

General Principles for Yield Optimization of Nucleoside Phosphorylase-Catalyzed Transglycosylations

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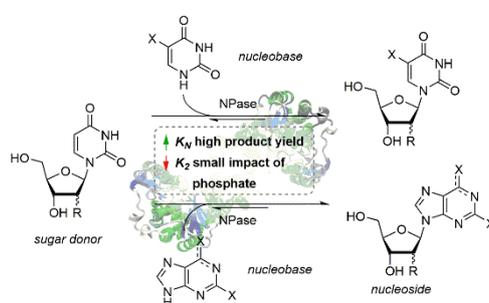
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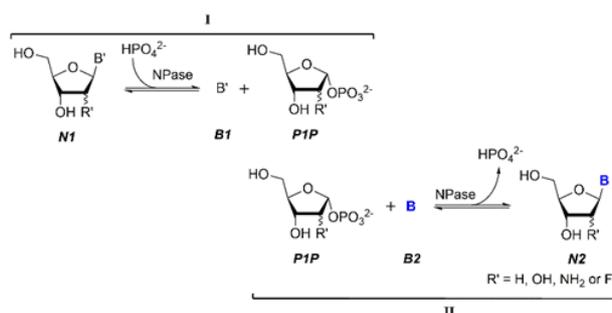
The biocatalytic synthesis of natural and modified nucleosides with nucleoside phosphorylases offers the protecting group-free direct glycosylation of free nucleobases in transglycosylation reactions. This contribution presents guiding principles for nucleoside phosphorylase-mediated transglycosylations alongside mathematical tools for straightforward yield optimization. We illustrate how product yields in these reactions can easily be estimated and optimized using the equilibrium constants of phosphorolysis of the nucleosides involved. Furthermore, the varying negative effects of phosphate on transglycosylation yields are demonstrated theoretically and experimentally with several examples. Practical considerations for these reactions from a synthetic perspective are presented, as well as freely available tools that serve to facilitate a reliable choice of reaction conditions to achieve maximal product yields in nucleoside transglycosylation reactions.

Graphical Abstract:



Nucleosides are highly functionalized biomolecules essential for the storage of information as DNA and RNA, cellular energy transfer and as enzyme cofactors. Modified nucleosides are widely employed as pharmaceuticals for the treatment of cancers and viral infections.^[1] Consequently, their synthetic accessibility is crucial. However, the preparation of nucleosides and nucleoside analogs by conventional synthetic methods heavily relies on protecting groups and, thus, suffers from poor atomic efficiency and low yields.^[2–5]

Biocatalytic methods offer the efficient and protecting group-free synthesis of pyrimidine and purine nucleosides. The use of nucleoside phosphorylases (NPases) for the preparation of nucleosides and their analogues in transglycosylation reactions is firmly established^[6] and numerous examples of enzymatic or chemoenzymatic syntheses can be found in the literature.^[7–14] NPases catalyze the reversible phosphorolysis of nucleosides to pentose-1-phosphates (Scheme 1, I). In transglycosylation reactions, a forward and a reverse nucleoside phosphorolysis are coupled *in situ* to glycosylate a free nucleobase with the pentose-1-phosphate generated by the first reaction (Scheme 1, I and II). Formally, this equals a direct glycosylation of the nucleobase to yield a nucleoside of interest. Conveniently, nature has provided an arsenal of robust biocatalysts that offer a broad substrate spectrum, excellent tolerance to harsh reaction conditions as well as perfect regio- and diastereoselectivity at the C1' position.^[11,12]



Scheme 1. Reaction sequence of a nucleoside transglycosylation.

Despite their great versatility, enzymatically catalyzed nucleoside transglycosylation reactions have previously suffered from an unclear interrelation between yields and the employed enzymes and starting materials. Particularly, the impact of different sugar donors and/or nucleobases as well as varying phosphate concentrations on the product yield had remained unclear until recently. The pioneering work of Alexeev *et al.*^[15] demonstrated that yields of nucleoside transglycosylation reactions involving uridine and adenosine can be accurately predicted based on the equilibrium constants of phosphorolysis of the sugar donor and the product nucleoside. They concluded that the ratio of the equilibrium constants of the sugar donor and the product nucleoside (K_1/K_2) determines maximum product yields and that an excess of sugar donor is further beneficial. On the other hand, increasing phosphate concentrations were shown to have a negative impact on product yields. However, Alexeev and colleagues^[15] based their calculations on the assumption that phosphate would not be consumed in the reaction and furthermore only investigated one example of a NPase-catalyzed transglycosylation. As a continuation of the considerations of Alexeev *et al.*^[15] we explored this reaction system from a practical synthetic perspective. In this work, we highlight the varying effect of excess phosphate on

product yield, an important feature of the reaction system that Alexeev and colleagues couldn't detect since they only investigated one example of a nucleoside transglycosylation. Furthermore, we provide a simplified equation for the estimation of product yield that allows for straightforward analytical solutions instead of the numerical solutions previously required.

Nucleoside transglycosylation reactions are generally considered as formal glycosylations of a nucleobase **B2**, which yields the corresponding nucleoside of interest, **N2**. Here, a starting nucleoside, **N1**, is used as a glycosylation agent with the purpose of donating the sugar moiety. In an enzyme cascade, the sugar donor **N1** is subjected to phosphorolysis yielding a pentose-1-phosphate **P1P**, which is consumed in the sequential reaction with nucleobase **B2** to produce **N2** (Scheme 1). The yield of this reaction is generally defined as the formation of **N2** in respect to **B2**, neglecting the other reagents **P1P**, **B1**, **N1** and phosphate. Indeed, inorganic phosphate only plays a catalytic role as it is used in the first step but liberated again in the following reaction.

Generally, yields in NPase-catalyzed transglycosylations are dictated by the equilibrium constraints of the two half reactions I and II:

$$K_1 = \frac{[B1][P1P]}{[N1][P]} \quad (1)$$

$$K_2 = \frac{[B2][P1P]}{[N2][P]} \quad (2)$$

where K_1 and K_2 are the apparent equilibrium constants of phosphorolysis of the sugar donor and product nucleoside, respectively, $[P]$ is the equilibrium concentration of phosphate, $[P1P]$ is the equilibrium concentration of the pentose-1-phosphate and $[N1]$, $[N2]$, $[B1]$ and $[B2]$ are the equilibrium concentrations of the nucleosides and bases. Alexeev *et al.*^[15] previously solved this system of equations by assuming a constant concentration of phosphate and numerically solving the resulting cubic equation. However, when we attempted to apply their equations to the synthesis of the pharmaceutically relevant nucleoside 5-ethynyluridine we were unable to obtain results that were in agreement with HPLC data (see Supplementary Table S1). Therefore, we sought establish a mathematical tool that allows general applicability and reaction optimization of all nucleoside transglycosylations. Bypassing the simplification made by Alexeev and coworkers, we implemented the system of equilibrium constraints (1) and (2) including all reagents as variables in a Python code to obtain more precise predictions (see externally hosted Python code and Table S1).^[16]

Numerical solutions of this system allowed theoretical examination of the effect of phosphate and sugar donor excess on the product yield, considering a reasonable range of equilibrium constants.^[17] Approaching zero phosphate concentration, the maximum (ideal) product yield can be obtained, but at higher phosphate concentrations an apparent loss of yield can be observed due to phosphorolysis (or non-synthesis) of the product nucleoside (Figure 1). While the K_1/K_2 ratio (equal to K_N) dictates the maximal yield with minimal phosphate, K_2 determines the extent of yield loss in the presence of phosphate. A high K_N in the order of 5–15 promises good to excellent yields (i.e. > 90%) with only moderate excesses (i.e. 2–fold) of the sugar donor. On the other

hand, reactions with a low K_N require a great excess of sugar donor to facilitate yields upwards of 50%. Interestingly, the effect of phosphate varies between systems with the same K_N , which results from the fact that high K_2 values dictate a greater degree of phosphorolysis of **N2** at non-negligible phosphate concentrations – even at great excess of the sugar donor **N1** (Figure 1). Notably, while potential formation of intermediate pentose-1-phosphate needs to be considered for a realistic assessment and prediction of synthetic yield, we only observed less than 4 percentage points of deviation from the ideal yield for any nucleoside transglycosylation with < 0.3 eq. of phosphate in the reaction conditions we covered with our considerations.^[16]

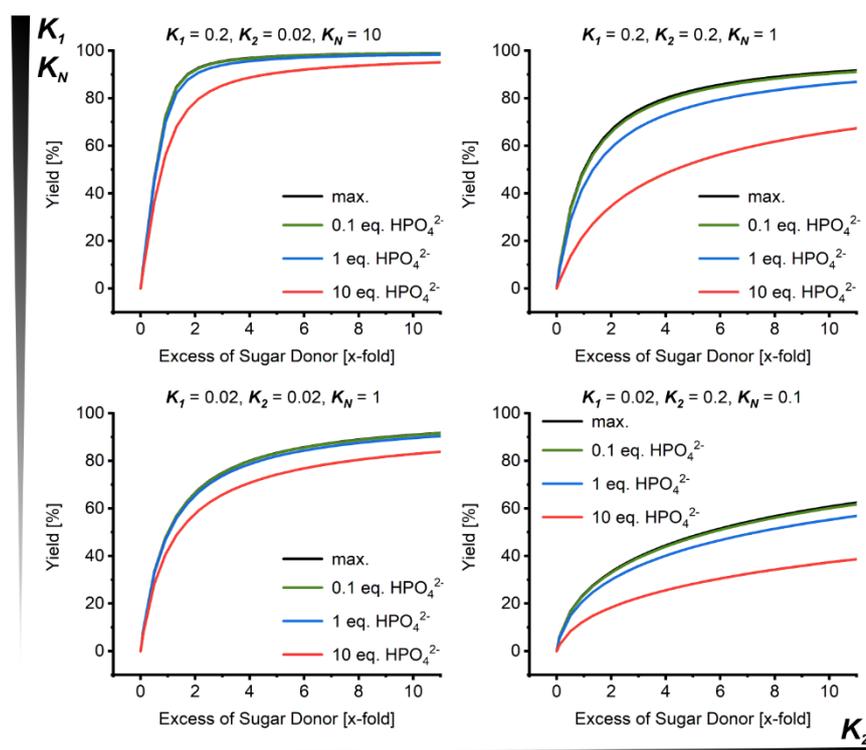


Figure 1. Impact of different K_1 and K_2 values on transglycosylation yield and phosphate gap. Realistic K_1 and K_2 values were assumed based on recently reported equilibrium constants.^[17] The graphs for maximal yield (max.; black), 0.1 eq. (green), 1 eq. (blue) and 10 eq. (red) of phosphate were plotted using numerical solutions of the system of equilibrium constraints (1) and (2) calculated with the Python code described in the external supplementary material.^[16]

To validate these predictions experimentally and demonstrate the varying impact of phosphate on the product yield, we prepared a series of natural and base-modified ribosyl nucleosides from their respective nucleobases, using uridine as a sugar donor. Fitting of the experimental data to the equilibrium constraints^[15,16] yielded equilibrium constants K_1 and K_2 very similar to those reported previously^[17] and revealed a great range of apparent equilibrium constants K_2 (0.01 to 0.35 at 60°C, pH 9) and K_N (0.4 to 16.0). In all cases, the experimental yields determined by HPLC agreed well with the predictions obtained for different phosphate concentrations (Figure 2). Our data emphasize that, as illustrated in Figure 1, particularly the yields of transglycosylation reactions with high K_2 values suffer enormously from phosphate concentrations higher than strictly necessary.

For instance, adenosine formation ($K_2 = 0.02$) was impacted only minorly by the addition of 10 eq. of phosphate (92% ideal yield vs. 88% experimental yield with 10 eq. of phosphate), but 5-ethynyluridine yield ($K_2 = 0.35$) dropped by more than 30 percentage points under the same conditions (53% ideal yield vs. 22% experimental yield with 10 eq. of phosphate; Figure 2). Thus, steep losses in yield should be expected for products with a high K value (K_2), whereas the synthesis of nucleosides with low K values tolerates significant amounts of phosphate (Figure 2). Consistent with our predictions, we only observed small deviations from the maximal yield in the experiments with 0.2 equivalents of phosphate. Thus, the concentration of phosphate should be kept low in synthetic nucleoside transglycosylations to obtain maximal yield. For these cases, calculation of maximal (ideal) conversion provides a close approximation of the yield and allows for the use of a simplified formula.

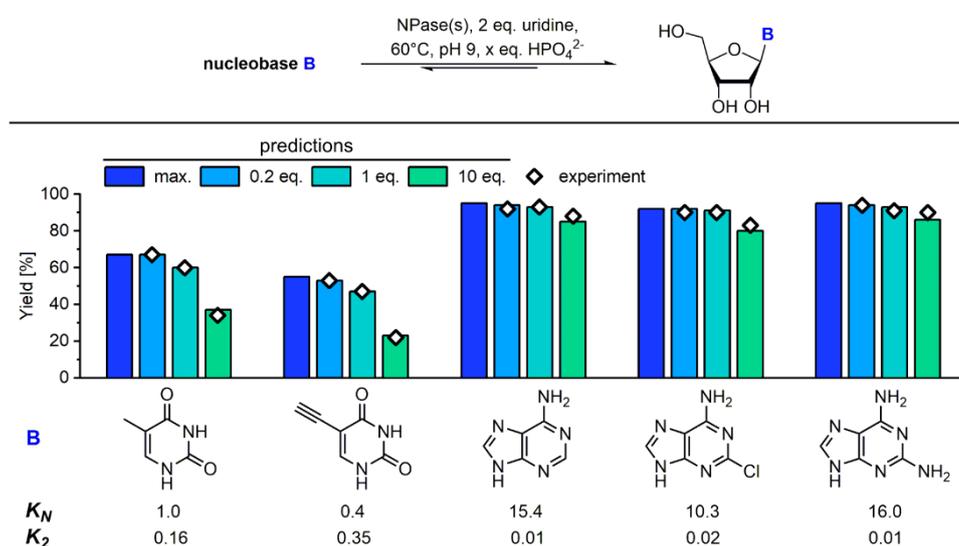


Figure 2. Biocatalytic synthesis of nucleosides by transglycosylation. Reactions were performed with 1 mM uridine as sugar donor ($K_1 = 0.16$), 0.5 mM nucleobase, 32 $\mu\text{g mL}^{-1}$ pyrimidine NPase (2.5 U mL^{-1}) and 66 $\mu\text{g mL}^{-1}$ purine NPase (5.0 U mL^{-1}) in 50 mM glycine buffer at pH 9 and 60 °C with either 0.1 mM (0.2 equivalents in respect to the starting base), 0.5 mM (1 eq.) or 5 mM (10 eq.) K_2HPO_4 in a total volume of 1 mL. Experimental yield (empty diamonds) was determined by HPLC considering conversion of the free nucleobase to its corresponding ribosyl nucleoside. Predictions (blue, light blue, turquoise and green columns) were carried out with the Python code described in the external supplementary material.^[16] The values for the maximal yield (max.; blue) can also be obtained via equation (4).

Considering ideal (intermediate-free) coupling of the two half reactions, I and II, the terms for phosphate and **PIP** would cancel in the mathematical consideration of this system, as the production of these in one half reaction is compensated by the consumption in the other. Considering the net reaction, one may therefore define:

$$K_N = \frac{K_1}{K_2} = \frac{[N2][B1]}{[N1][B2]} \quad (3)$$

with definitions from above. Solving this equation for the concentration of the product nucleoside, **N2**, yields only one physically possible solution that can be used to calculate ideal (phosphate- and **P1P**-free) yields of nucleoside transglycosylations with variable initial concentrations of the sugar donor **N1** and the nucleobase **B2**, $[N1]_0$ and $[B2]_0$, respectively:

$$[N2] = \frac{K_N([N1]_0 + [B2]_0) - \sqrt{K_N(K_N[N1]_0^2 - 2K_N[N1]_0[B2]_0 + K_N[B2]_0^2 + 4[N1]_0[B2]_0)}}{2(K_N - 1)} \quad (4)$$

Thus, the maximal yield (at zero phosphate) can be calculated easily *via* equation (4) to reflect a realistic estimate of the experimental yield if < 0.3 eq. of phosphate are used. Ideal yields for conversions employing a range of pyrimidine and purine ribosyl and 2'-deoxyribosyl nucleosides^[17] with different reaction conditions including sugar donor excess and temperature, can be calculated with an Excel sheet freely available from the externally hosted supplementary material.^[18]

These considerations and previous findings^[15,16] bear several practical implications for NPase-catalyzed nucleoside transglycosylations. First, a high K_1/K_2 ratio (high K_N) leads to excellent yields which can be achieved with moderate excess of the sugar donor, as mentioned by Alexeev and colleagues,^[15] and estimated easily with equation (4). Second, pyrimidine nucleosides serve better as sugar donors than purine nucleosides.^[17] From a practical point of view, uridine and thymidine recommend themselves as ribosyl and 2'-deoxyribosyl donor, respectively, due to their simple commercial availability and high K value. Third, phosphate concentration in the transglycosylation reaction should generally be kept as low as possible to prevent loss of product yield. This becomes especially important in the synthesis of nucleosides with high K values, such as pyrimidine nucleosides. Thus, 0.1–0.3 equivalents of phosphate in respect to the starting base may present an appropriate trade-off between reaction speed and maximal yield. A potential workflow for the fruitful application of the methodology presented in this work is suggested in the supporting information.

Given the easy accessibility of apparent equilibrium constants of phosphorolysis of any nucleoside of interest, the tools for yield prediction presented in this work aid the straightforward design and optimization of nucleoside transglycosylations to facilitate high yields in NPase-catalyzed reactions. Exact yield prediction of transglycosylations may be performed with our Python code considering phosphate^[16] and practical estimations for ideal yield can easily be obtained via equation (4).^[18]

Experimental Section

Enzymatic nucleoside transglycosylations were performed with 0.5 mM nucleobase, 1 mM uridine as sugar donor, 32 $\mu\text{g mL}^{-1}$ pyrimidine NPase (2.5 U mL^{-1} ; E-PyNP-0002, BioNukleo GmbH, Berlin, Germany) and 66 $\mu\text{g mL}^{-1}$

purine NPase (5.0 U mL⁻¹; E-PNP-0002, BioNukleo GmbH) in 50 mM glycine buffer at pH 9 and 60 °C with either 0.1 mM (0.2 equivalents in respect to the starting base), 0.5 mM (1 eq.) or 5 mM (10 eq.) K₂HPO₄ in a total volume of 1 mL. Reaction mixtures were prepared from stock solutions and started by the addition of the enzyme(s). Time to equilibrium was approximated *via* UV/Vis spectroscopy.^[19] Allowing for additional time after apparent reaction completion, the reactions were stopped after 1 h by quenching samples of 100 µL in an equal volume of MeOH and analyzed by HPLC. All experimental and calculated data are available online.^[16,18]

Acknowledgements

We would like to thank Sarah von Westarp for initial support. We are grateful for the support of R.T.G. by the German Research Foundation (DFG) under Germany's Excellence Strategy – EXC 2008/1 – 390540038 (UniSysCat), and by the Einstein Foundation Berlin (ESB) - Einstein Center of Catalysis (EC²). K.H. is funded by the Deutsche Forschungsgemeinschaft (DFG), grant number 392246628.

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Supplementary Information

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Author contributions (with definitions as recommended by Brand *et al.*^[1])

Conceptualization, F.K., R.T.G., P.N., A.W. and M.G.; Data curation, F.K. and R.T.G.; Formal analysis, F.K. and R.T.G.; Funding acquisition, R.T.G., P.N. and A.W.; Investigation, F.K. and K.H.; Methodology, F.K. and R.T.G.; Project administration, F.K., R.T.G. and A.W.; Resources, R.T.G., K.H., A.W. and P.N.; Software, R.T.G. ; Supervision, R.T.G., P.N. A.W. and M.G.; Validation, - ; Visualization, F.K.; Writing—original draft, F.K. and R.T.G.; Writing—review & editing, F.K., R.T.G., K.H., P.N., A.W. and M.G.

Conflict of Interest

A.W. is CEO of the biotech company BioNukleo GmbH. F.K. is a researcher at BioNukleo GmbH and P.N. is a member of the advisory board.

Sample analysis by HPLC

HPLC analysis was carried out with an Agilent 1200 Series HPLC System, employing a Phenomenex Kinetex Evo C18 100 Å column (250 mm x 4.6 mm). Separation was achieved using an isocratic elution at 3% MeCN in 20 mM NH₄Ac for 5 min followed by a linear gradient of 3–40% MeCN in 20 mM NH₄Ac over 20 min. The typical retention times for the nucleosides and bases in this work were 4.2 min (2-aminoadenine), 4.4 min (5-methyluracil), 4.6 min (5-ethynyluracil), 5.1 min (adenine), 5.6 min (5-methyluridine), 7.5 min (5-ethynyluridine), 9.3 min (2-aminoadenosine), 10.3 min (adenosine), 10.9 min (2-chloroadenine) and 13.2 min (2-chloroadenosine). For the calculation of concentration from peak area, calibration coefficients α [$\mu\text{M}\cdot\text{mV}^{-1}\cdot\text{s}^{-1}$] of 0.5625 (5-methyluracil), 0.3693 (5-methyluridine), 0.7267 (5-ethynyluracil), 0.9091 (5-ethynyluridine), 0.2680 (adenine), 0.2486 (adenosine), 0.2738 (2-chloroadenine), 0.2515 (2-chloroadenosine), 0.6150 (2-aminoadenine) and 0.3758 (2-aminoadenosine) were used.

Yield (Y) was calculated as

$$Y = \frac{P}{S + P}$$

where S and P are the concentrations determined from HPLC peak areas of the substrate and the product, respectively.

Table S1. Comparison of HPLC data and Calculations with Alexeev's formula^[3] for the synthesis of 5-ethynyluridine by transglycosylation^[a]

| Phosphate [eq.] | HPLC yield [%] | Calculated yield [%] Alexeev's formula | Calculated yield [%] our solutions ^[b] |
|-----------------|----------------|---|--|
| 0.2 | 53 | 48 | 53 |
| 1 | 47 | 21 | 47 |
| 10 | 22 | -185 | 23 |

[a] We used reaction conditions of 0.5 mM 5-ethynyluracil, 1 mM uridine as sugar donor, x equivalents of phosphate in respect to the starting base, pH 9 and 60 °C. Reactions were performed as described in the Experimental section. Equilibrium constants of $K_1 = 0.16$ and $K_2 = 0.35$ were used for calculation. [b] as described in the main text and the externally hosted Python code.

Table S2. HPLC data

| product | phosphate [equivalents] | peak area [mVs] | | concentration [mM] | | Yield of N2 [%] |
|------------------------|----------------------------|-----------------|-----------|--------------------|-----------|---------------------------|
| | | N2 | B2 | N2 | B2 | |
| 5-methyl- uridine | 0.2 | 689 | 224 | 0.254 | 0.126 | 66.9 |
| | 1 | 686 | 294 | 0.253 | 0.165 | 60.5 |
| | 10 | 411 | 524 | 0.152 | 0.295 | 34.0 |
| 5-ethynyl- uridine | 0.2 | 277 | 308 | 0.252 | 0.224 | 52.9 |
| | 1 | 265 | 369 | 0.241 | 0.268 | 47.3 |
| | 10 | 115 | 522 | 0.105 | 0.379 | 21.6 |
| adenosine | 0.2 | 1811 | 152 | 0.450 | 0.041 | 91.7 |
| | 1 | 1913 | 137 | 0.476 | 0.037 | 92.8 |
| | 10 | 1769 | 231 | 0.440 | 0.062 | 87.7 |
| 2-chloro- adenosine | 0.2 | 1826 | 191 | 0.459 | 0.052 | 89.8 |
| | 1 | 1887 | 189 | 0.475 | 0.052 | 90.2 |
| | 10 | 1719 | 330 | 0.432 | 0.090 | 82.7 |
| 2-amino- adenosine | 0.2 | 1103 | 41 | 0.414 | 0.025 | 94.3 |
| | 1 | 1125 | 67 | 0.423 | 0.041 | 91.1 |
| | 10 | 1083 | 76 | 0.407 | 0.047 | 89.7 |

Suggested workflow for NPase-catalyzed nucleoside synthesis

For maximal yields in NPase-mediated transglycosylations, phosphate should always be kept minimal. Therefore, we recommend abstaining from adding more than catalytic amounts of phosphate to reaction mixtures (e.g. 0.1 equivalents in respect to the starting base).

Step 1: Selection of the sugar donor

A sugar donor should be selected based on price and equilibrium constant of phosphorolysis. Ideally, K_1 should be as high as possible. If, however, price prevents the use of the “ideal” sugar donor, a trade-off needs to be made to ensure profitability, since, in most cases, the sugar donor needs to be applied in excess to grant satisfactory yields. Additionally, solubility needs to be considered and reaction conditions may need to be adjusted. Generally, we recommend uridine and thymidine, or, if available, 7-methylated guanosyl nucleosides.

Step 2: Determination of the equilibrium constant of phosphorolysis of the product nucleoside (K_2)

If the nucleoside of interest is available in pure form, the equilibrium constant of phosphorolysis K_2 can easily be determined in a phosphorolysis experiment, monitored either by HPLC or UV spectroscopy.^[2] Here, the phosphate concentration should be selected so that between 20 and 80% conversion of the nucleoside are observed to obtain reliable results. Equilibrium should be clearly visible and potential decay of reaction products (pentose-1-phosphates) should be considered if either no equilibrium is observable or apparent conversion is visible after an initial quick equilibrium. To ensure pentose-1-phosphate stability, it may be recommended to perform these experiments, as well as transglycosylation reactions, at alkaline pH. However, if the working space of the enzyme precludes application at pH 8–9, phosphorolysis may be performed under the same conditions (temperature, pH) as the anticipated transglycosylation.

If the nucleoside of interest is not at hand, K_2 may also be estimated by transglycosylation if the corresponding nucleobase is available. In a transglycosylation employing 1 eq. of sugar donor and 0.1 eq. of phosphate, the yield can be approximated and K_2 can be estimated with equation (3), if K_1 is known (if K_1 is unknown, see above).

Step 3: Consideration of the reaction conditions

Using equation (4), the required sugar donor excess to achieve satisfactory yields can be calculated. In some cases 2–3 eq. of sugar donor are sufficient to reach >90% yield, in other cases as much as 20 eq. may be needed. In the latter case, settling for less yield or the use of a different sugar donor may need to be considered.

Step 4: The Transglycosylation

The concentration of the main reactants (sugar donor, base) can be adjusted as desired as confirmed by Alexeev and colleagues,^[3] as long as 1) their ratio is maintained and 2) the solubility of the reactants

allow scaling up of the reaction (e.g. the reaction can be carried out on a 2 mM or 50 mM scale, as long as all components are soluble).

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