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Synthesis of azide congeners of preQ$_1$ as potential substrates for tRNA guanine transglycosylase

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Abstract

PreQ$_1$ (**2**) is a precursor of queuine (**1**) that in eubacteria is incorporated into transfer RNA (tRNA) by tRNA guanine transglycosylase (TGT) before being further elaborated into queuine. The queuine modification is unusual and occurs across all eukaryotes and eubacteria with few exceptions, but its function remains unclear. As the modified nucleotide occurs through incorporation of a specially synthesized nucleotide instead of via modification of a genetically encoded base, a study of the sites of modification by prepared probes is possible. We report the synthesis of two novel azide congeners (**3**,**4**) of preQ$_1$ for this purpose. The evaluation of their interaction with TGT shows that both probes act as weak competitive inhibitors of guanine exchange of guanine(34) tRNA^{Tyr} with a K_i of ~70 μM. However, we could not show that these are substrates for TGT-catalyzed incorporation into tRNA. We believe the reason for this is a marked loss of binding due to the azide functionality of **3** and **4** abrogating the possibility of two hydrogen bonds to the carbonyl group of Leu231 and Met260 of TGT, previously observed for the terminal methylene amine of preQ$_1$ by x-ray crystallography.

1 | INTRODUCTION

Queuine (**1**; Figure 1) makes up one of approximately 100 base-modified nucleotides found in RNA.[1] Since its discovery many details of its incorporation into RNA have been described and its potential role examined. Unlike the majority of modified nucleotides that result from modifications of the genetically encoded nucleotides, queuine is incorporated into transfer RNA (tRNA) by transglycosylation.[2] Eukaryae incorporates queuine obtained through their diet, whereas eubacteria biosynthesize and incorporate the queuine precursor preQ$_1$ (**2**; Figure 1) via multistep modifications from guanine (34) tRNA to yield queuine(34) tRNA. As a result, the enzyme responsible for the incorporation of the modification into tRNA, tRNA guanine transglycosylase (TGT), differs between eukaryal and eubacterial organisms.[3] The pathway for the biosynthesis of queuine has been fully elucidated with all the involved enzymes identified. The queuine modification is known to be present in the wobble position of the anticodon loop of tRNA^{Tyr}, tRNA^{His}, tRNA^{Asp}, and tRNA^{Asn}.[4] Investigations have determined that its presence has an effect on codon recognition and promoting translational fidelity.

Base modification by transglycosylation is unusual and represents an interesting entry point for study with small molecule probes. Previous studies from our laboratory with tritium-labeled queuine and preQ$_1$ have shown differences between the eukaryotic and eubacterial versions of TGT with the prevalence of queuine extending beyond the previously known four tRNAs.[3,5] To further build on this finding, we turned our attention to finding a means to identify the specific tRNA species that are modified. Toward that end, we have designed two novel azide probes (**3** and **4**), based on the heterocyclic core of queuine and preQ$_1$, for studies showing them to be

substrates for TGT. These included bioorthogonal Staudinger and/or click ligation chemistry to isolate modified strands of tRNA. Structurally, **3** is more closely related to $preQ_1$, which is important for recognition by TGT for its incorporation into tRNA. Since the benzylic disposition of the azide function raised questions about its potential stability, we decided to mitigate this concern by also making a more stable one-carbon homolog **4**. This paper describes the synthesis of **3** and **4** followed by studies to determine if these are substrates for TGT.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

The synthesis of benzylic azide probe **3** is shown in Scheme 1 with aldehyde **9** as the key intermediate. Prior synthesis of precursor **8** ($preQ_0$) proceeded via in situ generation of a 2-chloro-3-oxopropanenitrile from the base-catalyzed condensation of methyl formate (**5**) and chloroacetonitrile followed by acidic workup. The resultant oil,

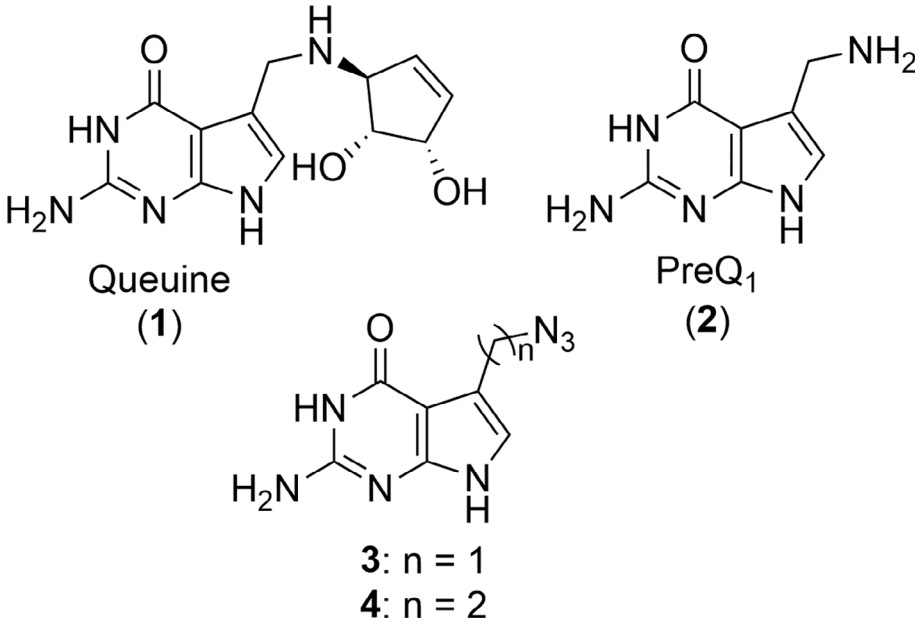
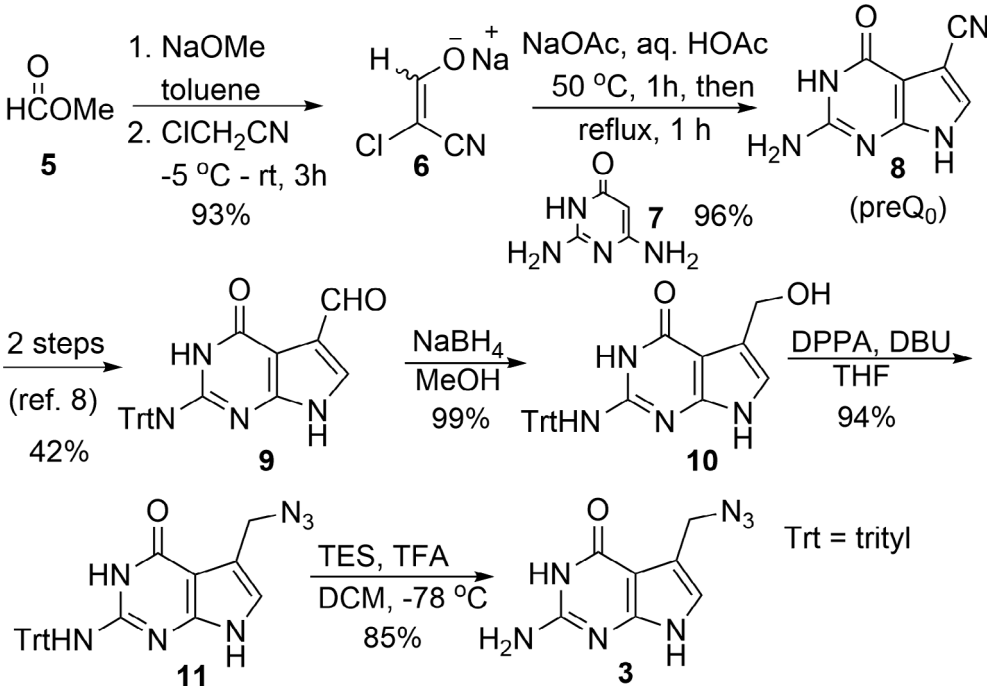


FIGURE 1 Queuine (**1**), precursor PreQ1 (**2**) and target azide probes (**3, 4**)



SCHEME 1 Synthetic pathway to azide probe **3**

without isolation, was then further reacted with 2,6-diaminopyrimidin-4(3*H*)-one (**7**) to give pyrrolopyrimidinone nitrile **8**.[6] Since the yield of **8** was variable from run to run (never exceeding 70%), we decided to try pre-isolation of 2-chloro-3-oxopropanenitrile as its sodium salt (**6**) prior to reaction with **7**. This was successfully accomplished on a 0.2 mole scale in 93% yield as a 7:1 mixture of *E*:*Z* isomers by ^{1}H NMR integration. The salt is a stable white solid and with storage in a desiccator maintained its physical and chemical properties for over a year, giving comparable yields of **8** in subsequent runs. Condensation of **6** with **7** then provided **8** in 96% yield. Following our work, a patent disclosed a similar preparation of **6** in 88% yield on a 0.265 scale.[7] We believe that **6** will serve as a valuable 3-carbon synthon for the synthesis of related 4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidine-5-carbonitrile heterocycles. With a reproducible synthesis of nitrile **8**, it was then parlayed to aldehyde **9** in 42% yield in two standard steps (trityl protection then DIBAL-H reduction) by our previously reported procedure, each involving in situ silylation to provide solution solubility.[8]

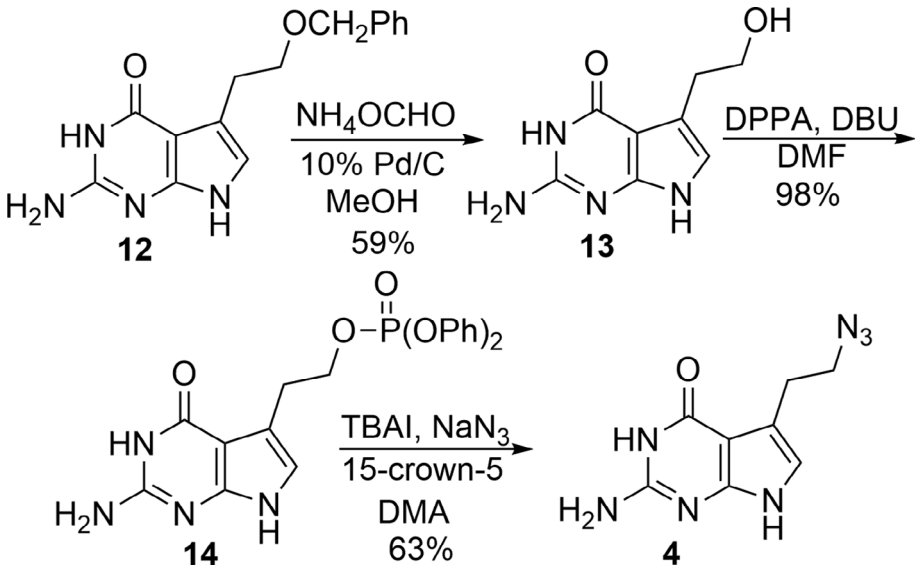
There is only one report in the literature of the synthesis of a benzylic azide of the 4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidine ring system found in **3**.[9] This proceeded via azide displacement of a benzylic bromide on a heterocyclic framework requiring multiple protection/deprotection steps of each nitrogen. There are no reports of the synthesis of an azide related to **4**.

Our strategy to **3** was to employ a much more readily available benzylic alcohol. Thus, reduction of aldehyde **9** to alcohol **10** proceeded uneventfully with sodium borohydride and set the stage for generation of target benzylic azide **3**. Standard conditions of mesylation/azide displacement unsurprisingly failed. A Lewis acid-catalyzed method (boron trifluoride etherate/trimethylsilyl azide)[10] provided a small amount of **11** with the remainder of products as decomposition materials. Several other Lewis and mineral acids of this reaction were evaluated, but all fared poorly. It is likely the above conditions generate a benzylic-type cation, which readily leads to polymerization.

We then turned to an alternate method for substituting a benzylic-type alcohol with an azide under basic conditions.[11] Hence, treatment of alcohol **10** with diphenylphosphoryl azide (DPPA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) resulted in a high-yield transformation to azide **11**. The reaction likely proceeds through a diphenylphosphoryl intermediate with the released azide displacing the now activated alcohol. Azide **11** was then converted at low temperature to probe **3** in 85% yield by removal of the trityl protecting group with trifluoroacetic acid in the presence of triethylsilane as a scavenger. This scheme yielded probe **3** in an overall 32% yield from the starting salt **6**.

The synthesis of the second azide probe **4**, which features an additional methylene spacer between the heterocyclic ring and the azide, is shown in Scheme 2. It begins with the benzyl ether **12**, which was prepared in a straightforward four-step process in 24% overall yield as reported by Hornillo-Aruajo et al.[12] Their removal of the benzyl protecting group to give alcohol **13** utilized boron trichloride. In our hands, we found this to be cumbersome due to the resulting boronates that made extracting the product difficult upon workup. We determined that transfer hydrogenation with ammonium formate and 10% palladium on carbon was cleaner and much easier for protecting group removal, giving **13** in 59% yield.

Standard methods to generate probe **4** from alcohol **13** failed. These included mesylation/azide displacement and Mitsunobu conditions. A successful method was to



SCHEME 2 Synthetic pathway to azide probe **4**

employ similar conditions used to synthesize azide **3**. As **13** is a primary alkyl alcohol rather than a benzylic type, modifications to the reaction conditions were necessary. Thus, treatment of **13** with DPPA and DBU in DMF did not give an expected azide, but cleanly yielded the phosphoryl ester (**14**) in 98% yield, which could be isolated and purified. To displace the ester with azide, we utilized a literature method that describes the transformation of the 6-position alcohol of a sugar to an azide.[13] Thus, the phosphoryl ester (**14**) was treated with sodium azide, 15-crown-5 ether, and tetrabutylammonium iodide to provide the desired azide probe **4** in 63% yield and an overall 36% yield from **12**.

All compounds were rigorously purified by flash chromatography or crystallization, and their structural assignments were supported by diagnostic peaks in NMR spectra and by mass spectrometry.

2.2 | Biology

All biological studies were conducted with *E. coli* TGT and *E. coli* $tRNA^{Tyr}$.

Our initial goal was to determine if azide probe **3** is an inactivator of TGT. This was based on concerns that its benzylic-like connection could cause it to unravel to an electrophilic trapping species. Following protocols by Hoops et al.,[14] we determined that **3** is not an inactivator of TGT, and thus we proceeded with incorporation experiments with this and homolog **4**.

Studies with ^{14}C-guanine (Figures S1 and S2, Supplementary Information) followed by kinetic analysis indicated that both azide probes act as weak competitive inhibitors of guanine exchange by TGT with a K_i of ~70 μM compared to 0.35 μM for guanine and 0.05 μM for its natural substrate $preQ_1$.[3] In one approach, to test for incorporation into tRNA as competitive substrates, the azide probes at 200 μM (approximately 3× $K_{i,apparent}$) were incubated overnight with tRNA, and TGT was added every 6 h. Two tRNAs were used as substrate: (i) a mini-hairpin of tRNA, utilized in earlier work, to demonstrate $preQ_1$ incorporation by mass spectrometry,[15] and (ii) the other is a full-length tRNA under the same conditions. Mass spectrometry analysis of an untreated sample of each tRNA was performed concurrently. The reactions were quenched by ethanol precipitation and the samples were desalted and submitted for electrospray ionization mass spectrometry analysis. In neither experiment was incorporation of either azide probe observed with the intact mass of starting tRNA observed instead. Additionally, preliminary experiments with the azide probes showed them to react with Staudinger and copper-free click ligation reagents bound to biotin in the presence of TGT and tRNA, but without incorporation into tRNA (data not shown). Thus, cumulative data confirm that our azide probes are not substrates for TGT-catalyzed incorporation into tRNA.

3 | CONCLUSIONS

We have synthesized two novel azide congeners (**3**,**4**) of $preQ_1$ and evaluated their interaction with TGT. Both compounds were made in good overall yield with the sequence to azide **3** featuring a scalable synthesis of 2-chloro-2-cyanoethen-1-olate, sodium salt (**6**), a stable, storable solid that promises to serve as a valuable 3-carbon synthon for the synthesis of related 4-oxo-4,-7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidine-5-carbonitrile heterocycles. Both probes act as weak competitive inhibitors of guanine exchange of guanine(34) tRNA with a K_i of ~70 μM with additional experiments showing that these are not substrates for TGT-catalyzed incorporation into tRNA. We believe the reason for this is a marked loss of binding due to the azide functionality of **3** and **4** abrogating the possibility of two hydrogen bonds to the carbonyl group of Leu231 and Met260 of TGT, previously observed for the terminal methylene amine of $preQ_1$ by x-ray crystallography.[16] This hypothesis is supported by recent publications from the Kelly lab on the synthesis of a chain-extended congener of $preQ_1$ that is a TGT-specific substrate that rapidly reverses disease in murine experimental autoimmune encephalomyelitis,[17] and the Devaraj lab[18] on the synthesis of TGT-specific probes to label RNA. Each of these structures incorporates the basic benzylic amine of $preQ_1$ and is readily incorporated into RNA.

While disappointed that our azide probes are not substrates for tRNA guanine transglycosylase, we feel that these can serve as valuable intermediates for click reactions with alkynes to provide a wide range of analogues incorporating substituted 1,2,3-triazines as replacements for heterocycloalkyl or benzenoid moieties that are tethered to the 5-alkyl-2-amino-3,7-dihydro-4*H*-pyrrolo[2,3-*d*]pyrimidin-4-one scaffold. Potential applications to broad-spectrum antiproliferative agents[19] or thymidylate synthase inhibitors[20] exemplify these possibilities.

4 | EXPERIMENTAL

4.1 | Chemistry

All reagents were obtained from commercial suppliers and used as received. Toluene and pyridine were distilled over calcium hydride. Glassware was oven-dried before use for

reactions run under anhydrous conditions. Melting points were determined in open capillary tubes on a Laboratory Devices Mel-Temp apparatus and are uncorrected. NMR spectra were recorded on a Bruker instrument at 500 MHz for ^{1}H and 125 MHz for ^{13}C spectra. Chemical shift values are recorded in δ units (ppm). Mass spectra (MS) were recorded on a Micromass TofSpec-2E Matrix-Assisted, Laser-Desorption, Time-of-Flight Mass Spectrometer in the positive ESI mode unless otherwise noted. High-resolution mass spectrometry (HRMS) analysis was performed on an Agilent Q-TOF system. TLC was performed on EM Science aluminum baked silica gel (SiO_2) plates. Visualization was performed with 254 nm UV light and 2,4-dinitrophenylhydrazine (2,4-DNP) stain to identify aldehydes. Extraction solutions were dried over magnesium sulfate or sodium sulfate prior to concentration.

4.1.1 | 2-Chloro-2-cyanoethen-1-olate, sodium salt (6)

A dry 250 ml three-neck RB flask was equipped with a mechanical stirrer and an addition funnel. The flask was charged with a suspension of sodium methoxide (10.8 g, 200 mmol) in toluene (100 ml), which was cooled to −5 °C in a water ice-salt bath. Methyl formate (**5**; 37.0 ml, 600 mmol) was added slowly over 5 min and the solution temperature was maintained for 15–20 min. Chloroacetonitrile (12.66 ml, 200 mmol) was added dropwise over ∼1 h from the addition funnel with each droplet resulting in a local discharge of yellow color. The mixture was kept at −5 °C for 3 h and then allowed to come to room temperature, becoming a thick buttery yellow suspension after 16 h. The suspension was filtered on a glass Buchner fritted funnel and the collected white solid was washed with ethyl acetate and dried under vacuum overnight to give 23.2 g (93%) of product: ^{1}H NMR (DMSO-d_6) δ 8.50, 8.16 (integrates for 7:1 mixture of *E*:*Z* isomers); ^{13}C NMR (DMSO-d_6) δ 168.23, 126.55, 67.78; The material was stored in a desiccator at room temperature. The material maintained its physical and chemical properties for over a year, giving comparable yields in subsequent reactions.

4.1.2 | 2-Amino-4,7-dihydro-4-oxo-3*H*-pyrrolo[2,3-*d*]pyrimidine-5-carbonitrile (8; preQ$_0$ from sodium salt 6)

An RB flask was charged with a stir bar, anhydrous sodium acetate (0.065 g, 0.79 mmol), 2,6-diaminopyrimidin-4-one (**7**; 0.10 g, 0.79 mmol), sodium salt **6** (0.25 g, 2 mmol), glacial acetic acid (0.124 ml), and distilled water (3.2 ml) at 25 °C. The rapidly stirring suspension was heated at 50 °C for 16 h and then at reflux for 1 h. The resulting suspension was cooled to room temperature and collected on a fritted funnel. The light brown solid was washed with portions of water and then acetone. Vacuum drying provided 0.133 g (96%) of **3**: mp 360 °C (dec); ^{1}H NMR (DMSO-d_6) δ 11.98 (br s, 1H) 10.74 (br s, 1H), 7.59 (s, 1H), 6.43 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 158.0, 154.3, 152.1, 128.2, 116.4, 99.2, 86.0.

4.1.3 | 2-Amino-4,7-dihydro-4-oxo-3*H*-pyrrolo[2,3-*d*]pyrimidine-5-carbonitrile (8; preQ$_0$ from free form of 6)

Reaction of 2,6-diaminopyrimidin-4-one (**7**; 13.32 g, 105.6 mmol) was carried out as previously described[6] to give a 68% yield of **8** with spectral data identical to the preparation using sodium salt **6**.

4.1.4 | 5-(Hydroxymethyl)-2-(tritylamino)-3*H*-pyrrolo[2,3-*d*]pyrimidin-4(7*H*)-one (10)

An ice-cold suspension of 4-oxo-2-(tritylamino)-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidine-5-carbaldehyde[8] (**9**; 0.10 g; 0.24 mmol) in methanol (1 ml) was treated with sodium borohydride (0.027 g; 0.71 mmol) and the mixture was stirred for 3 h. The mixture was quenched with brine and extracted with dichloromethane (3x). The combined extracts were dried and concentrated to leave **10** (0.1 g, 99%) as a white powder: R_f 0.33 (EtOAc); ^{1}H NMR (DMSO-d_6) δ 10.69 (br s, 1H), 10.51 (br s, 1H), 7.41 (br s, 1H), 7.31–7.20 (m, 15H), 6.34 (s, 1H), 4.95 (br s, 1H), 4.41 (d, J = 5.6, 2H); MS *m/z* 445.1 $(M + Na)^+$.

4.1.5 | 5-(Azidomethyl)-2-(tritylamino)-3*H*-pyrrolo[2,3-*d*]pyrimidin-4(7*H*)-one (11)

An ice-cold solution of alcohol **10** (0.05 g, 0.12 mmol), diphenylphosphoryl azide (DPPA; 0.031 ml, 0.142 mmol), and tetrahydrofuran (1 ml) was treated dropwise with 1,8-diazabicycloundec-7-ene (DBU) (0.021 ml, 0.142 mmol). The mixture was stirred for 4 h followed by additional equivalent charges of DPPA and DBU. After 18 h of total reaction time, the solution was quenched with water and then extracted with diethyl ether (3x). The combined extracts were washed successively with water (2x) and brine, dried, and concentrated to leave a solid that was purified by flash silica gel chromatography (gradient elution from 8:2 to 1:1 hexanes/ethyl acetate). Product

fractions were combined and concentrated to leave **11** (0.05 g, 94%) as a white powder: R_f 0.52 (1:1 hexanes/ethyl acetate); ^{1}H NMR (DMSO-d_6) δ 10.94 (br s, 1H), 10.36 (br s, 1H), 7.42 (br s, 1H), 7.31–7.20 (m, 15H), 6.58 (s, 1H), 4.31 (br s, 2H); MS *m/z* 459.1 $(M + Na)^+$, 895.3 $(2\,M + H)^+$.

4.1.6 | 2-Amino-5-(azidomethyl)-3*H*-pyrrolo[2,3-*d*]pyrimidin-4(7*H*)-one (3)

A stirring solution of azide **11** (0.10 g, 0.022 mmol) and triethylsilane (3.57 µl, 0.022 mmol) in dichloromethane (0.5 ml) was cooled to −78 °C, and treated with trifluoroacetic acid (3.32 µl, 0.045 mmol). The mixture was maintained at −78 °C for 3 h, and then allowed to slowly warm to room temperature. A formed precipitate was collected and dried to leave **3** (0.039 g, 85%) as an off-white powder: ^{1}H NMR (DMSO-d_6) δ 11.11 (br s, 1H), 10.44 (br s, 1H), 6.76 (s, 1H), 6.20 (br s, 2H), 4.38 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 158.69, 157.95, 152.39, 116.95, 111.07, 98.32, 45.65; MS *m/z* 228.0 $(M + Na)^+$, 433.1 $(2\,M + Na)^+$; HRMS *m/z* $(M + Na)^+$: calcd for $C_7H_9N_5ONa^+$, 228.0610; found, 228.0611.

4.1.7 | 2-Amino-5-(2-hydroxyethyl)-3*H*-pyrrolo[2,3-*d*]pyrimidin-4(7*H*)-one (13)

A mixture of benzyl ether **12**[12] (0.5 g, 1.76 mmol), ammonium formate (1.11 g, 17.6 mmol), and methanol (7 ml) was heated to reflux followed by the addition of 10% palladium on carbon (0.187 g, 0.176 mmol). After 8 h at reflux, the mixture was filtered through a pad of Celite followed by a methanol washing. The concentration of the combined filtrates left a solid that was triturated in hot ethyl acetate to give **13** (0.2 g, 59%) as a white powder: R_f 0.40 (4:1 ethyl acetate/methanol). The ^{1}H NMR was the same as previously described.[12]

4.1.8 | 2-(2-Amino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl diphenyl phosphate (14)

An ice-cold solution of alcohol **13** (0.162 g, 0.834 mmol), diphenyl phosphoryl azide (0.539 ml, 2.5 mmol), and *N*,*N*-dimethylformamide (6.7 ml) was treated with DBU (0.374 ml, 2.5 mmol). The mixture was stirred for 16 h at room temperature, diluted with water, and extracted with ethyl acetate (3×). The combined organic extracts were washed with brine, dried, and concentrated to a solid that was triturated in ∼9:1 ethyl acetate/methanol to give **14** (0.350 g, 98%) as a white solid: R_f 0.76 (4:1 ethyl acetate/methanol); ^{1}H NMR (DMSO-d_6) 10.80 (br s, 1H), 10.25 (br s, 1H), 7.39 (t, J = 7.7, 3H), 7.24 (t, J = 7.7, 2H), 7.17 (d, J = 7.7, 3H), 6.46 (s, 1H), 6.04 (br s, 2H), 4.52 (q, J = 6.8, 2H), 2.96 (t, J = 6.8, 2H); MS *m/z* 449.0 $(M + Na)^+$, 875.0 $(2\,M + Na)^+$.

4.1.9 | 2-Amino-5-(2-azidoethyl)-3*H*-pyrrolo[2,3-*d*]pyrimidin-4(7*H*)-one (4)

A mixture of **14** (0.186 g, 0.436 mmol), sodium azide (0.142 g, 2.181 mmol), tetra-*n*-butylammonium iodide (0.016 g, 0.044 mmol), 15-crown-5 ether (10 µl, 0.044 mmol), and *N*,*N*-dimethylacetamide (3.5 ml) was stirred at 80 °C for 18 h. The mixture was concentrated in vacuo to give a brown solid that was dissolved in methanol and adsorbed onto a small amount of flash silica gel for dry loading onto a chromatography column. The product was then purified by flash silica gel chromatography using gradient elution (ethyl acetate to 4:1 ethyl acetate/methanol). Product fractions were pooled and concentrated to leave **4** (0.060 g, 63%) as a yellow-white powder: R_f 0.63 (4:1, ethyl acetate/methanol); ^{1}H NMR (DMSO-d_6) δ 10.77 (br s, 1H), 10.22 (br s, 1H), 6.48 (s, 1H), 6.03 (s, 2H), 3.56 (t, J = 6.6, 2H), 2.82 (t, J = 6.6, 2H); ^{13}C NMR (DMSO-d_6) δ 159.69, 152.74, 151.99, 115.20, 114.52, 100.00, 51.53, 26.42; MS *m/z* 220.1 $(M + H)^+$; HRMS *m/z* $(M + H)^+$: calcd for $C_8H_{10}N_7O^+$, 220.0947; found, 220.0949.

4.2 | Biology

All biological studies were conducted with *E. coli* TGT and *E. coli* $tRNA^{Tyr}$. Biotin-bound reagents for Staudinger and copper-free click ligation chemistry were synthesized or commercially available. These were CAS 608514-42-7[21] and 1255942-07-4,[22] respectively.

4.2.1 | Inactivation study of azide 3 with TGT ^{14}C-guanine exchange assay

Experimental protocols by Hoops et al. were followed.[14] Azide **3** at 150 µM was incubated with 5 µM TGT in the presence of tRNA at 20 µM in bicine buffer for 15, 30, 60, and 120 min. A control preincubation of TGT without any azide **3** was also performed. Following incubation, TGT was diluted into a ^{14}C-guanine exchange assay with the following conditions in a bicine buffer: 20 µM tRNA, 0.1 µM TGT (from preincubation), 20 µM ^{14}C-guanine. Experiments were run in duplicate and time points were taken at 2, 4, 6, 8, and 10 min.

4.2.2 | TGT [^{14}C] guanine exchange assay of azide probes 3 and 4

Assays were performed with the azide probes under the following conditions: 0.1 μM TGT; 10 μM tRNA; 0.75 μM, 1.50 μM, or 5.00 μM ^{14}C-guanine at six concentrations of azide probe. These included 5, 50, 100, 250, and 500 μM for **3** and 10, 100, 175, 250, 500, and 1000 μM for **4**. The controls (without azide) were also run for each concentration. Experiments were performed in duplicate in bicine buffer. Data were obtained at time points for 2, 4, 6, 8, and 10 min by quenching and precipitating 70 μl of reaction mixture in 5% TCA. The resulting precipitate was collected by filtration. Liquid scintillation counting provided raw data for analysis. Data are shown in Figures S1 and S2 (Supplementary Information).

4.2.3 | Azide incorporation into tRNA

TGT mediated incorporation of azide probes **3** and **4** into tRNA was attempted with the following conditions: 80 μM tRNA, 200 μM azide probe, and 1.4 μM TGT in bicine buffer. An additional aliquot of TGT was added at 6 h and 12 h, and the reaction was quenched by ethanol precipitation at 18 h. The resulting tRNA was pelleted by centrifugation at 13 k rpm for 10 min in a tabletop Eppendorf centrifuge. The pellet was washed twice with 70% ethanol and air-dried 10 min before being suspended in solvent for analysis by mass spectrometry.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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