Selenium-modified nucleosides are powerful tools to study the structure and function of nucleic acids and their protein interactions. The wide-spread application of 2-seleno pyrimidine nucleosides is currently limited by low yields in established synthetic routes. Here, we describe the optimization of the synthesis of 2-Se-uridine and 2-Se-thymidine derivatives by thermostable nucleoside phosphorylases in transglycosylation reactions using natural uridine or thymidine as sugar donors. Reactions were performed at 60 or 80 °C and at pH 9 under hypoxic conditions to improve the solubility and stability of the 2-Se-nucleobases in aqueous media. To optimize the conversion, the reaction equilibria in analytical transglycosylation reactions were studied. The equilibrium constants of phosphorolysis of the 2-Se-pyrimidines were between 5 and 10 and thus belong to the highest described so far. Thus, a ten-fold excess of sugar donor was needed to achieve 40−48% conversion to the target nucleoside. Scale-up of the optimized conditions provided four Se-containing nucleosides in 6−40% isolated yield which compares favorably to established chemical routes.
Scheme 1. Approaches to the synthesis of 2-Se pyrimidine nucleosides. Previous work has established introduction of selenium via a methylated sulfo-nucleoside or selenation of isocytidine. Our work aims for biocatalytic direct glycosylation of a selenium-containing nucleobase.

from the natural nucleosides uridine or thymidine and a selenium-containing nucleobase.

For synthetic applications, a higher substrate loading than previously employed (1 mM at pH 7) would be highly desirable to reduce solvent use and facilitate purification. Based on the pKₐ of 2-Se-uracil (1) (7.18 at RT)⁹ we hypothesized that a slightly higher reaction pH would facilitate dissolution of 1 and 2-Se-thymine (2) as their corresponding anions. Since it was not available from the literature, we determined the pKₐ of 2 through analysis of its UV absorption spectra at different pH values and found a similar value (7.49 ± 0.01, Figure S1) to that of 1. Hence, compared to the natural nucleobases, the Se-containing analogues were more acidic.³⁴,³⁵ Next, we investigated the solubility of 1 and 2 at pH values below and above their pKₐ to see if a pH shift would allow higher substrate loading. At pH 7, 1 was not soluble at 10 mM, as indicated by the presence of precipitate (Figure S2). In contrast, full dissolution was observed at pH 9, confirming that a higher reaction pH can increase the solubility of the free nucleobase through deprotonation and solvation of the corresponding salt. Nonetheless, this effect was modest, as only 2 was fully soluble at 20 mM at pH 9 and RT.

A reaction temperature of 80 °C was initially chosen for the synthesis of Se-modified nucleosides as higher reaction rates are observed with the enzyme and it can be assumed that a high reaction temperature would further improve the solubility of 1 and 2. Initial studies, however, revealed product losses during the synthesis of 2'-deoxyribonucleosides (data not shown) which can be correlated to the hydrolysis of the transglycosylation intermediate b' at higher temperatures.³⁶,³⁷ Hence, reaction temperatures of 80 °C were applied for the synthesis of ribonucleosides, while 60 °C was chosen for reactions involving 2'-deoxynucleosides.

As the pH and temperature might influence the activity of PyNP-04, we determined specific activities of the enzyme at pH 7 or 9. To this end, we performed phosphorolysis reactions (Figure S3A) with the sugar donors uridine (a) and thymidine (b) in a MOPS (pH 7) or glycine buffer (pH 9). PyNP-04 was active under all conditions and, for instance, catalyzed the phosphorolysis of a at 80 °C with activities of approximately 80 U mg⁻¹ (Figure S3B). We additionally

Scheme 2. Biocatalytic synthesis of 2-Seleno pyrimidine nucleosides via transglycosylation reactions using a thermostable pyrimidine nucleoside phosphorylase (PyNP) as biocatalyst.
performed the glycosylation of 2 with the sugar phosphate anion to confirm that the higher reaction pH did not inhibit the second reaction step (reverse phosphorolysis). We observed no significant difference in glycosylation activity at pH 7 or 9, indicating that the deprotonated Se-base is well-accepted by the enzyme (Figure S4). Taken together, these data show that a reaction pH of 9 improves the solubility of 1 and 2 and thus permits higher substrate loadings, while still allowing excellent enzymatic activity, with reaction temperatures of 60 °C or 80 °C presumably facilitating additional solubility.

**Hypoxic conditions for the stability of the 2-Se-bases**

The application of an alkaline reaction environment and high temperatures guided us to explore the stability of the 2-Se-bases under these rather harsh conditions. In early experiments we noticed significant oxidation of the starting materials 1 and 2 which manifested itself as a loss of substrate and product, as observed by HPLC, as well as discoloration of the reaction mixtures from colorless to red and black (data not shown). To prevent oxidation and deselenation, reducing agents, such as dithiothreitol (DTT) and ascorbic acid, and inert gases like argon and nitrogen have previously been applied in chemical syntheses and redox studies. This led us to examine whether and for how long the stability of the 2-Se-bases can be increased by the application of DTT and/or a nitrogen atmosphere, which appeared compatible with our reaction system. To this end, we incubated 1 and 2 in different buffer systems (glycine/NaOH pH 9, with or without DTT and with or without a nitrogen atmosphere) at 80 °C and analyzed samples at different time points to check for the integrity of the starting material. Without any additives, oxidation products were already detected after 2 h, with most of the Se-base being degraded after 24 h (Figure 1A and S4). The addition of DTT had a slightly conserving effect, since no oxidation was apparent after 2 h, but significant deselenation was apparent after 4 and 24 h (Figure 1B and S5). In contrast, saturation of the solution with nitrogen almost completely prevented oxidation of 1 and 2, with only minimal oxidation product being detectable after 24 h (Figure 1C and S5). As expected, combining DTT and nitrogen prevented oxidation completely and conserved the starting material for 24 h (Figure 1D and S5).

Having established that DTT and nitrogen aid in stabilization of 1 and 2, we questioned if these conditions would affect the activity of PyNP-04. Therefore, we performed phosphorolysis experiments at 80 °C with either a nitrogen-saturated buffer or the model substrate 5-iodouridine (c) in buffer with 5 mM DTT, as the latter nucleoside allowed us to obtain UV spectra that could be deconvoluted despite the heavy background absorption of DTT at lower wavelengths (see Figure S3 of ref. [34]). In contrast to the comparable activity observed in nitrogen-saturated buffer, the application of DTT caused a >40% drop in enzymatic activity with our model substrate c (160 U mg⁻¹ to 95 U mg⁻¹; Figure S3B). However, due to the prevention of oxidation by DTT, we considered this decrease in enzymatic activity tolerable and proceeded with these conditions. In summary, the application of hypoxic conditions combined with the reducing agent DTT improved the stability of 1 and 2 in aqueous solution with no detectable degradation after 24 h at 80 °C, while only slightly affecting the enzymatic activity.

**Optimization of the enzymatic synthesis of 2-Se-pyrimidine nucleosides based on thermodynamic calculations**

With hypoxic and alkaline reaction conditions in hand which served well to ensure solubility and stability of the nucleobases 1 and 2, we turned our attention to the improvement of the previously observed low conversions. Recent work from our group had demonstrated the application of analytical-scale experiments and thermodynamic calculations for the yield optimization in NP-catalyzed transglycosylations. Therefore, we sought to transfer the same principles which had succeed for the synthesis of dihalogenated nucleosides to the preparation of Se-pyrimidines to obtain improved yields. Since nucleoside phosphorolysis (and consequently also the reverse reaction, glycosylation) is a thermodynamically controlled reaction, one may calculate the equilibrium state of transglycosylations via the corresponding equilibrium constants. Thus, when both equilibrium constants in the system are known, conversions can be optimized in silico to suggest conditions that enable the desired extent of product formation (for further details, please see [37]).

Equilibrium constants for the phosphorolysis of the sugar donors have been described recently for a broad temperature range, but the corresponding values for 2-Se-nucleosides have not been reported yet. Since these constants are...
predictions using different sugar donor to nucleobase ratios were carried out as described previously. Similar results were obtained for the other products (Table 1). Please see the externally hosted Supporting Information for raw data and calculations.\cite{34}

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**Table 1.** Equilibrium state thermodynamic calculations were used to determine appropriate reaction conditions for the synthesis of 1a–2b

| Product | Product formation [%] at equilibrium for 5-fold sugar donor excess\[^a\] | Equilibrium constant of phosphorolysis | Product formation [%] at equilibrium for 10-fold sugar donor excess calculated\[^b\] | experimental (1 mM nucleobase)\[^c\] | experimental (5 mM nucleobase)\[^c\] |
|---------|-------------------------------------------------|----------------------------------|-------------------------------------------------|-----------------|-----------------|
| 1a      | 30.5                                            | 9.37                             | 39                                               | 40              | 46 (4 h)        |
| 1b      | 30                                              | 6.07                             | 40                                               | 40              | 39 (3 h)        |
| 2a      | 35                                              | 6.75                             | 44                                               | 47              | 48 (4 h)        |
| 2b      | 33                                              | 4.85                             | 44                                               | 43              | 45 (3 h)        |

\[^a\] Transglycosylation reactions were performed in a total volume of 1 mL in 50 mM glycine/NaOH buffer pH 9 and 5 mM DTT at 60 °C (b) or 80 °C (a). \[^b\] 1 mM 2-Se-nucleobase (1 or 2), 50.4 µg mL\(^{-1}\) (around 4 U) PyNP-04, 5 mM sugar donor (a or b). \[^c\] 1 mM 2-Se-nucleobase (1 or 2), 50.4 µg mL\(^{-1}\) (around 4 U) PyNP-04, 10 mM sugar donor (a or b).\cite{37} Please see the externally hosted Supporting Information for raw data and calculations.\cite{34}

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required for yield optimization via equilibrium thermodynamics, we performed small-scale transglycosylation reactions to determine these indirectly. Using a five-fold excess of the sugar donor over the Se-nucleobase and 0.09 equivalents of phosphate, equilibrium conversions between 30 and 35% were observed (Table 1, Figure 2A for 2b as a visual example). This yielded equilibrium constants of the phosphorolysis of the Se-nucleosides 1a–2b in the range of 5 to 10 (Table 1). Interestingly, these equilibrium constants surpass those of other natural and modified pyrimidine nucleosides (0.1–0.8)\cite{37–39} by around an order of magnitude. While the reason for these high equilibrium constants is unclear to date, we hypothesize that the increased electron density near the glycosylation site may contribute to a weaker C1’-N1 bond.

Next, we used thermodynamic calculations to predict the maximum conversions in transglycosylation reactions employing different sugar donor excesses (Figure 2A, Table 1). These predictions revealed that the high equilibrium constants of phosphorolysis of the target nucleosides limited the maximum conversion severely. For example, to obtain 50% conversion to the Se-nucleoside 1a, an approximately 20-fold excess of the sugar donor a would be necessary. However, such a high sugar donor excess is not suitable for preparative experiments due to high substrate costs and waste. In addition, we expected that this would also prevent an efficient workup and purification. Thus, we applied a sugar donor excess of 10 as a compromise to the aforementioned issues and maximum conversion. Although other sugar donors such as 5-ethynyluridine or 7-methylguanosine or direct glycosylation approaches with the sugar phosphates a’\(^a\) or b’\(^b\) might provide higher conversions than the application of natural uridine (a) and thymidine (b), these starting materials are considerably more expensive and would render this synthetic approach unfeasible (Table S1). Therefore, we decided to employ cheaply available a and b as sugar donors, which conveniently also offer some of the more favorable equilibrium constants of phosphorolysis among the natural nucleosides.\cite{38}

Finally, we confirmed our predictions for 10 equivalents of a or b and evaluated the reaction times until equilibrium in analytical-scale experiments under optimized conditions.
The predicted extent of product formation was observed for all four 2-Se-nucleosides (Figure 2B, Table 1) with reaction completion occurring within 3 to 4 h. Taken together, our thermodynamic characterization of the reaction system revealed unfavorable equilibrium constants of phosphorolysis of all Se-containing target nucleosides, severely limiting the maximum conversion achievable in transglycosylation reactions. However, a 10-fold excess of the sugar donors allowed for 40–48% conversion for all Se-nucleosides.

Enzymatic synthesis and purification in semi-preparative scale

After optimizing the synthesis under hypoxic and alkaline reaction conditions in small-scale experiments, we aimed to synthesize and purify the four 2-Seleno pyrimidines in semi-preparative scale. Therefore, the optimized reaction conditions were up-scaled to a volume of 50 mL with 5 mM 2-Se-nucleobase. The observed product formations in the larger scale of 41–47% after 3 (2b), 4 (1b) or 5 h (1a, 2a) (Table 2) were in good accordance with the small-scale experiments. Purification of the target compounds from these reaction mixtures, however, proved rather challenging due to the presence of large quantities of unreacted sugar donor. In fact, our attempts to purify any of the products by preparative HPLC were unsuccessful as the obtained material persistently contained sugar donor starting material. Therefore, we applied an initial silica chromatography step on normal phase to remove most of the sugar donor, followed by a second purification step via preparative HPLC. Using this two-step process, we obtained 4.9–29.5 mg of the target nucleosides, corresponding to isolated yields of 6–40% (Table 2, Figure S6-S9). Despite the incomplete conversions and product losses during purification, our biocatalytic route compares favorably to its counter parts where yields of less than 10% are typically achieved.[22]

| Table 2. Yields and purity of 2-Se-pyrimidine nucleosides |
|-----------------|-----------------|------------------|------------------|
| Product | Conversion [%] (Time [h]) | Isolated yield [mg] (%[%]) | Purity [%][a] |
| 1a | 44.5 | 4.9 (6.3) | 98 |
| 1b | 41.4 | 29.5 (40.5) | 94 |
| 2a | 47.5 | 20.8 (25.9) | 99 |
| 2b | 45.3 | 9.6 (12.5) | 99 |

The 50 mL reaction mixture consisted of 5 mM 2-Se-base (1 or 2), 50 mM sugar donor (a or b), 5 mM DTT and 24.6 µg mL\(^{-1}\) around 98 U PyNP-04 in 50 mM glycine/NaOH buffer pH 9 saturated with nitrogen. The reaction temperature was 60 °C (1b, 2b) or 80 °C (1a, 2a). [a] Purity was determined via analytical HPLC. Please see the externally hosted Supporting Information for raw data.

Conclusion

This work presents a biocatalytic synthesis of 2-Se-pyrimidine nucleosides via transglycosylation from uridine or thymidine. Following a thermodynamic characterization of the phosphorolysis of the target nucleosides on a small scale, four selenium-containing pyrimidine nucleosides were obtained in scale-up experiments where alkaline and hypoxic conditions, as well as high temperatures, enabled sufficient solubility and stability of the starting materials. Although the present route delivers improved yields compared to previous efforts, product purification currently represents a major bottleneck and needs to be addressed in future studies.

Experimental Section

General Information

All chemicals and solvents were of analytical grade or higher and purchased, if not stated otherwise, from Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), TCI Deutschland (Eschborn, Germany), Carbosynth (Berkshire, UK) or VWR (Darmstadt, Germany). 2-Se-uracil (1) and 2-Se-thymine (2) were prepared according to literature procedures.[32]

The thermostable nucleoside phosphorylase PyNP-04 (E-PyNP-0004)[31] was obtained from BioNukleo GmbH (Berlin, Germany) and used as recommended by the manufacturer. PyNP-04 was heterologously expressed in Escherichia coli and purified by affinity chromatography. The provided stock solution (1.12 mg mL\(^{-1}\)) was stored at 4 °C. The enzyme originates from a thermophilic bacterium with an optimum growth temperature of 80 °C.

All UV/Vis absorption spectra were recorded with a BioTek PowerWave HT plate reader using UV/VIS-transparent 96-well plates (UV-STAR F-Bottom #655801, Greiner Bio-One). All raw and calculated data described in this article are freely available from an external online repository.[34]

Determination of the dissociation constant (pK\(_a\)) of 2-Se-modified nucleobases

The pK\(_a\) of 1 and 2 was determined by analysis of their UV/Vis absorption spectra between pH 4 and pH 10 at RT. In a total volume of 10 mL, the nucleobases were dissolved to a concentration of 100 µM in 50 mM MOPS buffer (initially pH 9). Desired pH values were adjusted with HCl and NaOH and samples of 200 µL were transferred to a UV/Vis-transparent 96-well plate to record the UV absorption spectra in the range of 250 to 350 nm in steps of 1 nm. The spectra were analyzed via spectral unmixing as previously described\(^{35,40}\) using a fully protonated (pH 4) and a fully deprotonated (pH 10) spectrum as substrate and product reference for the deprotonation reaction, as spectra obtained near these pH values displayed identical shape (indicating no further reaction). For the determination of the pK\(_a\), the experimental data were fitted to equation (1), whereby the pH was set as the input variable, α as the dependent variable and pK\(_a\) as the parameter to fit.[41]

\[
\alpha = \frac{x_{deprot}}{x_{deprot} + x_{prot}} = \frac{10^{pH-pK_a}}{1 + 10^{pH-pK_a}} \tag{1}
\]
Solubility of the 2-Se-modified nucleobases
The solubility of 1 and 2 was tested in 50 mM MOPS/NaOH buffer pH 7 and 50 mM glycine/NaOH buffer pH 9 at RT. In a total volume of 0.1 mL, the nucleobases were dissolved to concentrations of 1 mM, 5 mM, 10 mM and 20 mM. The solutions were observed for the occurrence of precipitate.

Stability of the 2-Se-modified nucleobases
The stability of 1 and 2 was analyzed at 80 °C in Pyrex® glass tubes with screw caps. Therefore, the nucleobases were dissolved to a concentration of 5 mM in the following four buffers: (a) 50 mM glycine/NaOH buffer pH 9, (b) 50 mM glycine/NaOH buffer pH 9 with 5 mM 1,4-dithioreitol (DTT), (c) 50 mM glycine/NaOH buffer pH 9, saturated with nitrogen and (d) 50 mM glycine/NaOH buffer pH 9 with 5 mM DTT, saturated with nitrogen. Samples were taken after 0 h, 2 h, 4 h and 24 h, and diluted to a final concentration of 1 mM nucleobase with MeOH. After centrifugation (4 °C and 21,500 g for 20 min), samples were analyzed by analytical HPLC as described below.

Enzyme activity assays
The activity of PyNP-04 was determined by performing phosphorolysis reactions with the substrates uridine (a), thymidine (b) and the model substrate 5-iodouridine (c). Samples were analyzed by the UV/Vis spectroscopy-based assay described recently.[34,44] Briefly, a 500 µL reaction mixture consisting of 1 mM nucleoside and 50 mM K₂HPO₄ in 50 mM buffer (glycine/NaOH pH 9 or MOPS/NaOH pH 7) was preheated to the desired temperature (60 °C or 80 °C). The reaction was started by the addition of the enzyme (typically 10 µL of enzyme stock solution pre-diluted in 2 mM potassium phosphate buffer pH 7). Final concentrations of PyNP-04 from 150 to 750 ng mL⁻¹ were applied. At timely intervals, samples of 60 µL were taken and quenched in 450 µL 100 mM NaOH and 200 µL of the diluted sample was transferred to a UV/Vis-transparent 96-well plate to record the UV absorption spectra from 250 to 350 nm in steps of 1 nm. Spectral analysis was carried out as described previously,[34,40] with software[42] and reference spectra[43] freely available online.

To assay PyNP-04 for the glycosylation of the Se-nucleobase 2, reactions consisting of 1 mM 2, 10 mM b' and 70 µg mL⁻¹ enzyme in 50 mM glycine/NaOH buffer pH 9 were monitored. For analysis, samples of 60 µL were withdrawn, quenched in an equal volume of MeOH and diluted with 400 µL 100 mM glycine/NaOH buffer pH 10. The experimental spectra were fitted with corresponding reference spectra of 2 and 2b obtained under the same conditions.

One unit (U) of enzyme activity was defined as the amount of the enzyme catalyzing the conversion of 1 µmol of substrate per minute under the described assay conditions.

Optimization of the enzymatic synthesis of 2-Se-modified nucleosides
The 2-Se-modified nucleosides 1a–2b were accessed in a one-pot transglycosylation reaction using the pyrimidine nucleoside phosphorylase PyNP-04 as biocatalyst. The thermodynamic characterization was performed as described previously.[36,37] Reaction equilibria were determined from reactions with 1 mM 2-Se-nucleobase (1 or 2), 5 mM sugar donor (a or b), 5 mM DTT, 50 mM glycine/NaOH buffer pH 9 and 50.4 µg mL⁻¹ (around 4 U) PyNP-04 in a total volume of 1 mL. The nitrogen-saturated reaction mixtures were incubated at 60 °C for the deoxyribosides 1b and 2b and 80 °C for the ribosides 1a and 2a until reaction completion (indicated by no further product formation; within 30 min under these conditions). Samples were diluted to 1 mM sugar donor concentration in MeOH, centrifuged (4 °C, 21,500 g, 20 min) and analyzed by HPLC.

The equilibria constants of phosphorolysis of the 2-Se-pyrimidine nucleosides were calculated via equilibrium state thermodynamics and numerical solutions. Nucleoside phosphorolysis is a tightly thermodynamically controlled reaction and transglycosylations behave as coupled equilibrium reactions as they comprise a forward and a reverse phosphorolysis. Thus, knowledge of the equilibrium constant of the sugar donor, the concentrations of the starting materials as well as the degree of conversion to the product nucleoside allows calculation of the equilibrium constants of phosphorolysis of the product nucleoside via numerical solutions of the system of coupled equilibrium constraints (see[36,37]). These numerical solutions can either be obtained via the Python code described in our previous work[44] or, more conveniently, the Excel sheet presented in the externally hosted Supplementary Information of this publication. Herein, we used the previously published equilibrium constants of phosphorolysis of the sugar donors a and b for calculation.[38,45] Based on the obtained equilibrium constants, expected conversions for the 2-Se-nucleosides using a 10-fold sugar donor excess were calculated (please see the externally hosted Supplementary Information for details).[34,44] To verify the theoretical calculations and to monitor the reaction progression, reactions with 5 mM 2-Se-base, 50 mM sugar donor, 5 mM DTT in 50 mM glycine/NaOH buffer pH 9 saturated with nitrogen and 2.46 µg mL⁻¹ (around 2 U) PyNP-04 in a total volume of 1 mL were performed at 60 °C for the deoxyribosides and 80 °C for the ribosides. Samples were diluted 50-fold in MeOH, centrifuged (4 °C, 21,500 g, 20 min) and analyzed by HPLC.

Analytical High-performance liquid chromatography (HPLC)
Analytical HPLC analyses were carried out with an Agilent 1200 series system equipped with an Agilent DAD detector using a Phenomenex (Aschaffenburg, Germany) reversed phase Kinetex EVO C18 column (250 x 4.6 mm). Samples were analyzed at two wavelengths (λ = 260 nm, 307 nm) at 25 °C and a flow rate of 1 mL min⁻¹. Isocratic elution was performed using 97% 20 mM ammonium acetate buffer and 3% acetonitrile for 7 min followed by a linear gradient to 60% 20 mM ammonium acetate buffer and 40% acetonitrile over 8 min. Afterwards, the initial conditions were restored and maintained for 4 min.

Conversions were determined by quantifying the 2-Se-nucleosides 1a–2b and 2-Se-nucleobases 1 and 2 at 307 nm using equation (2), whereby $P_X$ is the peak area of compound X and $P_{total}$ is the sum of all peak areas at 307 nm. Substrates and products were identified based on their retention time and UV absorption spectra by...
pH 9 and 24.6 µg mL\(^{-1}\) was dissolved in preparative HPLC.

The reaction mixture was saturated with nitrogen. Deoxyribosyl derivatives were prepared at 60 °C and ribosyl derivatives at 80 °C. The reactions were stopped after 3 to 5 h by a pH shift to 13 through addition of 10 M NaOH (monitored with a pH-electrode). The pH was shifted back to 9 using 25% HCl and proteins were removed by filtration at room temperature via column chromatography (10% MeOH in DCM) using a vacuum pump and 0.45 µm cellulose. The collected product was analyzed at 210 nm. Deionized water and acetonitrile were UV-active and cannot be detected with a DAD system.

**Synthesis of 2-Se pyrimidine nucleosides in semipreparative scale**

2-Se nucleosides were synthesized in a reaction volume of 50 mL consisting of 5 mM 2-Se base (I or 2), 50 mM sugar donor (a or b), 5 mM DTT, 50 mM glycine/NaOH buffer pH 9 and 24.6 µg mL\(^{-1}\) (a: 98.5 U, b: 97.4 U) PyNP-04. The reaction mixture was saturated with nitrogen. Deoxyribosyl derivatives were prepared at 60 °C and ribosyl derivatives at 80 °C. The reactions were stopped after 3 to 5 h by a pH shift to 13 through addition of 10 M NaOH (monitored with a pH-electrode). The pH was shifted back to 9 using 25% HCl and proteins were removed by filtration at room temperature using a vacuum pump and 0.45 µm cellulose. The collected product was analyzed at 210 nm. Deionized water and acetonitrile were UV-active and cannot be detected with a DAD system.

**Conversion (X) [%] = \( \frac{P_x}{P_{total}} \times 100 \)**

**Purification of 2-Se pyrimidine nucleosides by Silica column chromatography**

The first purification step was performed at room temperature via column chromatography (10% MeOH in DCM) using a Sigma-Aldrich silica gel (pore size 60; mesh particle size 220-440; particle size 35-75 µm). The reaction mixture was adsorbed onto silica gel by addition of 1.8 g of silica to the aqueous reaction mixture and evaporation of the mixture was adsorbed onto silica gel by addition of 1.8 g of silica to the aqueous reaction mixture and evaporation of the solution in vacuo. The resulting powder was stored at -20 °C until loading on a silica column for purification. Collected fractions were analyzed by TLC (10% MeOH in DCM) using UV detection. Fractions containing the 2-Se-nucleoside were combined and dried under reduced pressure and stored at -20 °C until further purification by semipreparative HPLC.

**Purification of 2-Se pyrimidine nucleosides by semipreparative HPLC**

The purification was performed using a KNAP HPLC system equipped with a Smartline Detector 2600 and an Agilent P2, 1 L pump. A reversed phase Kinetex® 5 µm Evo C18 column (250 x 21.2 mm) and a flow rate of 21.24 mL min\(^{-1}\) were used. Samples were analyzed at 210 nm. Deionized water and acetonitrile were applied as eluents, while the gradient was modified from the analytical method as summarized in Table S2. The product purified by silica chromatography was dissolved in deionized water (Table S2) and filtered either with a 0.45 µm PES syringe filter or with a 0.45 µm cellulose nitrate filter using a vacuum pump. The collected product fractions were kept on ice to prevent product degradation during the purification process. Collected 2-Se-nucleoside fractions were dried using a Christ Gamma 1-20 freeze-dryer (Osterode am Harz, Germany).

**LC-MS analysis**

For LC-MS analysis, 0.1 mg sample was dissolved in 1 mL water in a HPLC vial. Samples were analyzed using a HPLC Agilent 1200 series system coupled to an ESI-Orbitrap-MS (Thermo Fisher LTQ Orbitrap XL). The LC analyses were carried out using a Grace reversed phase GROM-Sil-ODS-4-HE column (50 x 2 mm, 3 µm). Samples were analyzed at 20 °C and at three wavelengths (λ = 215 nm, 280 nm, 350 nm). A flow rate of 0.3 mL min\(^{-1}\) was applied. The gradient was linearly increased from 80% 0.1% HCOOH in water and 20% 0.1% HCOOH in ACN to 100% 0.1% HCOOH in ACN in 10 min. The mobile phase composition was held for 3 min at 100% 0.1% HCOOH in ACN. Finally, the initial conditions were restored and maintained for 5 min. The raw data was analyzed using FreeStyle (Thermo Scientific).

**Compound characterization**

2'-Deoxy-2-Seadenosine (1a): off-white powder, \( R_f = 0.29 \) (DCM/MeOH 9:1); UV/Vis (4.5% ACN in 20 mM NH\(_4\)Ac pH 6.8): \( \lambda_{max} = 307 \) nm; HRMS (ESI): m/z calcd for C\(_{10}\)H\(_{15}\)N\(_2\)O\(_2\)Se+H\(^{+}\): 308.9984 [M+H]\(^{+}\); found: 308.9986

2'-Deoxy-2-Seadenosine (1b): yellow powder, \( R_f = 0.39 \) (DCM/MeOH 9:1); UV/Vis (26% ACN in 20 mM NH\(_4\)Ac pH 6.8): \( \lambda_{max} = 307 \) nm; HRMS (ESI): m/z calcd for C\(_{10}\)H\(_{15}\)N\(_2\)O\(_2\)Se+H\(^{+}\): 308.9984 [M+H]\(^{+}\); found: 308.9986

5-Methyl-2'-selenouridine (2a): slightly cream-colored powder, \( R_f = 0.38 \) (DCM/MeOH 9:1); UV/Vis (26% ACN in 20 mM NH\(_4\)Ac pH 6.8): \( \lambda_{max} = 307 \) nm; HRMS (ESI): m/z calcd for C\(_{10}\)H\(_{14}\)N\(_2\)O\(_2\)Se+H\(^{+}\): 323.0141 [M+H]\(^{+}\); found: 323.0144

5-Methyl-2'-selenouridine (2b): off-white powder, \( R_f = 0.43 \) (DCM/MeOH 9:1); UV/Vis (30% ACN in 20 mM NH\(_4\)Ac pH 6.8): \( \lambda_{max} = 307 \) nm; HRMS (ESI): m/z calcd for C\(_{10}\)H\(_{14}\)N\(_2\)O\(_2\)Se+H\(^{+}\): 307.0192 [M+H]\(^{+}\); found: 307.0194

**Acknowledgements**

We thank the Mass Spectrometry Analytical Center of the Institute of Chemistry of Technische Universität Berlin for analysis of our samples. K.F.H. was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - grant number 392246628.

**Conflict of Interest**

A. K. is CEO of the biotech company BioNukleo GmbH. F. K. is a scientist at BioNukleo GmbH and P. N. is a member of the advisory board. These affiliations constitute no conflict of interest with the results presented and discussed in this report.

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