tRNA-Guanine Transglycosylase from *Escherichia coli*: Structure−Activity Studies Investigating the Role of the Aminomethyl Substituent of the Heterocyclic Substrate $PreQ_1$[†]

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ABSTRACT: A series of 5-substituted 2-aminopyrrolo[2,3-*d*]pyrimidin-4(3*H*)-ones have been synthesized in order to study the substrate specificity of the tRNA-guanine transglycosylase (TGT) from *Escherichia coli*. A number of these compounds were initially examined as inhibitors of radiolabeled guanine incorporation into tRNA catalyzed by TGT [Hoops, G. C., Garcia, G. A., & Townsend, L. B. (1992) 204th National Meeting of the American Chemical Society, Washington, DC, August 23−28, 1992, Division of Medicinal Chemistry, Abstract 113]. The kinetic parameters of these analogues as substrates in the TGT reaction have been determined by monitoring the loss of radiolabeled guanine from 8-[^{14}C]G34-tRNA. This study reveals that the tRNA−guanine transglycosylase from *E. coli* will tolerate a wide variety of substituents at the 5-position. The role of the 5-substituent appears to be entirely in binding/recognition with no apparent effects upon catalysis. A correlation between N7 pK_a and V_{max} suggests the deprotonation of N7 during the reaction, which must occur prior to subsequent glycosidic bond formation, appears to be partially rate-determining for the natural substrate. Comparison of the K_is of 7-methyl-substituted competitive inhibitors to the K_ms of their corresponding substrates suggests that some substrates (including $preQ_1$) are kinetically "sticky" (*i.e.*, K_m is equivalent to K_d) and other substrates have K_ms that reflect catalytic rates as well as binding.

To date, over 90 modified nucleosides have been discovered in RNA, over 70 in transfer RNA alone (Limbach *et al.*, 1994). In many cases their structures, positions in specific tRNAs, and biosynthetic pathways have been elucidated. However, relatively little is known about their biological roles and the molecular mechanisms by which those roles are performed. One of the hypermodified nucleoside bases found in tRNA is the guanine derivative queuine [2-amino-5-[[(4,5-*cis*-dihydroxy-1-cyclopenten-3-yl)amino]methyl]pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one, **1**, Figure 1]. This is the first modified base in which the basic (parent) ring system (purine or pyrimidine) has been modified. tRNA-guanine transglycosylase (TGT)[1] is the enzyme responsible for the post-transcriptional modification of specific tRNAs (Tyr, His, Asp, and Asn) with queuine. There appear to be two functionally different classes of the enzyme. The first class, represented by the enzyme from *Escherichia coli*, does not recognize queuine itself but exchanges a queuine precursor, $preQ_1$ (**2**, Figure 1), which lacks the cyclopentene diol moiety, for guanine-34. Further,

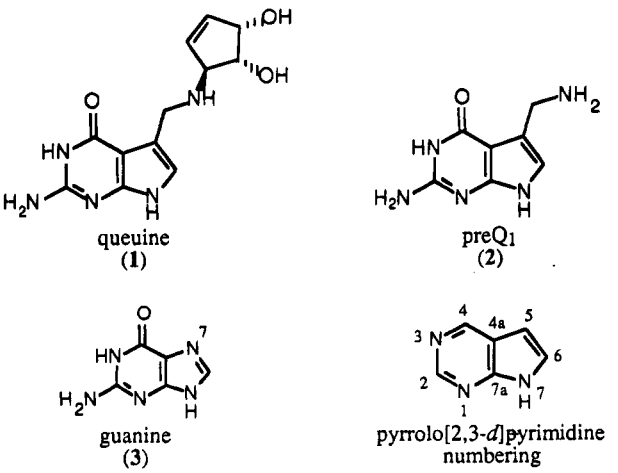

FIGURE 1: Queuine, $preQ_1$, guanine, and the numbering system for the pyrrolo[2,3-*d*]pyrimidine ring system. Note that the 7-position of guanine corresponds to the 5-position of the pyrrolo-[2,3-*d*]pyrimidine.

the *E. coli* TGT does not recognize tRNA containing queuine, however, it will exchange free guanine (**3**, Figure 1) for guanine-34. The second class of TGT, represented by the enzyme isolated from rat liver, recognizes queuine in addition to its precursors.

A number of purine analogues have been evaluated as substrates or inhibitors of the TGT reaction (Figure 2). 6-Thioguanine (**4**, Figure 2) and 8-azaguanine (**5**, Figure 2) were found to be substrates for the TGT from rat liver (Shindo-Okada *et al.*, 1980). Of the analogues tested with the enzyme from *E. coli*, only 7-methylguanine (**6**, Figure 2) was a potent inhibitor (80% inhibition at a concentration

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[1] Abbreviations: TGT, tRNA-guanine transglycosylase; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; HEPES, (hydroxyethyl)piperazineethanesulfonate; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; NTP, ribonucleotide triphosphate; BSA, bovine serum albumin; Piv, trimethylacetyl; and TLC, thin-layer chromatography.

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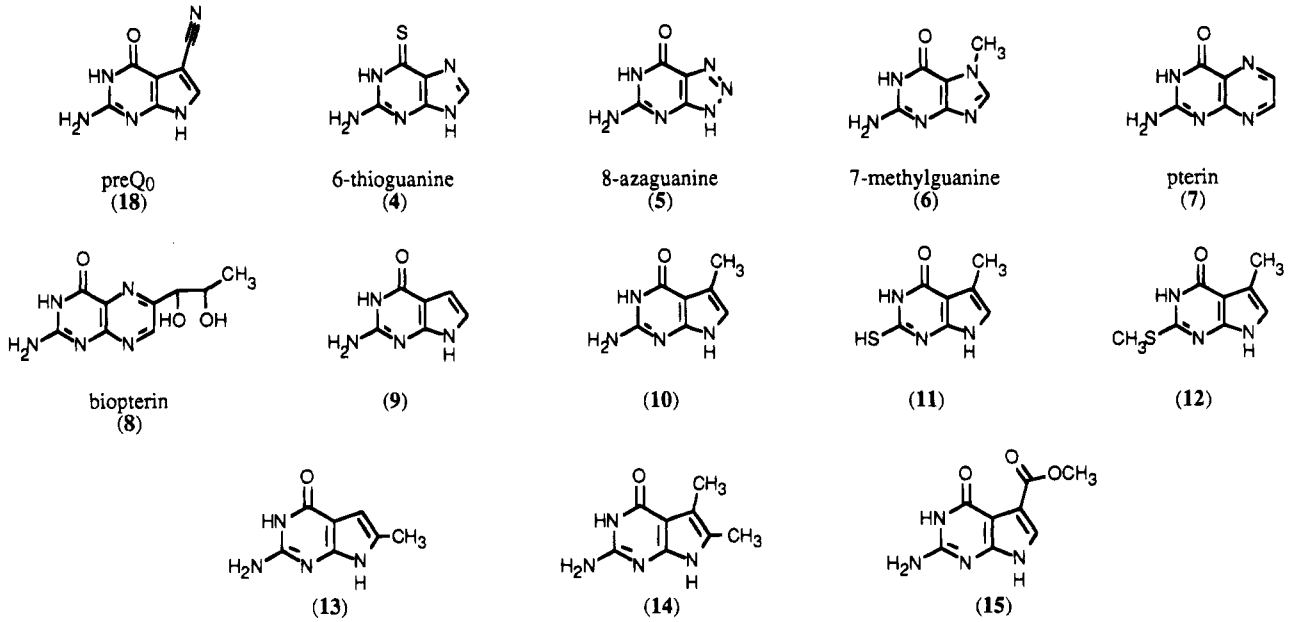


FIGURE 2: Purine analogues previously investigated as substrates/inhibitors of various TGTs. Note that compounds **9**−**12** are named as guanine derivatives. Compounds **13**−**15** have been studied with TGT only in this report.

25 times greater than guanine) of [^{14}C]guanine incorporation into tRNA (Okada & Nishimura, 1979). 7-Methylguanine has an apparent K_i of 1 mM with the rabbit reticulocyte TGT (Farkas *et al.*, 1984). In either case, 7-methylguanine was not incorporated into tRNA and therefore is not a substrate for either enzyme.

A low level of Q-tRNA was found in the skin of xiphophorine fish (Kersten *et al.*, 1983). The level of hypomodification was found to vary with the level of pteridines in the fish skin. A number of pterins (**7** and **8**, Figure 2) were reported to be inhibitors of the TGT from rabbit erythrocytes (Jacobson *et al.*, 1981). Pterin (**7**) itself was competitive with respect to guanine with a K_i of 90 nM and was a more potent inhibitor than biopterin (**8**) *in vitro*. However, *in vivo*, biopterin was a more potent inhibitor of queuine modification. Tetrahydrobiopterin is a more potent inhibitor of TGT from LM cells than biopterin. It has been speculated that the inhibitory effect of biopterin on queuine modification may be related to the oxidation state of biopterin and its role as a coenzyme of monooxygenases (Kersten *et al.*, 1983). Interestingly, 7-deazaguanine [**9**, 2-aminopyrrolo-[2,3-*d*]pyrimidin-4(3*H*)-one], 7-methyl-7-deazaguanine (**10**), and the 2-thio- (**11**) and 2-(methylthio)-7-methyl-7-deaza-guanine (**12**) analogues (Figure 2) were reported to be neither substrates for the TGT from *E. coli* (Okada & Nishimura, 1979) nor inhibitors of the TGT from rat liver (Shindo-Okada *et al.*, 1980). However, Farkas *et al.* (1984) have found that 7-deazaguanine is a substrate, albeit a poor one, for the TGT from LM cells.

These studies have been illuminating, but they have involved TGT enzymes from numerous sources, both prokaryotic and eukaryotic, and in no instance have all of the compounds been evaluated with a TGT from a single source. In order to elucidate the interaction of the heterocyclic substrate with TGT we have focused our efforts upon the TGT from *E. coli*. We have synthesized a series of 3-, 5-, 6-, and 7-substituted 2-aminopyrrolo[2,3-*d*]pyrimidin-4-ones as analogues of the physiological substrate for the *E. coli* TGT, preQ$_1$. Our studies indicate that the *E. coli* TGT is very tolerant of relatively small substitutions at the 5-position but that the 3-methyl and 6-methyl analogues are inactive. 7-Methyl analogues are competitive inhibitors of TGT. The role of the 5-substituent appears to be entirely in binding/recognition with no apparent effects upon catalysis. A correlation between N7 pK_a and V_{max} suggests the deprotonation of N7 during the reaction, which must occur prior to subsequent glycosidic bond formation, appears to be partially rate-determining for the natural substrate.

MATERIALS AND METHODS

Reagents and Enzymes. Buffers and 7-methylguanine were purchased from Sigma. 8-[^{14}C]Guanine was from Moravek Biochemicals. Centricon-10 concentrators were from Amicon. Wild-type *E. coli* TGT was isolated from an overexpressing clone as described previously (Chong & Garcia, 1994; Garcia *et al.*, 1993). T7 RNA polymerase was isolated from *E. coli* BL21/pAR1219 following a literature procedure (Grodberg & Dunn, 1988).

Syntheses. 2-Amino-5-(aminomethyl)-7*H*-pyrrolo[2,3-*d*]-pyrimidin-4(3*H*)-one (**2**, preQ$_1$, Figure 1) (Akimoto *et al.*, 1988), 2-amino-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**9**, Figure 2) (Cheng *et al.*, 1976), 2-amino-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**10**, Figure 2) (Secrist & Liu, 1978), 2-amino-6-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**13**, Figure 2) (Secrist & Liu, 1978), 2-amino-5,6-dimethyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**14**, Figure 2) (Secrist & Liu, 1978), 5-methyl-2-amino-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one-5-carboxylate (**15**, Figure 2) (Ramasamy *et al.*, 1989), 2-amino-5-[(*N*,*N*-dimethylamino)methyl]-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**16**, Figure 3B) (Akimoto *et al.*, 1988), and 2-amino-7*H*-pyrrolo-[2,3-*d*]pyrimidin-4(3*H*)-one-5-carboxamide (**17**, Figure 3c) (Ramasamy *et al.*, 1989) were produced via modifications of published procedures. 2-Amino-5-cyano-7*H*-pyrrolo-[2,3-*d*]pyrimidin-4(3*H*)-one (**18**, preQ$_0$, Figure 2) was produced via a procedure developed in this laboratory for which a manuscript is in preparation.[2]

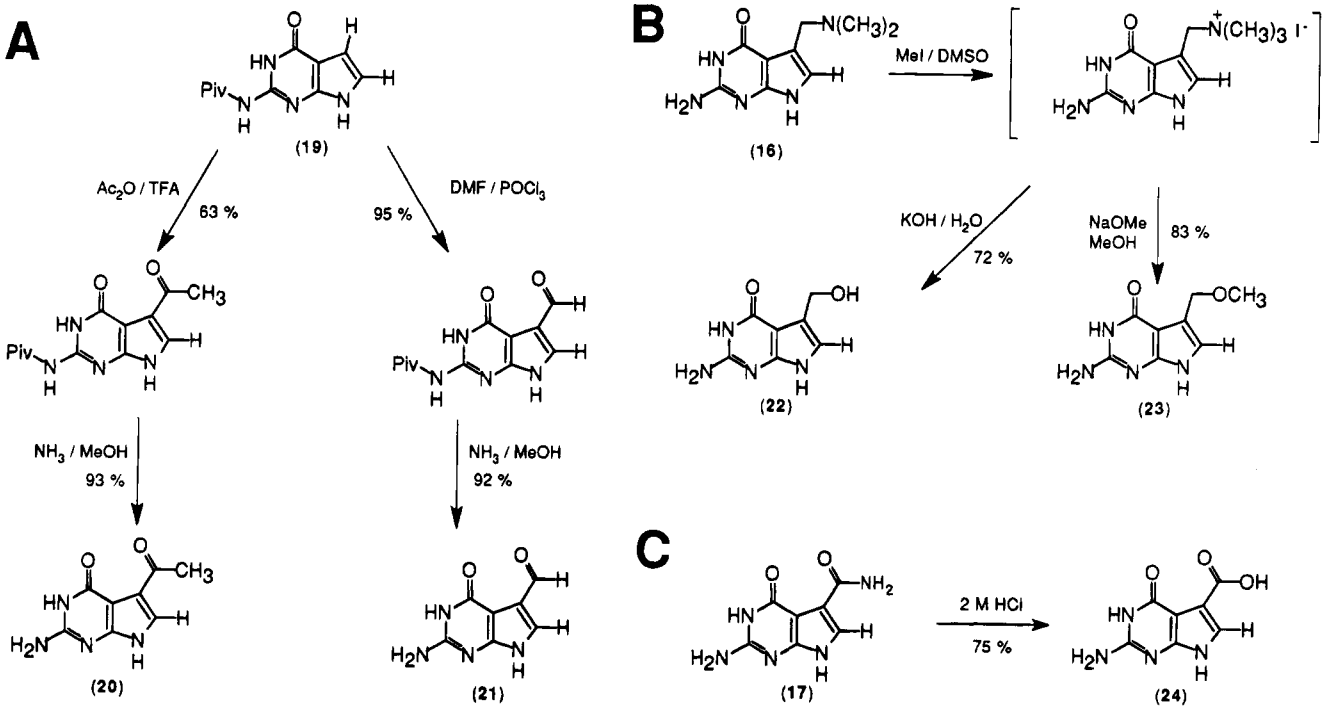


FIGURE 3: Synthetic schemes. (A) Scheme for 5-acetyl and 5-formyl analogues. (B) Scheme for 5-(hydroxymethyl) and 5-(methoxymethyl) analogues. (C) Scheme for the 5-carboxy analogue.

5-Acetyl-2-amino-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**20**) was produced by treating 2-[*N*-(trimethylacetyl)-amino]-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**19**) (Taylor *et al.*, 1992) with acetic anhydride in trifluoroacetic acid followed by ammonolysis of the trimethylacetyl protecting group (Figure 3A). A Vilsmeyer–Haack reaction of **19** (Taylor *et al.*, 1992) followed by removal of the trimethylacetyl protecting group with NH_3/MeOH gave 2-amino-5-formyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**21**, Figure 3A). 2-Amino-5-(hydroxymethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**22**) and 2-amino-5-(methoxymethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (**23**) were produced by displacement of trimethylamine from the methyliodide salt of (**15**) with hydroxide and methoxide, respectively (Figure 3B). 2-Amino-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one-5-carboxylic acid (**24**) was produced by the acid hydrolysis of **17** (Figure 3C) (Ramasamy *et al.*, 1989).

The detailed synthetic procedures can be found in the Supporting Information.

tRNA Preparation. Fully unmodified *E. coli* $tRNA^{Tyr}$ (ECY2) was prepared as previously described (Curnow & Garcia, 1994; Curnow *et al.*, 1993). A 10 mL transcription reaction consistently yielded 2.5 mg of tRNA after purification. This tRNA was stored at −20 °C as an ethanol precipitate. Tyrosine acceptance assays (data not shown) indicate that ca. 67% of the tRNA produced has the correct CCA 3′ terminus.

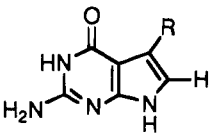
Production of 8-[^{14}C]Guanine-34 tRNA (tRNA).* tRNA labeled with 8-[^{14}C]guanine in the anticodon was produced by incubating a mixture of 10 μM tRNA, 10 μM 8-[^{14}C]-guanine, and 500 nM TGT in HEPES assay buffer [100 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 5 mM DTT] at 37 °C for 5 h. The reaction mixture was then extracted with equal volumes of phenol and chloroform/isoamyl alcohol (24:1). The tRNA* was then precipitated by the addition of 0.1 vol of 3 M sodium acetate and 2 vol of ethanol and incubation at −20 °C overnight. The tRNA* was pelleted by centrifugation (10 000 rpm in a Beckman JA-18 rotor at 4 °C for 30 min), and the ethanol was decanted away. The tRNA* pellet was air-dried and then stored at −20 °C. The tRNA* pellet was resuspended to a concentration of 100 μM in 10 mM HEPES (pH 7.5) and 1 mM $MgCl_2$. The tRNA* was repeatedly centrifuged in a Centricon-10 concentrator (Beckman JA-10 rotor, 4000 rpm, 30 min, 4 °C), readjusting the concentration to 100 μM until the specific activity remained constant (as determined by scintillation counting).

Initial Screens of preQ$_1$ Analogues. The assay mixtures contained the following: 70 mM Tris (pH 7.5), 20 mM $MgCl_2$, 10 μM 8-[^{14}C]guanine, 100 μM unfractionated yeast tRNA, 375 nM TGT, and 100 μM preQ$_1$ (or analogue). The incubations were performed at 37 °C with aliquots taken at various time points up to 60 min. The aliquots were quenched in 2 mL of 5% trichloroacetic acid, and the precipitated tRNA was collected on a glass fiber (GF/C) filter, air-dried, and counted by liquid scintillation counting. Due to low solubility in water, the solutions of preQ$_1$ and analogues contained DMSO. Control experiments found that concentrations of DMSO up to 1% (the maximum that would be found in any assay) in the incubation mixture had no effect upon TGT activity.

TGT-Catalyzed Washout of 8-[^{14}C]Guanine from tRNA.* TGT-catalyzed washout of 8-[^{14}C]guanine from tRNA* was performed as follows. A 400 μL mixture of 10 μM tRNA* (see above), 50 nM TGT, and preQ$_1$ or analogue thereof at various concentrations in HEPES assay buffer was incubated at 37 °C. Aliquots (5−70 μL) were taken at various time points to 15 min (longer time courses were employed for certain experiments; see Results). The aliquots were quenched, filtered, and counted as described above. These time courses

[2] M. T. Migawa, G. C. Hoops, J. M. Hinkley, and L. B. Townsend, unpublished.

Table 1: Kinetic Parameters for Substrate Analogues[a]



R	compd no.	K_i (apparent)[b] (μM)	K_m (μM)	V_{max} (μM s^{-1} mg^{-1})	V_{max}/K_m (s^{-1} mg^{-1})
$-CH_2NH_2$[c]	**2**	0.2(0.1)	0.39(0.05)	2.6(0.1)	6.6(0.5)
$-CH_2OH$[c]	**22**	52(8)	23.0(1.4)	2.6(0.1)	0.114(0.003)
$-H$	**9**	210(40)	172(10)	2.6(0.1)	0.015(0.001)
$-CH_3$	**10**	506(100)	255(30)	2.6(0.4)	0.007(0.001)
$-CH_2OCH_3$	**23**	nd[d]	57(3)	2.7(0.2)	0.047
$-CH_2N(CH_3)_2$	**16**	nd	75(7)	2.5(0.2)	0.034
guanine (G)	**3**	4.9(0.6)	2.2(0.2)	3.55(0.05)	1.4(0.1)
7-methylG	**6**	2.1(0.3)	nda[e]	nda	nda
$-CONH_2$	**17**	88(16)	26(3)	4.0(0.2)	0.16(0.02)
$-CN$[c]	**18**	0.8(0.2)	2.35(0.1)	4.16(0.1)	1.77(0.03)
$-COCH_3$	**15**	nd	87(4)	4.2(0.2)	0.049
$-COCH_3$	**20**	nd	26(2)	4.2(0.1)	0.161
$-CHO$	**21**	nd	22(4)	4.2(0.2)	0.188
$-CO_2H$	**24**	nd	126(20)	1.2(0.1)	0.009
$-CH_3$ (6-CH_3)	**13**	ndi[f]	nd	nd	nd
$-H$ (6-CH_3)	**14**	ndi	nd	nd	nd

[a] The values reported in parentheses are the standard errors of nonlinear fits of the single sets of data. [b] These analogues were tested for their ability to inhibit the incorporation of radiolabeled guanine into unfractionated yeast tRNA as described in Materials and Methods. This assay does not distinguish between competitive inhibitors and competitive substrates. [c] The K_m values for these analogues have been determined in triplicate, and the errors reported are the standard deviation of the means. [d] nd, not determined. [e] nda, no detectable activity. [f] ndi, no detectable inhibition for the 6-methyl analogues at concentrations up to 100 μM.

were linear, and the final time point typically corresponded to less than 10% of the total possible reaction. Linear regression of these time courses gave initial velocities which were fit by nonlinear regression to the Michaelis–Menten equation. The determinations for preQ$_1$ (**2**), preQ$_0$ (**18**), and the 5-hydroxymethyl analogue (**22**) were performed in triplicate. The values for the remaining analogues were calculated from single sets of initial velocity data.

Inhibition of TGT by 7-Methyl-Substituted Analogues. The 7-methyl-substituted analogues of preQ$_1$ (**25**), preQ$_0$ (**26**), and the 5-hydroxymethyl analogue (**27**) were synthesized, along with numerous other 7- and 3-methyl analogues, to determine the ionization coinstants of the N7 proton in this ring system [Hoops *et al.* (1994) and a manuscript in preparation].[3] (Note: the structures of these analogues are represented in Table 3.) The K_i of **26** was determined by following the TGT-catalyzed incorporation of 8-[^{14}C]guanine into tRNA in the presence of **26** while varying the concentrations of both guanine and **26**. A 400 μL mixture of 20 μM tRNA, 50 nM TGT, and both 8-[^{14}C]guanine and **26** at various concentrations in HEPES assay buffer was incubated at 37 °C with 75 μL aliquots taken at 2 min intervals up to 10 min. The aliquots were quenched, filtered, and counted as described above. A double-reciprocal plot of the data (Figure 5) indicated a competitive mode of inhibition. The K_i was therefore determined by a computer fit of the data to eq 1. The K_is for **25** and **27** were determined as for **26** but

$$v_i = \frac{V_{max} S}{K_m(1 + I/K_i) + S} \quad (1)$$

with only one concentration of 8-[^{14}C]guanine (5 μM), assuming competitive inhibition.

[3] G. C. Hoops, L. B. Townsend, and G. A. Garcia, unpublished.

RESULTS

Initial Screening of Analogues as Inhibitors of Guanine Exchange. A number of analogues were initially evaluated for their ability to inhibit the TGT-catalyzed incorporation of radiolabeled guanine into unfractionated yeast tRNA. Double-reciprocal plots ($1/v_i$ vs $1/[S]$) indicate that the analogues are competitive with respect to guanine (data not shown). The K_is determined for these analogues are listed in Table 1. As controls, the K_is for guanine and for 7-methylguanine were determined in the same manner. The K_is for the analogues varied from 0.2 to 500 μM. Interestingly, all of the 5-substituted pyrrolopyrimidines tested do interact with the enzyme; however, the two 6-methylpyrrolopyrimidine analogues are inactive at concentrations up to 100 μM.

Kinetic Parameters of Substrate Analogues. The kinetic parameters for all of the substrate analogues acting as substrates for TGT were determined using a guanine washout assay. The incorporation of the analogues was followed by monitoring the decrease in ^{14}C radioactivity in the tRNA* due to the exchange of the radiolabeled guanine (at position 34) out of the tRNA*. The results of these assays are also presented in Table 1. The kinetic parameters for preQ$_1$ (**2**), preQ$_0$ (**18**), and the 5-(hydroxymethyl) analogue (**22**) were performed in triplicate to validate the assay. For example, the hyperbolic plots of the determinations for preQ$_1$ are shown in Figure 4. The kinetic parameters for the remainder of the analogues were determined from single sets of initial velocity data; however, additional data points were measured as needed to ensure that the hyperbolic curve was well defined in all cases.

A control experiment was performed in which the washout of 8-[^{14}C]guanine from tRNA* was examined in the absence of any preQ$_1$ analogue. It was found that background

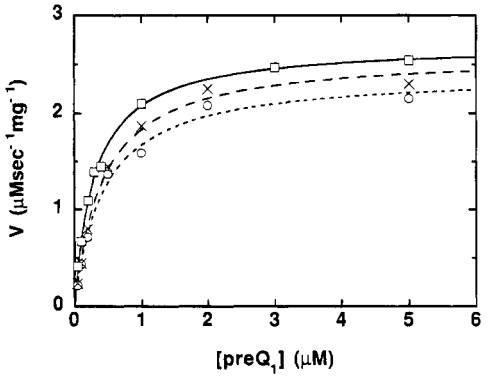


FIGURE 4: Michaelis–Menton plot for $preQ_1$ incorporation into tRNA catalyzed by TGT. $PreQ_1$ incorporation was monitored by following the loss of radiolabel from 8-[^{14}C]G34-tRNA as described in Materials and Methods. The symbols represent each of three independent determinations.

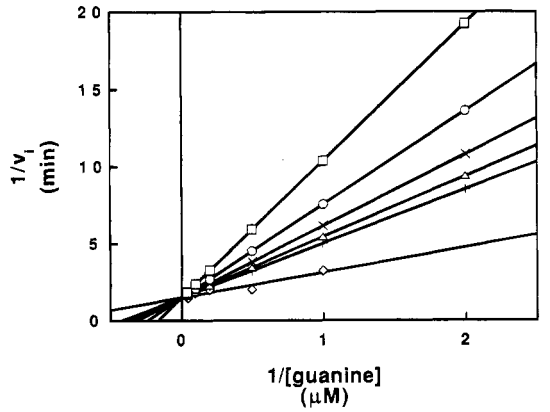


FIGURE 5: Double-reciprocal plot of 7-methyl-$preQ_0$ inhibition of guanine exchange catalyzed by TGT. The compound was assayed for the ability to inhibit the incorporation of 8-[^{14}C]guanine into tRNA as described in Materials and Methods. The symbols correspond to the following concentrations of inhibitor: ◇, no inhibitor; +, 1 μM; ▲, 5 μM; ×, 10 μM; ○, 20 μM; and □, 50 μM.

washout, presumably due to hydrolysis, of 8-[^{14}C]guanine by TGT alone does occur at a significant rate relative to control (no TGT present). Longer time courses were required (to 60 min) in order to accurately measure this washout rate. This rate is ca. 10^{-5} s^{-1} and is 3 orders of magnitude less than the rate of the guanine exchange reaction of ca. 0.01 s^{-1} (calculated assuming that all of the enzyme is active with one active site per monomer). Therefore, no significant amount of background guanine washout occurs during the time course (typically 15 min) of the experiments performed with the substrate analogues. The velocity of the TGT reaction is linear with respect to enzyme concentration over a 50-fold range of TGT concentration (data not shown).

Inhibition Constants of 7-Methyl-Substituted Analogues. The 7-methyl-substituted analogues of $preQ_1$ (**25**), $preQ_0$ (**26**), and the 5-(hydroxymethyl) analogue (**27**) were evaluated as competitive inhibitors of guanine exchange by TGT. A full inhibition profile for the 7-methyl $preQ_0$ analogue (**26**) was performed to establish that the inhibition was competitive with respect to guanine (Figure 5). Assuming competitive inhibition, the K_is for **25** and **27** were determined, in duplicate, by fitting a set of initial velocities for varying concentrations of inhibitor and a fixed concentration of guanine (2 μM, equal to K_m) to eq 1. These values are reported in Table 3 along with the K_i for **26**, also determined in the same manner.

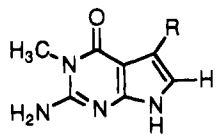
DISCUSSION

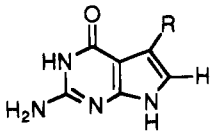
The interactions between synthetic $preQ_1$ analogues and the tRNA-guanine transglycosylase from *E. coli* were initially investigated by evaluating their ability to inhibit TGT-catalyzed incorporation of guanine into unfractionated yeast tRNA (Hoops *et al.*, 1992). Yeast does not express TGT, and yeast tRNA does not contain queuine (Walden *et al.*, 1982); however, yeast tRNA can be utilized as a substrate by the *E. coli* TGT. A series of apparent K_is were determined (Table 1). It is reasonable to assume that these analogues should act as competitive substrates and not true inhibitors. Owing to the fact that the K_m for a competitive substrate is mathematically equivalent to the K_i for a competitive inhibitor when assayed in this manner (Cornish-Bowden & Wharton, 1988), we have utilized an 8-[^{14}C]-guanine washout assay (Okada & Nishimura, 1979) to determine substrate activity for these analogues. We have observed that TGT will catalyze the washout of 8-[^{14}C]-guanine from 8-[^{14}C]G34-tRNA in the absence of any heterocyclic substrate. However, the rate of this hydrolysis reaction (ca. 10^{-5} s^{-1}) is insignificant during the time course of these assays. The mechanistic implications of this washout reaction are under investigation.

The K_ms determined in the guanine washout assays are in good agreement with the apparent K_is determined in the guanine exchange inhibition assay (Table 1). This agreement is all the more striking due to the fact that the guanine exchange inhibition assays (K_is) were performed using unfractionated yeast tRNA, in which the TGT–substrate tRNAs were most likely not present at saturating concentrations, whereas the washout experiments were performed using homogeneous, *in vitro*-generated substrate tRNA, which was present in a saturating concentration. Interestingly, all of the 5-substituted analogues interact with TGT having K_ms varying over 3 orders of magnitude, with $preQ_1$ being the most potent analogue. Analogues that were substituted with a methyl group at the 6-position show no detectable inhibition of guanine exchange at concentrations up to 100 μM and were not evaluated in the guanine washout assay. As a control, incubation of 7-methylguanine at 10× its K_i with [^{14}C]G34-tRNA in the presence of TGT did not result in any significant washout of guanine. This result confirms a previous report in the literature (Okada & Nishimura, 1979) that 7-methylguanine is a true competitive inhibitor of TGT rather than an alternate substrate.

The analogues in Table 1 fall into two categories based upon the V_{max} exhibited. Those analogues having an electron-donating substituent at the 5-position exhibited a lower V_{max} (ca. 2.7 μM s^{-1} mg^{-1}) than analogues with an electron-withdrawing substituent (V_{max} ca. 4.0 μM s^{-1} mg^{-1}). This suggests that deprotonation of N7 (or the equilibrium between protonated and deprotonated forms) may be partially rate limiting in the TGT reaction. Within the electron-donating group of analogues, the K_ms vary by 3 orders of magnitude. Given the consistency of V_{max} for these analogues, we interpret the changes in K_m as reflecting changes in binding of the analogues. This suggests that the primary, if not exclusive role of the 5-substituent for these analogues, including the physiological substrate $preQ_1$, is in binding/recognition and not in catalysis.

Table 2: Correlation Between the N7 pK_a for 3-Methyl-Substituted Analogues and the V_{max} for the Corresponding Substrates[a]





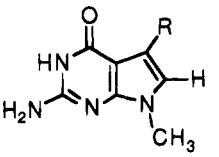
R	N7 pK_a	V_{max} (μM s^{-1} mg^{-1})
$-CH_2NH_2$	15(1)	2.6(0.1)
$-CH_2OH$	>13	2.6(0.1)
$-H$	14.5(0.8)	2.6(0.1)
$-CH_3$	14.6(0.9)	2.6(0.4)
$-CH_2OCH_3$	>13	2.7(0.2)
$-CH_2N(CH_3)_2$	>13	2.5(0.2)
$-CONH_2$	12.1(0.2)	4.0(0.2)
$-CN$	12.2(0.4)	4.16(0.1)
$-CO_2CH_3$	11.5(0.6)	4.2(0.2)
guanine	10.5[b]	3.55(0.05)

[a] The pK_a values listed are for the 3-methyl analogues (from Hoops *et al.*, unpublished.[3] The V_{max} values are for the corresponding, unmethylated analogues (from Table 1). [b] The pK_a value for guanine is for the N9 proton on 1-methylguanine (Pfleiderer, 1961).

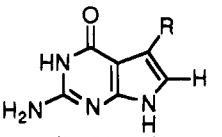
A manuscript in preparation[3] describes investigations to determine the ionization constant of the N7 proton for this 2-amino-7*H*-pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one ring system. In this study, a number of N3- and N7-methylated analogues were synthesized and their pK_as were determined. The N3-methyl analogues show no detectable activity, either as substrates or inhibitors, with TGT (data not shown). Therefore, the ionization constants (pK_as) for a series of N3-methyl analogues are correlated with the V_{max} for the corresponding substrate analogues in Table 2. The pK_as fall into two categories consistent with expectations based upon the electron-donating/withdrawing potential of the 5-substituent. These pK_as correlate with the V_{max} for the corresponding substrate analogue, with those having pK_as greater than 13 having the lower V_{max}. This observation is consistent with our conclusion that deprotonation of N7 is partially rate-determining. However, the difference in V_{max} observed is not large enough to account for the change in pK_a for the analogues if the deprotonation step were entirely rate-determining. We conclude that the transition state for the deprotonation must be only slightly higher than the transition state for some other step in the reaction (*e.g.*, loss of G34 to form the abasic tRNA intermediate, product release, etc.). This would account for the small but significant increase in V_{max} to the point where this other step is now rate-determining.

A number of 7-methyl analogues synthesized in the course of the pK_a investigations inhibit the TGT reaction, three of which exhibited greater than 50% inhibition at 100 μM (data not shown). The K_is for these three analogues (**25**−**27**, Table 3) were determined. (The intersecting lines in the inhibition profile (Figure 5) for the 7-methyl-preQ$_0$ analogue (**26**) are indicative of competitive inhibition with respect to guanine.) The K_is for the 7-methyl analogues of preQ$_1$ (**25**) and the 5-(hydroxymethyl) analogue (**27**) match the K_ms for their corresponding substrates (**2** and **22**), consistent with our earlier conclusion that these K_ms reflect binding of the substrates and are equal to the K_ds for these compounds. The K_i for **26** is significantly different from the K_m for preQ$_0$ (**18**), suggesting that the K_m for this substrate analogue is

Table 3: Correlation Between the K_i for N7-Methyl-Substituted Inhibitors and the K_m for Corresponding Substrates[a]



(25, 27, 26)



(2, 22, 18)

R	compd no.	K_i (μM)	compd no.	K_m (μM)
$-CH_2NH_2$	**25**	0.51(0.02)	**2**	0.39(0.05)
$-CH_2OH$	**27**	30(2)	**22**	23.0(1.5)
$-CN$	**26**	11(2)	**18**	2.35(0.1)

[a] The K_m values are from Table 1. For reference, the K_i for 7-methylguanine in the yeast tRNA assay (Table 1) is 2.1(0.3) μM.

reflecting not only binding, but also contains catalytic rate components.

Archaeosine is a 5-carboximidamide(or amidine)-substituted analogue of preQ$_1$ that has been found in position 15 in archaeal tRNA (Gregson *et al.*, 1993). Given the relatively broad specificity of the *E. coli* TGT regarding the 5-substituent, it is possible that an ancestral *tgt* gene has diverged to incorporate archaeosine into archaeal tRNA and preQ$_1$ into *E. coli* tRNA. It remains to be determined whether the *E. coli* TGT will recognize the archaeosine free base as a substrate for exchange into tRNA.

This study reveals that the tRNA-guanine transglycosylase from *E. coli* will tolerate a wide variety of substituents at the 5-position, including a cyano substituent corresponding to preQ$_0$ (**18**), a molecule found as both the free base and as a base incorporated into tRNA *in vivo* (Noguchi *et al.*, 1978). The role of the 5-substituent appears to be entirely in binding/recognition with no apparent effects upon catalysis. The correlation between N7 pK_a and V_{max} suggests that deprotonation of N7 during the reaction, which must occur prior to subsequent glycosidic bond formation, is partially rate-determining for the natural substrate. Comparison of the K_is of 7-methyl-substituted competitive inhibitors to the K_ms of their corresponding substrates suggests that some substrates (including preQ$_1$) are kinetically "sticky" (*i.e.*, K_m is equivalent to K_d) and other substrates have K_ms that reflect catalytic rates as well as binding.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Supporting information describing the details of the syntheses of the compounds reported is available (5 pages). Ordering information is given on any current masthead page.

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