



## Biosynthesis of pyrrolopyrimidines

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### ABSTRACT

Pyrrolopyrimidine containing compounds, also known as 7-deazapurines, are a collection of purine-based metabolites that have been isolated from a variety of biological sources and have diverse functions which range from secondary metabolism to RNA modification. To date, nearly 35 compounds with the common 7-deazapurine core structure have been described. This article will illustrate the structural diversity of these compounds and review the current state of knowledge on the biosynthetic pathways that give rise to them.

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### 1. Introduction

Pyrrolopyrimidine-containing compounds, also known as 7-deazapurines, have been a source of research interest since the discovery of toyocamycin in 1956 [1]. Their broad distribution in biological samples, their potential uses as antibiotic, antifungal, antiviral, and antineoplastic agents, and the cryptic biosynthetic pathways that give rise to them, have fueled significant interest in 7-deazapurines in the intervening five decades since they were first described. This review will catalog known, naturally occurring pyrrolopyrimidines and highlight enzymatic transformations leading to the 7-deazapurine core and the unique tailoring steps required for production of several naturally occurring pyrrolopyrimidines.

### 2. Biological distribution of 7-deazapurine metabolites

7-Deazapurines are widely distributed in nature and occur in sources as disparate as bacteria and man and function as secondary metabolites or modified bases in RNA. This section will introduce the sources of naturally occurring 7-deazapurine-containing secondary metabolites.

#### 2.1. 7-Deazapurines as secondary metabolites from terrestrial sources

Soil bacteria produce an array of structurally diverse compounds; though not essential for the primary metabolic processes, these so-called 'secondary metabolites' confer selective advantage to the pro-

ducing host. The pioneering studies by Selman Waksman [2] that eventually led to discovery of streptomycin – the first treatment for tuberculosis – underscored a need to examine the biosynthetic potential of soil bacteria and inspired the discovery of a slew of other antibiotics, including compounds that have 7-deazapurine core structures (see Fig. 1 for structures and Table 1 for summary).

Studies on the identification, therapeutic efficacy, and biosynthesis of 7-deazapurines began with the isolation of an anti *Candida* agent, toyocamycin (1), from culture filtrates of *Streptomyces* No. 278. The organism was renamed *Streptomyces toyocaensis* [1] for the compound that it produces and the structure of toyocamycin was ultimately confirmed by total synthesis in 1968 [3], revealing for the first time a 7-deazapurine core structure in a natural product. Soon thereafter, tubercidin (2) was isolated from culture filtrates of *Streptomyces tubercidicus* obtained from a soil sample from the Chiba Prefecture in Japan [4]. The third 7-deazapurine, sangivamycin (3), was isolated from culture filtrates of *Streptomyces rimosus* [5]. Interestingly, *S. rimosus* (ATCC 14673) also produces toyocamycin [6], suggesting that both compounds are made by a common pathway. Indeed, this was confirmed when the gene cluster involved in the biosynthesis of deazapurines by *S. rimosus* was uncovered [7]. Each of these three compounds contains a 7-deazaadenosine core structure with the only structural diversification occurring at C-7 of the base. Toyocamycin and sangivamycin bear a cyano or amido substituent at C-7, respectively, while in tubercidin the C-7 substituent is hydrogen.

Toyocamycin is a powerful anti-tumor compound both *in vitro* and *in vivo*; however, it also shows high levels of host toxicity [8]. Tubercidin exhibits potent antibiotic activity against *Candida albicans* and *Mycobacterium tuberculosis* but does not inhibit the growth of Gram-positive bacteria, and fungi. Tubercidin shows cytotoxic activity towards NF-mouse sarcoma cells in culture [8], cultured mouse fibroblasts [9], and human tumor specimens [10]. Antiviral activity toward Vaccinia, Reovirus III, and Mengiavirus [9], which

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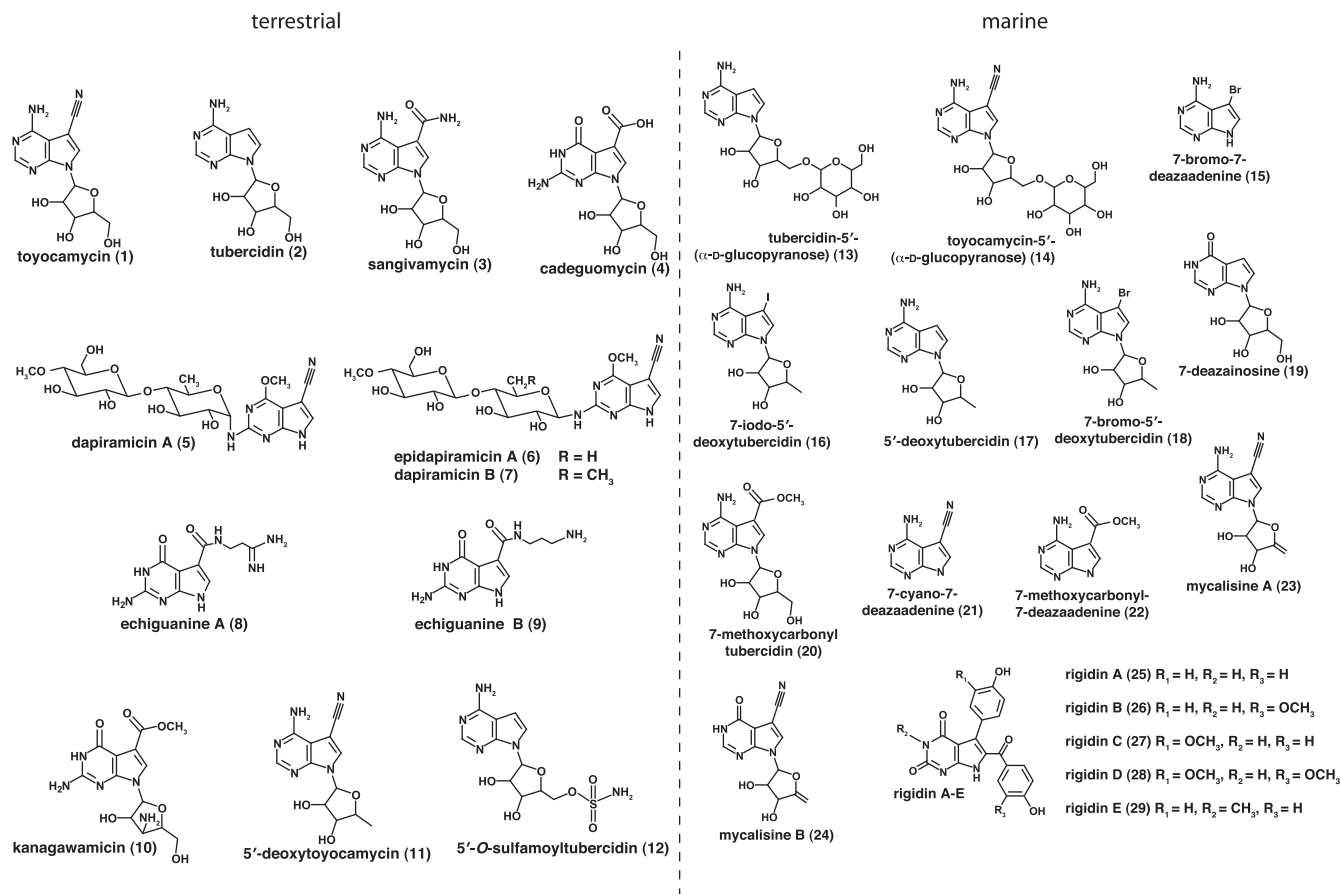


Fig. 1. Deazapurine-containing secondary metabolites isolated from terrestrial (1–12) and marine (13–29) sources.

contain genomes composed of DNA, double stranded RNA, and single stranded RNA, respectively, have also been noted. Sangivamycin is highly cytotoxic to HeLa cells in culture and leukemia L1210 in mice [5].

Toyocamycin, tubercidin, and sangivamycin have served as prototypes for 7-deazapurines; the few instances in which their molecular modes of action have been interrogated are described here. As adenosine analogs, toyocamycin, tubercidin, and sangivamycin would be expected to participate in and possibly interfere with cellular processes involving adenine nucleosides and nucleotides. Indeed, studies with partially purified mammalian adenosine kinases show that toyocamycin and tubercidin are substrates for this enzyme [11]. Furthermore, toyocamycin can be phosphorylated to the mono-, di- and tri-phosphorylated forms in cultured Ehrlich ascites tumor cells and become incorporated into RNA and DNA, indicating that toyocamycin triphosphate is a substrate for mammalian RNA and DNA polymerases and implying that ribonucleotide reductase acts upon toyocamycin nucleotide to produce the corresponding deoxyribonucleotide [12]. Tubercidin, toyocamycin, and sangivamycin triphosphates are not substrates for amino acid aminoacylation, but toyocamycin and sangivamycin are competitive inhibitors of this process [13]. While all three of these deazapurines can be added to the 3'-termini of tRNA by the action of tRNA nucleotidyltransferase, the presence of toyocamycin and sangivamycin in the acceptor stem inhibits the aminoacylation of tRNA [13]. Toyocamycin is incorporated into 45S rRNA [14], which in mammalian systems is cleaved to give ribosomal subunits 5.8S, 18S, and 28S [15]. However, 45S rRNA containing toyocamycin is not further processed to 18S and 45S rRNA. It has been speculated that the presence of toyocamycin in 45S rRNA alters its tertiary structure rendering it unrecognizable to the nucleases that are

required for downstream processing. More recently, sangivamycin was demonstrated to be a powerful ATP-competitive inhibitor of protein kinase C ( $K_i = 11 \mu\text{M}$ ) [16,17].

In summary, toyocamycin, tubercidin, and sangivamycin seem to exert their cytotoxic effects by interfering with cellular metabolism on a variety of levels rather than acting on a specific cellular target. Self-resistance mechanisms have yet to be identified in 7-deazapurine producing organisms; however, since these compounds are isolated from culture filtrates, it is likely that export of the molecules from the cells protects the producing strains.

In the years since the discovery of toyocamycin, a range of structurally diverse deazapurine-based compounds have been isolated from terrestrial or marine sources, often by taking advantage of toxicity of these compounds to various strains of bacteria, fungi, plants, or mammalian cell lines. Table 1 is a comprehensive list of deazapurine secondary metabolites that have been isolated to date. We note that some of these compounds were isolated from marine specimens, such as tunicates; however, it is not clear whether the compounds are produced by the eukaryotic host, or other organisms engaged in symbiotic relationships with them.

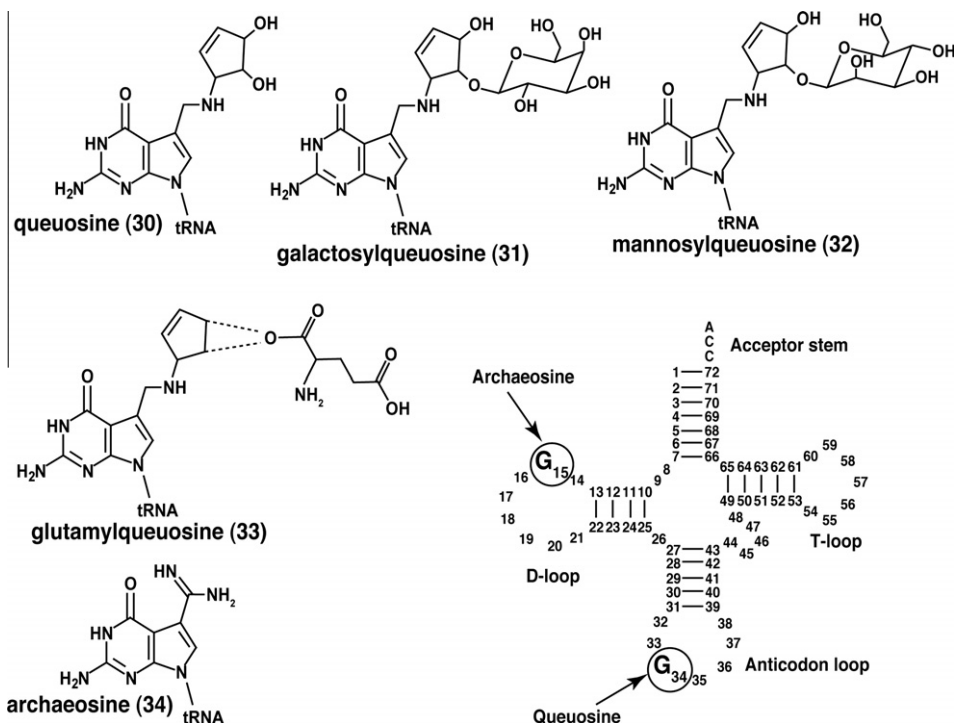
## 2.2. 7-Deazapurines as hypermodified bases in tRNA

In addition to secondary metabolites, 7-deazapurines are also found as modified bases in tRNA (see Fig. 2 for structure of queuosine and related modified RNA bases). RNA is one of the most heavily post-transcriptionally modified biological molecules. As of 2011, 109 modifications have been identified of which 93, 31, and 13 are modified bases located in tRNA, rRNA, mRNA, respectively [18]. One of the most highly elaborated RNA bases, queuosine (30), is located in tRNA and contains a 7-deazaguanine core. Pioneering

**Table 1**

Deazapurine secondary metabolites from terrestrial (1–12) and marine (13–29) sources.

Compound	Source	Refs.
Toyocamycin (1)	<i>Streptomyces rimosus</i>	[1]
Tubercidin	<i>Streptomyces tubercidicus</i>	[4]
Sangivamycin	<i>Streptomyces rimosus</i>	[5]
Cadeguomycin (4)	<i>Streptomyces hygroscopicus</i>	[121–123]
Dapiramicin A (5)	<i>Micromonospora</i> sp. SF-1917	[124,125]
Epidapiramicin A (6)	Epimerization of Dapiramicin A	[126]
Dapiramicin B (7)	<i>Micromonospora</i> sp. SF-1917	[126]
Echiguanine A (8)	<i>Streptomyces</i> sp. MI698-50F1	[127]
Echiguanine B (9)	<i>Streptomyces</i> sp. MI698-50F1	[127]
Kanagawamycin (10)	<i>Actinoplanes kanagawaensis</i>	[128]
5'-Deoxytoyocamycin (11)	<i>Streptomyces</i> sp. A14345	[129]
5'-O-sulfamoyltubercidin (12)	<i>Streptomyces mirabilis</i>	[130]
Tubercidin-5'- $\alpha$ -D-glucopyranose (13)	<i>Tolypotrix tenuis</i>	[131]
Toyocamycin-5'- $\alpha$ -D-glucopyranose (14)	<i>Tolypotrix tenuis</i>	[131]
7-Bromo-7-deazaadenine (15)	<i>Echinodictyum</i>	[132]
7-Iodo-5'-deoxytubercidin (16)	<i>Hypnea valendiae</i>	[132]
5'-Deoxytubercidin (17)	<i>Didemnum voeltzkowi</i>	[133]
7-Bromo-5'-deoxytubercidin (18)	<i>Didemnum voeltzkowi</i>	[133]
7-Dezainosine (19)	<i>Aplidium pantherinum</i>	[135]
7-(Methoxycarbonyl)tubercidin (20)	<i>Jaspis johnstoni</i>	[134]
7-Cyano-7-deazaadenine (21)	<i>Jaspis johnstoni</i>	[134]
7-Methoxycarbonyl-7-deazaadenine (22)	<i>Jaspis johnstoni</i>	[134]
Mycalisine A (23)	<i>Mycale</i> (marine sponge)	[136]
Mycalisine B (24)	<i>Mycale</i> (marine sponge)	[136]
Rigidins A-E (25–29)	<i>Eudistoma rigida</i>	[137–139]



**Fig. 2.** Deazapurines in tRNA. Queuosine and archaeosine analogs are found in the anticodon loop or the D-loop of tRNA, respectively. Archaeosine is only found in archaea. The site of glutamate esterification of glutamylqueuosine remains to be established.

work by RajBhandary and coworkers revealed the presence of a non-canonical nucleotide in tyrosyl tRNA, which was designated Q [19]. The corresponding nucleoside of Q is referred to as queuosine, while the free base is called queuine. Queuosine was subsequently found to reside in the wobble position of asparaginyl, tyrosyl, histidyl and aspartyl tRNA, which contain the genetically encoded 5'-GUN-3' in the anticodon loop (where 'N' is any canonical nucleoside) [20]. Queuosine-containing tRNAs bind the codons NA<sub>U</sub><sup>C</sup>, coding for asparagine, tyrosine, histidine, and aspartate, respectively. Soon after its initial discovery, queuosine was isolated on a preparative

scale and its unique cyclopentenediol-appended 7-deazapurine structure was determined [21]. Parallels between the 7-deazaguanine core structure of queuosine and 7-deazapurine containing nucleoside antibiotics were immediately noted. Queuosine, appended with galactose or mannose bound by an O-glycosidic linkage to the C2 position of the cyclopentenediol substituent, are also found in animal Tyr-tRNA and Asp-tRNA, respectively [22,23].

Queuosine is distributed broadly throughout biology in both prokaryotes and eukaryotes [23,24], but it is absent in archaea and, notably, *Saccharomyces cerevisiae* [25]. Despite its conservation

throughout biology, the precise physiological role of this modified base has eluded investigators for decades, but a few key findings are worthy of note. In an experiment where wildtype *Escherichia coli* were cultured together with a strain that lacks a key gene for the incorporation of the deazapurine into RNA, the deletion strain comprises <1% of the total cell population at deep stationary phase [26], suggesting that the RNA modification may confer growth advantage. In another intriguing report, Tyr-tRNA containing queuosine was demonstrated to suppress read-through of the viral UAG stop codon in the tobacco mosaic virus [27], suggesting a role for the modification in viral infectivity. In the context of viral infections, the genome of phage Dp-1, which is a *Streptococcus pneumoniae* virulent phage, encodes nearly all of the genes for the biosynthesis of the modified base; this is surprising considering the propensity for compact viral genomes, suggesting a possible role for queuosine in virulence [28]. From its prominent location in the wobble position of tRNA, one may suppose that queuosine could modify the base-pairing characteristics of the tRNA in which it is found. Indeed, His-tRNA containing guanine in the wobble position displays a preference for the codon CAC over CAU while His-tRNA containing queuosine does not [29], suggesting that queuosine could enhance translational efficiency.

Queuosine has been implicated as a requirement in the biosynthesis of tyrosine in mammals [30]. A recent study demonstrated that liver extracts from mice that lack the enzymes for incorporating Q into RNA contained decreased levels of tetrahydrobiopterin, the cofactor required for the conversion of phenylalanine to tyrosine by phenylalanine hydroxylase [31].

Cultured cells derived from various tumors have consistently been observed to contain decreased queuosine levels. Hypomodification by queuosine is often correlated with a higher incidence of tumor metastasis and a lower patient survival rate. The precise meaning of this phenomenon is not well understood [32,33].

Archaeosine (**34**) is a non-canonical 7-deazapurine nucleoside identified during sequencing of the Met-tRNA from *Thermoplasma acidophilum* [34] and is widely distributed in archaeal species [35]. It is located at position 15 of the dihydrouridine loop (the D-loop) in select tRNA molecules. As with queuosine, genes for tRNA species that contain archaeosine encode guanosine for the position in which archaeosine is found. The precise physiological role of archaeosine remains unknown but it is thought to enhance stability of the tertiary structure of tRNA [36].

### 3. Biosynthesis of deazapurines: insights from radiotracer experiments

The fact that purines are precursors to deazapurines was established by following the fate(s) of various trace radioisotope labeled potential precursors in feeding experiments; efficient uptake of the

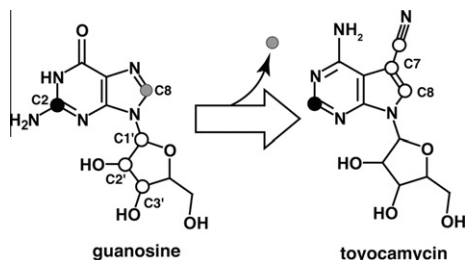
precursor, however, was not confirmed in every case. Nevertheless, pyrimidines were eliminated as plausible precursors to deazapurines by showing that no radioactivity is incorporated into tubercidin that is isolated from fermentation media of *S. tubercidicus* cells grown in the presence of [6-<sup>14</sup>C]-orotic acid. A pre-formed 7-deazapurine was eliminated from consideration by showing that [U-<sup>3</sup>H]-7-deazaadenine is also not a precursor to tubercidin. Finally, no radioactivity was incorporated into tubercidin when the cells were grown in the presence of [1,4-<sup>14</sup>C]-succinate, or [1-<sup>14</sup>C]-propionate, demonstrating that deazapurines are not derived from C<sub>3</sub> or C<sub>4</sub> carboxylic acid pools [37]. By contrast, 7-deazaadenine base and not the ribosyl moiety of tubercidin isolated from cells grown in the presence of [U-<sup>14</sup>C]-adenine contained radioactivity, implying that deazapurines are derived from purines. Intriguingly, cells grown in the presence of [2-<sup>14</sup>C]-adenine and not [8-<sup>14</sup>C]-adenine incorporated the proffered radiolabel into the 7-deazaadenine base of tubercidin. Collectively, these results suggested that the 7-deazapurine core is derived from a purine nucleoside precursor with the loss of purine carbon 8 [38]. Similar radiotracer results were obtained for the biosynthesis of toyocamycin by *S. rimosus* [39].

Additional feeding studies established the origin of pyrrole carbons (7 and 8), as well as the cyano carbon in toyocamycin [38]. *S. rimosus* cells that were grown in the presence of either [1-<sup>14</sup>C]-ribose or [U-<sup>14</sup>C]-ribose incorporated radioactivity into the deazapurine base and the ribose moiety of toyocamycin. Moreover, toyocamycin that was isolated from cultures grown in the presence of either [1-<sup>3</sup>H]-ribose or [3-<sup>3</sup>H]-ribose revealed tritium incorporation into the deazapurine base only when the proffered ribose was tritiated at carbon 1. Radioactivity in toyocamycin isolated from culture filtrates of cells that were grown in the presence of either [U-<sup>14</sup>C]-adenosine or adenosine in which a majority of the total <sup>14</sup>C radiolabel was located in the ribose was localized carbons 1', 2', and 3'. These observations led to the remarkable insight that ribose from a purine nucleoside precursor is rearranged to form carbons 7 and 8, as well as the cyano carbon in toyocamycin, respectively (see Fig. 3).

While the structures of tubercidin, toyocamycin, and sangivamycin resemble adenosine more closely than guanosine, feeding experiments with either [2-<sup>14</sup>C]-guanine or adenine give rise to radiolabeled deazapurine nucleosides, so it remained unclear which of the two was the true deazapurine precursor because interconversion of purines by endogenous salvage pathways could not be ruled out. Similar biosynthetic insights were obtained in studies aimed at understanding the biosynthesis of queuosine. [40].

The radiotracer experiments showing retention of carbon-2 and loss of carbon-8 in the biosynthesis of the 7-deazapurine backbone suggested a rearrangement analogous to the first step in the biosynthesis of folic acid, pterins, and toxoflavin, which are all synthesized from GTP in a process that requires GTP cyclohydrolase I (GCH I) [41–44,52,53]. GCH I catalyzes the conversion of GTP to 7,8-dihydro-neopterin triphosphate (H<sub>2</sub>NTP), in a reaction that leads to loss of carbon-8, retention of carbon-2, and incorporation of the ribose carbons to form a new ring system. Consistent with the radiotracer experiments, dialyzed cell lysates from *S. rimosus*, which produces both sangivamycin and toyocamycin, contained GCH I activity [45,46]. While the lysate affected the loss of radioactivity from [8-<sup>14</sup>C]-GTP as formic acid, similar activity was not observed toward [8-<sup>14</sup>C]-ATP or ITP. Moreover, the activity was not constitutively expressed; it increased dramatically from 30 to 48 h post inoculation, and tracked closely with the production of sangivamycin. These results suggest a common first step in the biosynthetic pathways for folic acid, tetrahydropterin and deazapurines.

In summary, the radiotracer isotope experiments established the outline of the biosynthetic pathway. These results demonstrated that a purine, likely GTP, is the precursor to all deazapurines and that in the course of biosynthesis, C-2 of the purine



**Fig. 3.** Summary of radiotracer experiments for the conversion of guanosine to toyocamycin. In general, biosynthesis of all deazapurine-containing compounds from purines entails loss of carbon 8, retention of carbon 2, incorporation of carbons 1', 2' and 3'. In some cases, the 6-membered ring of the proffered purine undergoes further modifications. Refer to Figs. 4, 5 and 7 for the specific reactions in the biosynthesis of toyocamycin.



base is retained but C-8 is eliminated. Moreover, ribose carbons 1'–3' are utilized for the newly formed pyrrole ring. These are summarized in Fig. 3.

#### 4. Enzymes involved in the biosynthesis of deazapurines

The enzymatic transformations that lead to the core 7-deazapurine structure are common to all of these compounds. In this section, the biosynthetic steps to the 7-deaza core are discussed, followed by subsections on the unique steps that tailor the 7-deazapurine to queuosine, archaeosine, and toyocamycin/sangivamycin.

##### 4.1. Biosynthesis of the 7-deazapurine core

Nature's strategy for assembly of the 7-deazapurine core was elucidated in two independent studies that were focused on identifying the biosynthetic pathways for queuosine and the pyrrolo-pyrimidine nucleosides sangivamycin and toyocamycin, respectively. In the first, comparative genomic approaches led to identification of a subset of the genes required for the production of queuosine in *Acanitobacter calcoaceticus* [47]. Since GTP cyclohydrolase activity had been implicated in queuosine biosynthesis as discussed above, and because genes involved in a single biosynthetic pathway are sometimes co-localized in bacterial genomes, the search for genes in the proximity of GCH I homologs led to discovery of four open reading frames (*ykjv*, *ykjK*, *ykjL*, and *ykjM*) that encoded proteins of unknown function. This search was limited to genomes containing the *tgt* and *queA* genes, which encode two enzymes involved in late steps of the biosynthesis of queuosine (see Section 4.2). The genes, *ykjv*, *ykjK*, *ykjL*, and *ykjM* were subsequently renamed *queC*, *queD*, *queE*, and *queF* for their role in queuosine biosynthesis.

Identification of the genes required for the biosynthesis of toyocamycin and sangivamycin in *S. rimosus* [7] provided substantial clues to the biosynthesis of the 7-deazapurine core found in nucleoside antibiotics, in queuosine, and in archaeosine. Suhadolnik and coworkers had shown the presence of a toyocamycin nitrile hydratase (TNHase) activity in *S. rimosus*, which produces both toyocamycin and sangivamycin [6,48]. TNHase catalyzes the conversion of toyocamycin to sangivamycin. Purification of the nitrile hydratase and N-terminal sequencing of the three subunits of the protein led to the identification of three genes encoding TNHase. The remainder of the genes required for the biosynthesis of toyocamycin and sangivamycin were located adjacent to those encoding TNHase, within a single gene cluster of ~13 kbp. The entire set of genes involved in toyocamycin and sangivamycin biosynthesis was designated *toyA-L* (*toy* designation was employed to denote toyocamycin biosynthesis). As was suspected from the similarities in radiotracer data for biosynthesis of deazapurines (see Section 3), *toyM*, *toyB*, and *toyC* are homologous to the essential genes for the biosynthesis of queuosine, *queC*, *queD*, and *queE*, respectively. *In vitro* reconstitution of the biosynthesis of 7-cyano-7-deazaguanine (preQ<sub>0</sub>), a previously identified intermediate in the biosynthesis of

queuosine [49], was achieved subsequently in four steps from GTP using recombinantly expressed and purified QueC, QueD and QueE homologs.

The steps from GTP to preQ<sub>0</sub> are likely common to almost all 7-deazapurines and will be discussed first. In the sections that follow, the reactions that are unique to the biosynthesis of queuosine, archaeosine and sangivamycin/toyocamycin, will be highlighted. The core reactions involved in the biosynthesis of preQ<sub>0</sub> are summarized in Fig. 4 and discussed below (Sections 4.1.1–4.1.4).

##### 4.1.1. GTP cyclohydrolase I (GCH I)

The role of GCH I in biosynthesis of 7-deazapurines was first demonstrated *in vitro* with its homolog, ToyD, from the toyocamycin biosynthesis pathway of *S. rimosus* [50]. The reaction catalyzed by *S. rimosus* ToyD is identical to that of the *E. coli* GCH I. The X-ray crystal structure of the *E. coli* homolog has revealed the presence of a Zn<sup>2+</sup> metal ion, which activates a water molecule in each of the two half reactions that are involved in hydrolytic removal of carbon-8 [50]. Opening of the ribose ring is followed by a series of Amadori rearrangements and subsequent re-cyclization into the pteridine structure of H<sub>2</sub>NTP [51]. All relevant sequence features that have been shown to be essential for the *E. coli* GCH I are retained in ToyD.

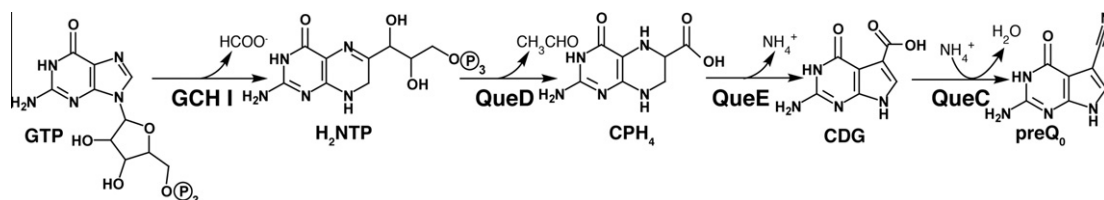
The discovery that GCH I in *S. rimosus* is co-localized with the toyocamycin biosynthetic genes is entirely consistent with the radiotracer experiments, where available, which have shown that in the course of conversion of a purine to a deazapurine C-8 of the base is lost whereas C-2 is retained [38,39]; moreover, it accounts for the observation that carbon atoms that are originally part of the sugar are incorporated into the 7-deazapurine core. GCH I is required for biosynthesis of queuosine in *E. coli* and archaeosine in *Haloferax volcanii* as well [54].

##### 4.1.2. 6-Carboxy-5,6,7,8-tetrahydropterin synthase (QueD)

The second step in the pathway is catalyzed by 6-carboxy-5,6,7,8-tetrahydropterin (CPH<sub>4</sub>) synthase (QueD or ToyB), which converts H<sub>2</sub>NTP produced by GCH I to 6-carboxy-5,6,7,8-tetrahydropterin (CPH<sub>4</sub>). The reaction entails the loss of 2' and 3' carbons of the substrate as acetaldehyde. The enzyme is homologous to the mammalian 6-pyruvoyltetrahydropterin (PPH<sub>4</sub>) synthase (mPTPS), which catalyzes the second step in the biosynthesis of tetrahydrobiopterin in eukaryotes [55], namely, the conversion of H<sub>2</sub>NTP to 6-pyruvoyl-5,6,7,8-tetrahydropterin (PPH<sub>4</sub>). However, the new activity appears to have evolved in the same scaffold. Intriguingly, CPH<sub>4</sub> synthase also catalyzes the conversion of PPH<sub>4</sub> and 6-lactoyl-7,8-dihydropterin to CPH<sub>4</sub> suggesting the possible intermediacy of these compounds in the catalytic cycle, though chemical and kinetic competence has not been shown yet [56].

##### 4.1.3. CDG synthase (QueE)

The next enzyme in the pathway, 7-carboxy-7-deazaguanine (CDG) synthase (QueE/ToyC), catalyzes the conversion of CPH<sub>4</sub> to



**Fig. 4.** Biosynthesis of preQ<sub>0</sub>. Biosynthesis of 7-cyano-7-deazaguanine core of deazapurines from GTP is accomplished by the successive actions of GTP cyclohydrolase I (GCH I/ToyD), CPH<sub>4</sub> synthase (QueD/ToyB), CDG synthase (QueE/ToyC) and preQ<sub>0</sub> synthetase (QueC/ToyM). The loss of three carbon atoms by the successive actions of GCH I and QueD, elimination of a nitrogen by QueE and incorporation of ammonia by QueC have been demonstrated by high resolution mass spectrometry [56,62]. Loss of the nitrogen as ammonia from CPH<sub>4</sub> catalyzed by QueE has not been demonstrated explicitly.

CDG [56]. CDG synthase is a member of the radical *S*-adenosyl-L-methionine (SAM) enzyme superfamily [57,58]. Radical SAM enzymes harbor a [4Fe–4S] cluster in which three of the four iron atoms are ligated to cysteine thiolates that are typically located in a conserved CX<sub>3</sub>CX<sub>2</sub>C motif. The reduced form of the cluster (+1 oxidation state) donates an electron to affect the reductive cleavage of the C5'-sulfur bond in SAM to generate methionine and 5'-deoxyadenosyl radical (5'-dAdo•). The highly reactive 5'-dAdo• abstracts a hydrogen atom from substrate and the resulting substrate radical undergoes rearrangement resulting in a chemical transformation. Members of this enzyme superfamily catalyze an array of diverse reactions, which are involved in a variety of biological processes including cofactor and antibiotic biosynthesis, tRNA modification, anaerobic oxidation, DNA repair, and protein radical formation [59–61]. The mechanism of CDG synthase remains to be established. However, it is hypothesized that the conversion of CPH<sub>4</sub> to CDG is accomplished by H-atom abstraction from either the C-6 or C-7 position of the substrate, and that the resulting activated molecule undergoes a complex radical mediated ring contraction to form CDG [62].

#### 4.1.4. PreQ<sub>0</sub> synthetase (QueC)

CDG is converted to 7-cyano-7-deazaguanine (preQ<sub>0</sub>) by the action of preQ<sub>0</sub> synthetase (QueC or ToyM), which catalyzes conversion of the carboxylic acid moiety of CDG to a nitrile with ammonium as a nitrogen source in an ATP-dependent reaction. The putative mechanism for preQ<sub>0</sub> synthetase is that the two molecules of ATP are utilized to activate the carboxylate oxygen atoms, allowing for successive half reactions involving addition of ammonia and collapse to the alkyl cyanide. The X-ray crystal structure of QueC from *Bacillus subtilis* was reported [63] before its enzymatic activity had been established [56]. In QueC, the conserved residues, SGGXDS, comprise a pyrophosphate-binding loop that is found in many enzymes that carry out hydrolysis of the α-β phosphoanhydride bond in ATP [64]. The preQ<sub>0</sub> synthetase reaction is a rare example of nitrile production in living systems.

The biosynthetic pathways for 7-deazapurine containing tRNA modifications diverge from those for toyocamycin and sangivamycin from this point forward. The remaining tailoring steps during which queuosine, archaeosine, and toyocamycin/sangivamycin are derived from preQ<sub>0</sub> will be discussed next.

### 4.2. Biosynthesis of queuosine

The pathway to incorporation and maturation of queuosine begins with preQ<sub>0</sub>, which is formed by the successive actions of GTP cyclohydrolase I, CPH<sub>4</sub> synthase, CDG synthase and preQ<sub>0</sub> synthetase, as described above. The pathway to queuosine is detailed in Fig. 5 and discussed below (Sections 4.2.1–4.2.5).

#### 4.2.1. PreQ<sub>0</sub> reductase (QueF)

PreQ<sub>0</sub> reductase catalyzes the NADPH-dependent reduction of the nitrile group in preQ<sub>0</sub> to the aminomethyl group found in preQ<sub>1</sub>

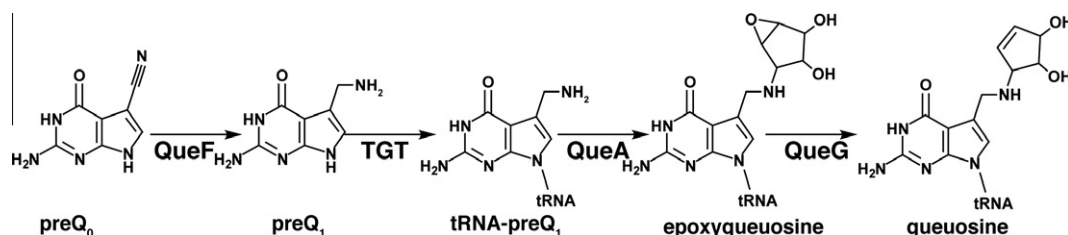
(7-aminomethyl-7-deazaguanine) [65]. QueF is homologous to GCH I and had been annotated initially as an 'enzyme related to GCH I' prior to identification of its true biochemical function. QueF, however, lacks His and Cys residues [65] that are required for binding and activation of an essential zinc metal ion in *E. coli* GCH I [50]. Interestingly, an additional Cys residue, which is also involved in coordination of the metal ion in GCH I, is retained but utilized in covalent catalysis. QueF catalyzes covalent attachment of this Cys thiolate sidechain to the carbon atom of the cyano group of preQ<sub>0</sub> to form a thioimide, which is converted to preQ<sub>1</sub> in two successive NADPH-dependent reactions [66]. Homology modeling [67] and crystallographic structure determinations have confirmed the structural similarities between QueF and GCH I, showing that it adopts a tunnel fold and the position of the catalytically essential cysteine residue [68].

#### 4.2.2. tRNA:guanine transglycosylase (TGT)

Bacterial TGT catalyzes the posttranscriptional exchange of guanine in the wobble position of tRNA anticodons with the sequence 3'-GUN-5' (where 'N' is any canonical nucleoside) with preQ<sub>1</sub> base [49,69]. Modified 7-deazapurine bases in tRNA are unique in that they are incorporated by a base-exchange mechanism without cleavage of the phosphodiester linkage [49]. While preQ<sub>1</sub> is the physiological substrate, exogenously supplied guanine and preQ<sub>0</sub> are also exchanged for guanine by TGT; by contrast, queuine, the free base of queuosine, is not a substrate for the bacterial TGT. Truncated RNA hairpin stem-loop structures corresponding to the anticodon loop of tRNA are also alternate substrates, so long as a U flanks the 3'-end of the GUN anticodon [70–72].

The mechanism of TGT has been probed by biochemical and structural methods. The most illuminating mechanistic insight was from the structure of the *Zymomonas mobilis* protein, which when crystalized with tRNA and the catalytically inactive substrate analog 9-deazazaguanine revealed the structure of a ternary complex [73]. In the structure the C-1' of the tRNA base in position 34 is attached to the protein through covalent linkage to the carboxylate moiety of an active Asp. Moreover, the captured covalent intermediate is converted to the product by soaking preQ<sub>1</sub> into the crystals, demonstrating its intermediacy in the catalytic cycle [73]. Site-directed and kinetic studies have confirmed the role of the Asp and have demonstrated kinetic competence for the covalent intermediate [74,75].

Unlike the bacterial TGT, the eukaryotic TGT homolog (QTRT1) catalyzes the incorporation of queuine (the base of queuosine) into tRNA instead of preQ<sub>1</sub> [76,77]. Eukaryotes do not synthesize queuosine precursors *de novo* and obtain queuosine from the diet or from intestinal flora as the free base, queuine. Queuine is a common nutrient factor and is found in many sources, including milk, serum, wheat germ and tomatoes [78]. Purified QTRT1 from several laboratories has been found to contain, varying subunit compositions [79–83]. QTRT1 associates with another protein, QTRTD1, which is a splice variant of QTRT1 and shares significant sequence identity with it; the two associate *in vivo* and appear to



**Fig. 5.** Biosynthesis of queuosine. PreQ<sub>0</sub>, synthesized as shown in Fig. 4 is reduced by preQ<sub>0</sub> reductase (QueF), incorporated into tRNA by tRNA:guanine transglycosylase (TGT), and elaborated by epoxyqueuosine synthase (QueA) and oQ reductase (QueG) to the hypermodified base queuosine.

localize to the outer membrane of the mitochondria [84]. Complex formation, either by co-expression [84] or *in vitro* mixing [85] leads to active protein. The bacterial and mammalian proteins have been proposed to have evolved via divergent evolution, and there is evidence that small changes in active site architecture are responsible for their differential substrate recognition properties [86,87].

#### 4.2.3. S-Adenosylmethionine:tRNA ribosyltransferase-isomerase (QueA)

QueA was first identified as a protein encoded by the *queA* gene, which in *E. coli* co-localized in the chromosome with *tgt* and encoded a protein that could complement a methyl-deficient strain of *E. coli* K12, which accumulates preQ<sub>1</sub> in tRNA [88,89]. The so-called QueA enzyme utilizes SAM as a substrate and the C-4 of the ribosyl moiety of SAM is transferred to the aminomethyl group of preQ<sub>1</sub> and isomerized to the cyclopentendiol epoxide [90–92]. The enzyme employs an ordered sequential bi-ter kinetic mechanism, in which tRNA substrate is bound first, followed by SAM, and products are released in the order of adenine, methionine, and modified RNA [93]. As with TGT, truncated tRNA molecules that contain only the anti-codon stem loop are substrates [94]. X-ray crystal structures of the ligand-free proteins from *Thermotoga maritima* and *B. subtilis* have been solved recently [95,96]. The structures should facilitate studies of the fascinating mechanism by which SAM serves as a ribosyl donor instead of its more common role as a methyl group donor in biology.

#### 4.2.4. oQ reductase (QueG)

The final step of queuosine biosynthesis, the conversion of oQ to queuosine, had remained elusive until very recently, but vitamin B<sub>12</sub> had been implicated previously as a cofactor required for the reaction [97]. A screen of tRNA from over 1700 *E. coli* strains from the Keio collection [98], that harbored a deletion in a single gene of unknown function (so-called y-genes), led to the identification of the gene *yjeS* (renamed *queG* after the queuosine biosynthesis genes) that encodes oQ reductase [99]. QueG is homologous to a family of vitamin B<sub>12</sub> dependent, iron-sulfur cluster containing reductive dehalogenases that catalyze the dechlorination of compounds such as tetrachloroethene to form ethane [100]. The purified, recombinant QueG catalyzes the conversion of oQ–Q in a synthetic, oQ containing RNA stem loop corresponding to the anticodon loop of Tyr-tRNA, as well as isolated cellular tRNA from the  $\Delta$ *queG* strain [99]. Mechanistic studies on this protein are on-going to define the role(s) of the complex cofactors required for conversion of oQ to Q.

#### 4.2.5. Glutamylqueuosine synthetase

A recent investigation into a truncated aminoacyl-tRNA synthetase homolog in *E. coli* (*yadB*) that contains a catalytic core but lacks a conserved anticodon recognition domain resulted in the unexpected finding that it does not catalyze the aminoacylation of any tRNA species [101,102]. Rather, *YadB* catalyzes the ATP-dependent addition of glutamate to the cyclopentendiol moiety

of Asp-tRNA (see Fig. 2 for the structure). *YadB* is not widely distributed in nature, suggesting that it is not essential; the biological function of this modification remains unknown.

#### 4.2.6. Mannosyl/galactosyl queuosine

Queuosine in tRNA isolated from mammalian sources is modified at the 4-position of the cyclopentendiol moiety with a mannosyl or galactosyl group. The identity of the sugar appears to be related to the amino acid encoded by the tRNA, with tRNA<sup>Tyr</sup> being galactosylated and tRNA<sup>Asp</sup> being mannosylated [22,23,103]. The enzyme that catalyzes mannosylation of Asp tRNA was partially purified from rat liver and shown to utilize GDP-mannose as substrate [104]. Microinjection studies with *Xenopus laevis* show that the modification can occur *in vivo* given exogenous tRNA [105–107].

### 4.3. Biosynthesis of archaeosine

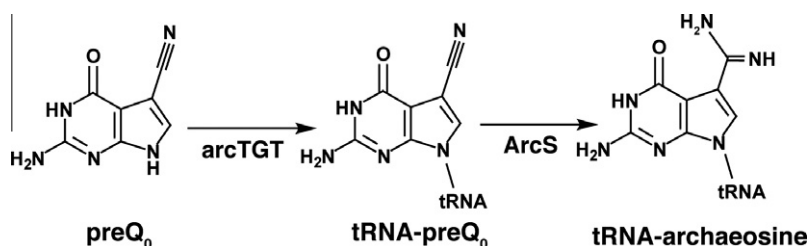
The biosynthesis of archaeosine is essentially identical to that of queuosine up to the formation of preQ<sub>0</sub>. In *H. volcanii*, knock-out and complementation studies have shown that GCH I is required for appearance of archaeosine in tRNA [54]; moreover, preQ<sub>0</sub> has been detected in acid-soluble cell extracts from *H. volcanii* and archaeosine is present in tRNA isolated from cells grown in Q-deficient media [108]. This section will discuss the two steps that are required for incorporation of preQ<sub>0</sub> into tRNA and its amidination to archaeosine (see Fig. 6 for summary).

#### 4.3.1. Archaeal tRNA:guanine transglycosylase (*arcTGT*)

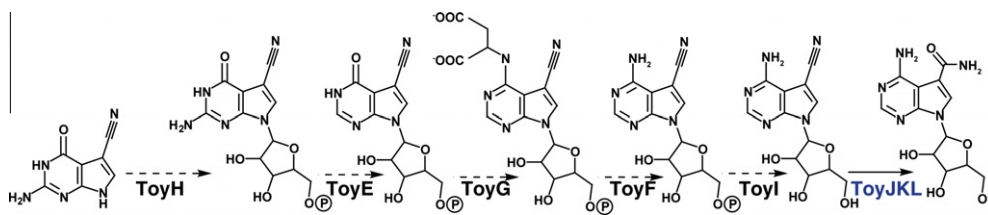
As with bacterial and eukaryotic TGT, the archaeal TGT (*arcTGT*) catalyzes exchange of guanosine in RNA for archaeosine [108,109]. However, unlike the other proteins, the substrate appears to be preQ<sub>0</sub>, the 7-cyanoguanine base, and the reaction takes place not at the wobble position of RNA but in the D-loop. *ArcTGT* is composed of a single type of subunit and appears to be homologous to the bacterial protein. Interestingly, the proteins from archaea appear to have three additional C-terminal domains (Domain C1–3), one of which, domain C3, is homologous to PUA domains (for pseudouridine synthases and archaeosine tRNA transglycosylase) [110]. In some archaea, the C-terminal extension is encoded by a separate gene [111]. While comparison of the apo [111] and tRNA-bound [112] X-ray crystal structure of *arcTGT* suggested that the PUA domain may be responsible for proper orientation of the substrate, namely, exposing the modification site by disrupting the D-loop, biochemical studies have indicated that deletion of the entire C-terminal region does not impair the reaction [113]. The catalytic efficiency of the protein is reduced, primarily due to an effect on *K<sub>m</sub>*.

#### 4.3.2. Archaeosine synthase

The final step in the biosynthesis of archaeosine is catalyzed by archaeosine synthase (*ArcS*) [114]. The enzyme catalyzes the hydrolysis of glutamine (glutaminase), which is utilized to carry



**Fig. 6.** Biosynthesis of archaeosine. PreQ<sub>0</sub>, synthesized as shown in Fig. 4, is incorporated into RNA by the archaeal TGT (*arcTGT*) and elaborated by archaeosine synthase (*ArcS*) to archaeosine.



**Fig. 7.** Biosynthesis of toyocamycin and sangivamycin from preQ<sub>0</sub>. Dashed arrows denote hypothetical reactions that are based on sequence similarity. PreQ<sub>0</sub> is presumably converted to toyocamycin by the successive actions of ToyH, ToyE, ToyG, ToyF and ToyI. Toyocamycin nitrile hydratase (ToyJKL) catalyzes the conversion of toyocamycin to sangivamycin.

out amidination of preQ<sub>0</sub> to generate archaeosine. ATP is not required for the process. ArcS homologs are widely distributed in archaea. Sequence alignments suggest that it is homologous to ArcTGT but lacks the catalytic residues that are essential for the base exchange reaction, as would be expected from the unique reaction that it catalyzes. As with ArcTGT, ArcS has a PUA domain whose function is not understood.

#### 4.4. Biosynthesis of toyocamycin/sangivamycin

PreQ<sub>0</sub> is an intermediate in the biosynthesis of toyocamycin/sangivamycin (see Fig. 7 for pathway). The identification of the gene cluster for the biosynthesis of these compounds revealed that in addition to enzymes required for the biosynthesis of the 7-deazapurine core, the cluster also houses a series of purine biosynthesis/salvage genes, which presumably encode enzymes involved in appending preQ<sub>0</sub> to a new ribose and those for converting the guanine-like preQ<sub>0</sub> to the adenine-like base moiety found in toyocamycin. The purine salvage genes are predicted, based on bioinformatics, to encode homologs of guanine/hypoxanthine phosphoribosylpyrophosphate transferase (PRPTase) (ToyH), GMP reductase (ToyE), adenylosuccinate synthetase (ToyG), adenylosuccinate lyase (ToyF), and haloacid dehalogenase (ToyI). These proteins are collectively hypothesized to convert preQ<sub>0</sub> to toyocamycin [7]. It will be interesting to determine the extent to which these proteins have diverged from their counterparts in primary metabolism in order to accept substrates that have a substituent appended to the newly installed carbon, which in purines is a nitrogen.

##### 4.4.1. Toyocamycin nitrile hydratase (TNHase)

Conversion of toyocamycin to sangivamycin is catalyzed by toyocamycin nitrile hydratase (TNHase) [7]. TNHase belongs to a family of metalloenzymes that ligate either a non-heme Fe<sup>3+</sup> ion or non-corrin Co<sup>3+</sup> ion to three cysteine sulfurs and two backbone amide nitrogens in a conserved active site motif, V-C-(S/T)-L-C-S-C [115]. The presence of Ser or Thr correlates with coordination to iron or cobalt, respectively. Two of the cysteines in the conserved motif (C<sub>2</sub> and C<sub>3</sub>) are post translationally oxidized to cysteine sulfenic (–SO<sub>2</sub>H) and sulfenic acid (–SOH), respectively (therefore TNHase is expected to ligate cobalt). Most NHases are composed of two subunits and the active site post-translationally modified cysteine residues reside in the α-subunit. TNHase is composed of three subunits – the β and γ subunits are homologous to the N- and C-terminal halves of a typical NHase beta subunit.

## 5. Regulation of biosynthesis of deazapurines

As with other secondary metabolites produced by microorganisms, it appears that the biosynthesis of deazapurine containing compounds is regulated. The compounds are often isolated from culture filtrates 24–48 h after inoculation, suggesting that the biosynthetic pathways for their production are turned-on in the

stationary phase of growth. The biosynthetic cluster for toyocamycin/sangivamycin also encodes a LuxR-like transcriptional activator [7]. However, the mode of regulation, and whether the activation of the pathway is tied to general transcriptional regulators in *Streptomyces*, remains to be established.

Substantially more is known about the regulation of appearance of queuosine in RNA. The queuosine precursor, preQ<sub>1</sub>, down-regulates the transcription of queuosine biosynthetic genes through the binding of a preQ<sub>1</sub>-specific riboswitch [116,117]. To date, two distinct, non-homologous classes of preQ<sub>1</sub>-binding riboswitches have been confirmed in prokaryotes and several structural studies have provided the molecular basis of the interactions [118–120] that may discriminate for the modified base.

## 6. Perspectives and future directions

The burst of research in the deazapurine biosynthesis pathway has been spurred in the last 10 years by the availability of genome sequences and emergence of comparative genomic tools. The complete outline of the pathway to various deazapurines has revealed the key biosynthetic steps, and chemical intuition suggests that all known deazapurines (Fig. 1) can be biosynthesized from pathways that have in common a GTP cyclohydrolase I, CPH<sub>4</sub> synthase and CDG synthase. In the near future, coupling of recent advances in sequencing technologies with bioinformatics tools will hasten discovery of biosynthetic steps that lead to some of the more highly decorated deazapurines. If the present state of the field is any indication, there are numerous unprecedented chemical transformations that will emerge from these studies in the future.

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