A Modular Approach to Phosphoglycosyltransferase Inhibitors Inspired by Nucleoside Antibiotics

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A Modular Approach to Phosphoglycosyl Transferase Inhibitors Inspired by Nucleoside Antibiotics

Marthe T. C. Walvoort,[b] Vinita Lukose, and Barbara Imperiali*[a]

Abstract: Phosphoglycosyl transferases (PGTs) represent “gatekeeper” enzymes in complex glycan assembly pathways by catalyzing transfer of a phospho-sugar from an activated nucleotide diphospho-sugar to a membrane-resident polyprenol phosphate. The unique structures of selected nucleoside antibiotics, such as tunicamycin and mureidomycin A, which are known to inhibit comparable biochemical transformations, are exploited as the foundation for the development of modular synthetic inhibitors of PGTs. Herein we present the design, synthesis and biochemical evaluation of two readily manipulatable modular scaffolds as inhibitors of monotopic bacterial PGTs. Selected compounds show IC50 values down to the 40 µM range, thereby serving as lead compounds for future development of selective and effective inhibitors of diverse PGTs of biological and medicinal interest.

Introduction

Phosphoglycosyl transferases (PGTs), also designated as “priming glycosyl transferases”, act at a membrane interface and catalyze the transfer of C1-phosphosugars to membrane-associated polyprenol phosphates to afford polyprenol diphosphate-linked glycans for glycoprotein, proteoglycan and glycolipid biosynthesis. Since PGTs catalyze the first membrane-committed step in many complex glycoconjugate biosynthesis pathways, they serve as “gatekeepers” of glycoconjugate production across all domains of life.[1] For example, MraY is a bacterial PGT that catalyzes transfer of phospho-MurNAc-pentapeptide to undecaprenol phosphate, mediating a key step in peptidoglycan biosynthesis,[2] and WecA is a bacterial phospho-GlcNAc transferase, which catalyzes the first step in the biosynthesis of lipopolysaccharide O-antigen.[3] Nucleoside antibiotics[4] that feature a common uridinyl motif, have been demonstrated to be potent inhibitors of selected PGTs, and MraY[5] and WecA[6] are potently inhibited by tunicamycin and mureidomycin A respectively (Figure 1). Recently, the importance of MraY in bacterial peptidoglycan synthesis has prompted major interest in the development of simplified nucleoside antibiotic analogues in the search for novel antimicrobial strategies.[7-11]

PGTs also feature in eukaryotic biology, most prominently at the initiation of the dolichol-dependent glycosylation pathway, where a GlcNAc-1-phosphate transferase (GPT), designated as Alg7 in Saccharomyces cerevisiae and other eukaryotes, transfers phospho-GlcNAc to dolichol phosphate.[12] Sequence analysis of WecA and Alg7 reveals that these enzymes are both integral membrane proteins with 11 predicted transmembrane helices (TMHs), and that they share key predicted active site residues, therefore it is unsurprising that Alg7 is also potently inhibited by tunicamycin[13] (Figure 1).

Unfortunately, despite the tantalizing inhibition properties of the uridinyl nucleoside antibiotics, their biological activities are hard to predict for other PGT targets, and must be empirically determined. This is exemplified by the large differences in inhibition properties of mureidomycin A and tunicamycin (Figure 1). Furthermore, the complexity of the natural product structures makes it very challenging to repurpose the structures of the natural products, by synthesis[14-16] or semisynthesis,[17-22] to target alternative PGTs with different substrate specificities. This challenge is further exacerbated with PGTs belonging to structural classes other than the well-studied MraY and WecA-type integral membrane proteins, which feature 10 and 11 predicted TMHs respectively. For example, recent bioinformatics and biochemical analysis has revealed thousands of homologous small bacterial PGTs with only a single TMH and a soluble globular domain within a 20-25 kDa protein.[23] While these PGTs catalyze the same biochemical processes and also play important roles at the initiation of diverse glycoconjugate biosynthetic pathways, there are currently no small molecules inhibitors that can be used to inform on the biology and essentiality of particular pathways and which may ultimately represent novel targets for therapeutic intervention.

A prototypic example of a small PGT is PglC from Campylobacter jejuni, a Gram-negative food-borne pathogen that is the major cause of gastroenteritis worldwide. PglC catalyzes the first membrane-committed step in the biosynthesis of virulence-associated N-linked glycoproteins in the

Figure 1. Structures of mureidomycin A and tunicamycin
Pathogen.\textsuperscript{[24]} Specifically, PgIC, acting at the cytoplasmic face of the inner bacterial membrane, commences assembly of an undecaprenol-diphosphate-linked heptasaccharide, which is the ultimate substrate for asparagine-linked glycosylation in the periplasm. PgIC catalyzes transfer of a modified sugar phosphate, di-N-acetylcytbiosamine-phosphate, from the corresponding UDP-sugar donor, onto undecaprenol-phosphate with release of UMP (Scheme 1).\textsuperscript{[25]} The PGT reaction is isoenergetic: a diphosphate product is formed from a diphosphate starting material (UDP-sugar). However, it is likely that in nature flux through the pathway is promoted by the action of the subsequent glycosyl transferases, which elaborate the PGT product into the polyprenolphosphate-linked glycan.

Currently, there is no structural information available to aid in the design of inhibitors towards small PGTs such as PgIC. The only PGT that has been structurally characterized is MraY,\textsuperscript{[26]} and the complete absence of sequence homology to the small PGTs reinforces that very little insight can be gleaned to address inhibitor development for the small PGTs from the MraY structure. Therefore, we have turned our attention to the nucleoside antibiotics as inspiration for the blueprints of new modular scaffolds for PGT inhibitor design. Examination of the structures and reported biological activities of mureidomycin A and tunicamycin suggests important design criteria. First the uridinylo moiety is a key “placeholder” for PGTs that exploit UDP-sugar substrates and therefore this moiety should feature in projected inhibitors. Second, as illustrated in Figure 1, interception of a divalent cation-binding site, either through a strategic metal ion coordinating group(s) on the inhibitor (the tunicamycin model)\textsuperscript{[1]} or by metal ion displacement with a protonated primary amine (the mureidomycin A model)\textsuperscript{[22]} represents an important design feature. In this context, despite the diversity of PGT structures, Mg\textsuperscript{2+}-dependence, presumably to coordinate to the uridine diphosphate to prime the substrate for nucleophilic attack at the β-phosphate, is common to small PGTs, such as PgIC, as well as the multi-TMH PGTs exemplified by MraY, WecA and Alg7. Finally, the design should be compatible with extended binding determinants, including glycan, peptidic, and lipophilic components, to mimic the characteristics of the native substrates. Based on these criteria, we have devised two modular synthetic strategies, inspired by mureidomycin A and tunicamycin. Key features of both strategies include 1) a uridinylo moiety, 2) a functionality to either displace or coordinate to the metal ion cofactor, 3) an aromatic moiety that may intercept sugar binding, and, 4) a hydrophobic acyl moiety to either bind into the undecaprenol-phosphate-binding site, or potentially direct the inhibitor to the membrane interface. Strategy 1 (mureidomimetic) is based on mureidomycin A in that it includes a modification at the CS'-site to display three different lengths of alkylation to explore placement of a positive charge adjacent to the uridine, and strategy 2 (tunicamimetic) is based on the incorporation of amino acid building blocks, once again conjugated to the uridinylo moiety, to explore an alternative, metal ion-coordinating functionality adjacent to the uridine.

The development of novel small PGT (e.g. PgIC) inhibitors has also demanded attention to practical details including the establishment of reliable heterologous expression and purification procedures for the target membrane-bound enzyme\textsuperscript{[27]} and the application of robust assays to provide consistent feedback on inhibitor development. One of the standard approaches for assessing PGTs relies on radioactivity-based strategies that use tritium or $[^{14}C]$-labeled UDP-sugars and undecaprenol phosphate together with liquid/liquid extraction of lipophilic undecaprenol-diphosphosugar products. For example, PgIC may be assayed in a coupled assay, in which the PgIC product is further elaborated by PgIa, an N-acetylgalactosamine transferase that uses UDP-[H]GalNAc, from the pgI pathway of C. jejuni.\textsuperscript{[28]} In this way, tritium-labeled GalNAc is incorporated into the undecaprenol-diphosphosaccharide (Und-PP-Bac-GalNAc) and enzymatic activity is quantified by extraction of the hydrophobic product followed by scintillation counting. In order to test for PgIC activity and inhibition directly, $[^{14}N]$-labeled UDP-di-N-acetylcytbiosamine was prepared via chemoenzymatic synthesis using $[^{14}N]$-labeled AcCoA (Supporting Information). Since the radioactivity-based assays are cumbersome and labor intensive, we also present the use of a prototype UMP/CMP-Glo assay from Promega, which is a luminescent UMP detection assay, that greatly increased the reliability and throughput of activity and inhibition assays. In all cases, inhibition assays were carried out in the presence of 10% DMSO and 0.1% Triton X-100 in order to ensure that observed inhibition behavior was due to specific binding to the enzyme rather than promiscuous binding activity resulting from inhibitor aggregation effects.\textsuperscript{[29]}

**Results and Discussion**

**Strategy 1 – Mureidomimetics**

The first modular strategy takes its cue from mureidomycin A and includes a primary amine adjacent to the uridinylo moiety (Figure 1). Starting from free uridine, the 2',3'-syn diol of the ribose was protected with an isopropylidene group, and the 5'-hydroxyl was tosylated (Scheme 2). Substitution of the tosylate with mono-Boc-protected alkyldiamines (C$_2$, C$_4$ and C$_6$) was accomplished under basic conditions after prolonged reaction times to yield intermediates 2-4 in good yields. Different spacer lengths between the uridine core and amino group were incorporated to investigate potential structure/function...
dependencies in subsequent inhibition assays (vide infra). Removal of the N-Boc and isopropylidene protecting groups was accomplished simultaneously using aqueous TFA, and products 5-7 were obtained in quantitative yield after lyophilization. Initial inhibition assays revealed that these first generation compounds 5-7 inhibited PglC reaction completely at 5 mM, while activity was almost completely recovered at 1 mM (data not shown). Qualitatively, small differences were observed with the three congeners, so all series were forwarded for further elaboration.

To reduce flexibility and potentially increase the opportunity for productive binding interactions with the enzyme, the C5'-secondary amine position was derivatized via coupling with N-acetyl-alanine using standard coupling conditions (EDC, HOBT). Although the use of the N-acetyl derivative of alanine under these conditions resulted in epimerization at the C-α center, the diastereomeric mixture was carried forward for inhibition analysis. While epimerization could have been prevented using the N-α-Fmoc-protected amino acid, deprotection under basic conditions was known to result in decomposition of the resulting tertiary amide at the 5'-N in related compounds (vide infra). Acidic treatment to remove the isopropylidene group afforded compounds 8-10, which showed improved inhibition potency, with 78±14% inhibition at 1 mM for compound 10 (the C6-dialkyl amine, Figure 2A), with substrate concentrations of 20 μM UDP-dNAcBac (Kᵢ = 7.2 ± 1.1 μM) and 20 μM Und-P (Kᵢ, app = 15.6 ±

![Scheme 2. Synthesis of dyad inhibitors, varying alkyl length and alkyne. A) H₂SO₄, acetone (96%); B) p-TsCl, DMAP, DCM/pyridine (89%); C) N-Boc-alkylamine, K₂CO₃, THF (2: 78%, 3: 91%, 4: 98%); D) TFA, H₂O (quant.); E) Ac-L-Ala-OH, EDC, HOBr, DMF (towards 8: 54%, 9: 37%, 10: 74%); F) i. chloroacetic anhydride, DCM; ii. Na₂SO₄, DMF (toward 11: quant., toward 12: 84%, toward 13: quant.); G) alkyne, Cs₂CO₃, sodium ascorbate, H₂O/H₂O.](image)

For the PglC reaction, a targeted screen was performed to identify a moiety that could catalyze the [3+2] cycloaddition click reaction. Reaction progress was monitored using LC-MS and purification was performed using SepPak C18 cartridges. For this study, the C6 compound, 13 was used as the parent compound because the alanine-conjugated equivalent, 10, had shown the best activity in inhibition assays. Indeed, a broad range of activities was observed that the uridine core alone is not sufficient for binding at this concentration (Figure 2A). In contrast, uridine monophosphate (UMP), which is also a side product of the PglC reaction, shows comparable inhibition relative to 10 at 1 mM. This is in line with our observations that the PglC reaction saturates at 30% conversion, supposedly due to UMP product inhibition. Although uridyl-phosphates are successfully elaborated towards potent glycosyl transferase inhibitors, in the long term, the amine-substituted derivatives would be advantageous due to the positive charge, which would enhance cell permeability relative to negatively charged analogues, as has been an inspiration in several inhibitor design strategies.

Encouraged by these initial inhibition results, the mureidomimetic strategy was expanded to create ‘dyad inhibitors’ that would display two binding modules. Instead of the alanine that had principally been incorporated to increase rigidity, a targeted screen was performed to identify a moiety that could potentially bind in the carbohydrate-binding site of the UDP-sugar substrate, and therefore our attention was focused on mono- and bicyclic aromatic compounds that could engage in π-stacking interactions often identified in sugar-binding sites. The synthesis of azide-functionalized alkylamine-uridines 11-13 involved N-acylation with chloroacetic anhydride, followed by chloride displacement with sodium azide in DMF and global deprotection under acidic conditions. Next, a selection of commercially available terminal alkyne (a-i, Scheme 2) was conjugated to 13 using standard Cu(I)/ascorbate conditions to catalyze the [3+2] cycloaddition click reaction. Reaction progress was monitored using LC-MS and purification was performed using SepPak C18 cartridges. For this study, the C6 compound, 13 was used as the parent compound because the alanine-conjugated equivalent, 10, had shown the best activity in inhibition assays. Indeed, a broad range of activities was observed.
observed when compounds 13a-i were assayed, with 13a resulting in complete inhibition of PglC activity at 3 mM (Figure 2B). Interestingly, the parent compound 13 showed only modest inhibition at 3 mM, reinforcing the importance of the naphthyl moiety on binding. These results demonstrate the added value of the dyad design, and might also suggest the importance of a stereocenter appended to 5'-N which is lacking in 13 but present in 10. With the aim of distinguishing between the different spacer lengths at this stage of inhibitor design, we conjugated the alkynes that had showed the greatest inhibition potential (a, f-i) to the shorter parent compounds 11 and 12. Interesting trends in inhibition properties were observed (see Figure S2. SI), and although alkyn e also yielded inhibitors with improved properties, the overall conclusion was that inhibitor 13a, in which the naphthyl moiety was combined with the C6 alkylamine, showed the highest activity.

Because the dyad inhibitor strategy left little opportunity for further functionalization, the inhibitor scaffold was modified to allow further derivatization toward ‘triad inhibitors’. As illustrated in Scheme 3, N-α-Fmoc-protected azido-alanine (Aza) was coupled to protected hexylamine-uridine 4, using standard amide coupling conditions. In this way, the scaffold has the potential to be modified first by click reactions at the azide moiety with different alkynes and second by coupling different carboxylic acid derivatives to the liberated amine. Ensuing Fmoc deprotection of compound 14 using 20% piperidine in DMF gave a high amount of the β-eliminated product, as well as decomposition back to starting compound 4 (as identified using LC-MS), which was inseparable from the product. While shorter reaction times and reduced levels of base suppressed the formation of the elimination product, decomposition was still detected. Ultimately, a systematic study of potential conditions resulted in the use of piperazine immobilized on polystyrene resin, which significantly reduced the amount of compound 4 regenerated. Subsequently, myristic acid was coupled to the amine in 15, followed by acidic hydrolysis to afford inhibitor 17. Additionally, compound 16 was subjected to click chemistry conditions using 2-ethyl-6-methoxynaphthyl to give fully functionalized triad inhibitor 18 after acidic hydrolysis in good yields.

Inhibition assays using the UMP/CMP-Glo system revealed that attaching the long chain (C18) fatty acid had a positive effect on the inhibition potency, as exemplified by 90±3% inhibition of PglC at 250 µM inhibitor with substrate concentrations of 20 µM UDP-diNAcBac and 20 µM Und-P in the assay. The IC_{50} of compound 17 was established to be 85 ± 28 µM. This reinforces the importance of a hydrophobic acyl moiety in the design of PGT inhibitors, to either compete with the undecaprenol phosphate acceptor for binding, or to direct the inhibitor to the membrane interface proximal to where catalysis is performed. Interestingly, the inhibition potency decreased on going from 17 to 18, which has the added methoxynaphthyl moiety, to 75±1% inhibition at 250 µM inhibitor. This corresponds to an IC_{50} value of ~ 207 µM for compound 18. This decrease in inhibition potency with the added naphthyl moiety is most likely explained by the poorer solubility of inhibitor 18 in the assay conditions. In addition, the exact distance between the uridine core and the naphthyl group is slightly longer in 18 than in the precursor 13a, which could indicate a slight clash in the binding site and therefore creating a suboptimal fit. However, it is clear from these results that the increasing complexity of the modular scaffold results in increased inhibitory potency, until an unfavourable interaction is introduced. Most importantly, the modular design of the mureidomycin mimetic strategy allows rapid differentiation at three positions, which will be extremely valuable in future optimization of this scaffold towards specific PGT targets.

**Strategy 2 - Tunicamimetics**

Having established that the combination of an alkylamine, a hydrophobic acyl group, and an aromatic moiety appended to a uridine core produced inhibitors with three points of variation, revealing micromolar IC_{50} values, we then investigated an alternative modular scaffold that would display similar functionalities, but would facilitate easier differentiation at three modular positions. As depicted in Scheme 4, this strategy started off with isopropylidene-protected 5'-amino-uridine (compound 19), and involved amide coupling chemistry to introduce the various modules. In this case, instead of a primary
amine that would displace the metal in the active site, the strategy exploits a strategic carboxylic acid functionality to investigate if such compounds might offer advantages in binding by directly coordinating to a bound divalent cation at the active site. Such a strategy would mimic the proposed effect of tunicamycin-like nucleosides, which are proposed to intercept such compounds might offer advantages in binding such as directly coordinating to a bound divalent cation at the active site. Such a strategy would mimic the proposed effect of tunicamycin-like nucleosides, which are proposed to intercept such compounds might offer advantages in binding.

Scheme 4. Synthesis and biological evaluation of triad inhibitors based on a peptidic backbone, using the UMP/CMP-Glo assay. A) Fmoc-L-Asp(tBu)-OH, EDC, HOBt, DMF; B) 20% piperidine, DMF (62% 2 steps); C) AcOH, Et3N, MeOH (24: 89%); D) TFA, H2O (21: 86% 2 steps, 25: 74% 3 steps, 26: 61% 2 steps, 28: quant., 29: 66%). E) Fmoc-L-Aza-OH, EDC, HOBt, DMF (71%); F) immobilized piperazone, DCM; G) myristic acid, EDC, HOBt, DMF (63% 2 steps); H) 2-ethynyl-6-methoxynaphthalene, CuSO4, sodium ascorbate, DMF (loward 29: 53%). Error bars indicate mean ±SD.

subjected to click conditions with 2-ethynyl-6-methoxynaphthalyl to generate a new class of tunicamimetic dyad inhibitor, 26, after acidic hydrolysis of the protecting groups. To generate the other dyad inhibitor displaying the hydrophobic acyl group, myristic acid was coupled to compound 23 using standard amide-coupling conditions. This resulted in compound 27, which was either fully deprotected using aqueous TFA to give dyad inhibitor 28, or subjected to click conditions with 2-ethyl-6-methoxynaphthalyl to produce the triad inhibitor 29. Because this second synthetic strategy was more straightforward than the first strategy, it allowed for thorough purification and isolation of intermediate compounds throughout the sequence of reactions. Advantageously, evaluating the inhibitory potential of the intermediates would provide information about the additive effects of the different modules on the overall potency. The results of the comparative inhibition assays are displayed in Scheme 4. Using the UMP/CMP-Glo assay, it was demonstrated that acid-functionalized uridine 21 inhibited PglC activity by 57±6% at 250 µM. Notably, this inhibitory potential is

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In Scheme 4, the synthesis and biological evaluation of triad inhibitors based on a peptidic backbone are illustrated. The process involves reactions such as Fmoc-L-Asp(tBu)-OH, EDC, HOBt, DMF; 20% piperidine, DMF (62% 2 steps); AcOH, Et3N, MeOH (24: 89%); TFA, H2O (21: 86% 2 steps, 25: 74% 3 steps, 26: 61% 2 steps, 28: quant., 29: 66%). Each step is carefully described, including the use of immobilized piperazine, DCM, and myristic acid, EDC, HOBt, DMF (63% 2 steps). The final product, 2-ethynyl-6-methoxynaphthalene, CuSO4, sodium ascorbate, DMF (loward 29: 53%). Error bars indicate mean ±SD.
considerably higher than the inhibition observed with first-generation inhibitors based on the alkylamine module (Scheme 1, compound 7). The addition of the Azac building block did not influence the inhibitory potency to a great extent (25 in Scheme 4). In contrast, the addition of either a 2-ethylthio-6-methoxynaphthyl through click chemistry (compound 26), or a fatty acid moiety (compound 28) improved the inhibition of PgiC, resulting in 77±10% and 88±5% inhibition at 250 µM respectively. Using the UMP/CMP-Glo assay, we were able to determine the IC50 values to be ~205 µM for 26 and 43 ± 5 µM for 28. This implies a modest improvement over the analogous dyad inhibitor 17 from the start. Interestingly, a similar trend in inhibition potential was observed with the triad inhibitor 29, which showed 81±3% inhibition at 250 µM, a slight decrease in potency compared to 28. However, this corresponded to an IC50 of 64 ± 10 µM, which is an order of magnitude better than the corresponding alkylamine inhibitor 18.

Conclusions

The development of inhibitors for PGTs is a relatively unexplored field, and it holds great potential to aid our understanding of this important class of enzymes, and to generate target molecules that inhibit crucial bacterial enzymes. Apart from diverse nucleosides that have been targeted to inhibit MraY, there is little known about molecular scaffolds that inhibit other PGTs specifically. Bioinformatics and structural investigations have revealed that PGTs display highly diverse topologies, hinting at diverse active site architectures to catalyze a similar reaction. Herein, we have presented the de novo design of two modular scaffolds that resulted in compounds with low micromolar activity for PgiC, the monotopic PGT that is the gatekeeper enzyme of N-linked glycoprotein biosynthesis in C. jejuni. The modular approach allowed the evaluation of adding different modules on the inhibitory potential of PgiC. Thus, it was clear that, starting from uridine, the attachment of aspartate (21, tunicamimetic strategy) was superior to the hexamethylene moiety (7, mureidomimetic strategy), resulting in 57% inhibition at 250 µM with the former. Attachment of an aromatic moiety (i.e. naphthyl group in 26), and to a greater extent a hydrophobic long-chain acyl group (17 and 28) resulted in a further increase in inhibitory potential, while the combination of both modules resulted in a slight decrease (18 and 29). This detrimental effect might be attributed to a steric clash of the elaborated scaffold in the active site, or to the poor solubility of the triad inhibitors. Interestingly, the inhibition potentials of these scaffolds are similar to that of tunicamycin, which was found to have an IC50 of 100 ± 8 µM for PgiC (see Supporting Information). Together, these studies provide the proof-of-concept for our approach, and pave the way for increasing the potency and specificity for diverse monotopic PGTs and potentially the more complex polytopic PGTs by performing structure optimization at the diverse point of contact with the enzyme. Of paramount importance in these studies was the establishment of a reproducible enzyme purification protocol, and the development of a reliable activity assay, which in contrast to existing fluorescence-based assays for MraY, [41-42] represent a significant challenge. Our efforts in both of these arenas have greatly contributed to the generation of PgiC inhibitors. In conclusion, the chemical and biochemical studies lay the foundation for a diversity-oriented approach to explore the chemical space allowed for inhibitors in the active site of PGTs. Moreover, the results will direct diversification of existing uridine libraries. [43-44]

Experimental Section

General experimental procedures. All chemicals were used as received unless stated otherwise. 1H and 13C NMR spectra were recorded on a Bruker DPX-400 (400/100 MHz), a Varian 500 (500/125 MHz), and a Bruker AV-600 (600/150 MHz) spectrometer. Chemical shifts (δ) are given in ppm and coupling constants are given in Hz. All given 13C spectra are proton decoupled. Flash chromatography was performed on Silicycle Sillflash P80 silica gel (40-60µm). TLC-analysis was conducted on Agela Technologies TLC plates with detection by UV-absorption (254 nm) where applicable and by spraying with 20% sulfuric acid in ethanol followed by drying at ~150 °C or by spraying with a solution of (NH4)2MoO4, H2O (25 g/l) and (NH4)2Ce(SO4)3 2H2O (10 g/l) in 10% sulfuric acid in water followed by drying at ~150 °C. LC-MS analysis was performed on an HP 1000 series HPLC system and Finnegan LCQDeca mass spectrometer. Standard eluents used were A: 0.1% TFA in H2O, B: 0.1% TFA in acetonitrile. The column used was a YMC-Pack ODS-AQ column (3 µm, 100 x 3.0 mm I.D.). All analyses were 15 min, with a flow-rate of 0.1 ml/min. The UMP/CMP-Glo assay (Promega) was used as received. Luminescent readings were performed on a Synergy HT platereader (Biotek) according to protocol from Promega. Scintillation counting was performed on a Beckman Coulter LS6500 scintillation counting system.

General procedure A: Amino acid coupling. Fmoc-protected amino acid (2 eq) was pre-activated by mixing with EDC-HCl (2 eq) and HOBt (2 eq) in DMF (0.3 M) for 10 min at RT, and this mixture was added to the amine (1 eq). The mixture was stirred overnight, diluted with DCM and washed with H2O (4x). The organic fraction was dried over Na2SO4, concentrated in vacuo and re-dissolved in DMF (0.3 M). Piperidine (20 vol%) was added, and the mixture was stirred until complete consumption of the starting material was observed using LC-MS. The mixture was concentrated in vacuo and purified using flash column chromatography (silica gel, DCM/MeOH) to obtain the desired product.

General procedure B: Click reaction in t-BuOH/H2O. The azide (2 µmol, stock in DMSO) and alkyne (2.4 µmol, 100 mM stock in DMSO) were together dissolved in t-BuOH/H2O (800 µL, 1/1, v/v), CuSO4 (2 µmol, 125 mM stock in H2O), and sodium ascorbate (4.8 µmol, 250 mM stock in H2O) were added and the resulting solution was incubated on a shaker at RT until complete consumption of the azide was observed using LC-MS (24-48h). The mixture was lyophilized, redissolved in H2O/MeCN and purified using SepPak C18 cartridges. Product fractions were lyophilized and redissolved in DMSO to give 50 mM stock solutions.

General procedure C: Click reaction in DMF. A solution of the azide (1 eq) in DMF (0.025 M) was treated with 2-ethylthio-6-methoxynaphthalene (4 eq), CuSO4 (1 eq, 1M stock in H2O) and sodium ascorbate (2 eq, 1M stock in H2O). Complete consumption of the azide was confirmed using LC-MS (1-3 h) and the mixture was diluted with DCM, washed with NH4Cl (4x), dried over Na2SO4 and concentrated in vacuo. The product was purified using flash column chromatography (silica gel, DCM/MeOH).
General procedure D: Acidic hydrolysis. Protected compound (10 mg) was treated with TFA/H₂O (500 μL, 3/2, v/v) until complete removal of the protecting groups was observed using LC-MS (3-24 h). The mixture was lyophilized repeatedly to obtain the final compound.

Radioactivity-based activity assay with PglC. Assays contained 20 μM Und-P, 10% DMSO, 0.1% Triton X-100, 50 mM HEPES pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 20 μM [³²P]-UDP-dinAcBac, and 1 nM PglC in a final volume of 60 μL. Inhibitors were added in DMSO, in a volume such that the total concentration of DMSO in the reaction was equal to 10% (v/v). PglC was pre-incubated in the reaction mixture lacking [³²P]-UDP-dinAcBac for five minutes at RT. After initiation of the reaction with [³²P]-UDP-dinAcBac, aliquots (20 μL) were taken at twenty minute time points and quenched in 1 mL CHCl₃:MeOH. The organic layer was washed three times with 400 μL PSUP (Pure Solvent Upper Phase, composed of 15 mL CHCl₃, 240 mL MeOH, 1.83 g KCl in 235 mL H₂O). The resulting aqueous layers were combined with 5 mL EcoLite (MP Biomedicals) liquid scintillation cocktail. Organic layers were combined with 5 mL OptiFluor (PerkinElmer). Both layers were analyzed using scintillation counting. The data was plotted as percentage remaining activity compared to the positive control (no inhibitor), and the IC₅₀ values were obtained by plotting residual activity versus concentration (GraphPad Prism).

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Keywords: phosphoglycosyl transferase • nucleoside antibiotic • modular approach • inhibitor • glycoconjugate biosynthesis


Inspired by the functional characteristics of mureidomycin A and tunicamycin, two novel scaffolds are synthesized that display low-micromolar IC$_{50}$ against the monotopic phosphoglycosyl transferase PgiC (see scheme).
A Modular Approach to Phosphoglycosyl Transferase Inhibitors Inspired by Nucleoside Antibiotics

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Figure S1. Biological evaluation of compounds 8-10 at 1 mM (light grey) and 500 µM (dark grey) in the PglC activity assay, using the radioactivity-based extraction assay. Data represented are obtained in duplicate. Error bars indicate mean ± SD.

Figure S2. Biological evaluation of clicked products based on compounds 11 (A) and 12 (B) in a PglC activity assay using the radioactivity-based extraction assay. Data represented are obtained in duplicate. Error bars indicate mean ± SD.
$IC_{50}$ curves (generated using GraphPad Prism)
Experimental details

2',3'-O-isopropylidene-5'-O-(p-toluenesulfonyl)uridine (1). 2',3'-O-isopropylidene uridine\(^1\) (1.0 g, 3.52 mmol) was dissolved in DCM/pyridine (20 mL, 3/1, v/v) under a nitrogen atmosphere. p-Toluenesulfonyl chloride (1.34 g, 7.04 mmol) and 4-dimethylaminopyridine (43 mg, 0.35 mmol) were added, and the resulting solution was stirred at RT for 24 h. The reaction was quenched by the addition of methanol (2 mL), diluted with DCM and washed with sat. aq. NaHCO\(_3\) and brine. The organic layer was dried over Na\(_2\)SO\(_4\), concentrated and the title compound was isolated after flash column chromatography (silica gel, 33% hexane in EtOAc) as a colorless foam (Yield: 1.37 g, 3.12 mmol, 89%). The analytical data were in accord with those previously reported.\(^2\)

5'-N-(tert-butyl) N-[2-aminooethyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (2). Tosyl uridine 1 (200 mg, 0.46 mmol) was dissolved in dry THF (2 mL) and treated with N-Boc-ethylenediamine (0.14 mL, 0.91 mmol) and K\(_2\)CO\(_3\) (0.13 g, 0.91 mmol). The resulting suspension was stirred for 7 days, after which time the mixture was diluted with DCM and washed with H\(_2\)O (2x). The organic layer was dried over Na\(_2\)SO\(_4\), concentrated in vacuo and purified using flash column chromatography (silica gel, 10% MeOH in DCM) to afford the title compound as a colorless foam (Yield: 151 mg, 0.35 mmol, 78%). TLC: \(R_f = 0.38\) (DCM/MeOH, 9/1, v/v); \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta 7.21\) (d, 1H, J = 7.4 Hz, H-6) 7.05 (s, 1H, NH), 5.72 (d, 1H, J = 7.6 Hz, H-5), 5.56 (s, 1H, NH), 5.19 (d, 1H, J = 3.9 Hz, H-1'), 4.95 (dd, 1H, J = 1.8, 6.5 Hz, H-3'), 4.90 (dd, 1H, J = 4.6, 6.4 Hz, H-2'), 4.28 (d, 1H, J = 1.3 Hz, H-4'), 3.90 (dd, 1H, J = 1.8, 12.2 Hz, H-5'), 3.85 (d, 1H, J = 11.4 Hz, H-5'), 3.42 – 3.55 (m, 2H, CH\(_2\)), 3.20 – 3.33 (m, 2H, CH\(_2\)), 1.52 (s, 3H, CH\(_3\)), iPr), 1.32 (s, 9H, CH\(_3\)), tBu), 1.29 (s, 3H, CH\(_3\)), iPr). \(^13\)C NMR (CDCl\(_3\), 100 MHz): \(\delta 171.9\) (C=O Boc), 156.7 (C-4), 153.2 (C-2), 141.1 (C-6), 114.9 (C\(_q\) iPr), 106.0 (C-5), 99.0 (C-1'), 85.6 (C-4'), 81.9, 80.7 (C-2', C-3'), 79.3 (C\(_q\) tBu), 61.0 (C-5'), 41.1, 39.9 (CH\(_2\)), 28.4 (CH\(_3\) tBu), 27.3, 25.2 (CH\(_3\)), iPr), LC: \(R_f = 6.36\) min; ESI-MS: \(m/z = 427.13\) (M+H\(^+\)).

5'-N-(tert-butyl) N-[4-aminobutyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (3). Tosyl uridine 1 (110 mg, 0.25 mmol) was dissolved in dry THF (2 mL) and treated with N-Boc-1,4-butanediamine (94 mg, 0.50 mmol) and K\(_2\)CO\(_3\) (138 mg, 1.0 mmol). The resulting suspension was stirred for 3 days, after which time the mixture was diluted with DCM and washed with H\(_2\)O (2x). The organic layer was dried over Na\(_2\)SO\(_4\), concentrated in vacuo and purified using flash column chromatography (silica gel, 8% MeOH in DCM) to afford the title compound as a colorless oil (Yield: 103 mg, 0.23 mmol, 91%). TLC: \(R_f = 0.37\) (DCM/MeOH, 9/1, v/v); \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta 7.18\) (d, 1H, J = 7.7 Hz, H-6), 7.12 (t, 1H, J = 4.6 Hz, NH), 6.31 (bs, 1H, NH), 5.75 (d, 1H, J = 7.6 Hz, H-5), 5.30 (t, 1H, J = 5.0 Hz, NH), 5.17 (d, 1H, J = 4.8 Hz, H-1'), 4.97 (dd, 1H, J = 1.9, 6.6 Hz, H-3'), 4.92 (dd, 1H, J = 5.0, 6.4 Hz, H-2'), 4.30 (s, 1H, H-4'), 3.92 (d, 1H, J = 11.7 Hz, H-5'), 3.88 (dd, 1H, J = 1.3, 11.8 Hz, H-5'), 3.31 – 3.38 (m, 2H, CH\(_2\)), 2.98 – 3.07 (m, 2H, CH\(_2\)), 1.53 – 1.62 (m, 2H, CH\(_2\)), 1.54 (s, 3H, CH\(_3\)), iPr), 1.38 – 1.47 (m, 2H, CH\(_2\)), 1.38 (s, 9H, CH\(_3\)), tBu), 1.30 (s, 3H, CH\(_3\)), iPr). \(^13\)C NMR (CDCl\(_3\), 100 MHz): \(\delta 172.0\) (C=O Boc), 156.5 (C-4), 152.9 (C-2), 141.3 (C-6), 114.9 (C\(_q\) iPr), 105.9 (C-5), 99.5 (C-1'), 85.2 (C-4'), 81.2, 80.7 (C-2', C-3'), 79.2 (C\(_q\) tBu), 60.9 (C-5'), 40.8, 40.0 (CH\(_2\)), 28.4 (CH\(_3\) tBu), 27.3 (CH\(_3\)), iPr), 27.2, 25.8 (CH\(_2\)), 25.1 (CH\(_3\)), iPr); LC: \(R_f = 6.65\) min; ESI-MS: \(m/z = 455.13\) (M+H\(^+\)).

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5′-N-(tert-butyl N-[6-aminohexyl] carbamate)-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (4). Tosyl uridine 1 (200 mg, 0.46 mmol) was dissolved in dry THF (2 mL) and treated with N-Boc-1,6-hexanediamine (230 mg, 0.91 mmol) and KHCO₃ (378 mg, 2.74 mmol). The resulting suspension was stirred for 7 days, after which time the mixture was diluted with DCM and washed with H₂O (2x). The organic layer was dried over Na₂SO₄, concentrated in vacuo and purified using flash column chromatography (silica gel, 8% MeOH in DCM) to afford the title compound as a colorless oil (Yield: 210 mg, 0.44 mmol, 96%). TLC: Rᵣ = 0.46 (DCM/MeOH, 9/1, v/v); ¹H NMR (CDCl₃, 400 MHz): δ 7.27 (d, 1H, J = 7.8 Hz, H-6), 6.76 (t, 1H, J = 4.8 Hz, NH), 6.81 (t, 1H, J = 4.6 Hz, NH), 6.33 (bs, 1H, NH), 5.69 (d, 1H, J = 7.6 Hz, H-5), 5.18 (d, 1H, J = 4.8 Hz, H-1′), 4.97 (bs, 1H, NH), 4.95 (dd, 1H, J = 1.8, 6.4 Hz, H-3′), 4.85 (dd, 1H, J = 5.1, 6.0 Hz, H-2′), 4.31 (d, 1H, J = 1.3 Hz, H-4′), 3.87 (s, 2H, H-5′), 3.22 – 3.38 (m, 2H, CH₂), 2.94 – 3.02 (m, 2H, CH₂), 1.52 (s, 3H, CH₃ iPr), 1.46 – 1.54 (m, 2H, CH₂), 1.35 (s, 9H, CH₃ tBu), 1.32 – 1.40 (m, 2H, CH₂), 1.28 (s, 3H, CH₃ iPr), 1.18 – 1.25 (m, 4H, CH₂); ¹³C NMR (CDCl₃, 100 MHz): δ 172.1 (C=O Boc), 156.2 (C-4), 152.9 (C-3), 140.6 (C-6), 114.7 (C₂ iPr), 105.6 (C-5), 98.7 (C-1′), 85.6 (C-4′), 81.9, 81.1 (C-2′, C-3′), 79.0 (C₄ tBu), 61.1 (C-5′), 41.5, 40.4, 29.7, 28.5 (CH₂), 28.4 (CH₃ tBu), 27.3 (CH₃ iPr), 26.4, 26.2 (CH₂), 25.2 (CH₃ iPr); LC: Rᵣ = 7.48 min; ESI-MS: m/z = 483.20 (M+H⁺).

5′-N-(2-aminomethyl)-5′-amino-5′-deoxy-uridine (5). The title compound was obtained from compound 2 (43 mg, 0.10 mmol) using general procedure D (Yield: 28 mg, 0.10 mmol, quant.). ¹H NMR (D₂O, 400 MHz) δ 8.11 (d, 1H, J = 7.7 Hz, H-6), 6.28 (d, 1H, J = 7.7 Hz, H-5), 5.64 (d, 1H, J = 6.0 Hz, H-1′), 4.44 (dd, 1H, J = 6.1, 5.0 Hz, H-2′), 4.15 – 4.24 (m, 2H, H-3′, H-4′), 3.65 – 3.84 (m, 4H, H-5′, CH₂), 3.20 (t, 2H, J = 5.9 Hz, CH₂); ¹³C NMR (D₂O, 100 MHz): δ 170.7 (C-4), 153.9 (C-2), 144.6 (C-6), 102.1 (C-5), 93.1 (C-1′), 86.7 (C₄′), 73.1 (C-2′), 70.0 (C-3′), 60.5 (C-5′), 39.3, 38.4 (CH₂).

5′-N-(4-aminobutyl)-5′-amino-5′-deoxy-uridine (6). The title compound was obtained from compound 3 (53 mg, 0.12 mmol) using general procedure D (Yield: 36 mg, 0.12 mmol, quant.). ¹H NMR (D₂O, 400 MHz) δ 7.91 (d, 1H, J = 8.1 Hz, H-6), 6.08 (d, 1H, J = 8.0 Hz, H-5), 5.59 (d, 1H, J = 6.4 Hz, H-1′), 4.42 (dd, 1H, J = 6.4, 5.3 Hz, H-2′), 4.17 – 4.25 (m, 2H, H-3′, H-4′), 3.78 (ddd, J = 2.8, 12.5 Hz, H-5′), 3.72 (dd, 1H, J = 3.1, 12.7 Hz, H-5′), 3.42 – 3.48 (m, 2H, CH₂), 2.90 – 2.96 (m, 2H, CH₂), 1.60 – 1.70 (m, 4H, CH₂); ¹³C NMR (D₂O, 100 MHz) δ 164.2 (C-4), 151.4 (C-2), 143.1 (C-6), 103.8 (C-5), 93.5 (C-1′), 87.0 (C-4′), 72.9 (C-2′), 70.2 (C-3′), 60.5 (C-5′), 41.9, 38.9, 24.5, 23.9 (CH₂).

5′-N-(6-aminohexyl)-5′-amino-5′-deoxy-uridine (7). The title compound was obtained from compound 4 (63 mg, 0.13 mmol) using general procedure D (Yield: 44 mg, 0.13 mmol, quant.). ¹H NMR (D₂O, 400 MHz) δ 7.86 (dd, 1H, J = 3.9, 8.2 Hz, H-6), 6.02 (dd, 1H, J = 4.3, 8.1 Hz, H-5), 5.55 (dd, 1H, J = 4.2, 6.5 Hz, H-1′), 4.35 – 4.44 (m, 1H, H-2′), 4.13 – 4.23 (m, 2H, H-3′, H-4′), 3.68 – 3.79 (m, 2H, H-5′), 3.32 – 3.42 (m, 2H, CH₂), 2.82 – 2.89 (m, 2H, CH₂), 1.48 – 1.60 (m, 4H, CH₂), 1.23 – 1.32 (m, 4H, CH₂); ¹³C NMR (D₂O, 100 MHz) δ 163.1 (C-4), 150.9 (C-2), 142.9 (C-6), 103.9 (C-5), 93.7 (C-1′), 87.1 (C-4′), 72.8 (C-2′), 70.3 (C-3′), 60.5 (C-5′), 42.8, 39.3, 27.1, 26.8, 25.3, 25.1 (CH₂).

5′-N-(N-acetyl-d/L-alanine)-5′-N-(tert-butyl N-[2-aminomethyl] carbamate)-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (30). Ac-L-Ala-OH (81 mg, 0.62 mmol) in DMF (300 µL) was pre-activated with EDC·HCl (119 mg, 0.62 mmol) and HOBT (95 mg, 0.62 mmol) for 15 min at RT, followed by addition of the mixture to compound 2 (88 mg, 0.21 mmol) in DMF (200 µL). The resulting mixture was stirred for 6 days, after which time the mixture was diluted with DCM, washed with H₂O (4x), dried over
Na$_2$SO$_4$ and concentrated in vacuo. Flash column purification (silica gel, 15% MeOH in DCM) yielded the title compound as a colorless oil (Yield: 60 mg, 0.11 mmol, 54%). Due to epimerization and rotamerization, the $^1$H and $^{13}$C spectra were difficult to interpret. Based on the purity by LC-MS, compound 30 was used in the next step, and fully characterized thereafter. TLC: $R_f$ = 0.40 (DCM/MeOH, 9/1, v/v); LC: $R_t$ = 6.78 min; ESI-MS: m/z = 540.27 (M+H$^+$).

5'-N-(N-acetyl-D/L-alanine)-5'-N-(2-aminoethyl)-5'-amino-5'-deoxy-uridine (8). Compound 30 (23 mg, 43 µmol) was deprotected using general procedure D to give the product as white solids (Yield: 16.7 mg, 42 µmol, quant., A : B = 0.3 : 1). $^1$H NMR (D$_2$O, 600 MHz) δ 8.02 (d, 0.3H, $J$ = 7.7 Hz, H-6), 7.94 (dd, 1H, $J$ = 4.8, 7.7 Hz, H-6b), 6.14 – 6.22 (m, 1.3H, H-5A, H-5B), 5.61 (dd, 1H, $J$ = 1.9, 5.8 Hz, H-1'A), 5.53 (d, 0.3H, $J$ = 6.3 Hz, H-1'α), 4.20 – 4.36 (m, 4.3H, H-2'A, H-2'B, H-4'A, H-5'B), 4.05 – 4.18 (m, 2.9H, H-3'A, H-3'B, H-4'A, CH-Ala, CH-Ala), 3.62 – 3.68 (m, 3.2H, H-5'A, CH$_2$), 3.09 – 3.13 (m, 2.6H, CH$_2$), 1.83 (s, 0.9H, CH$_3$ NHAc-α), 1.80 (s, 3H, CH$_3$ NHAc-β), 1.19 – 1.24 (m, 3H, CH$_3$-Ala), 1.13 – 1.18 (m, 0.9H, CH$_3$-Ala); $^{13}$C NMR (D$_2$O, 150 MHz) δ 174.2, 174.1, 173.9, 173.8 (C=O), 171.1 (C-4B), 170.9 (C-4A), 154.2 (C-2B), 154.0 (C-2A), 144.4 (C-6A), 142.5 (C-6B), 102.7 (C-5B), 102.3 (C-5A), 93.0 (C-1', C-1''), 91.1 (C-1'A', C-1'B'), 86.6 (C-4'A), 83.7 (C-4'B), 74.3 (C-2'B), 73.0 (C-2'A), 70.2 (C-3'B), 70.0 (C-3'A), 64.3 (C-5'B), 60.5 (C-5'A), 48.8 (CH-Ala), 48.6 (CH-Ala), 39.3, 39.2, 38.5 (CH$_2$), 21.4, 21.3 (CH$_3$ NHAc), 16.0, 15.7 (CH$_3$ Ala); LC: $R_t$ = 1.96 min; ESI-MS: m/z = 400.27 (M+H$^+$).

5'-N-(N-acetyl-D/L-alanine)-5'-N-(tert-butyl N-[4-aminoethyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (31). Ac-L-Ala-OH (74 mg, 0.56 mmol) in DMF (300 µL) was pre-activated with EDC·HCl (109 mg, 0.56 mmol) and HOBr (87 mg, 0.56 mmol) for 15 min at RT, followed by addition of the mixture to compound 3 (86 mg, 0.19 mmol) in DMF (200 µL). The resulting mixture was stirred for 6 days, after which time the mixture was diluted with DCM, washed with H$_2$O (4x), dried over Na$_2$SO$_4$ and concentrated in vacuo. Flash column purification (silica gel, 8% MeOH in DCM) yielded the title compound as a colorless oil (40 mg, 70.5 µmol, 37%). Due to epimerization and rotamerization, the $^1$H and $^{13}$C spectra were difficult to interpret. Based on the purity by LC-MS, compound 31 was used in the next step, and fully characterized thereafter. TLC: $R_f$ = 0.34 (DCM/MeOH, 9/1, v/v); LC: $R_t$ = 6.71, 6.78 min; ESI-MS: m/z = 590.70 (M+Na$^+$).

5'-N-(N-acetyl-D/L-alanine)-5'-N-(4-aminobutyl)-5'-amino-5'-deoxy-uridine (9). Compound 31 (14 mg, 24 µmol) was deprotected using general procedure D to give the product as white solids (Yield: 9.7 mg, 23 µmol, quant., A : B = 1 : 0.14). $^1$H NMR (D$_2$O, 600 MHz) major species: δ 7.87 (dd, 1H, $J$ = 8.1, 9.8 Hz, H-6), 6.11 (d, 1H, $J$ = 8.1 Hz, H-5), 5.66 (dd, 1H, $J$ = 1.9, 5.9 Hz, H-1'), 4.38 – 4.42 (m, 2H, H-2', H-3'), 4.36 (q, 1H, $J$ = 4.36 Hz, CH Ala), 4.26 – 4.32 (m, 1H, H-4'), 4.18 – 4.24 (m, 2H, H-5'), 3.41 – 3.48 (m, 2H, CH$_2$), 2.89 (m, 2.94 (m, 2H, CH$_2$), 1.87 (s, 3H, CH$_3$ NHAc), 1.60 – 1.69 (m, 4H, CH$_2$), 1.26 (t, 3H, $J$ = 7.4 Hz, CH$_3$ Ala); $^{13}$C NMR (D$_2$O, 150 MHz) major species: δ 174.2, 173.9 (C=O), 164.4 (C-4), 151.8 (C-2), 141.0 (C-6), 104.0 (C-5), 91.3 (C-1'), 84.2 (C-4'), 74.4 (C-2'), 70.3 (C-3'), 64.4 (C-5'), 48.8 (CH Ala), 42.0, 38.9, 24.5, 23.9 (CH$_2$), 21.3 (CH$_3$ NHAc), 15.7 (CH$_3$ Ala); LC: $R_t$ = 1.65 min; ESI-MS: m/z = 450.33 (M+Na$^+$).
5′-N-(N-acetyl-d/l-alanine)-5′-N-[6-aminohexyl] carbamate-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (32). Ac-l-Ala-OH (72 mg, 0.55 mmol) in DMF (300 µL) was pre-activated with EDC-HCl (86 mg, 0.55 mmol) and HOBT (69 mg, 0.55 mmol) for 15 min at RT, followed by addition of the mixture to compound 4 (72 mg, 0.15 mmol) in DMF (200 µL). The resulting mixture was stirred for 6 days, after which time the mixture was diluted with DCM, washed with H2O (4x), dried over Na2SO4 and concentrated in vacuo. Flash column purification (silica gel, 10% MeOH in DCM) yielded the title compound as a colorless oil (Yield: 66 mg, 0.11 mmol, 74%). Due to epimerization and rotamerization, the 1H and 13C spectra were difficult to interpret. Based on the purity by LC-MS, compound 32 was used in the next step, and fully characterized thereafter. TLC: Rf = 0.34 (DCM/MeOH, 9/1, v/v); LC: Rf = 7.36, 7.43 min; ESI-MS: m/z = 618.33 (M+Na+).

5′-N-(N-acetyl-d/l-alanine)-5′-N-[6-aminohexyl]-5′-amino-5′-deoxy- uridine (10). Compound 32 (16 mg, 27 µmol) was deprotected using general procedure D to give the product as white solids (Yield: 12.3 mg, 27 µmol, quant., A : B = 1 : 0.13). 1H NMR (D2O, 600 MHz) major species: δ 7.84 (dd, 1H, J = 8.2, 9.6 Hz, H-6), 6.07 (d, 1H, J = 8.1 Hz, H-5), 5.65 (dd, 1H, J = 2.2, 5.8 Hz, H-1'), 4.37 – 4.44 (m, 2H, H-2', H-3'), 4.34 – 4.37 (m, 1H, CH Ala), 4.26 – 4.31 (m, 1H, H-5'), 4.18 – 4.24 (m, 3H, H-4', H-5'), 3.37 – 4.43 (m, 2H, CH2), 2.84 – 2.89 (m, 2H, CH2), 1.87 (s, 3H, CH3 NHAc), 1.52 – 1.53 (m, 4H, CH2), 1.23 – 1.34 (m, 7H, CH2, CH3 Ala); 13C NMR (D2O, 150 MHz) major species: δ 174.2, 173.9 (C=O), 163.3 (C-4), 151.3 (C-2), 140.7 (C-6), 104.3 (C-5), 91.4 (C-1'), 84.3 (C-4'), 74.4 (C-2'), 70.4 (C-3'), 64.4 (C-5'), 48.8 (CH Ala), 42.7, 39.3, 27.1, 26.5, 25.2, 25.2 (CH2), 21.3 (CH3 NHAc), 15.7 (CH3 Ala); LC: Rf = 1.80 min; ESI-MS: m/z = 456.40 (M+H+).

5′-N-azidoacetyl-5′-N-[2-aminomethyl] carbamate-5′-amino-5′-deoxy- uridine (33). Compound 2 (40 mg, 94 µmol) was dissolved in dry DMF (800 µL) and treated with chloroacetic anhydride (32 mg, 187 µmol) and pyridine (15 µL, 187 µmol) for 20 min at RT. The mixture was diluted with DCM and quenched by the addition of aq. NaHCO3. The organic layer was separated, dried over Na2SO4 and concentrated in vacuo. The intermediate was re-dissolved in DMF (800 µL) and treated with NaCN overnight at RT. The mixture was diluted with DCM and washed with sat. aq. NaCl. The organic layer was dried over Na2SO4, concentrated in vacuo, and the product was purified using flash column chromatography (silica gel, 5% MeOH in DCM) (Yield: 48 mg, 93 µmol, quant.). 1H NMR (CDCl3, 400 MHz) δ 7.37 (d, 1H, J = 7.8 Hz, H-6), 5.91 (d, 1H, J = 7.5 Hz, H-5), 5.59 (d, 1H, J = 3.9 Hz, H-1'), 5.52 (bs, 1H, NH), 4.89 (dd, 1H, J = 4.0, 6.7 Hz, H-3'), 4.81 (dd, J = 6.7, 2.7 Hz, H-2'), 4.48 – 4.55 (m, 3H, H-4', H-5'), 3.99 (s, 2H, CH2-N3), 3.45 – 3.62 (m, 2H, CH2), 3.25 – 3.44 (m, 2H, CH2), 1.65 (s, 3H, CH3 iPr), 1.43 (s, 9H, CH3 tBu), 1.40 (s, 3H, CH3 iPr); 13C NMR (CDCl3, 100 MHz) δ 167.9 (C=O), 93.8 (C-1'), 83.7 (C-4'), 82.5, 80.4 (C-2', C-3'), 79.5 (C q tBu), 64.4 (C-5'), 50.3 (CH2-N3), 28.4 (CH3 tBu), 27.0, 25.3 (CH3 iPr); LC: Rf = 8.11 min; ESI-MS: m/z = 510.20 (M+H+).

5′-N-azidoacetyl-5′-N-[2-aminomethyl]-5′-amino-5′-deoxy-uridine (11). Compound 33 (48 mg, 93 µmol) was deprotected using general procedure D to give the product as a colorless oil (Yield: 34 mg, 93 µmol, quant.). 1H NMR (DMSO-d6, 500 MHz): δ 8.02 (d, 1H, J = 7.7 Hz, H-6), 5.92 (d, 1H, J = 7.8 Hz, H-5), 5.58 (d, 1H, J = 5.8 Hz, H-1'), 4.68 (d, 1H, J = 5.3 Hz, H-2'), 4.15 – 4.20 (m, 2H, H-3', H-4'), 3.95 – 4.00 (m, 2H, H-5'), 3.97 (s, 2H, CH2-N3), 3.47 – 3.52 (m, 2H, CH2), 2.94 – 3.05 (m, 2H, CH2); 13C NMR (DMSO-d6, 125 MHz): δ 171.2 (C=O), 169.7 (C-4), 155.9 (C-2), 142.5 (C-6), 105.2 (C-5), 95.6 (C-1'), 87.4 (C-4'), 74.7 (C-2'), 70.9 (C-3'), 61.6 (C-5'), 50.6 (CH2-N3), 39.5, 39.4 (CH2); LC: Rf = 3.09 min; ESI-MS: m/z = 370.13 (M+H+).
5′-N-azidoacetyl-5′-N-(tert-butyl N-[4-aminobutyl] carbamate)-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (34). Compound 3 (87 mg, 192 μmol) was dissolved in dry DMF (800 μL) and treated with chloroacetic anhydride (65 mg, 383 μmol) and pyridine (313 μL, 383 μmol) for 20 min at RT. The mixture was diluted with DCM and quenched by the addition of aq. NaHCO₃. The organic layer was separated, dried over Na₂SO₄ and concentrated in vacuo. The intermediate was re-dissolved in DMF (800 μL) and treated with NaN₃ (37 mg, 569 μmol) overnight at RT. The mixture was diluted with DCM and washed with sat. aq. NaCl. The organic layer was dried over Na₂SO₄, concentrated in vacuo, and the product was purified using flash column chromatography (silica gel, 5% MeOH in DCM) (Yield: 97 mg, 181 μmol, 94%). ¹H NMR (CDCl₃, 400 MHz): δ 7.35 (d, 1H, J = 3.9 Hz, H-6), 5.55 – 5.59 (m, 2H, H-5, NH), 5.53 (d, 1H, J = 3.9 Hz, H-1′), 4.55 – 4.58 (m, 3H, H-2′, H-3′, NH), 4.48 – 4.52 (m, 3H, H-4′, H-5′), 3.94 (s, 2H, CH₂-N₃), 3.40 – 3.48 (m, 2H, CH₂), 3.05 – 3.15 (m, 2H, CH₂), 1.56 – 1.63 (m, 5H, CH₂, CH₃ iPr), 1.50 – 1.56 (m, 2H, CH₂), 1.41 (s, 9H, CH₃ tBu), 1.38 (s, 3H, CH₃ iPr); ¹³C NMR (CDCl₃, 100 MHz): δ 167.8, 156.1 (C=O), 136.2 (C-6), 116.2 (C₄ iPr), 106.3 (C-5), 93.6 (C-1′), 84.0 (C-4′), 82.5, 80.8 (C’-2, C-3′), 79.0 (C₄ tBu), 64.5 (C-5′), 50.3 (CH₃-N₃), 41.8, 40.1 (CH₂), 28.4 (CH₃ tBu), 27.5 (CH₂), 27.0 (CH₃ iPr), 26.1 (CH₂), 25.2 (CH₃ iPr); LC: R₉ = 7.93 min; ESI-MS: m/z = 538.13 (M+H⁺).

5′-N-azidoacetyl-5′-N-(4-aminobutyl)-5′-amino-5′-deoxy-uridine (12). Compound 34 (79 mg, 148 μmol) was deprotected using general procedure D to give the product as a colorless oil (Yield: 57 mg, 146 μmol, quant.). ¹H NMR (DMSO-d₆, 500 MHz): δ 8.06 (d, 1H, J = 7.8 Hz, H-6), 6.02 (d, 1H, J = 7.7 Hz, H-5), 5.57 (d, 1H, J = 6.4 Hz, H-1′), 4.60 (d, 1H, J = 5.3 Hz, H-2′), 4.16 – 4.20 (m, 1H, H-3′), 4.01 – 4.04 (m, 1H, H-4′), 3.97 (s, 2H, CH₂-N₃), 3.62 – 3.66 (m, 2H, H-5′), 3.27 – 3.33 (m, 2H, CH₂), 2.75 – 2.85 (m, 2H, CH₂), 1.50 – 1.58 (m, 4H, CH₂); ¹³C NMR (DMSO-d₆, 125 MHz): δ 171.2 (C=O), 169.6 (C-4), 153.6 (C-2), 144.4 (C-6), 104.1 (C-5), 94.0 (C-1′), 87.9 (C-4′), 73.9 (C-2′), 71.1 (C-3′), 61.7 (C-5′), 50.6 (CH₂-N₃), 41.4, 39.6, 26.4, 25.4 (CH₂); LC: R₉ = 2.32 min; ESI-MS: m/z = 398.20 (M+H⁺).

5′-N-azidoacetyl-5′-N-(tert-butyl N-[6-aminohexyl] carbamate)-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (35). Compound 4 (112 mg, 232 μmol) was dissolved in dry DMF (1 mL) and treated with chloroacetic anhydride (80 mg, 464 μmol) and pyridine (37 μL, 464 μmol) for 40 min at RT. The mixture was diluted with DCM and quenched by the addition of aq. NaHCO₃. The organic layer was separated, dried over Na₂SO₄ and concentrated in vacuo. The intermediate was re-dissolved in DMF (1 mL) and treated with NaN₃ (45 mg, 692 μmol) overnight at RT. The mixture was diluted with DCM and washed with sat. aq. NaCl. The organic layer was dried over Na₂SO₄, concentrated in vacuo, and the product was purified using flash column chromatography (silica gel, 5% MeOH in DCM) (Yield: 129 mg, 230 μmol, quant.). ¹H NMR (CDCl₃, 400 MHz): δ 7.23 – 7.30 (m, 1H, H-6), 5.74 (d, J = 7.7 Hz, H-5), 5.66 (bs, 1H, NH), 5.43 (d, 1H, J = 4.0 Hz, H-1′), 4.83 (bs, 1H, NH), 4.72 – 4.77 (m, 1H, H-3′), 4.66 – 4.72 (m, 1H, H-2′), 4.45 – 4.50 (m, 1H, H-4′), 4.32 – 4.44 (m, 2H, H-5′), 3.87 (s, 2H, CH₂-N₃), 3.29 – 3.38 (m, 2H, CH₂), 2.93 – 3.05 (m, 2H, CH₂), 1.44 – 1.55 (m, 5H, CH₂, CH₃ iPr), 1.20 – 1.40 (m, 18H, CH₂, CH₃ iPr, CH₃ tBu); ¹³C NMR (CDCl₃, 100 MHz): δ 167.8, 156.0 (C=O), 135.9 (C-6), 115.8 (C₄ iPr), 106.4 (C-5), 93.4 (C-1′), 84.0 (C-4′), 82.4, 80.8 (C-2′, C-3′), 78.7 (C₄ tBu), 64.5 (C-5′), 50.2 (CH₂-N₃), 41.8, 40.2, 29.9, 28.7 (CH₂), 28.3 (CH₃ tBu), 27.0 (CH₃ iPr), 26.3, 26.2 (CH₂), 25.1 (CH₃ iPr); LC: R₉ = 9.49 min; ESI-MS: m/z = 565.33 (M+H⁺).
5'-N-azidoacetyl-5'-N-(6-aminohexyl)-5'-amino-5'-deoxy-uridine (13). Compound 35 (119 mg, 211 µmol) was deprotected using general procedure D to give the product as a colorless oil (Yield: 89 mg, 208 µmol, quant.). 1H NMR (DMSO-d6, 500 MHz): δ 8.15 (d, 1H, J = 7.7 Hz, H-6), 6.11 (d, 1H, J = 7.6 Hz, H-5), 5.62 (d, 1H, J = 6.3 Hz, H-1'), 4.20 – 4.23 (m, 1H, H-2'), 4.02 – 4.05 (m, 2H, H-3', H-4'), 3.97 (s, 2H, CH2-N3), 3.64 (d, 2H, J = 2.4 Hz, H-5'), 3.30 – 3.40 (m, 2H, CH2), 2.70 – 2.80 (m, 2H, CH2), 1.47 – 1.55 (m, 4H, CH2), 1.24 – 1.35 (m, 4H, CH2); 13C NMR (DMSO-d6, 125 MHz): δ 171.2 (C=O), 169.5 (C-4), 153.5 (C-2), 145.2 (C-6), 103.3 (C-5), 94.3 (C-1'), 88.2 (C-4'), 74.0 (C-2'), 71.1 (C-3'), 61.6 (C-5'), 50.6 (CH2-N3), 42.6, 39.9, 29.0, 28.0, 26.7, 26.5 (CH2); LC: Rf = 1.64 min; ESI-MS: m/z = 425.47 (M+H+).

5'-N-(N-[9-fluorenylmethoxycarbonyl]-3-azido-L-alanine)-5'-N-( tert-butyl N-[6-aminohexyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (14). Fmoc-L-Aza-OH (119 mg, 338 µmol) was dissolved in DMF (250 µL) and pre-activated by the addition of EDC-HCl (69 mg, 360 µmol) and HOBt (55 mg, 360 µmol) and the solution was stirred for 10 min at RT. The mixture was subsequently added to a solution of compound 4 in DMF (200 µL), and the resulting solution was stirred overnight. The mixture was diluted with DCM and washed with H2O (4x), dried over Na2SO4 and concentrated in vacuo. Flash column chromatography (silica gel, 4% MeOH in DCM) afforded the pure title compound as a colorless oil (Yield: 82 mg, 101 µmol, 45%). TLC: Rf = 0.53 (DCM/MeOH, 9/1, v/v); 1H NMR (CDCl3, 400 MHz): δ 7.67 (d, 2H, J = 7.6 Hz, CH2om), 7.51 (d, 2H, J = 7.4 Hz, CH2om), 7.31 (t, 3H, J = 7.9 Hz, CH2om, H-6), 7.18 – 7.25 (m, 2H, CH2om), 6.28 (d, 1H, J = 6.0 Hz, NH), 5.79 (d, 1H, J = 7.3 Hz, H-5), 5.35 (bs, 1H, NH), 5.24 (d, 1H, J = 3.5 Hz, H-1'), 4.64 – 4.75 (m, 2H, H-2', H-3'), 4.34 – 4.49 (m, 4H, H-4', CH2 Fmoc, CHH Aza), 4.25 – 4.32 (m, 2H, CH Aza, CHH Aza), 4.12 (t, 1H, J = 6.9 Hz, CH Fmoc), 3.64 – 3.75 (m, 2H, H-5'), 3.25 – 3.37 (m, 2H, CH2), 2.94 – 3.03 (m, 2H, CH2), 1.52 (s, 3H, CH3 iPr), 1.42 – 1.50 (m, 2H, CH2), 1.35 (s, 9H, CH3 tBu), 1.30 – 1.40 (m, 2H, CH2), 1.28 (s, 3H, CH3 iPr), 1.16 – 1.28 (m, 4H, CH2); 13C NMR (CDCl3, 100 MHz): δ 171.1 (C=O), 169.5 (C-4), 156.0, 155.9 (C=O), 152.5 (C-2), 143.8, 143.5 (Cq, C-6), 141.3 (Cq), 135.7, 127.8, 127.2, 125.1, 120.0 (CH2om), 116.2 (Cq iPr), 106.8 (C-5), 93.3 (C-1'), 83.9 (C-2'), 82.3 (C-4'), 80.9 (Cq tBu), 79.0 (C-3'), 67.4 (CH2 Fmoc), 64.5 (C-5'), 54.0, 52.0 (CH Aza, CH2 Aza), 47.0 (CH Fmoc), 41.9, 40.3, 30.0, 28.8 (CH2), 28.4 (CH3 tBu), 27.1 (CH3 iPr), 26.5, 26.3 (CH2), 25.2 (CH3 iPr); LC: Rf = 10.19 min; ESI-MS: m/z = 817.33 (M+H+).

5'-N-(3-azido-L-alanine)-5'-N-( tert-butyl N-[6-aminohexyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (15). Compound 14 (35 mg, 43 µmol) was dissolved in DCM (3 mL) and treated with immobilized piperazine (430 mg, 430-860 µmol) for 24 h, after which time the solution was filtered through a syringe filter and concentrated at RT. The resulting amine was directly used in the next step without further purification. TLC: Rf = 0.29 (DCM/MeOH, 9/1, v/v + 1% Et3N); LC: Rf = 7.32 min; ESI-MS: m/z = 595.27 (M+H+).

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3 The extent of labeling of commercially available immobilized piperazine is reported to range between 1-2 mmol/g.
5'-N-(N-myristoyl-3-azido-1-alanine)-5'-N-(tert-butyl N-[6-aminohexyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (16). Myristic acid (20 mg, 86 μmol) was dissolved in DMF (250 μL) and stirred with EDC·HCl (16.4 mg, 86 μmol) and HOBT (13 mg, 86 μmol) at RT for 15 min. This solution was added to the crude amine 15 (~43 μmol) in DMF (350 μL) and the resulting solution was stirred until complete consumption of the starting material was observed using LC-MS (2 h). The mixture was diluted with DCM, washed with H2O (4x), dried over Na2SO4, and concentrated in vacuo. Purification using flash column chromatography (silica gel, 10% MeOH in DCM) afforded the title compound as a colorless oil (Yield: 16.7 mg, 21 μmol, 48% over two steps). TLC: Rf = 0.50 (DCM/MeOH, 9/1, v/v); 1H NMR (CDCl3, 400 MHz): δ 7.32 (d, 1H, J = 7.7 Hz, H-6), 6.86 (bs, 1H, NH), 5.84 (d, 1H, J = 6.7 Hz, H-5), 5.45 (bs, 1H, NH), 5.31 (s, 1H, H-1'), 4.68 – 4.75 (m, 2H, H-2', H-3'), 4.65 (dt, 1H, J = 4.3, 7.1 Hz, CH Aza), 4.56 (bs, 1H, NH), 4.43 – 4.51 (m, 2H, H-4', H-5'), 4.35 – 4.42 (m, 1H, H-5'), 3.68 (d, 2H, J = 4.3 Hz, CH2 Aza), 3.30 – 3.43 (m, 2H, CH2), 2.97 – 3.09 (m, 2H, CH2), 2.10 – 2.25 (m, 2H, CH2), 1.57 (s, 3H, CH3 iPr), 1.48 – 1.57 (m, 4H, CH2), 1.37 (s, 9H, CH3 tBu), 1.33 (s, 3H, CH3 iPr), 1.27 – 1.43 (m, 6H, CH2), 1.12 – 1.25 (m, 2OH, CH2), 0.81 (t, 3H, J = 6.6 Hz, CH3); 13C NMR (CDCl3, 100 MHz): δ 173.6, 171.2 (C=O), 169.7 (C-4), 156.1 (C-2), 136.1 (C-6), 116.2 (Cq iPr), 106.5 (C-5), 93.6 (C-1'), 83.9 (C-2' or C-3'), 82.5 (C-4'), 81.0 (C-2' or C-3'), 79.1 (Cq tBu), 64.4 (C-4'), 52.3, 51.9 (CH Aza, CH2 Aza), 42.0 (CH2), 40.3 (CH2), 36.2, 31.9, 30.0, 29.6, 29.5, 29.4, 29.3, 28.4, 27.1, 26.5, 26.3, 25.5, 25.2 (CH2, CH3 tBu, CH3 iPr), 22.7 (CH2), 14.1 (CH3); LC: Rf = 11.50 min; ESI-MS: m/z = 805.40 (M+H+).

5'-N-(N-myristoyl-3-azido-1-alanine)-5'-N-(6-aminohexyl)-5'-amino-5'-deoxy uridine (17). Compound 16 (4.5 mg, 5.6 μmol) was deprotected according to general procedure D to give the title compound as a white solid (Yield: 4.2 mg, 5.35 μmol, 96%). 1H NMR (DMSO-d6, 600 MHz): δ 8.54 (d, 1H, J = 7.8 Hz, NH), 7.73 (d, 1H, J = 7.8 Hz, H-6), 5.94 (d, 1H, J = 7.6 Hz, H-5), 5.65 (d, 1H, J = 5.8 Hz, H-1'), 4.52 (dt, 1H, J = 7.1, 7.1 Hz, CH Aza), 4.30 (d, 2H, J = 3.7 Hz, H-5'), 4.16 – 4.20 (m, 1H, H-4'), 4.14 (t, 1H, J = 5.5 Hz, H-2'), 4.00 (dd, 1H, J = 4.0, 4.8 Hz, H-3'), 3.65 – 3.70 (m, 2H, CH2 Aza), 3.30 – 3.36 (m, 2H, CH2), 2.75 – 2.83 (m, 2H, CH2), 2.12 – 2.17 (m, 2H, CH2), 1.51 – 1.59 (m, 4H, CH2), 1.44 – 1.51 (m, 2H, CH2), 1.28 – 1.37 (m, 4H, CH2), 1.18 – 1.27 (m, 2OH, CH2), 0.86 (t, 3H, J = 6.7 Hz, CH3); 13C NMR (DMSO-d6, 150 MHz): δ 173.3, 170.1 (C=O), 169.1 (C-4), 153.3 (C-2), 140.3 (C-6), 105.0 (C-5), 90.0 (C-1'), 83.0 (C-4'), 74.2 (C-2'), 70.0 (C-3'), 65.0 (C-5'), 52.3 (CH Aza), 51.8 (CH2 Aza), 41.5, 39.3, 35.4, 31.8, 29.5, 29.4, 29.3, 29.2, 29.0, 28.3, 27.3, 26.0, 25.7, 25.5, 22.6 (CH2), 14.4 (CH3); LC: Rf = 9.84 min; ESI-MS: m/z = 665.40 (M+H+).

5'-N-(N-myristoyl-3-[4-(6-methoxyphosphoryl)-1H-1,2,3-triazol-1-yl]1-alanine)-5'-N-(tert-butyl N-[6-aminohexyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (36). Compound 16 (9.6 mg, 11.9 μmol) was converted to the title compound using general procedure C (Yield: 9.1 mg, 9.2 μmol, 77%)). TLC: Rf = 0.46 (DCM/MeOH, 9/1, v/v); LC: Rf = 11.90 min; ESI-MS: m/z = 987.60 (M+H+). Due to rotamerization, the 1H and 13C spectra were difficult to interpret. Based on the purity by LC-MS, compound 36 was used in the next step, and fully characterized thereafter.
5′-N-(N-myristoyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alanine)-5′-N-(6-aminohexyl)-5′-amino-5′-deoxy uridine (18). Compound 36 (-9.2 µmol) was fully deprotected according to general procedure D to give the title compound as a slightly colored solid (Yield: 6.9 mg, 7.8 µmol, 85%). 1H NMR (DMSO-d6, 600 MHz): δ 8.54 (s, 1H, CHtriazole), 8.50 (d, 1H, J = 7.8 Hz, NH), 8.29 (s, 1H, CHarom), 7.85 – 7.92 (m, 3H, CHarom), 7.73 – 7.82 (m, 2H, NH), 7.52 (d, 1H, J = 7.8 Hz, H-6), 7.34 (d, 1H, J = 2.5 Hz, CHarom), 7.15 – 7.20 (m, 2H, CHarom, NH), 5.70 (d, 1H, J = 7.8 Hz, H-5), 5.52 – 5.62 (m, 3H, H-1′, 2′-OH, 3′-OH), 4.88 – 4.93 (m, 1H, CH Aza), 4.86 (dd, 1H, J = 4.7, 14.0 Hz, C/H Aza), 4.75 (dd, 1H, J = 9.1, 13.9 Hz, CHH Aza), 4.29 – 4.36 (m, 2H, H-5′), 4.13 – 4.16 (m, 1H, H-4′), 4.08 – 4.12 (m, 1H, H-2′), 3.99 – 4.03 (m, 1H, H-3′), 3.89 (s, 3H, CH3 OMe), 3.21 – 3.27 (m, 2H, CH2), 2.75 – 2.83 (m, 2H, CH2), 2.05 (t, 2H, J = 7.3 Hz, CH2), 1.49 – 1.60 (m, 4H, CH2), 1.28 – 1.40 (m, 4H, CH2), 1.00 – 1.25 (m, 2H, CH2), 0.86 (t, 3H, J = 7.1 Hz, CH3); 13C NMR (DMSO-d6, 150 MHz): δ 173.2, 170.0 (C=O), 169.7 (C-4), 157.9 (Cq), 153.6 (C-2), 146.9 (Cq), 138.1 (C-6), 134.4 (Cq), 130.0 (CHarom), 129.0 (Cq), 127.8 (CHarom), 126.3 (Cq), 124.5, 123.8 (CHarom), 122.5 (CHtriazole), 119.6 (CHarom), 106.8 (C-5), 106.4 (CHarom), 89.3 (C-1′), 82.5 (C-2′), 73.8 (C-3′), 69.9 (C-3′), 65.3 (C-5′), 55.7 (CH3 OMe), 52.6 (CH Aza), 49.9 (CH2 Aza), 40.5, 39.3, 31.8, 29.6, 29.5, 29.3, 29.2, 28.9, 28.4, 27.1, 25.9, 25.6, 25.4, 22.6 (CH2), 14.4 (CH3); LC: Rf = 10.35 min; ESI-MS: m/z = 847.53 (M+H+).

5′-N-(O-tert-butyl-L-aspartate)-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (20). Starting from 5′-amino-5-deoxy-2′,3′-O-isopropylidene uridine 19, compound 20 was obtained using general procedure A (Yield: 82 mg, 180 µmol, 62%). TLC: Rf = 0.24 (DCM/MeOH, 9/1, v/v + 1% Et3N); 1H NMR (CDCl3, 400 MHz): δ 7.65 (t, 1H, J = 5.6 Hz, NH), 7.22 (m, 1H, H-6), 5.67 (d, 1H, J = 8.0 Hz, H-5), 5.46 (d, 1H, J = 2.2 Hz, H-1′), 4.98 (dd, 1H, J = 2.2, 6.6 Hz, H-2′), 4.71 (dd, 1H, J = 4.7, 6.4 Hz, H-3′), 4.29 (bs, 2H, NH2), 4.11 (dd, 1H, J = 4.7, 9.5 Hz, H-4′), 3.68 (dd, 1H, J = 4.4, 7.6 Hz, CH Asp), 3.61 (dt, 1H, J = 6, 14.2 Hz, H-5′), 3.48 (dt, 1H, J = 4.8, 14.2 Hz, H-5′), 2.78 (dd, 1H, J = 4.3, 16.6 Hz, C/H Asp), 2.51 (dd, 1H, J = 7.9, 16.6 Hz, CHH Asp), 1.47 (s, 3H, CH3 iPr), 1.37 (s, 9H, CH3 tBu), 1.26 (s, 3H, CH3 iPr); 13C NMR (CDCl3, 100 MHz): δ 173.7, 171.2 (C=O), 163.6 (C-4), 150.3 (C-2), 143.1 (C-6), 114.7 (Cq iPr), 102.8 (C-5), 95.1 (C-1′), 85.5 (C-4′), 83.9 (C-2′), 81.3, 81.1 (C-3′, Cq tBu), 52.1 (CH Asp), 40.6, 40.1 (C-5′, CH2 Asp), 28.1 (CH3 tBu), 27.2, 25.3 (CH3 iPr); LC: Rf = 5.77 min; ESI-MS: m/z = 454.93 (M+H+).

5′-N-(N-acetyl-L-aspartate)-5′-amino-5′-deoxy uridine (21). Amine 20 (10 mg, 22 µmol) was dissolved in MeOH (300 µL) and treated with Ac2O (10 µL, 110 µmol) and Et3N (15 µL, 110 µmol) for 2 h, after which time the mixture was diluted with toluene and concentrated in vacuo. Flash column chromatography (silica gel, 5% MeOH in DCM) gave the acetylated intermediate (LC: Rf = 5.91 min; ESI-MS: m/z = 496.93 (M+H+)), which was subsequently treated with TFA/H2O (500 µL, 1/1, v/v) overnight. LC-MS confirmed full consumption of the acetylated intermediate, and the reaction mixture was lyophilized to give the title compound as a colorless oil (Yield: 7.8 mg, 19 µmol, 86%). 1H NMR (D2O, 600 MHz): δ 7.54 (d, 1H, J = 8.1 Hz, H-6), 5.77 (d, 1H, J = 8.1 Hz, H-5), 5.68 (d, 1H, J = 4.6 Hz, H-1′), 4.59 (dd, 1H, J = 5.7, 7.4 Hz, CH Asp), 4.23 (t, 1H, J = 4.7 Hz, H-2′), 3.98 – 4.02 (m, 2H, H-3′, H-4′), 3.45 – 3.51 (m, 2H, H-5′), 2.80 (dd, 1H, J = 5.7, 17.1 Hz, C/H Asp), 2.73 (dd, 1H, J = 7.4, 17.0 Hz, CHH Asp), 1.92 (s, 3H, CH3 NHAc); 13C NMR (D2O, 150 MHz): δ 174.1, 174.0, 172.9 (C=O), 166.1 (C-4), 151.5 (C-2), 142.2 (C-6), 102.2 (C-5), 90.2 (C-1′), 81.9 (C-4′), 73.0 (C-2′), 70.3 (C-3′), 50.2 (CH Asp), 40.6 (C-5′), 35.5 (CH2 Asp), 21.8 (CH3 NHAc); LC: Rf = 1.68 min; ESI-MS: m/z = 400.93 (M+H+).

5'-N-(N-[9-fluorenylmethoxycarbonyl]-3-azido-L-alaninyl-O-tert-butyl-L-aspartate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (22). Fmoc-L-Aza-OH (70 mg, 198 µmol) was dissolved in DMF (400 µL) and treated with EDC·HCl (41 mg, 216 µmol) and HOBT (33 mg, 216 µmol) for 10 min at RT. This mixture was added to compound 20 (82 mg, 180 mmol) and the resulting solution was stirred until complete consumption of compound 20 was observed using LC-MS (~1 h). The mixture was diluted with DCM, washed with H₂O (4x), dried over Na₂SO₄ and concentrated in vacuo. Flash column chromatography (silica gel, 5% MeOH in DCM) afforded the title compound as a colorless oil (Yield: 101 mg, 129 µmol, 71%). TLC: Rf = 0.47 (DCM/MeOH, 9/1, v/v); ¹H NMR (CDCl₃, 400 MHz): δ 9.83 (bs, 1H, NH), 7.68 (d, 2H, J = 7.5 Hz, CH₆arom), 7.48 – 7.59 (m, 2H, CH₆arom), 7.32 (t, 2H, J = 7.5 Hz, CH₆arom), 7.23 (t, 2H, J = 7.5 Hz, CH₆arom), 7.10 (d, 1H, J = 8.0 Hz, H-6), 5.76 (bs, 1H, NH), 5.67 (dd, 1H, J = 2.0, 8.0 Hz, H-5), 5.24 (s, 1H, H-1'), 5.00 (d, 1H, J = 6.1 Hz, H-2'), 4.68 – 4.77 (m, 2H, H-3', CH Asp), 4.50 – 4.58 (m, 1H, CH Aza), 4.35 – 4.50 (m, 2H, CH₂ Fmoc), 4.16 (t, 1H, J = 6.7 Hz, CH Fmoc), 4.10 (s, 1H, H-4'), 3.78 (dd, 1H, J = 3.6, 11.8 Hz, CHH Aza), 3.62 – 3.71 (m, 1H, H-5'), 3.52 (dd, 1H, J = 5.2, 11.9 Hz, CHH Aza), 3.37 – 3.45 (m, 1H, H-5'), 2.82 – 2.90 (m, 1H, CHH Asp), 2.57 (dd, 1H, J = 5.3, 16.8 Hz, CHH Asp), 1.45 (s, 3H, CH₃ iPr), 1.30 (s, 9H, CH₃ tBu), 1.24 (s, 3H, CH₃ tPr); ¹³C NMR (CDCl₃, 100 MHz): δ 170.9, 170.5 (C=O), 169.3 (C-4), 162.7, 156.5 (C=O), 150.7 (C-2), 143.6, 143.5 (C₉, C-6), 141.3 (C₂a), 127.8, 127.2, 125.0, 120.0 (CH₆arom), 114.8 (C₉ iPr), 102.9 (C-5), 96.2 (C-1'), 84.7 (C-4'), 83.5 (C-2'), 81.8 (C₂ tBu), 80.3 (C-3'), 67.7 (CH₂ Fmoc), 54.3, 52.0, 50.0 (CH Aza, CH Asp, CH₂ Aza), 47.1 (CH Fmoc), 40.8 (C-5'), 36.9 (CH₂ Asp), 28.0 (CH₃ tBu), 27.3, 25.3 (CH₃ iPr); LC: Rf = 9.51 min; ESI-MS: m/z = 789.07 (M+H⁺).

5'-N-(3-azido-L-alaninyl-O-tert-butyl-L-aspartate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (23). Compound 22 (35 mg, 44 µmol) was dissolved in DCM (2.5 mL) and treated with immobilized piperezine (440 mg, 440-880 µmol) for 24 h, after which time the solution was filtered through a syringe filter and concentrated at RT. The resulting amine was directly used in the next step without further purification. LC: Rf = 5.95 min; ESI-MS: m/z = 567.07 (M+H⁺).

5'-N-(N-acetyl-3-azido-L-alaninyl-O-tert-butyl-L-aspartate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (24). The crude amine 23 (9 mg, 16 µmol) was dissolved in MeOH (400 µL) and treated with Ac₂O (7.7 µL, 82 µmol) and Et₃N (11.4 µL, 82 µmol) for 2 h, after which time the mixture was diluted with toluene and concentrated in vacuo. Flash column chromatography (silica gel, 5% MeOH in DCM) gave the title compound as a white solid (Yield: 9 mg, 14.6 µmol, 89%). TLC: Rf = 0.44 (DCM/MeOH, 9/1, v/v); ¹H NMR (CDCl₃, 400 MHz): δ 9.92 (s, 1H, NH), 7.47 (d, 1H, J = 8.9 Hz, NH), 7.23 (dd, 1H, J = 4.3, 5.6 Hz, NH), 7.14 (d, 1H, J = 8.1 Hz, H-6), 6.49 (d, 1H, J = 8.4 Hz, NH), 5.70 (dd, 1H, J = 2.1, 8.0 Hz, H-5), 5.27 (d, 1H, J = 2.4 Hz, H-1'), 5.05 (dd, 1H, J = 2.3, 6.6 Hz, H-2'), 4.82 (ddd, 1H, J = 4.6, 5.7, 8.4 Hz, CH Aza), 4.72 – 4.77 (m, 2H, H-3', CH Asp), 4.15 (dt, 1H, J = 3.3, 4.9 Hz, H-4'), 3.77 (dd, 1H, J = 4.6, 12.4 Hz, CH Aza), 3.67 (ddd, 1H, J = 3.3, 6.3, 14.6 Hz, H-5'), 3.53 (dd, 1H, J = 5.7, 12.4 Hz, CHH Aza), 3.47 (dd, 1H, J = 3.7, 14.7 Hz, H-5'), 2.85 (dd, 1H, J = 5.1, 16.7 Hz, CHF H Asp), 2.58 (dd, 1H, J = 5.3, 16.7 Hz, CHH Asp), 2.03 (s, 3H, CH₃ NHAc), 1.48 (s, 3H, CH₃ iPr), 1.34 (s, 9H, CH₃ tBu), 1.27 (s, 3H, CH₃ tPr); ¹³C NMR (CDCl₃, 100 MHz): δ 171.0, 170.5 (C=O), 169.2 (C-4), 163.0 (C=O), 150.9 (C-2), 143.5 (C-6), 114.9 (C₂ tPr), 103.0 (C-5), 96.7 (C-1'), 84.8 (C-4'), 83.6 (C-2'), 81.9 (C₂ tBu), 80.1 (C-3'), 52.3, 51.7, 50.0 (CH Aza, CH Asp, CH₂ Aza), 40.8 (C-5'), 37.0 (CH₂ Asp), 28.0 (CH₃ tBu), 27.3, 25.3 (CH₃ iPr), 23.2 (CH₃ NHAc); LC: Rf = 6.55 min; ESI-MS: m/z = 680.93 (M+H⁺).
5'-N-(N-acetyl-3-azido-L-alanine-L-aspartate)-5'-amino-5'-deoxy uridine (25). Compound 24 was fully deprotected using general procedure D to afford the title compound as a white solid (Yield: 1.75 mg, 3.42 μmol, 83%). 1H NMR (D2O, 600 MHz): δ 7.57 (d, 1H, J = 8.1 Hz, H-6), 5.81 (d, 1H, J = 8.1 Hz, H-5), 5.69 (d, 1H, J = 4.4 Hz-1'), 4.60 – 4.75 (m, 1H, CH Asp), 4.43 (t, 1H, J = 5.5 Hz, CH Aza), 4.26 (t, 1H, J = 4.7 Hz, H-2'), 4.97 – 4.05 (m, 2H, H-3', H-4'), 3.66 (dd, 1H, J = 6.0, 12.9 Hz, CH/CH Aza), 3.59 (dd, 1H, J = 5.2, 12.9 Hz, CH/CH Aza), 3.48 – 3.53 (m, 2H, H-5'), 2.87 (dd, 1H, J = 5.9, 16.9 Hz, CH/CH Asp), 2.76 (dd, 1H, J = 7.4, 16.9 Hz, CH/CH Asp), 1.95 (s, 3H, CH3 NHAc); 13C NMR (D2O, 150 MHz): δ 174.6, 174.0, 172.2, 171.2 (C=O), 166.2 (C-4), 151.5 (C-2), 142.4 (C-6), 102.3 (C-5), 90.5 (C-1'), 81.8 (C-4'), 73.0 (C-2'), 70.2 (C-3'), 53.2 (CH Aza), 50.9 (CH2 Aza), 50.1 (CH Asp), 40.6 (C-5'), 35.2 (CH2 Asp), 21.6 (CH3 NHAc); LC: Rf = 1.61 min; ESI-MS: m/z = 513.07 (M+H').

5'-N-(N-acetyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alanine-L-aspartate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (37). Compound 24 was converted to the title compound using general procedure C. TLC: Rf = 0.41 (DCM/MeOH, 9/1, v/v); LC: Rf = 8.26 min; ESI-MS: m/z = 791.20 (M+H'). In spite of extensive purification, the NMR spectra were difficult to interpret. Purity and identity of compound 37 were assessed through LC-MS, and the compound was extensively characterized after the subsequent reaction step.

5'-N-(N-acetyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alanine-L-aspartate)-5'-amino-5'-deoxy uridine (26). The title compound was obtained from compound 37 using general procedure D (Yield: 2.1 mg, 3.0 mmol, 61% over two steps). 1H NMR (DMSO-d6, 600 MHz): δ 11.36 (s, 1H, NH), 8.59 (d, 1H, J = 7.7 Hz, NH), 8.54 (s, 1H, CHtriazole), 8.32 (s, 1H, CHarom), 8.29 (d, 1H, J = 7.9 Hz, NH), 8.11 (t, 1H, J = 5.8 Hz, NH), 7.88 – 7.94 (m, 3H, CHarom), 7.68 (d, 1H, J = 8.0 Hz, H-6), 7.35 (d, 1H, J = 2.0 Hz, CHarom), 7.19 (dd, 1H, J = 2.4, 8.9 Hz, CHarom), 5.76 (d, 1H, J = 6.1 Hz, H-1'), 5.62 (dd, 1H, J = 1.9, 8.0 Hz, H-5), 5.42 (d, 1H, J = 5.8 Hz, 2'-OH), 5.19 (d, 1H, J = 4.8 Hz, 3'-OH), 4.81 – 4.86 (m, 1H, CH Aza), 4.79 (dd, 1H, J = 1.6, 14.0 Hz, CHH Aza), 4.64 (dd, 1H, J = 7.4, 14.2 Hz, CHH Aza), 4.57 – 4.61 (m, 1H, CH Asp), 4.05 – 4.09 (m, 1H, H-2'), 3.86 – 3.93 (m, 5H, H-3', H-4', CH3 OMe), 3.30 – 3.45 (m, 2H, H-5'), 2.73 (dd, 1H, J = 5.2, 16.7 Hz, CHH Asp), 2.57 (dd, 1H, J = 8.0, 16.7 Hz, CHH Asp), 1.85 (s, 3H, CH3 NHAc); 13C NMR (DMSO-d6, 150 MHz): δ 172.2, 171.1, 170.3 (C=O), 169.0 (C-4), 163.5, 157.9 (Cq), 151.2 (C-2), 146.8 (Cq), 141.7 (C-6), 134.3 (Cq), 132.0, 131.9, 130.0, 129.3, 129.2, 129.0, 127.9 (CHarom), 126.4 (Cq), 124.6, 123.9 (CHarom), 122.6 (CHtriazole), 119.6, 106.5 (CHarom), 102.4 (C-5), 88.3 (C-1'), 83.0 (C-4'), 72.9 (C-2'), 71.2 (C-3'), 55.7 (CH3 OMe), 53.2 (CH Aza), 51.1 (CH2 Aza), 50.3 (CH Asp), 41.6 (C-3'), 36.5 (CH2 Asp), 22.9 (CH3 NHAc); LC: Rf = 6.43 min; ESI-MS: m/z = 695.20 (M+H').

5'-N-(N-myristoyl-3-azido-L-alanine-L-aspartate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (27). Myristic acid (19 mg, 82 μmol) was dissolved in DMF (200 μL) and stirred with EDC·HCl (16 mg, 82 μmol) and HOBT (13 mg, 82 μmol) at RT for 15 min. This solution was added to the crude amine 23 (~41 μmol) in DMF (200 μL) and the resulting solution was stirred until complete consumption of the starting
material was observed using LC-MS (2 h). The mixture was diluted with DCM, washed with H2O (4×), dried over Na2SO4 and concentrated in vacuo. Purification using flash column chromatography (silica gel, 5% MeOH in DCM) afforded the title compound as a colorless oil (Yield: 20 mg, 26 µmol, 63% over two steps). TLC: Rf = 0.41 (DCM/MeOH, 9/1, v/v); 1H NMR (CDCl3, 400 MHz): δ 10.04 (s, 1H, NH), 7.54 (d, 1H, J = 8.9 Hz, NH), 7.24 (dd, 1H, J = 4.0, 6.2 Hz, NH), 7.15 (d, 1H, J = 8.1 Hz, H-6), 6.55 (d, 1H, J = 6.9 Hz, NH), 5.69 (d, 1H, J = 8.0 Hz, H-5), 5.29 (d, 1H, J = 2.3 Hz, H-1'), 5.03 (dd, 1H, J = 2.2, 6.6 Hz, H-2'), 4.82 (dt, 1H, J = 5.2, 8.0 Hz, CH Aza), 4.72 – 4.78 (m, 2H, H-3', CH Aza), 4.10 – 4.15 (m, 1H, H-4'), 3.75 (dd, 1H, J = 4.7, 12.5 Hz, CHH Aza), 3.65 (ddd, 1H, J = 3.3, 6.2, 14.7 Hz, H-5'), 3.53 (dd, 1H, J = 5.7, 12.4 Hz, CHH Aza), 3.47 (dt, 1H, J = 3.6, 14.6 Hz, H-5'), 2.85 (app dd, 1H, J = 5.0, 16.8 Hz, CHH Aza), 2.56 (dd, 1H, J = 5.3 Hz, 16.8 Hz, CHH Aza), 2.18 – 2.25 (m, 2H, CH2), 1.52 – 1.63 (m, 2H, CH2), 1.48 (s, 3H, CH3 iPr), 1.15 – 1.26 (m, 20H, CH2), 0.81 (t, 3H, J = 6.8 Hz, CH3); 13C NMR (CDCl3, 100 MHz): δ 170.9, 170.6 (C=O), 169.4 (C-4), 163.1, 162.6 (C=O), 150.9 (C-2), 143.4 (C-6), 114.8 (Cq iPr), 103.0 (C-5), 96.4 (C-1'), 84.7 (C-4'), 83.6 (C-2'), 81.8 (Cq tBu), 80.1 (C-3'), 52.2 (CH Aza), 51.7 (CH2 Aza), 50.0 (CH Asp), 40.8 (C-5'), 36.9 (CH2 Asp), 36.5, 31.9, 29.7, 29.6, 29.5, 29.3, 29.1 (CH2), 28.0 (CH3 tBu), 27.3 (CH3 iPr), 25.5, 25.3 (CH3 iPr, CH2), 22.7 (CH2), 14.1 (CH3); LC: Rf = 11.41 min; ESI-MS: m/z = 777.07 (M+H+).

5'-N-(N-myristoyl-3-azido-L-alaninyl-L-aspartate)-5'-amino-5'-deoxy uridine (28). Compound 27 was fully deprotected using general procedure D to afford the title compound as an off-white solid (Yield: 3.0 mg, 4.4 µmol, quant.). 1H NMR (DMSO-d6, 600 MHz): δ 8.33 (d, 1H, J = 7.3 Hz, NH), 8.26 (d, 1H, J = 8.0 Hz, NH), 7.92 (t, 1H, J = 5.7 Hz, NH), 7.66 (d, 1H, J = 8.1 Hz, H-6), 5.73 (d, 1H, J = 5.8 Hz, H-1'), 5.65 (d, 1H, J = 8.0 Hz, H-5), 5.39 (d, 1H, J = 5.6 Hz, 2'-OH), 4.50 – 4.57 (m, 2H, CH Asp, CH Aza), 3.98 – 4.03 (m, 1H, H-2'), 4.83 – 4.87 (m, 1H, H-3'), 3.77 – 3.82 (m, 1H, H-4'), 3.53 (dd, 1H, J = 4.5, 12.6 Hz, CHH Aza), 3.40 – 3.46 (m, 1H, CHH Aza), 3.28 – 3.39 (m, 2H, H-5'), 2.66 (dd, 1H, J = 5.6, 16.6 Hz, CHH Asp), 2.53 – 2.56 (m, 1H, CHH Asp), 2.12 – 2.17 (m, 2H, CH2), 1.45 – 1.53 (m, 2H, CH2), 1.20 – 1.30 (m, 20H, CH2), 0.86 (t, 3H, J = 6.7 Hz, CH3); 13C NMR (DMSO-d6, 150 MHz): δ 173.3, 172.2, 171.0 (C=O), 169.6 (C-4), 163.5 (C=O), 151.2 (C-2), 141.7 (C-6), 102.4 (C-5), 88.4 (C-1'), 83.0 (C-2'), 72.9 (C-2'), 71.0 (C-3'), 52.7, 52.0 (CH Aza, CH2 Aza), 50.1 (CH Asp), 41.5 (C-5'), 36.6 (CH Asp), 35.6, 31.8, 29.5, 29.4, 29.3, 29.2, 29.1, 25.5, 22.6 (CH2), 14.4 (CH3); LC: Rf = 9.84 min; ESI-MS: m/z = 681.20 (M+H+).

5'-N-(N-myristoyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-alaninyl-O-tert-butyl-L-aspartate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (38). The title compound was obtained from compound 27 according to general procedure C (8.4 mg, 8.8 µmol, 93%). TLC: Rf = 0.60 (DCM/MeOH, 9/1, v/v); 1H NMR (CDCl3/MeOH-d4, 400 MHz): δ 8.17 (s, 1H, CH arom), 7.97 (s, 1H, CH triazole), 7.81 (dd, 1H, J = 1.6, 8.5 Hz, CH arom), 7.72 – 7.77 (m, 2H, CH arom), 7.25 (d, 1H, J = 8.0 Hz, H-6), 7.09 – 7.15 (m, 2H, CH arom), 5.64 (d, 1H, J = 8.0 Hz, H-5), 5.40 (d, 1H, J = 2.5 Hz, H-1'), 4.98 – 5.03 (m, 2H, H-2', CH Aza), 4.86 (dd, 1H, J = 5.6, 14.2 Hz, CHH Aza), 4.70 – 4.81 (m, 3H, H-3', CH Aza, CHF Aza), 4.15 – 4.20 (m, 1H, H-4'), 3.90 (s, 3H, CH3 OMe), 3.64 (dd, 1H, J = 3.8, 14.4 Hz, H-5'), 3.53 (dd, 1H, J = 4.1, 14.4 Hz, H-5'), 2.70 (dd, 1H, J = 6.0, 16.7 Hz, CHH Aza), 2.63 (dd, 1H, J = 5.7, 16.7 Hz, CHH Aza), 2.21 (t, 2H, J = 7.5 Hz, CH2), 1.51 – 1.60 (m, 2H, CH2), 1.48 (s, 3H, CH3 iPr), 1.34 (s, 9H, CH3 tBu), 1.27 (s, 3H, CH3 iPr), 1.12 – 1.24 (m, 20H, CH2), 0.83 (t, 3H, J = 6.7 Hz, CH3); 13C NMR (CDCl3/MeOH-d4, 100 MHz): δ 174.8, 170.7 (C=O), 168.7 (C-4), 163.7 (C=O), 158.0 (Cq), 150.8 (C-2), 148.0 (Cq), 143.2 (C-6), 134.5 (Cq), 129.7 (CH arom), 128.9 (Cq), 127.5 (CH arom), 125.0 (Cq), 124.4, 124.1 (CH arom), 121.4 (CH triazole), 119.3 (CH arom), 114.7 (Cq iPr), 105.8 (CH arom), 102.7 (C-5), 95.5 (C-1'), 84.5 (C-4'), 83.5 (C-2'), 81.8 (Cq tBu), 80.5 (C-3'), 55.3 (OMe), 52.8 (CH Aza), 50.4 (CH2 Aza), 49.8 (CH Asp), 40.5 (C-5'), 36.8
5'-N-(N-myristoyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alaninyl-L-aspartate)-5'-amino-5'-deoxy uridine (29). Compound 38 was fully deprotected using general procedure D to afford the title compound as a slightly colored solid (Yield: 5.1 mg, 5.9 µmol, 66%). \(^1\)H NMR (DMSO-\(d_6\), 600 MHz): \(\delta\) 11.4 (s, 1H, NH), 8.53 (d, 1H, \(J = 7.7\) Hz, NH), 8.48 (s, 1H, CH\(_{\text{triazole}}\)), 8.28 (s, 1H, CH\(_{\text{arom}}\)), 8.21 (d, 1H, \(J = 8.2\) Hz, NH), 8.11 (t, 1H, \(J = 5.6\) Hz, NH), 7.86 – 7.92 (m, 3H, CH\(_{\text{arom}}\)), 7.68 (d, 1H, \(J = 8.0\) Hz, H-6), 7.33 (d, 1H, \(J = 1.9\) Hz, CH\(_{\text{arom}}\)), 7.18 (dd, 1H, \(J = 2.3, 8.9\) Hz, CH\(_{\text{arom}}\)), 5.75 (d, 1H, \(J = 5.9\) Hz, H-1'), 5.64 (dd, 1H, \(J = 1.3, 7.8\) Hz, H-5), 5.41 (d, 1H, \(J = 5.7\) Hz, 2'-OH), 5.18 (d, 1H, \(J = 4.4\) Hz, 3'-OH), 4.87 – 4.92 (m, 1H, CH Aza), 4.82 (dd, 1H, \(J = 4.1, 14.0\) Hz, CHH Aza), 4.57 – 4.62 (m, 2H, CH Asp, CHH Aza), 4.04 – 4.08 (m, 1H, H-2'), 3.85 – 3.92 (m, 5H, H-3', H-4', CH\(_{\text{3OMe}}\)), 3.40 – 3.45 (m, 2H, H-5'), 2.71 (dd, 1H, \(J = 4.5, 16.8\) Hz, CHH Asp), 2.57 – 2.61 (m, 1H, CHH Asp), 2.02 – 2.13 (m, 2H, CH\(_{\text{2}}\)), 1.29 – 1.38 (m, 2H, CH\(_{\text{2}}\)), 0.95 – 1.26 (m, 20H, CH\(_{\text{2}}\)), 0.86 (t, 3H, \(J = 7.0\) Hz, CH\(_{\text{3}}\)). \(^{13}\)C NMR (DMSO-\(d_6\), 150 MHz): \(\delta\) 173.1, 171.1 (C=O), 169.2 (C-4), 163.5 (C=O), 157.9 (C\(_{\text{q}}\)), 151.2 (C-2), 146.7 (C\(_{\text{q}}\)), 141.7 (C-6), 134.3 (C\(_{\text{q}}\)), 130.0, 129.0, 127.8 (CH\(_{\text{arom}}\)), 126.4 (C\(_{\text{q}}\)), 124.5, 123.8 (CH\(_{\text{arom}}\)), 122.5 (CH\(_{\text{triazole}}\)), 119.5, 106.4 (CH\(_{\text{arom}}\)), 102.4 (C-5), 88.4 (C-1'), 83.0 (C-4'), 73.0 (C-2'), 71.2 (C-3'), 55.7 (CH\(_{\text{3OMe}}\)), 52.9 (CH Aza), 50.9 (CH\(_{\text{2}}\) Aza), 50.3 (CH Asp), 41.6 (C-5'), 35.7 (CH\(_{\text{2}}\) Asp), 31.8, 29.5, 29.4, 29.3, 29.2, 29.0, 25.6, 22.6 (CH\(_{\text{2}}\)), 14.4 (CH\(_{\text{3}}\)). LC: \(R_t = 10.45\) min; ESI-MS: m/z = 863.3 (M+H\(^+\)).
Overview of the click library based on azides 11, 12 and 13 (synthesized using general procedure B)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>LC-MS data</th>
</tr>
</thead>
</table>
| 11a      | ![Structure 11a](image1.png) | R<sub>t</sub> = 7.35 min  
           m/z = 552.20 (M+H<sup>+</sup>) |
| 11f      | ![Structure 11f](image2.png) | R<sub>t</sub> = 6.15 min  
           m/z = 502.13 (M+H<sup>+</sup>) |
| 11g      | ![Structure 11g](image3.png) | R<sub>t</sub> = 6.61 min  
           m/z = 582.20 (M+H<sup>+</sup>) |
| 11h      | ![Structure 11h](image4.png) | R<sub>t</sub> = 6.07 min  
           m/z = 496.20 (M+H<sup>+</sup>) |
| 11i      | ![Structure 11i](image5.png) | R<sub>t</sub> = 2.00 min  
           m/z = 473.07 (M+H<sup>+</sup>) |
| 12a      | ![Structure 12a](image6.png) | R<sub>t</sub> = 8.04 min  
           m/z = 580.27 (M+H<sup>+</sup>) |
| 12f      | ![Structure 12f](image7.png) | R<sub>t</sub> = 6.13 min  
           m/z = 530.27 (M+H<sup>+</sup>) |
12g \[ R_t = 6.52 \text{ min} \]
\[ m/z = 611.27 \text{ (M+H)}^+ \]

12h \[ R_t = 5.89 \text{ min} \]
\[ m/z = 525.27 \text{ (M+H)}^+ \]

12i \[ R_t = 2.03 \text{ min} \]
\[ m/z = 501.20 \text{ (M+H)}^+ \]

13a \[ R_t = 6.98 \text{ min} \]
\[ m/z = 608.27 \text{ (M+H)}^+ \]

13b \[ R_t = 6.93 \text{ min} \]
\[ m/z = 650.33 \text{ (M+16+H)}^+ \]

13c \[ R_t = 8.18 \text{ min} \]
\[ m/z = 584.33 \text{ (M+H)}^+ \]

13d \[ R_t = 7.77 \text{ min} \]
\[ m/z = 604.33 \text{ (M+H)}^+ \]
13e  
\[ R_t = 4.95 \text{ min} \]
\[ m/z = 571.33 (M+H^+) \]

13f  
\[ R_t = 6.10 \text{ min} \]
\[ m/z = 558.33 (M+H^+) \]

13g  
\[ R_t = 6.70 \text{ min} \]
\[ m/z = 639.33 (M+H^+) \]

13h  
\[ R_t = 6.16 \text{ min} \]
\[ m/z = 553.27 (M+H^+) \]

13i  
\[ R_t = 1.94 \text{ min} \]
\[ m/z = 529.27 (M+H^+) \]
Chemo-enzymatic synthesis of radiolabeled UDP-diNacBac

[\textsuperscript{3}H]-UDP-diNacBac was prepared by previously described chemoenzymatic methods, with a modification at the final step to incorporate the tritium radiolabel.\textsuperscript{5} UDP-GlcNAc was converted to UDP-4-amino-NacBac using PglF and PglE. After purification, 5 mM UDP-4-amino-NacBac was incubated with [\textsuperscript{3}H]-Acetyl coenzyme A (20 Ci/mmol, American Radiolabeled Chemicals), 50 mM HEPES pH 7.5, 50 mM NaCl, and 260 µM PglD, followed by a chase with 5 mM unlabeled acetyl coenzyme A for 2 hours. The radiolabeled product was purified on a Phenomenex Synergi C18 reverse-phase HPLC column, to yield [\textsuperscript{3}H]-UDP-diNacBac (5.4 mCi/mmol).

Purification of PglC

PglC was cloned into the pET-SUMO vector (Life Technologies) using the BsaI and XhoI restriction sites and the following primers:

<table>
<thead>
<tr>
<th>PglC Forward Primer</th>
<th>5’-CGCCCGGTCTCCAGGTATGTATGAAAAAA-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>PglC Reverse Primer</td>
<td>5’-ATCGCTCGAGTTATGCCGTCCTTT-3’</td>
</tr>
</tbody>
</table>

The pET-SUMO-PglC plasmid was transformed into BL21-RIL cells (Agilent) for overexpression, using kanamycin and chloramphenicol for selection. Overexpression was performed using the Studier method.\textsuperscript{19} In this method, 1 mL of an overnight cell culture was added to expression media containing 30 µg/mL kanamycin and 30 µg/mL chloramphenicol in 1 L of autoinduction media (0.1% (w/v) tryptone, 0.05% (w/v) yeast extract, 2 mM MgSO\textsubscript{4}, 0.05% (v/v) glycerol, 0.005% (w/v) glucose, 0.02% (w/v) α-lactose, 2.5 mM Na\textsubscript{2}HPO\textsubscript{4}, 2.5 mM KH\textsubscript{2}PO\textsubscript{4}, 5 mM NH\textsubscript{4}Cl, 0.5 mM Na\textsubscript{2}SO\textsubscript{4}). Cells were allowed to grow with shaking for 3 h at 37 °C. After 3 h, the temperature was decreased to 16 °C, and the cells were incubated for 16 hours. Cells were harvested by centrifuging at 9000 x g, and cells were stored at -80 °C. Cell pellets were thawed in 10% of the original culture volume in 50 mM Tris pH 8.0, 150 mM NaCl, 40 µL protease inhibitor cocktail (Calbiochem). The cells were lysed by two rounds of sonication for 90 seconds each, at an amplitude of 50% with one-second on/off pulses. The cells were incubated on ice for ten minutes between rounds of sonication. Cellular debris was removed by centrifugation at 9000 x g for 45 minutes. The resulting supernatant was transferred to a clean centrifuge tube and subjected to centrifugation at 142,000 x g for 65 minutes to pellet the CEF. If the CEF was to be used for activity assays, it was homogenized into 1% of the original culture volume in 50 mM HEPES pH 7.5, 100 mM NaCl and stored at -80 °C. If protein was to be purified from the CEF, it was isolated and homogenized into 10% of the original culture volume in 50 mM HEPES pH 7.5, 100 mM NaCl, 1% n-dodecyl beta-D-maltoside (DDM), using a glass homogenizer. 20 µL protease inhibitor cocktail was added to prevent proteolysis. This sample was incubated at 4 °C with gentle rocking for 16 hours, after which it was centrifuged (145,000 x g) to remove insoluble material. The resulting supernatant was incubated with 1 mL Ni-NTA resin for 1-2 hours. The resin was washed with 30 ml Wash 1 buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.03% DDM, 20 mM imidazole), followed by a wash with 30 ml Wash 2 buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.03% DDM, 45 mM imidazole). PglC was eluted in 4 x 1 mL fractions of elution buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.03% DDM, 300 mM imidazole). Gel filtration analysis was performed using a Superdex S200 10/300 column (GE Healthcare) equilibrated with 50 mM HEPES pH 7.5, 100 mM NaCl, and 0.03% DDM.

Radioactivity-Based Activity Assays with PglC.
Assays contained 20 µM Und-P, 10% DMSO, 0.1% Triton X-100, 50 mM HEPES pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 20 µM [³H]-UDP-diNAcBac, and 1 nM PglC in a final volume of 60 µL. Inhibitors were added in DMSO, in a volume such that the total concentration of DMSO in the reaction was equal to 10% (v/v). PglC was pre-incubated in the reaction mixture lacking [³H]-UDP-diNAcBac for five minutes at RT. After initiation of the reaction with [³H]-UDP-diNAcBac, aliquots (20 µL) were taken at twenty minute time points and quenched in 1 mL CHCl₃:MeOH. The organic layer was washed three times with 400 µL PSUP (Pure Solvent Upper Phase, composed of 15 mL CHCl₃, 240 mL MeOH, 1.83 g KCl in 235 mL H₂O). The resulting aqueous layers were combined with 5 mL EcoLite (MP Biomedicals) liquid scintillation cocktail. Organic layers were combined with 5 mL OptiFluor (PerkinElmer). Both layers were analyzed using scintillation counting. Conversion was calculated by dividing the radioactive counts in the organic layer over the total number of counts. The data was plotted as percentage remaining activity compared to the positive control (no inhibitor), and the IC₅₀ values were obtained by plotting residual activity versus concentration (GraphPad Prism).

Luminescence assay using UMP/CMP-Glo.
The quenching solution was prepared as described by Promega. Assays contained 20 µM Und-P, 10% DMSO, 0.1% Triton X-100, 50 mM HEPES pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 20 µM UDP-diNAcBac, and 1 nM PglC in a final volume of 25 µL. Inhibitors were added in DMSO, in a volume such that the total concentration of DMSO in the reaction was equal to 10% (v/v). PglC was pre-incubated in the reaction mixture lacking UDP-diNAcBac for five minutes at RT. After initiation of the reaction with UDP-diNAcBac, the reaction was halted after 20 min by the addition of 25 µL quenching buffer. The mixture was transferred to a 96-well plate (white, half area, Corning) and placed in the plate reader. The plate was shaken at low speed for 16 min, and incubated at RT for 44, after which time the luminescence was read. Background inhibition of the UMP/CMP-Glo assay was established for each inhibitor,⁶ and the luminescent reads were adjusted accordingly. Conversion was calculated by dividing the luminescence units (RLU) in the sample with inhibitor over the RLU obtained for the positive control (no inhibitor). The data was plotted as percentage remaining activity compared to the positive control (no inhibitor), and the IC₅₀ values were obtained by plotting residual activity versus concentration (GraphPad Prism).

⁶ Some inhibitors, especially the first-and second-generation scaffolds, inhibited the UMP/CMP-Glo assay itself. Fortunately, the more elaborate compounds and end products were not inhibiting the assay.
Michaelis Menten plots for UDP-diNAcBac and Und-P

Kinetic parameters were determined for each of the substrates of the PglC reaction. The radioactivity-based assay was used to determine the $K_M$ of UDP-diNAcBac, at 20 µM Und-P, while the UMP/CMP-Glo assay was used to determine the $K_M$ of Und-P, at 20 µM UDP-diNAcBac. An important caveat for the determination of kinetic parameters for Und-P is that it is difficult to predict how this substrate is distributed among detergent micelles that contain PglC, and it is not apparent what the effective concentration of Und-P is in the microenvironment of the micelles. Thus, the reported parameters for Und-P are apparent values. The data was fit to a Michaelis Menten plot using non-linear regression.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ app</th>
<th>$V_{max}$ app</th>
<th>$k_{cat}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-diNAcBac</td>
<td>7.2 ± 1.1 µM</td>
<td>1.84 ± 0.09 µM/min</td>
<td>303 ± 90 min$^{-1}$</td>
</tr>
<tr>
<td>Und-P</td>
<td>15.6 ± 5.1 µM</td>
<td>0.3 ± 0.04 µM/min</td>
<td>460 ± 10 min$^{-1}$</td>
</tr>
</tbody>
</table>
5’-N-(tert-butyl N-[4-aminobutyl] carbamate)-5’-amino-5’-deoxy-2’,3’-O-isopropylidene uridine (3).
5'-N-(tert-butyl N-[6-aminohexyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (4).
5'-N-(2-aminoethyl)-5'-amino-5'-deoxy-uridine (5).
5’-N-(4-aminobutyl)-5’-amino-5’-deoxy-uridine (6).
$5'$-$N$-(6-aminohexyl)-$5'$-amino-$5'$-deoxy-uridine (7).
5'-N-(N-acetyl-D/L-alanine)-5'-N-(tert-butyl N-[2-aminoethyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (30).
$5'-(N\text{-acetyl-D/L-alanine})-5'-N\text{-}(2\text{-aminoethyl})-5'\text{-amino-5'}\text{-deoxy-uridine (8).}$
5′-N-(N-acetyl-D/L-alanine)-5′-N-(tert-butyl N-[4-aminobutyl] carbamate)-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (31).
5'-N-(N-acetyl-D/L-alanine)-5'-N-(4-aminobutyl)-5'-amino-5'-deoxy-uridine (9).
5’-\(N\text{-}N\text{-acetyl-D/L-alanine}\)\(-5’\text{-}\(N\text{-}(\text{term-butyl N-[6-aminohexyl] carbamate})\)\(-5’\text{-}\text{aamino-}5’\text{-deoxy-}2’,3’\text{-O-}
\text{isopropylidene uridine} (32).
5'-N-(N-acetyl-D/L-alanine)-5'-N-(6-aminohexyl)-5'-amino-5'-deoxy- uridine (10).
5'-N-azidoacyl-5'-N-(tert-butyl N-[2-aminoethyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (33).

![Chemical structures and spectra](image-url)
deoxy-uridine (11).
5′-N-azidoacetyl-5′-N-(tert-butyl N-[4-aminobuty] carbamate)-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (34).
5'-N-azidoacetyl-5'-N-(4-aminobutyl)-5'-amino-5'-deoxy-uridine (12).
5'-N-azidoacetyl-5'-N-(tert-butyl N-[6-aminohexyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (35).
5'-N-azidoacetyl-5'-N-(6-aminohexyl)-5'-amino-5'-deoxy-uridine (13).
5'-N-(N-[9-fluorenlymethoxycarbonyl]-3-azido-L-alanine)-5'-N-(tert-butyl N-[6-aminohexyl] carbamate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (14).
5’-N-(N-myristoyl-3-azido-L-alanine)-5’-N-(6-aminohexyl)-5’-amino-5’-deoxy uridine (17).

5’-N-(N-myristoyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alanine)-5’-N-(tert-buty1 N-[6-aminohexyl]carbamate)-5’-amino-5’-deoxy-2’,3’-O-isopropylidene uridine (36).
5’-N-(N-myristoyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alanine)-5’-N-(6-aminohexyl)-5’-amino-5’-deoxy uridine (18).

$5'-(N\text{-}acetyl\text{-}L\text{-}aspartate})-5'\text{-}amino\text{-}5'\text{-}deoxy~uridine\ (21)$. 

Acetylated intermediate

Final compound 21
5'-N-(N-[9-fluorenylmethoxycarbonyl]-3-azido-L-alaninyl-O-tert-butyl-L-aspartate)-5'-amino-5'-deoxy-2',3'-O-isopropylidene uridine (22).
$5'-(N$-acyethyl-3$'$-azido-L-alaninyl-L-aspartate)$-5'$-amino-5'$-deoxy uridine (25).
5'-N-(N-acetyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alaninyl-O-tert-butyl-L-aspartate)-5’-amino-5’-deoxy-2’,3’-O-isopropylidene uridine (37).
5'-N-(N-acetyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alaninyl-L-aspartate)-5'-amino-5'-deoxy uridine (26).
$5'-(N$-\text{myristoyl}-3$-$\text{azido-L-alaninyl-L-aspartate})-5'$$-$\text{amino-5'$$-\text{deoxy uridine (28).}$
5′-N-(N-myristoyl-3-[(4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl)-L-alaninyl-O-tert-butyl-L-aspartate)-5′-amino-5′-deoxy-2′,3′-O-isopropylidene uridine (38).
5'-(N-myristoyl-3-[4-(6-methoxynaphthyl)-1H-1,2,3-triazol-1-yl]-L-alaninyl-L-aspartate)-5'-amino-5'-deoxy uridine (29).