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# Biocatalytic Nucleobase Diversification of 4'-Thionucleosides and Application of Derived 5-Ethynyl-4'-thiouridine for RNA synthesis detection

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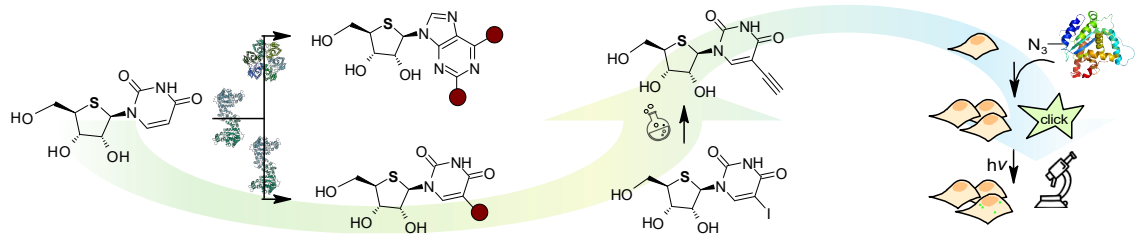


## Abstract

Nucleoside and nucleotide analogues have proven to be transformative in the treatment of viral infections and cancer. One branch of structural modification to deliver new nucleoside analogue classes explores replacement of canonical ribose oxygen with a sulfur atom. Whilst biological activity of such analogues has been shown in some cases, widespread exploration of this compound class is hitherto hampered by the lack of a straightforward and universal nucleobase diversification strategy. Herein, we present a synergistic platform enabling both biocatalytic nucleobase diversification from 4'-thiouridine in a one-pot process, and chemical functionalization to access new entities. This methodology delivers entry across pyrimidine and purine 4'-thionucleosides, paving a way for wider synthetic and biological exploration. We exemplify our approach by enzymatic synthesis of 5-iodo-4'-thiouridine on multi-milligram scale and from here switch to complete chemical synthesis of a novel nucleoside analogue probe, 5-ethynyl-4'-thiouridine. Finally, we demonstrate the utility of this probe to monitor RNA synthesis in proliferating HeLa cells, validating its capability as a new metabolic RNA labelling tool.

## Introduction

Analogues of nucleosides represent a successful strategy to perturb diverse biological processes and underpin the development of new therapeutics. This includes targeting DNA replication, transcription, translation, and cell signaling and is exemplified by the privileged and accomplished history of nucleoside analogues developed to fight viruses (e.g., entecavir, sofosbuvir) and cancer (e.g., clofarabine, forodesine).[1,2] Additionally, modified nucleoside components can be incorporated within synthetic nucleic acid sequences, providing a cornerstone building block within the emergent field of oligonucleotide therapeutics.[3,4]

Since its foundation in the 1950's, the field of nucleoside analogue chemistry has continually evolved, delivering generations of landscape defining molecules, including frontline mRNA treatments for COVID-19 containing modified nucleosides.[5–8] Furthermore, the recent identification of cGAMP as an agonist for STING activation has led to several nucleoside analogues being developed to act as innate immune modulators.[9]

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Given their broad biological remit, there is a continued requirement to explore new nucleoside chemotype structure-activity-relationship space. This is, however, often hampered by the challenging and lengthy chemical synthesis routes necessary to structurally modify the furanose sugar and/or heterobase components.[10] For example, access to purine- and pyrimidine-containing nucleosides typically requires a late-stage synthetic diversification to access all the required heterobase forms (A, G, U, T, C) for biological evaluation. This significantly reduces the overall synthetic route efficiency.

In this context, the use of enzymes to effect biocatalytic diversification offers a significant opportunity, through the adoption of biotransformations that can interchange nucleoside heterobase components. Importantly, such a strategy could be envisaged to synergise with the strengths of organic synthesis.[11] For example, first effecting constituent changes to the core D-ribose sugar using synthesis and therefrom delivering diverse, base-modified nucleosides using biocatalysis.[12] Such a tactic would bolster overall route sustainability, offering an environmentally friendly alternative.

Considering the importance of chemical modifications to the D-ribose ring in developing nucleoside analogue therapeutics, we recently disclosed an efficient gram-scale synthetic entry to 4'-thioribopyrimidines, where the furanose ring oxygen is substituted with a sulfur atom.[13–15] This work complimented wider attention from several academic and industrial groups exploring the chemical synthesis of 4′-thionucleosides, since the first disclosure of 4′-thioadenosine in the early 1960s.[16–19] Bioisosteric replacement of furanose oxygen with larger sulfur can impart changes to the furanose ring conformation, improving the hydrolytic stability of the glycosidic linkage and has delivered several biologically active 4'-thionucleosides (*Figure 1a*).[20–25] Additionally, locked 4'-thionucleoside monomers have recently been developed for inclusion within therapeutic oligonucleotide sequences.[26,27]

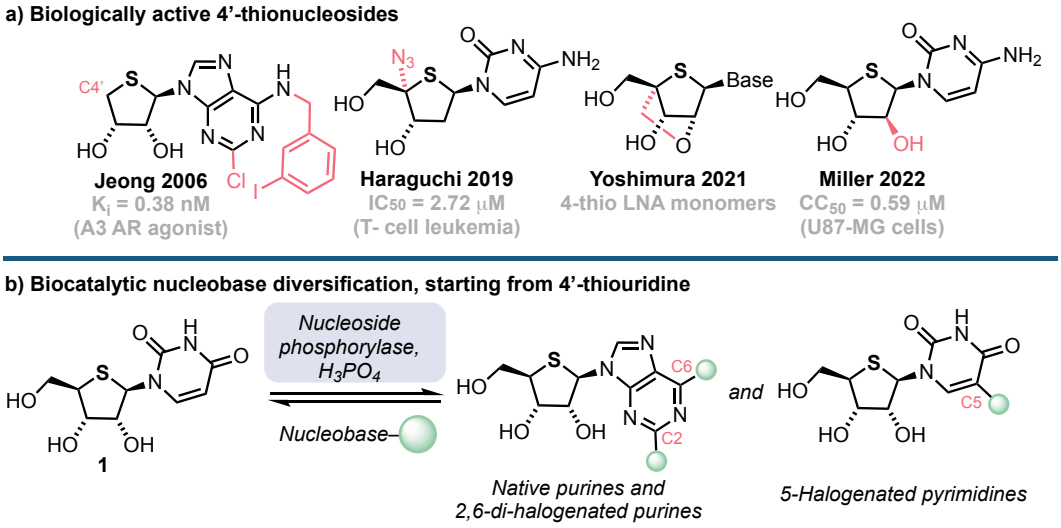



Figure 1 a) Examples of biologically relevant 4'-thionucleosides with a furanose ring modification replacing oxygen with a sulfur atom (bold) and additional structural modifications (in red); b) Strategy undertaken here to rapidly diversify 4'-thiouridine into pyrimidine and purine heterobase-modified 4'-thionucleosides using nucleoside phosphorylases.

The biocatalytic synthesis of nucleoside analogues is a burgeoning field,[4,6,28] and often applies enzymes from the class of nucleoside phosphorylases (NPs),[29] which are ubiquitous in nature and play a pivotal role in nucleoside salvage pathways.[30] Their mode of action involves cleavage of the *N*-glycosidic bond with phosphate, yielding the free heterobase and a pentose-α-1-phosphate (*Figure 2a*).[30] Since this is a reversable reaction, with a thermodynamically controlled position of the equilibrium, it is possible to steer the reaction towards nucleoside synthesis using low phosphate concentrations (0.1 equivalents).[31] By employing thermophilic enzymes with high temperature optima, solvent resistance and a broad substrate scope, multiple issues typically considered drawbacks of biocatalysis can be addressed.[32] The capabilities of nucleoside phosphorylases for the synthesis of

pharmaceutically relevant nucleoside analogues, such as islatravir, cladribine or ribavirin, have been described,[33] but their capabilities in accessing 4'-thionucleosides are hitherto unexplored.

Herein we harmonise the synthetic availability of 4'-thioribouridine **1** with nucleoside phosphorylases, to pioneer an enzymatic heterobase diversification, gaining scalable entry to a series of 4'-thiopurines and 4'-thiopyrimidines, including incorporation of halogenated nucleobases (*Figure 1b*). Subsequently, a halogen substituted 4'-thiouridine, **4a,** serves as a substrate for the chemical synthesis of 5-ethynyl-4'-thiouridine **6**, not accessible *via* biocatalytic transglycosylation, and thereby demonstrating the advantages of a chemoenzymatic approach. Finally, we show that 5-ethynyl-4'-thiouridine **6** can be utilised as a novel metabolic probe to detect RNA synthesis in proliferating HeLa cells.

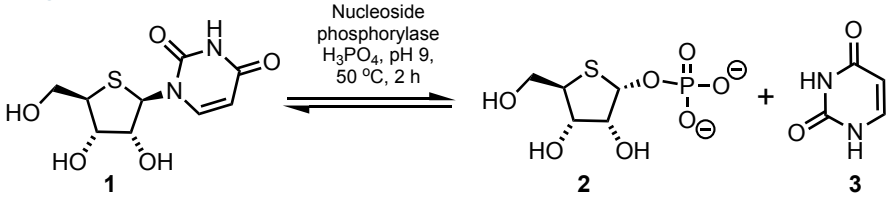
## Results and Discussion

### 4'-Thiouridine is a substrate of thermophilic pyrimidine nucleoside phosphorylases

Reports concerning the enzymatic metabolisation of 4'-thioribonucleosides are rare. In 1970 Bobek and colleagues tested a cell free lysate of *Escherichia coli* for phosphorolytic cleavage of 4'-thioribo-6-mercaptopurine.[34] Two years later Parks *et al* screened for human erythrocyte purine NP activity using 4'-thioinosine.[35] Both concluded that there was no (Bobek) or very slow (Parks) phosphorolytic cleavage. This underpinned our initial curiosity in screening thermophilic and hyperthermophilic pyrimidine nucleoside phosphorylases (PyNPs) from *Parageobacillus thermoglucosidasius* (formerly *Geobacillus thermoglucosidans*[36]) and *Thermus thermophilus* for phosphorolysis of **1** into 4-thioribo-α-1-phosphate **2** and uracil **3** (*Figure 2*). Concomitantly we screened commercially available PyNPs Y01 and Y04 and *Escherichia coli* uridine phosphorylase (*Ec*UP). Considering the previous reports,[34,35] it was surprising to observe that all the thermophilic PyNPs tested, as well as the mesophilic *Ec*UP, were able to perform the intended phosphorolysis reaction, as demonstrated by HPLC and UV spectroscopy (see Figures S1 and S2). The apparent equilibrium constant (K = 0.0013) was much lower than that of native uridine (K = 0.18),[37] but also indicates **1** as a good substrate for phosphorolytic cleavage and therefore a good starting material for subsequent transglycosylation reactions. Furthermore, we determined apparent specific activities (U $mg^{-1}$) of the hyperthermophillic enzymes *Pt*PyNP, *Tt*PyNP, Y01, Y04 and *Ec*UP with **1** and uridine. The comparatively low specific activity of the thermophilic *Tt*PyNP and Y04 for uridine is accredited to the reaction temperature being at least 30 °C below optimal temperature and a preference for thymidine (as stated by the manufacturer). However, even at these suboptimal conditions, PyNP Y04 retained around 5% of its activity with non-natural substrate **1**, as opposed to just 0.7% for *Ec*UP. Although *Tt*PyNP retained 14% of activity compared to uridine, it displayed the lowest specific activity for **1** under the chosen conditions.

**Enzyme Screening**

**a)**




**1** **2** **3**

| **b)** | **Apparent Specific Activity [U $mg^{-1}$]** | | **% Activity towards 1 relative to uridine** | **Temperature [°C]** |
|---|---|---|---|---|
| | **Uridine** | **1** | | |
| ***Pt*PyNP** | 32.27 ± 0.75 | 0.11 ± 0.01 | 0.34 | 50 |
| ***Tt*PyNP** | 0.29 ± 0.02 | 0.04 ± 0,01 | 14.25 | 50 |
| **PyNP Y01** | 120.17± 2.59 | 0.13 ± 0,01 | 0.10 | 50 |
| **PyNP Y04** | 2.01± 0.09 | 0.11 ± 0.01 | 5.33 | 50 |
| ***Ec*UP** | 73.60 ± 8.14 | 0.49 ± 0.04 | 0.67 | 37 |

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Figure 2 a) Phosphorolytic cleavage of 4'-thiouridine **1** into 4-thioribo-α-1-phosphate **2** and uracil **3** using thermophilic PyNPs and *Ec*UP b) Comparative apparent specific activities of all tested nucleoside phosphorylases for the phosphorolytic cleavage of **1** and uridine; U = units [µmol $min^{-1}$].

Whilst this observation cannot be directly compared to the above-mentioned experiments of Bobek on 4'-thioribo-6-mercaptopurine (requiring a purine nucleoside phosphorylase, PNP), it does caution against the prior indication that 4'-thioribonucleosides are not prone to enzymatic phosphorolysis. Having demonstrated capability for enzymatic phosphorolysis of **1**, we next explored the potential to install alternative nucleobases using transglycosylation.

**4'-Thiouridine hetereobase diversification by PyNP- and PNP-mediated transglycosylation**

Our biocatalytic approach towards heterobase diversification of **1** was built around the reversible nature of C-N anomeric cleavage by inorganic phosphate, catalysed by PyNP. Following phosphorolytic cleavage to 4-thioribo-α-1-phosphate **2**, the addition of different pyrimidine or purine bases to the system leads to the formation of novel nucleosides of interest. As such, within this two-reaction cascade inorganic phosphate functions as a catalyst and is only needed in small quantities (0.1 equivalents relative to **1**). To explore capability of using **1** as a donor, we first conducted transglycosylation experiments with the canonical purine bases adenine and guanine and the pyrimidine base thymine. Pleasingly, all three transglycosylation reactions led to the formation of the desired target nucleosides. The formation of 4'-thioadenosine and 4'-thioguanosine was particularly interesting, as it proved that **2** was also a substrate for a purine nucleoside phosphorylase (see SI, Figure S4) and therefrom offers a direct access to purine nucleosides that are otherwise difficult to assemble *via* conventional chemical synthesis.

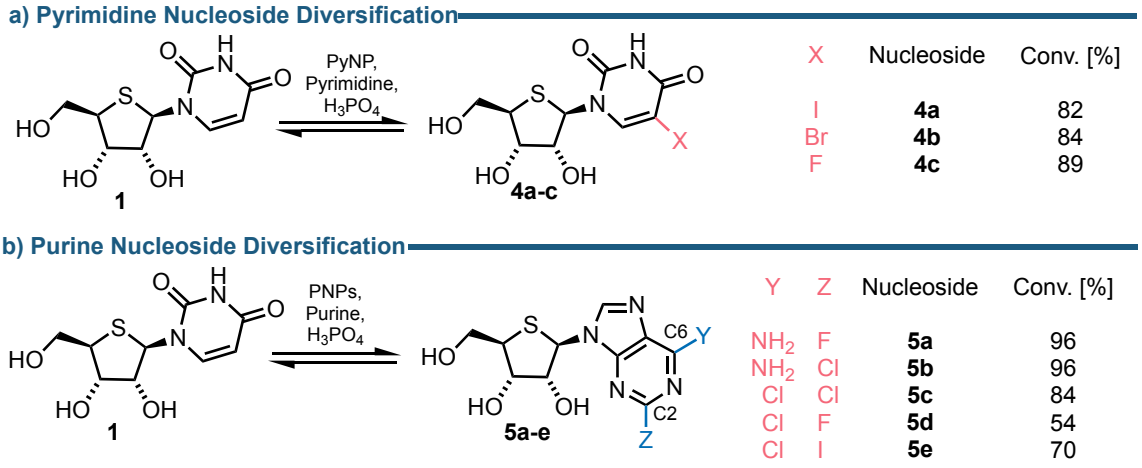


Figure 3. a) Halogenated pyrimidine nucleoside transglycosylation reactions b) Halogenated purine nucleoside transglycosylation reactions. Percentage conversions are shown towards the target molecules. 4'-Thiouridine (0.5 mM), 0.1 equiv. of $K_2HPO_4$ (50 µM), 1 mg $mL^{-1}$ enzyme (solely PyNP Y04 for pyrimidines, additionally *Pt*PNP for purine bases) were incubated with 2.0 equiv. (purines) or 4.0 equiv. (pyrimidines) of the nucleobase at 50 °C in 50 mM HEPES pH 7 (dihalogenated purines) or 50 mM glycine-OH pH9 (halogenated pyrimidines) for up to 50 h to reach reaction equilibrium (Figure S5). The conversions were calculated based on initial 4'-thiouridine concentrations (see SI, HPLC analysis and calculations).

Base-halogenated purine and pyrimidine nucleosides are important structural motifs, both within established nucleoside drugs and for providing conjugable handle points for further diversification (e.g., $S_NAr$, click reactions). Accordingly, we evaluated the synthesis of three C5-halogenated 4'-thiopyrimidine nucleosides in a one enzyme transglycosylation reaction (*Figure 3a*). Excellent conversion rates for **1** could be reached using four equivalents of C5-halogenated pyrimidine

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bases (C5-I = 82% for **4a**, C5-Br = 84% for **4b** and C5-F = 89% for **4c**). Furthermore, two C2-halogenated, and three C2,C6-dihalogenated 4'-thiopurine nucleosides were also synthesised using a two enzyme transglycosylation reaction (Y04 and *Pt*PNP) with two equivalents of the relevant purine bases (*Figure 3b*). Conversions were excellent towards 2-fluoroadenosine **5a** (96%), 2-chloroadenosine **5a** (96%) and C2,C6-dichloropurine **5c** (84%). Within dihalogenated purines, variation of the C2-halogen was explored (whilst retaining a C6-Cl), delivering C2-F,C6-Cl **5d** with a conversion of 54% and C2-I,C6-Cl **5e** with 70%. We attempted transglycosylation with 8-bromoguanine and 5-(trifluormethyl)-uracil, but no conversion was observed. The results shown in *Figure 3* demonstrate that the reaction thermodynamics favour purine derivatives over pyrimidines for 4'-thiouridine transglycosylation, as has been described for other natural nucleosides. The observed yields for these new halogenated 4'-thionucleosides compares well to the respective canonical systems.[37]

**Enzymatic synthesis of 5-iodo-4'-thiouridine enables access to a novel metabolic RNA labelling tool**

To illustrate a practicable use for 4'-thionucleoside transglycosylation, we selected 5-iodo-4'-thioribouridine **4a** for scale up and purification (*Figure 4a*). Increasing the concentration of **1** by a factor of 10 yielded slightly less of product **4a** (70% conversion over 96 hours *versus* 80% for a 1 mM reaction) in a 1 mL scale process (Figure S7). We reasoned that this could be caused by an instability of 4-thioribo-α-1-phosphate **2** or 5-iodouracil that only becomes apparent with longer incubation (Figure S7B). Therefore, for a multi-milligram reaction to convert **1** to **4a**, reaction speed was weighted against biocatalyst usage, to avoid unfavourable equilibria shifts due to substrate degradation. Accordingly, the enzyme concentration was increased four-fold over the course of the reaction by means of subsequent aliquot additions after 35 h, 64 h and 84 h, respectively. This 40 mL reaction reached 70% conversion after 104 h and **4a** was purified by semipreparative HPLC to >99% purity (Figure S7). We isolated 70 mg of **4a**, translating to an isolated yield of 87% for this preparative-scale enzymatic transformation.

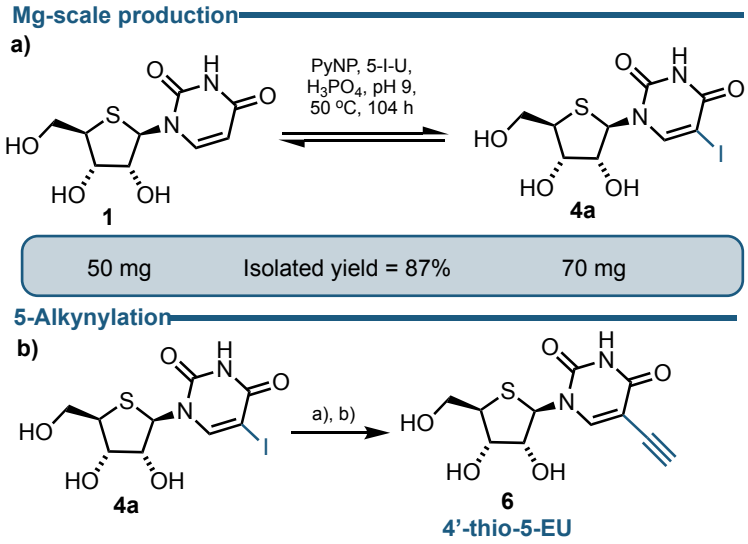


Figure 4 a) Preparative scale enzymatic synthesis of 5-iodo-4'-thiouridine **4a**; b) Chemical synthesis of 5-ethynyl-4'-thiouridine **6** from 5-iodo-4'-thiouridine **4a**. Reagents and conditions: a) TMS acetylene, $Pd(PPh_3)_4$, CuI, $Et_3N$, DMF, rt, 2 h, 85%; b) TBAF, THF, rt, 3 h, 80%.

With 70 mg of **4a** in hand, we next completed a Sonogashira cross-coupling to install TMS-acetylene, creating 5-ethynyl-4'-thiouridine **6**, following removal of the terminal TMS group with TBAF (*Figure 4b*). This was undertaken to establish an analogue of 5-ethynyl uridine (5-EU), a common nucleoside derivative used for metabolic RNA labelling. Furthermore, $K_{eq}$ for transglycosylation to access native 5-EU is poor (0.615),[31] supporting a change back to using synthetic chemistry to access **6**. The easy accessibility of **4a** described herein is expected to further support the development of post-transcriptional Suzuki–Miyaura cross-coupling reactions of RNA labelled with fluorogenic environment-sensitive probes.[38]

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**Metabolic labelling of RNA in HeLa cells using 5-ethynyl-4'-thiouridine**

5-ethynyluridine (5-EU) is a uridine analogue that is widely used for the detection of RNA synthesis in various cell lines from human, mice and zebrafish.[39–42] This metabolic labelling approach makes use of the promiscuity of nucleoside and nucleotide kinases and RNA polymerases, enabling phosphorylation of non-native nucleosides to a 5'-*O*-triphosphate and thus the incorporation of 5-ethynyluridine-5′-*O*-triphosphate into growing RNA.[41] The 5-ethynyl moiety can be labelled *in vitro* using copper(I)-catalysed click chemistry with a fluorescent azide.[43]

The incubation of HeLa cells with analogue **6** or 5-EU at concentrations of 1 and 2.5 mM, respectively, for 4.5 h and subsequent labelling with AlexaFluor®, confirmed that metabolic probe **6** is taken up into cells, activated by phosphorylation and incorporated into RNA (*Figure 5a, Figure S9*). Compared to 100% stained cells for 5-EU treated samples, not all cells treated with **6** displayed RNA fluorescence. The reasons for this might be a less efficient uptake of **6**, poor substrate binding or conversion by relevant kinases or strong dependence on enzymes that are differentially expressed during the cell cycle. Significant toxicity was neither observed for 5-EU nor **6**. The applied solvent, DMSO, however showed a significant effect on cell viability at concentrations above 2.5% (*Figure S11*). The most striking difference between samples treated with **6** or 5-EU, however, is the distinctly different location of the RNA signal (*Figure 5B*). Whilst 5-EU accumulates in the nucleolus in good accordance with literature,[44] the fluorescence for **6** was mainly observed in the cytoplasm either in close proximity to the nucleus or at the termini of the cells. Salic and co-workers[41] described a loss of 5-EU signal in the nucleoli upon inhibition of RNA polymerase I in NIH/3T3 cells, which might indicate that 5-EU and **6** are substrates of different RNA polymerases. Hence, probe **6** is a suitable additional tool to study RNA formation in cell culture, as it shows a different labelling preference compared to the more widely studied 5-EU.

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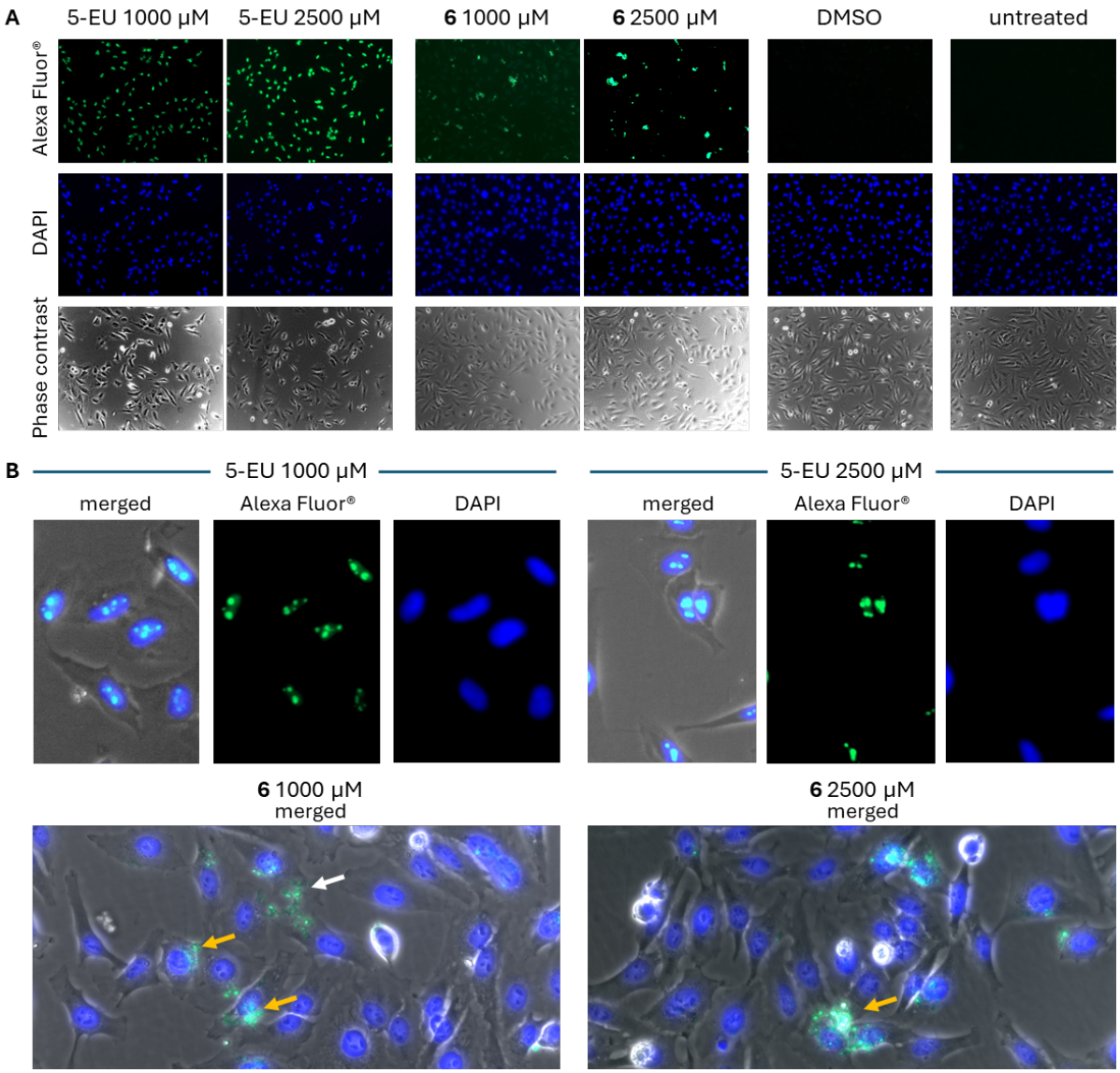


Figure 5. Microscopy images of HeLa cells treated with either 5-EU or **6**. HeLa cells were seeded with 20 000 cells per well in 48-well plates. Cells were grown to 50% confluency at 37 °C (around 24 h) and then incubated with either 1000 or 2500 µM of 5-EU or **6**. a) Alexa Fluor® and DAPI signals and phase contrast images were analysed. Untreated cells and cells treated with DMSO (solvent of 5-EU and **6**) served as controls (10x magnification). b) Localization of 5-EU (20x magnification) and **6** (40x magnification) signals. Arrows indicate location of **6** signals within the cells: white and orange arrows highlight location at the cell termini and close to the nuclei, respectively. Merged images show an overlay of Alexa Fluor® and DAPI signals and phase contrast images.

## Conclusion

In summary, we present a novel chemoenzymatic diversification strategy to access a variety of base modified 4'-thionucleosides starting from 4'-thiouridine. Harnessing nucleoside phosphorylases we introduce both canonical and halogenated purine and pyrimidine bases using transglycosylation, leading to the formation of 4'-thioribo-2-chloroadenosine, a cladribine analogue. Our platform posits 4'-thiouridine as a central compound to enable rapid and scalable access to a variety of base modified 4'-thionucleosides, simplifying a traditional bottleneck within purely chemical synthesis approaches and establishing nucleoside phosphorylases as valuable assets within biocatalytic nucleoside synthesis. Access to 5-halogenated pyrimidines enabled the chemical synthesis of a metabolic probe, 5-ethynyl-4'-thiouridine, which is confirmed as an analytical tool for monitoring RNA synthesis *in vitro* and

highlights a new potential for the non-native 4-thioribose motif as a substrate for cellular nucleic acid synthesis machineries. The enzymatic approach described herein is capable to expand the library of novel nucleosides accessible as potential therapeutic agents and offers new perspectives for the use chemoenzymatic synthesis to access novel molecular tools for potential application in RNA therapeutics.

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## Author contributions

Conceptualisation: SW, AK, GM; Data curation: SW; Investigation: SW, JM, CB, VR; Formal analysis: JM, SW, VR, CB; Writing-original draft: SW, GM; Visualisation: SW, JM, CB, AK, GM; Supervision: AK, GM, JK, PN; Project administration: GM & AK; Project funding: GM, AK, YS; Writing- Review & Editing: SW, CB, JM, VR, YS, PN, JK, AK, GM.

## Conflict of interest

AK is CEO of the biotech company BioNukleo GmbH, SW is a scientist at BioNukleo GmbH, and PN and JK are advisory board members. YS is president of Rasayan Inc. The authors declare no further conflict of interest.

## References

1 L. P. Jordheim, D. Durantel, F. Zoulim and C. Dumontet, *Nat Rev Drug Discov*, 2013, **12**, 447–464.
2 M. Guinan, C. Benckendorff, M. Smith and G. J. Miller, *Molecules*, 2020, **25**, 2050.
3 J. A. Kulkarni, D. Witzigmann, S. B. Thomson, S. Chen, B. R. Leavitt, P. R. Cullis and R. van der Meel, *Nat Nanotechnol*, 2021, **16**, 630–643.
4 K. J. D. Van Giesen, M. J. Thompson, Q. Meng and S. L. Lovelock, *JACS Au*, 2023, **3**, 13–24.
5 R. T. Eastman, J. S. Roth, K. R. Brimacombe, A. Simeonov, M. Shen, S. Patnaik and M. D. Hall, *ACS Cent Sci*, 2020, **6**, 672–683.
6 J. A. McIntosh, T. Benkovics, S. M. Silverman, M. A. Huffman, J. Kong, P. E. Maligres, T. Itoh, H. Yang, D. Verma, W. Pan, H.-I. Ho, J. Vroom, A. M. Knight, J. A. Hurtak, A. Klapars, A. Fryszkowska, W. J. Morris, N. A. Strotman, G. S. Murphy, K. M. Maloney and P. S. Fier, *ACS Cent Sci*, 2021, **7**, 1980–1985.
7 J. Motter, C. M. M. Benckendorff, S. Westarp, P. Sunde-Brown, P. Neubauer, A. Kurreck and G. J. Miller, *Nat Prod Rep*, , DOI:10.1039/D3NP00051F.
8 A. Abdullah Al Awadh, *Saudi J Biol Sci*, 2022, **29**, 103481.
9 L. Li, Q. Yin, P. Kuss, Z. Maliga, J. L. Millán, H. Wu and T. J. Mitchison, *Nat Chem Biol*, 2014, **10**, 1043–1048.
10 M. Meanwell, S. M. Silverman, J. Lehmann, B. Adluri, Y. Wang, R. Cohen, L.-C. Campeau and R. Britton, *Science (1979)*, 2020, **369**, 725–730.
11 S. Westarp, F. Kaspar, P. Neubauer and A. Kurreck, *Curr Opin Biotechnol*, 2022, **78**, 102829.
12 S. C. Cosgrove and G. J. Miller, *Expert Opin Drug Discov*, 2022, **17**, 355–364.
13 M. Guinan, N. Huang, M. Smith and G. J. Miller, *Bioorg Med Chem Lett*, 2022, **61**, 128605.
14 M. Guinan, N. Huang, C. S. Hawes, M. A. Lima, M. Smith and G. J. Miller, *Org Biomol Chem*, 2022, **20**, 1401–1406.
15 C. M. M. Benckendorff, Y. S. Sanghvi and G. J. Miller, *Current Protocols,* 2023*,* **3***, e878.*
16 T. Naka, N. Minakawa, H. Abe, D. Kaga and A. Matsuda, *J Am Chem Soc*, 2000, **122**, 7233–7243.

17 J. A. Secrist, K. N. Tiwari, J. M. Riordan and J. A. Montgomery, *J Med Chem*, 1991, **34**, 2361–2366.
18 D. J. Hoffman and R. L. Whistler, *Biochemistry*, 1970, **9**, 2367–2372.
19 E. J. Reist, D. E. Gueffroy and Leon. Goodman, *J Am Chem Soc*, 1964, **86**, 5658–5663.
20 H. W. Lee, H. O. Kim, W. J. Choi, S. Choi, J. H. Lee, S. Park, L. Yoo, K. A. Jacobson and L. S. Jeong, *Bioorg Med Chem*, 2010, **18**, 7015–7021.
21 S. M. Siddiqi, K. A. Jacobson, J. L. Esker, M. E. Olah, X. Ji, N. Melman, K. N. Tiwari, J. A. Secrist and S. W. Schneller, *J Med Chem*, 1995, **38**, 1174–1188.
22 L. S. Jeong, S. Pal, S. A. Choe, W. J. Choi, K. A. Jacobson, Z.-G. Gao, A. M. Klutz, X. Hou, H. O. Kim, H. W. Lee, S. K. Lee, D. K. Tosh and H. R. Moon, *J Med Chem*, 2008, **51**, 6609–6613.
23 D. Dukhan, E. Bosc, J. Peyronnet, R. Storer and G. Gosselin, *Nucleosides Nucleotides Nucleic Acids*, 2005, **24**, 577–580.
24 K. Haraguchi, H. Kumamoto, K. Konno, H. Yagi, Y. Tatano, Y. Odanaka, S. Shimbara Matsubayashi, R. Snoeck and G. Andrei, *Tetrahedron*, 2019, **75**, 4542–4555.
25 P. Dande, T. P. Prakash, N. Sioufi, H. Gaus, R. Jarres, A. Berdeja, E. E. Swayze, R. H. Griffey and B. Bhat, *J Med Chem*, 2006, **49**, 1624–1634.
26 R. Maeda, N. Saito-Tarashima, H. Wakamatsu, Y. Natori, N. Minakawa and Y. Yoshimura, *Org Lett*, 2021, **23**, 4062–4066.
27 M. Egli and M. Manoharan, *Nucleic Acids Res*, 2023, **51**, 2529–2573.
28 M. Pfeiffer, A. Ribar and B. Nidetzky, *Nat Commun*, 2023, **14**, 2261.
29 L. E. Iglesias, E. S. Lewkowicz, R. Medici, P. Bianchi and A. M. Iribarren, *Biotechnol Adv*, 2015, **33**, 412–434.
30 M. J. Pugmire and S. E. Ealick, *Biochemical Journal*, 2002, **361**, 1–25.
31 F. Kaspar, R. T. Giessmann, P. Neubauer, A. Wagner and M. Gimpel, *Adv Synth Catal*, 2020, **362**, 867–876.
32 S. Kamel, I. Thiele, P. Neubauer and A. Wagner, *Biochimica et Biophysica Acta- Proteins and Proteomics*, 2020, **1868**, 140304.
33 M. Rabuffetti, T. Bavaro, R. Semproli, G. Cattaneo, M. Massone, C. F. Morelli, G. Speranza and D. Ubiali, *Catalysts*, 2019, **9**, 355.
34 M. Bobek, R. L. Whistler and A. Bloch, *J Med Chem*, 1970, **13**, 411–413.
35 R. Parks Jr, J. Stoeckler, C. Cambor, T. Savarese, G. Crabtree and S. Chu, eds. A. Sartorelli, J. S. Lazo and J. R. Bertino, Academic Press, New York, 1981, pp. 229–252.
36 H. Aliyu, P. Lebre, J. Blom, D. Cowan and P. De Maayer, *Syst Appl Microbiol*, 2016, **39**, 527–533.
37 F. Kaspar, R. T. Giessmann, S. Westarp, K. F. Hellendahl, N. Krausch, I. Thiele, M. C. Walczak, P. Neubauer and A. Wagner, *ChemBioChem*, 2020, **21**, 2604–2610.
38 J. T. George and S. G. Srivatsan, *Chem Commun*, 2020, **56**, 12307–12318.
39 G. Akbalik, K. Langebeck-Jensen, G. Tushev, S. Sambandan, J. Rinne, I. Epstein, I. Cajigas, I. Vlatkovic and E. M. Schuman, *RNA Biol*, 2017, **14**, 20–28.
40 C. J. Bryant, M. A. McCool, L. Abriola, Y. V. Surovtseva and S. J. Baserga, *Open Biol*, DOI:10.1098/rsob.210305.
41 C. Y. Jao and A. Salic, *Proc Nat Acad Sci*, 2008, **105**, 15779–15784.
42 D. Qu, L. Zhou, W. Wang, Z. Wang, G. Wang, W. Chi and B. Zhang, *Anal Biochem*, 2013, **434**, 128–135.
43 J. T. George and S. G. Srivatsan, *Chem Commun*, 2020, **56**, 12307–12318.
44 M. Dvořáčková and J. Fajkus, *Front Plant Sci*, DOI:10.3389/fpls.2018.00177.