritten to equation (S14) and solved via equations (S15) and (S16).

\[ c^2 = K(1 - c)(x - c) = xK - cK - cxK + Kc^2 \]  
(S14)

\[ (1 - K)c^2 + (K + Kx)c - xK = 0 \]  
(S15)

\[ c_{1,2} = \frac{-K - Kx \pm \sqrt{(K + Kx)^2 + 4Kx(1 - K)}}{2(1 - K)} \]  
(S16)

Equation (S16) has only one physically reasonable solution, which is equivalent to equation (S4) and returns equilibrium constants of phosphorolysis for experimentally obtained equilibrium conversions \(c\) under known phosphate excesses \(x\).

The temperature-dependence of the phosphorolysis equilibria of \(1a\) was evaluated using reaction mixtures of 1 mM \(1a\), 2 mM potassium phosphate, 100 µg mL\(^{-1}\) TtPyNP (3.71 µM, 0.37 mol%) in 50 mM glycine/NaOH buffer at pH 9 in a total volume of 150 µL. The mixtures were incubated at 75 °C or 90 °C until the respective equilibria were reached and samples of 50 µL were taken after 30, 45 and 60 min and quenched in 200 µL 100 mM NaOH. The resulting alkaline samples were analyzed by spectral unmixing as described above. The obtained conversion data was transformed to equilibrium constants using equations (S5)–(S8) and fitted in an Arrhenius fashion to equation (S17),\(^{[10]}\)

\[ K = e^{-\frac{\Delta RH' - T\Delta RS'}{RT}} \]  
(S17)

where \(\Delta RH'\) is the apparent reaction enthalpy of phosphorolysis [J mol\(^{-1}\)], \(\Delta RS'\) is the apparent reaction entropy of phosphorolysis [J mol\(^{-1}\) K\(^{-1}\)], \(R\) is the universal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)) and \(T\) is the temperature [K]. The fit yielded \(\Delta RH' = 8.88 \pm 0.90\) kJ mol\(^{-1}\) and \(\Delta RS' = 11.66 \pm 2.56\) J mol\(^{-1}\) K\(^{-1}\).
(R² = 0.93). The raw data for this experiment are available from the externally hosted Supplementary Information.[2]

![Graph](image)

**Figure S2.** Temperature-dependence of K₁a. Data for 60, 75 and 90 °C were used for calculation.

The activity of the mutant PyNPs (Table S2, see below) was assessed with reaction mixtures of 1 mM 1a and 50 mM potassium phosphate in 50 mM glycine buffer pH 9 with PyNP (8 µg mL⁻¹ TtPyNP-S83T, 30 µg mL⁻¹ GtPyNP-T84S) at 60 °C in a total volume of 150 µL. Samples of 50 µL were taken after 20, 40 and 60 min, quenched in 300 µL 100 mM NaOH and analyzed by spectral unmixing. Kinetic constants were calculated as described above, using the extinction coefficient of 21,890 cm⁻¹ M⁻¹ of GtPyNP. The raw data and calculations for this experiment are available online.[2]

**Table S2.** Phosphorolytic activity of wild-type (wt) enzymes and mutants with 1a.[a]

| Enzyme            | kₐₛ[s⁻¹] |
|-------------------|----------|
| TtPyNP wt         | 0.59     |
| TtPyNP-S83T       | 0.25     |
| GtPyNP wt         | -        |
| GtPyNP-T84S       | 0.02     |

[a] Reactions conditions are detailed in the paragraph above

The thermodynamic control of the phosphorolysis of other methylated nucleosides (Figure 3) was determined via transglycosylation using reaction mixtures of 0.25 mM nucleobase (2b–2k), 0.075 mM potassium phosphate (0.3 equivalents), 50 µg mL⁻¹ TtPyNP (1.85 µM, 0.7 mol% with respect to the nucleobase) in 20 mM glycine buffer pH 9 at 60 °C with either 0.125 mM (0.5 eq.), 0.25 mM (1 eq.), 0.5 (2 eq.) or 1 mM (4 eq.) 1a in a total volume of 50 µL. Reaction mixtures with purine nucleobases (2g–2k) additionally contained 125 µg mL⁻¹ of the purine nucleoside phosphorylase from Geobacillus.
thermoglucosidasius. The reaction mixtures were incubated in a PCR cycler with lid heating (70 °C) for 4 h. After the reaction, the mixtures were quenched through addition of 50 µL MeOH, centrifuged (13,000 rpm, 10 min) and analyzed by HPLC. Conversion was calculated according to equation (S18), which assumes that the molar extinction coefficients of the nucleobase 2 and the corresponding nucleoside 1 are equal.

\[ c_T = 100 \frac{P_1}{P_1 + P_2} \quad \text{(S18)} \]

where \(c_T\) is the conversion in the transglycosylation reaction (i.e. conversion of the nucleobase to the target nucleoside), \(P_1\) is the peak area of the target nucleoside (1) and \(P_2\) is the peak area of the nucleobase (2). Typical retention times of the compounds used herein are given in Table S3. The identity of all target compounds was confirmed by high-resolution mass spectrometry (HRMS) as detailed below in Table S4. The raw data for all HPLC runs used for calculation of equilibrium constants are freely available online.\[^{[2]}\] Since opening and processing of the files requires Agilent software, all chromatograms are depicted below (Figure S3) and integration results are listed in Table S3. The equilibrium constants of phosphorolysis of the target nucleoside were determined by fitting the conversion of the nucleobase to the corresponding nucleoside as a function of the excess of \(1a\) according to equation (S19), which is derived below.

\[ c_T = 100 \frac{K_N(z + 1) - \sqrt{K_N(K_N z^2 - 2K_N z + K_N + 4z)}}{2(K_N - 1)} \quad \text{(S19)} \]

where \(K_N\) is the net equilibrium constant of transglycosylation as given by equation (S20) and \(z\) is the excess of \(1a\) over the nucleobase 2 as given by equation (S21).

\[ K_N = \frac{K_1}{K_2} \quad \text{(S20)} \]

\[ z = \frac{[B]_{z.0}}{[N]_{1.0}} \quad \text{(S21)} \]

where \(K_1\) and \(K_2\) are the equilibrium constants of phosphorolysis of the donor (1a) and acceptor nucleoside (1b–1k), \([B]_{z.0}\) is the initial concentration of the nucleobase 2 [mM] and \([N]_{1.0}\) the initial concentration of the donor 1a [mM]. The equilibrium constants of phosphorolysis of the target nucleosides \((K_2)\) were then obtained by employing equation (S20), using \(K_1 = K_{1a} = 0.16\).

Derivation of equation (S19) for the determination of phosphorolysis equilibrium constants from transglycosylation equilibria with different excesses of the donor nucleoside:

The basis for this equation is given by an expression reported and derived in our previous report (equation (4) in the original paper).\[^{[6]}\]

\[ c_T = 100 \frac{K_N([N]_{1.0} + [B]_{z.0})}{2(K_N - 1)} \quad \text{(S22)} \]
\[ \frac{\sqrt{K_N(K_N[N]_1^2 - 2K_N[N]_1[B]_2 + K_N[B]_2^2 + 4[N]_1[B]_2)} - 1}{2(K_N - 1)} \]

with definitions from above. Equation (S19) can be obtained from this expression by substituting \([N]_1\) and \([B]_2\) according to equations (S23) and (S24), which are consequences of equation (S21).

\[ [N]_1 = z \quad (S23) \]
\[ [B]_2 = 1 \quad (S24) \]

It should be noted that equation (S22), and in turn also (S19), inherently builds on the assumption of an ideal transglycosylation – i.e. a process during which no transglycosylation intermediate (pentose-1-phosphate, 3 in this case) contributes to the mass balance of the donor and target nucleoside. This in turn necessitates the assumption of a net zero phosphate concentration ([\(P1P\)] = [\(P\)] = 0), which is, of course, unrealistic. However, the observed deviation of the real conversions from those expected in an ideal case is almost imperceptibly small for low phosphate concentrations. For instance, we employed 0.3 equivalents of phosphate, which causes roughly a 0.2–0.5% deviation of the real (experimental) conversion from the ideal case, depending on the equilibrium constant of the target nucleoside (\(K_2\); as described in a previous work).\(^{[16]}\) Since this deviation is well within the inherent error of HPLC, equation (S19) provides an accurate output for realistic experimental data.
**Table S3.** HPLC data for determination of equilibrium constants of phosphorolysis.\(^{[a]}\)

| Entry | Excess \(z\) [x-fold] | Nucleobase 2 | Ret. time 2 [min] | Peak area 2 [mAU] | Ret. time 1 [min] | Peak area 1 [mAU] | Conv. 2 to 1 [%] | \(K_N\) | \(K_i\) |
|-------|------------------------|--------------|-------------------|-------------------|-------------------|-------------------|-----------------|--------|--------|
| 1     | 0.5                    |              |                   | 389               |                   | 190               | 32.8            |        |        |
| 2     | 1                      | 2b           | 4.5               | 318               | 10.9              | 349               | 52.3            | 1.28   | 0.12   |
| 3     | 2                      |              |                   | 199               |                   | 493               | 71.2            |        |        |
| 4     | 4                      |              |                   | 112               |                   | 587               | 84.0            |        |        |
| 5     | 0.5                    |              |                   | 418               |                   | 150               | 26.4            |        |        |
| 6     | 1                      | 2c           | 3.2               | 287               | 8.8               | 284               | 49.7            | 1.30   | 0.12   |
| 7     | 2                      |              |                   | 141               |                   | 429               | 75.3            |        |        |
| 8     | 4                      |              |                   | 77                |                   | 503               | 86.7            |        |        |
| 9     | 0.5                    |              |                   | 225               |                   | 82                | 26.7            |        |        |
| 10    | 1                      | 2d           | 5.2               | 175               | 12.4              | 147               | 45.7            | 0.76   | 0.21   |
| 11    | 2                      |              |                   | 120               |                   | 219               | 64.6            |        |        |
| 12    | 4                      |              |                   | 74.8              |                   | 263               | 77.9            |        |        |
| 13    | 0.5                    |              |                   | 282               |                   | 55                | 16.3            |        |        |
| 14    | 1                      | 2e           | 7.8               | 225               | 13.6              | 111               | 33.0            | 0.22   | 0.73   |
| 15    | 2                      |              |                   | 189               |                   | 139               | 42.4            |        |        |
| 16    | 4                      |              |                   | 131               |                   | 197               | 60.1            |        |        |
| 17    | 0.5                    |              |                   | 618               |                   | 415               | 40.2            |        |        |
| 18    | 1                      | 2g           | 4.8               | 297               | 12.6              | 772               | 72.2            | 7.38   | 0.02   |
| 19    | 2                      |              |                   | 83                |                   | 998               | 92.3            |        |        |
| 20    | 4                      |              |                   | 17                |                   | 1099              | 98.5            |        |        |
| 21    | 0.5                    |              |                   | 459               |                   | 433               | 48.5            |        |        |
| 22    | 1                      | 2h           | 10.5              | 198               | 15.2              | 720               | 78.4            | 13.78  | 0.01   |
| 23    | 2                      |              |                   | 61                |                   | 906               | 93.7            |        |        |
| 24    | 4                      |              |                   | 21                |                   | 948               | 97.8            |        |        |
| 25    | 0.5                    |              |                   | 260               |                   | 265               | 50.5            |        |        |
| 26    | 1                      | 2i           | 3.9               | 146               | 12.0              | 441               | 75.1            | 11.12  | 0.01   |
| 27    | 2                      |              |                   | 44                |                   | 564               | 92.8            |        |        |
| 28    | 4                      |              |                   | 9                 |                   | 660               | 98.7            |        |        |
| 29    | 0.5                    |              |                   | 477               |                   | 91                | 16.0            |        |        |
| 30    | 1                      | 2j           | 3.4               | 472               | 10.9              | 148               | 23.9            | 0.08   | 1.88   |
| 31    | 2                      |              |                   | 434               |                   | 186               | 30.0            |        |        |
| 32    | 4                      |              |                   | 375               |                   | 276               | 42.4            |        |        |
| 33    | 0.5                    |              |                   | 472               |                   | 106               | 18.3            |        |        |
| 34    | 1                      | 2k           | 3.2               | 419               | 9.9               | 145               | 25.7            | 0.11   | 1.51   |
| 35    | 2                      |              |                   | 355               |                   | 182               | 33.9            |        |        |
| 36    | 4                      |              |                   | 322               |                   | 256               | 44.3            |        |        |

*Chromatograms are shown below in Figure S3.*
Figure S3. HPLC-DAD data (260 nm) and fits for the determination of equilibrium constants of phosphorolysis from transglycosylation reactions (see below for full figure legend).
Figure S3 (continued). HPLC-DAD data (260 nm) and fits for the determination of equilibrium constants of phosphorolysis from transglycosylation reactions (see below for full figure legend).
Figure S3 (continued). HPLC-DAD data (260 nm) and fits for the determination of equilibrium constants of phosphorolysis from transglycosylation reactions. Data for 0.5 (black), 1 (purple), 2 (red) and 4 (orange) equivalents of the sugar donor 1a over the nucleobase are depicted. The chromatograms shown represent equilibrium states of the reactions.

Figure S4. Transglycosylation with the trifluorinated 2f. The nucleobase 2f is accepted by the enzyme and converted to the corresponding nucleoside 1f (A), but both the nucleobase and the nucleoside are labile to the alkaline conditions needed for stability of 3. Therefore, neither remaining starting material (as expected for a thermodynamically controlled reaction), nor product can be observed at pH 9 after 4 h (B). At pH 7 and with 4 equivalents of 2f, clear product formation is visible, which was confirmed by HRMS. However, significant hydrolysis is also apparent under these conditions, as is obvious by the large peak at the solvent front, corresponding to the hydrolysis product 2f*. Formation of 2f* as well as 1f* was also confirmed by HRMS analysis (Table S4, see below).
Table S4. HRMS data for transglycosylation reactions.

| Compound | Ion       | Molecular formula | Predicted m/z | Found m/z |
|----------|-----------|-------------------|---------------|-----------|
| 1b       | [M−H]    | C_{11}H_{15}N_{2}O_{6} | 271.0930     | 271.0941  |
| 1c       | [M−H]    | C_{10}H_{12}F_{2}N_{2}O_{6} | 275.0679     | 275.0690  |
| 1d       | [M−H]    | C_{10}H_{12}BrN_{2}O_{6} | 334.9879     | 334.9892  |
| 1e       | [M−H]    | C_{10}H_{12}N_{2}O_{6} | 382.9740     | 382.9750  |
| 1f\(^{[a]}\) | [M−H] | C_{11}H_{13}F_{2}N_{2}O_{6} | 325.0647     | 325.0658  |
| 1g       | [M+H]'   | C_{11}H_{16}N_{3}O_{4} | 282.1202     | 282.1201  |
| 1h       | [M+H]'   | C_{11}H_{15}ClN_{3}O_{4} | 316.0813     | 316.0815  |
| 1i       | [M+H]'   | C_{11}H_{16}N_{6}O_{4} | 297.1311     | 297.1312  |
| 1j       | [M−H]    | C_{11}H_{16}N_{6}O_{4} | 296.0995     | 296.1002  |
| 1k       | [M−H]    | C_{11}H_{13}N_{4}O_{5} | 281.0886     | 281.0898  |

\(^{[a]}\) The corresponding hydrolysis products 5-carboxyl-uracil (2f\(^{[a]}\), C_{5}H_{3}N_{2}O_{4} [M−H]’ : 155.0093; found: 155.0104) and 4′-methyl-5-carboxyluridine (1f\(^{[a]}\), C_{11}H_{13}N_{2}O_{8} [M−H]’ : 301.0672; found: 301.0686) were also weakly detected.

**Reactions with excess of nucleobase** (Figure 3D) were performed with reaction mixtures of 0.25 \(1a\), 0.075 mM potassium phosphate (0.3 equivalents), 1 mM nucleobase (2b or 2i) and 50 \(\mu\)g mL\(^{-1}\) TtPyNP (1.85 \(\mu\)M, 0.7 mol% with respect to the nucleobase) in 20 mM glycine buffer pH 9 at 60 °C in a total volume of 50 \(\mu\)L. The reaction mixture with 2i additionally contained 125 \(\mu\)g mL\(^{-1}\) of the purine nucleoside phosphorylase from *Geobacillus thermoglucosidasius*. The reaction mixtures were incubated in a PCR cycler with lid heating (70 °C) for 4 h. After the reaction, the mixtures were quenched through addition of 50 \(\mu\)L MeOH, centrifuged (13,000 rpm, 10 min) and analyzed by HPLC. Conversion was calculated based on remaining \(1a\). The mixture with 2b showed a residual peak area of \(1a\) of 104 mAU, corresponding to 86% consumption of \(1a\) (initial peak area of 719 mAU under identical conditions). The mixture with 2i showed full consumption of \(1a\). The HPLC chromatograms for these reactions are shown in Figure S5 and are available from the externally hosted Supplementary Information.\(^{[2]}\) The predicted conversions for these reactions were calculated via equation (S22) by setting \([N]_{1,0}\) to 1, \([B]_{2,0}\) to 4 and \(K_N\) to the values listed in Table S3.
The stability of the sugar phosphate 3 was assessed through an equilibrium shift experiment which provided stability information for this compound without having to isolate it or detect it directly (for details on the approach and equations, please see our method paper).\textsuperscript{[14]} To this end, reaction mixtures with 1 mM 1a, 5 mM potassium phosphate and 100 µg mL\textsuperscript{-1} TtPyNP (3.71 µM, 0.37 mol%) in 50 mM glycine/NaOH buffer at pH 9 in a total volume of 200 µL were incubated at 60 °C in a PCR cycler with lid heating (70 °C) for 2 h. This yielded a stable equilibrium of phosphorolysis at around 56.5% conversion of 1a to 2a, as determined by drawing samples of 40 µL from these mixtures, quenching in 200 µL 100 mM NaOH and analyzing as described above. This equilibrium is in accordance with the equilibrium constant of phosphorolysis ($K = 0.16$). The reaction mixtures were frozen overnight and then incubated at 98 °C for 1.5, 3 or 5 h to effect hydrolysis of 3 and denaturation of the enzyme (the half-life of TtPyNP under these conditions is around 5 min). These reaction mixtures with partially hydrolyzed 3 were frozen until analysis by re-equilibration. Re-equilibration was performed by addition of 20 µL of a 0.7 g L\textsuperscript{-1} stock of TtPyNP in 10 mM MOPS buffer (77.7 µg mL\textsuperscript{-1}, 2.89 µM, 0.28 mol% final concentration of active protein) and incubation at 60 °C. The reactions were allowed to reach their respective equilibria and samples of 50 µL were taken after 1, 1.5 and 2 h after enzyme addition and quenched in 200 µL 100 mM NaOH and analyzed via spectral unmixing as described above. The deviation of this second equilibrium from the first one was then used to calculate the hydrolytic loss of the sugar phosphate 3 according to equation (S25).

\[
[P1P]_h = \frac{K([P]_0 - c_2[N]_0 - c_2[P]_0 + c_2^2[N]_0) - c_2^2[N]_0}{Kc_2 - c_2 - K} \quad (S25)
\]
where \([P1P]_h\) is the concentration of hydrolyzed pentose-1-phosphate (3 in this case) [mM], \(c_2\) is the degree of conversion in the second (post-hydrolysis) equilibrium according to equation (S26), \([P]_0\) and \([N]_0\) are the initial phosphate and nucleoside concentrations [mM] and \(K\) is the equilibrium constant of phosphorolysis (0.16 for 1a).

\[
c_2 = \frac{[B]_2}{[N]_0} \quad \text{(S26)}
\]

where \([B]_2\) is the concentration of the nucleobase in the second equilibrium. The half-life of 3 was then obtained by fitting residual 3 as a function of time according to equations (S27) and (S28).

\[
[P1P](t) = [P1P]_1 - [P1P]_h \quad \text{(S27)}
\]
\[
[P1P](t) = [P1P]_1 e^{-\frac{t}{\tau}} \quad \text{(S28)}
\]

where \([P1P](t)\) is the pentose-1-phosphate concentration after incubation time \(t\) [h], \([P1P]_1\) is the pentose-1-phosphate concentration in the first equilibrium [mM] and \(\tau\) is the mean lifetime [h] from which the half-life \(t_{1/2}\) [h] was obtained via equation (S29).

\[
t_{1/2} = \tau \ln(2) \quad \text{(S29)}
\]

The fit of these equations to the experimental data yielded \([P1P]_1 = 0.54 \pm 0.01\) mM and \(\tau = 3.08 \pm 0.12\) h \((R^2 = 0.99)\) from which a half-life \(t_{1/2}\) of 2.13 \(\pm 0.08\) h was derived. The raw data and calculations for this experiment are freely available online\(^{[2]}\) and the fit results are shown in Figure S6. These results agree well both with the experimentally observed \([P1P]_1\) (0.56 mM) and the asymptotic approach to the maximum equilibrium shift predicted by equation (S30) (77% maximum conversion, 73.6% observed after 5 h hydrolytic incubation).

\[
c_{2,\text{max}} = \frac{-Y + \sqrt{Y^2 + 4[N]_0(1 - K)(c_1[N]_0K + [P]_0)}}{2[N]_0(1 - K)} \quad \text{(S30)}
\]

where \(c_{2,\text{max}}\) is the maximum possible conversion in the post-hydrolysis equilibrium, \(c_1\) is the conversion in the first (pre-hydrolysis) equilibrium, and \(Y\) is a factor described by equation (S31).

\[
Y = c_1[N]_0K - c_1[N]_0 + [N]_0K + [P]_0 \quad \text{(S31)}
\]

with definitions from above. For \([N]_0 = 1\) mM, \([P]_0 = 5\) mM, \(K = 0.16\) and \(c_1 = 0.56\) (56%), equation (S30) predicts \(c_{2,\text{max}} = 0.77\) (77%).

Considering the data reported by Bunton\(^{[13]}\) and us\(^{[14]}\) the half-life of 2.13 h at 98 °C found here suggests a half-life of >30 h at 60 °C, assuming that the hydrolysis of 3 approximately follows Eyring relationships. Considering further that the concentration of free 3 in a transglycosylation reaction cannot surpass the orthophosphate concentration (which we intentionally kept low), transglycosylations under “mild” conditions such as 60 °C run no risk of losing significant amounts of sugar phosphate to hydrolysis, unless extremely long reaction times are applied.

NMR experiments with a reaction mixture provided further insights into the stability of 3 under relevant conditions. In phosphate buffer at pH 7, we observed no loss of 3 from a reaction mixture.
incubated for 2 months at room temperature, indicating that the sugar phosphate is quite stable under moderate conditions. However, 3 is, like other sugar phosphates, labile to acidic conditions. At pH ≈1 (achieved via addition of HCl to a reaction mixture in equilibrium), full hydrolysis of 3 was apparent by disappearance of the signal corresponding to the anomeric proton after 1 month of incubation at room temperature (Figure S16).

**Figure S6.** Hydrolysis of 3 at 98 °C and pH 9.
4′-Methyl-5-iodouridine (1e) was prepared by TtPyNP-catalyzed transglycosylation. To this end, 5-iodouracil (2e, 13.9 mg, 0.059 mmol, 5 equivalents) and 4′-methyluridine (1a, 3 mg, 0.012 mmol, 1 equivalent) were dissolved in 40 mL 10 mM glycine buffer (pH 9) with 0.09 mM potassium phosphate (0.3 equivalents) and 4 µg mL\textsuperscript{-1} TtPyNP (0.15 µM, 0.05 mol%). The reaction mixture was intentionally kept very diluted since TtPyNP is inhibited by nucleobases such as 2e and more concentrated mixtures severely compromise the productivity of the enzyme. The reaction mixture was heated to 60 °C in a water bath. After 3 d, HPLC analysis revealed 68% conversion of 1a to the iodinated analogue 1e (please see Figure S7 and the externally hosted Supplementary Material for HPLC trace).\textsuperscript{[2]} The mixture was then concentrated to ≈7 mL \textit{in vacuo}, filtered to remove precipitated protein and injected into preparative HPLC. An HPLC method consisting of 10 min isocratic elution with 1% MeCN in water, followed by linear gradient to 10% MeCN over 40 min, cleanly afforded 1e after 35 min retention time. The fraction containing 1e was concentrated \textit{in vacuo}. Quantification of recovered 1e proved surprisingly difficult and inaccurate since the compound is quasi-intractable and practically insoluble in all solvents we tried. HRMS data were collected directly from the dilute eluate from the preparative HPLC and \textsuperscript{1}H-NMR analysis was performed with a saturated solution of 1e in D\textsubscript{2}O (ca. 0.5 mM, Figure S14). We estimate the isolated yield to be around 1.5−2 mg, corresponding to around 40% from 1a. HRMS (ESI): calculated for C\textsubscript{10}H\textsubscript{14}IN\textsubscript{2}O\textsubscript{6} [M+H]\textsuperscript{+}: 384.9897; found: 384.9895
\textsuperscript{1}H NMR (500 MHz, D\textsubscript{2}O): δ=8.35 (s, 1H; H-6), 5.98 (d, \textit{J}_{1',2'}=5.7 Hz, 1H; H-1'), 4.48 (t, \textit{J}_{1',2'}=\textit{J}_{2',3'}=5.8 Hz, 1H; H-2'), 4.23 (d, \textit{J}_{2',3'}=5.8 Hz, 1H; H-3'), 3.65 (m, 2H, H-5'), 1.28 (s, 3H; Me).

![Figure S7. HPLC chromatogram for the conversion of 1a to 1e with 5 equivalents of 2e. The sugar donor 1a has a peak area of 490 mAUs and the nucleobase 2a a total area of 1022 mAUs, indicating that 68% of 1a were converted. Please note that 1a/2a and 1e/2e have significantly different extinction coefficients at 260 nm.\textsuperscript{[5,6]}}](image-url)
Docking of uridine and 1a was performed by using the crystal structure of TtPyNP (PDB ID 2dsj) as a receptor structure. Dockings were performed in AutoDock VINA\textsuperscript{[17]} implemented in YASARA (Yet Another Scientific Artificial Reality Application). All water molecules were removed from the structure prior to the docking calculation. The receptor was treated as a rigid structure and the substrate was treated as a flexible molecule. Point charges on 2dsj were initially assigned according to the AMBER99\textsuperscript{[18]} force field and point charges on the nucleosides were generated with AM1-BCC.\textsuperscript{[19]} Docking results obtained for each ligand with the receptor were analyzed based on docking energy (kcal mol\textsuperscript{-1}). The best hit of 96 runs with $-5.94$ kcal mol\textsuperscript{-1} free energy of binding for uridine and $-5.07$ kcal mol\textsuperscript{-1} for 1a were used for subsequent docking of the phosphate using 32 runs. The crystal structure of GtPyNP (see below for crystallization; PDB ID 7m7k) was treated exactly as 2dsj. Here, uridine had a binding energy of $-5.70$ kcal mol\textsuperscript{-1} and 1a of $-5.60$ kcal mol\textsuperscript{-1}. The docking results are available as pdb files from the externally hosted Suppmentary Information.\textsuperscript{[2]}

Figure S8. Superposition of the proteins with docked (orange sticks) and cocrystallized uridine (white sticks) in the GtPyNP active site. Only the original crystal structure is shown. Residues interacting with the nucleobase (R168, S183, K187) and the relevant threonine (T84) are shown as grey sticks.
Crystallographic methods

The crystal structure of GtPyNP bound to uridine at a resolution of 1.9 Å was determined to enable a comparison of TtPyNP and GtPyNP via YARASA-docking of the uridine and 1a ligands (Table S5). The structure of GtPyNP revealed the typical two-domain architecture of a NP-II family PyNP enzyme, composed of an α-helical (α) domain and a mixed α-helical and β-sheet (α/β) domain (Figure S9A). Inspection of the active site after molecular replacement revealed positive density in the unrefined Fo-Fc density map, suggesting presence of the uridine ligand within the catalytic pocket (Figure S9B). Modelling of the uridine revealed that the substrate is recruited to the active site by a set of specific interactions, mediated by polar and aliphatic side chains extending from the cleft in between the α- and α/β-domains (Figure S9C). Notably, the positive electron density in the unrefined Fo-Fc density map is more pronounced for the uracil moiety, as compared to the ribose residue (Figure S9B), suggesting flexibility of the ribose, or degradation of the substrate. It is possible that sulfate, which was present in the crystallization solution, might have allowed for partial turnover of the substrate in crystallo. This phenomenon has been observed for the structurally only distantly related uridine phosphorylases, but is, to the best of our knowledge, unprecedented for pyrimidine nucleoside phosphorylases. Thus, we ascribe the lower electron density observed for the sugar moiety to the flexibility of this moiety in the open confirmation. Inspection of crystal packing contacts further revealed that GtPyNP homodimerizes via the α-domains, as suggested from the interaction with a symmetry mate (Figure S9D) and as expected for NP-II family PyNP enzymes.

The cloning, expression, and purification of GtPyNP were adjusted from the methods described above since the original construct did not crystallize. The gene encoding GtPyNP was amplified by PCR using oligonucleotides A&B (see below) and cloned into pET-24d (Novagen) using the restriction enzymes Ncol-HF (NEB) and BamHI-HF (NEB), yielding an N-terminally His6-tagged gene. Constructs were sequenced and subsequently transformed into E. coli BL21(DE3) cells (Novagen). Cells were inoculated in 4 L LB medium, supplemented with 12.5 g/L lactose and 50 mg/L kanamycin. Cells were incubated overnight with vigorous shaking (150 revolutions per minute) at 30 °C. Cells were harvested by centrifugation (3,500 g, 20 min, 4°C) and resuspended in 20 mL buffer A (20 mM HEPES-Na pH 8.0, 250 mM NaCl, 20 mM KCl, 20 mM MgCl2, 40 mM imidazole) before lysis in a M-110L Microfluidizer (Microfluidics). The lysate was heated for 30 min at 60 °C and subsequently cleared at 47,850 g for 20 min at 4°C. The supernatant was applied onto a 5 mL HisTrap FF column (GE Healthcare) for Ni-NTA affinity chromatography. After a wash step with ten column volumes (CV) of buffer A, GtPyNP was eluted with three CV of buffer B (20 mM HEPES-Na pH 8.0, 250 mM NaCl, 20 mM KCl, 20 mM MgCl2, 500 mM imidazole). GtPyNP was further purified by size exclusion chromatography (SEC) using a HiLoad 26/60 Superdex 200 column (GE Healthcare) equilibrated in buffer C (20 mM HEPES-K pH 7.5,
150 mM NaCl). Main peak fractions were concentrated to an absorbance at 280 nm of 31 arbitrary units (AU) measured on NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific), corresponding to an approximate concentration of 68 g L\(^{-1}\).

Oligonucleotides
A: 5'-TATACCATGGGCCATCACCATCACCATCACGTCGATTTAATTGCG-3'
B: 5'-TAATGGATCCTTATGAAATGGTTTCG-3'

**For crystallization**, uridine was dissolved in buffer C to a concentration of 25.8 mM. Purified GtPyNP (20 g L\(^{-1}\)) and uridine (10 mM) were combined in buffer C and subjected to crystallization screening by sitting-drop vapor diffusion at 20 °C using JCSG core suites I-IV (QIAGEN). Crystals appeared in a broad range of conditions within minutes up to a day. Well diffracting crystals grew within days in drops containing 0.75 µL GtPyNP-Uridine and 0.75 µL crystallization solution (0.2 M Ammonium sulfate and 20% PEG3350). Cryo-protectant was prepared by adding 20% (v/v) glycerol to the crystallization buffer. Crystals were transferred into the cryo-protectant solution, subsequently flash-frozen, and stored in liquid nitrogen.

**Diffraction data** were collected at Beamline ID29 of the European Synchrotron Radiation Facility (Grenoble, France).\(^{[22]}\) Data were processed with the XDS program package for data reduction,\(^{[23]}\) and merging and scaling was performed using the AIMLESS program as implemented in the CCP4 package.\(^{[24]}\) The data set was solved by molecular replacement using the crystal structure of *Bacillus stearothermophilus* PyNP (PDB ID 1brw, chain A)\(^{[25]}\) via the CCP4 implemented program Phaser.\(^{[26]}\) Coot\(^{[27]}\) in combination with phenix.refine, as implemented in the Phenix software suite,\(^{[28]}\) were used for iterative model building and refinement.
**Figure S9:** Crystal structure of uridine-bound GtPyNP. (A) Structure of uridine (orange sticks) bound to GtPyNP (grey cartoon) in two 180°-rotated orientations. (B) Unrefined F_o-F_c density map at 3.0 σ (above) and 1.8 σ (below), as observed after molecular replacement. Green mesh indicates positive density and red mesh indicates negative density. The refined GtPyNP-uridine model is shown as sticks. (C) Close-up on the active site formed in the cleft in between the α- and α/β-domains (coloring as in
(A)). (D) S-shaped homodimer formed by GtPyNP (dim grey) and symmetry mate GtPyNP' (silver; symmetry operator: -Y,-X,-Z-½). The dotted line highlights the dimerization interface of the α-domains.

Table S5: Crystallographic Table*

| GtPyNP-uridine (PDB-ID: 7m7k) |
|-------------------------------|
| **Method** | Molecular Replacement |
| **MR-Model** | PDB ID: 1brw, chain A, [25] |

**Data collection**
- **Wavelength (Å):** 0.979
- **Space group:** P 41 21 2
- **Cell dimensions:**
  - a, b, c (Å): 84.262, 84.262, 121.694
  - α, β, γ (Å): 90, 90, 90
- **Resolution (Å):** 49.33 - 1.893 (1.961 - 1.893)
- **Unique reflections:** 35436 (3340)
- **R_merge:** 0.193 (1.249)
- **R_pim:** 0.052 (0.362)
- **I / σI:** 13.26 (2.01)
- **CC1/2:** 0.999 (0.489)
- **Completeness (%)** 99.51 (95.29)
- **Redundancy:** 13.9 (12.6)

**Refinement**
- **Resolution (Å):** 49.33 - 1.893 (1.961 - 1.893)
- **No. reflections:**
  - Total: 35430 (3339)
  - Rfree: 1759 (174)
  - Rwork / Rfree: 18.9/22.7
- **No. atoms**
  - Macromolecules: 3246
  - Water: 270
  - Protein residues: 432
  - Ramachandran
    - Favoured (%): 98.6
    - Allowed (%): 1.4
    - Outliers (%): 0
    - Rotamer outliers (%): 2.03
    - Clashscore: 3.19
  - B-factors
    - Macromolecules: 29.03
    - Water: 32.93
    - R.m.s. Deviations
      - Bond lengths (Å): 0.007
      - Bond angles (°): 0.82

*Data was derived from a single crystal. Values in parentheses are for the highest resolution shell.
NMR spectra (the full raw data are freely available online\cite{2} and tabulated data are listed above)

Figure S10. $^1$H NMR (700 MHz, H$_2$O/D$_2$O, with water suppression, 5% (v/v) glycerol) of the phosphorolysis reaction mixture of 1a and TtPyNP prior to the reaction (described on page 8).

Figure S11. $^1$H NMR (700 MHz, H$_2$O/D$_2$O, with water suppression, 5% (v/v) glycerol) of the phosphorolysis reaction mixture of 1a and TtPyNP after the reaction (described on page 8).
Figure S12. $^1$H,$^3$P-HMQC (700 MHz, H$_2$O/D$_2$O, with water suppression, 5% (v/v) glycerol) spectrum of the phosphorolysis reaction mixture of 1a and TtPyNP after the reaction (described on page 8).

Figure S13. $^1$H,$^{13}$C-HMBC (700 MHz, H$_2$O/D$_2$O, with water suppression, 5% (v/v) glycerol) spectrum of the phosphorolysis reaction mixture of 1a and TtPyNP after the reaction (described on page 8).
Figure S14. $^1$H,$^{13}$C-HSQC (700 MHz, H$_2$O/D$_2$O, with water suppression, 5% (v/v) glycerol) spectrum of the phosphorolysis reaction mixture of 1a and TtPyNP after the reaction (described on page 8).

Figure S15. $^1$H NMR (500 MHz, D$_2$O) of a saturated solution of isolated 1e in D$_2$O (ca. 0.5 mM).
Figure S16. \textsuperscript{1}H NMR (500 MHz, H\textsubscript{2}O/D\textsubscript{2}O, with water suppression, 5\% (v/v) glycerol) of the phosphorolysis reaction mixture of 1a and TtPyNP (described on page 8) after addition of HCl and 1 month of incubation at pH 1.
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