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Identification of the *C*-Glycoside Synthases during Biosynthesis of the Pyrazole-*C*-Nucleosides Formycin and Pyrazofurin

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Abstract

C-Nucleosides are characterized by a C-C rather than a C-N linkage between the heterocyclic base and the ribofuranose ring. While the biosynthesis of pseudouridine-*C*-nucleosides has been studied, less is known about the pyrazole-*C*-nucleosides such as the formycins and pyrazofurin. Herein, genome screening of *Streptomyces candidus* NRRL 3601 led to the discovery of the pyrazofurin biosynthetic gene cluster *pyf*. *In vitro* characterization of gene product PyfQ demonstrated that it is able to catalyze formation of the *C*-glycoside carboxyhydroxypyrazole ribonucleotide (CHPR) from 4-hydroxy-1*H*-pyrazole-3,5-dicarboxylic acid and phosphoribosyl pyrophosphate (PRPP). Similarly, ForT, the PyfQ homologue in the formycin pathway, can catalyze the coupling of 4-amino-1*H*-pyrazole-3,5-dicarboxylic acid and PRPP to form carboxyaminopyrazole ribonucleotide. Finally, PyfP and PyfT are shown to catalyze amidation of CHPR to pyrazofurin 5′-phosphate thereby establishing the latter stages of both pyrazofurin and formycin biosynthesis.

Graphical Abstract

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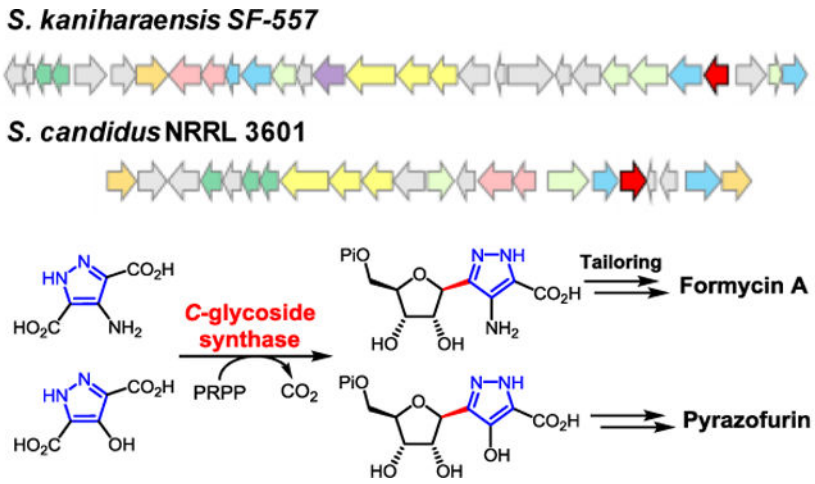
Experimental Section

Experimental procedures, genome sequencing and analysis, chemical syntheses of enzymatic substrates, and supporting figures and tables are available in Supporting Information. The genome sequence reported in this paper has been deposited into the GenBank database under the accession number MN305320.



Enzymatic C-glycoside bond formation: The C-glycoside synthases encoded in the formycin and pyrazofurin biosynthetic gene clusters are identified and compared by genomic analysis of the producing strains. Both enzymes catalyze decarboxylative C-glycoside bond formation between phosphoribosyl pyrophosphate and a pyrazole nucleobase in reactions likely to involve electrophilic aromatic substitution.

Keywords

C-nucleosides; biosynthesis; biosynthetic gene cluster; C-glycosidation reaction; enzyme mechanism

The *C*-nucleosides are a noncanonical class of nucleosides in which the heterocyclic base and the ribofuranosyl core are connected through a C-C bond rather than the more common C-N linkage that is found in the canonical nucleosides.[1–3] A wide variety of nucleobases and functional groups are found in the naturally occurring *C*-nucleosides including uracil in pseudouridine (**1**), [4] malayamycin (**2**) [5,6] and pseudouridimycin (**3**), [7,8] the oxazine dione group in minimycin (**4**), [9,10] the maleimide unit in showdomycin (**5**), [11–14] and the pyrazole moiety in pyrazofurins (**6**) [15] and formycins (**7** and **8**) [16,17] (see Figure 1). Many *C*-nucleosides found in nature are known to have antibiotic and antiviral activities mainly due to their structural resemblance to the *N*-nucleosides that results in their interference with primary metabolic processes. [5,7,9,18–27] Their biological activities have thus inspired the synthesis of many nonnatural *C*-nucleosides, some of which have been demonstrated to be effective therapeutic agents. [2,3,28] However, how the *C*-nucleosides are biosynthesized in nature remains largely unexplored.

Early studies have shown that pseudouridine (**1**) is derived from uridine (**10**) in a reaction catalyzed by pseudouridine synthase. [29] The catalytic cycle of this enzyme is initiated by C-N bond cleavage to release uracil (**11**) and an oxocarbenium ion (**12**) (or a 1,2-glycal species after C2 deprotonation). Reorientation of **11** in the active site followed by nucleophilic addition back to **12** can then lead to *C*-glycosidic bond formation in **1** as shown in Scheme 1A. [30–32] PumJ, a pseudouridine synthase equivalent, has been demonstrated to catalyze the same reaction during the biosynthesis of pseudouridimycin (**3**). [8] The

discovery of *sdmA*, which encodes a pseudouridine synthase homologue in the showdomycin (**5**) gene cluster, likewise suggested a similar mechanism of C-C bond formation in showdomycin assembly (see Scheme 1B). [14]

In contrast, little is known about the mode of *C*-glycosidic bond formation in the biosynthesis of pyrazofurin (**6**) and the formycins (**7**/**8**). As both carry a pyrazole-derived nucleobase that is different from those in **1**-**5**, enzymes catalyzing the *C*-glycosidation in **6**-**8** likely involve distinct mechanisms from that of pseudouridine synthase. The formycin biosynthetic gene cluster (i.e., the *for* cluster) from *Streptomyces kaniharaensis* SF-557 has already been identified. [33] *In vitro* experiments showed that carboxyaminopyrazole ribonucleotide (CAPR, **14**) is a late intermediate in the formycin A biosynthetic pathway and its maturation to formycin A 5'-phosphate (**18**) is sequentially catalyzed according to Scheme 2A by ForC, ForB, ForH, ForA, and ForB together with PurH, which originates in primary metabolism. [33,34] Importantly, the *forJ, forK* and *forL* genes in the *for* cluster were noted to be homologous to *spb38, spb39* and *spb*40 in the gene cluster of s56-p1 (**21**). [35] Enzymes encoded by the latter set of genes have been shown to catalyze the transformation of lysine and glycine to give hydrazino acetic acid (**20**) enroute to s56-p1 (Scheme 2B). [35] Hence, hydrazine formation may also be involved in the construction of the pyrazole group of formycins[33] and perhaps pyrazofurin as well (Scheme 2C). If correct, this hypothesis suggested a criterion by which the pyrazofurin gene cluster might be identified in a genome-wide search of the producing strain. Towards this goal, a comparative genomics approach was employed to find the responsible enzymes and investigate the mechanisms of C-C coupling in **6**-**8**. Reported herein is the discovery of novel enzyme-catalyzed *C*-glycosidation reactions that underlie biosynthesis of the pyrazole group in biological systems.

The genome of *Streptomyces candidus* NRRL 3601 was sequenced (9.2 Mbp with 301 contigs) (N50: 152,140 bp), and the genes *pyfG*, *pyfH*, and *pyfI*, which are homologous to *forJ, forK* and *forL*, were found clustered on the k141_289 contig. This cluster, designated as the *pyf* cluster, spans a ~25 kbp region between two ABC transporters which are the likely boundary of the *pyf* cluster (see Figure 2). BLAST (basic local alignment search tool) comparison of the *pyf* and *for* clusters not only revealed the conservation of the putative hydrazine-assembly genes (*forJ/forK/forL* versus *pyfG*/*pyfH*/*pyfI*) in each cluster, but also the presence of *forC* and *forB* homologues, denoted here as *pyfP* and *pyfT*, respectively. These findings are consistent with the *pyf* cluster being responsible for pyrazofurin biosynthesis and suggest that formycins and pyrazofurin may share the same pyrazole forming machinery. In addition, carboxyhydroxypyrazole ribonucleotide (CHPR, **19**) was considered to be a likely intermediate of pyrazofurin biosynthesis and specifically a substrate for sequential reactions catalyzed by the *pyfP* and *pyfT* gene products as shown in Scheme 2A.

Also noted was the absence of *forHAFI* homologs in the *pyf* cluster (see dashed box in Figure 2). This is consistent with the observation that ForH and ForA are required only for the construction of the second heterocycle in formycins, which is absent in pyrazofurin (see Scheme 2A). [33,34] Furthermore, the coformycin (**9**) biosynthetic genes (i.e., the *cof* cluster) were found to be adjacent to both the *for* and *pyf* main clusters. [36] This flanking arrangement is not surprising, because formycins (**7**/**8**) are produced synergistically with

coformycin (**9**), which is an inhibitor of adenosine deaminase (ADA) and can therefore prevent formycin A (**7**) deactivation by ADA. [16,37] Similarly, amide bond hydrolysis of pyrazofurin (e.g., **23** → **19**) was also observed when pyrazofurin (**6**) was incubated with ADA (type X) (see Figure S2 in the Supporting Information). This bioinformatic analysis collectively suggested that the *pyf* cluster encodes the enzymes of pyrazofurin biosynthesis.

To test the hypothesis that the *pyf* cluster is indeed responsible for pyrazofurin biosynthesis, selected *pyf* gene products were expressed and characterized *in vitro*. In particular, PyfQ as well as ForT from the *for* cluster are annotated as encoding a *β*-ribofuranosylaminobenzene 5'-phosphate (*β*-RFA-P) synthase. *β*-RFA-P synthase has been demonstrated to catalyze formation of the *C*-glycoside **27** from phosphoribosyl pyrophosphate (PRPP, **24**) and *p*-aminobenzoic acid (*p*ABA, **25**) during biosynthesis of methanopterin (Scheme 3A). [38–41] Furthermore, this reaction is believed to involve electrophilic aromatic substitution (EAS) of *p*ABA (**25**) with the oxocarbenium ion (**12**) from PRPP (**24**, Scheme 3A). [38,39] Therefore, it was proposed that an analogous substitution reaction also occurs between PRPP and an aromatic nucleobase in the catalytic cycles of ForT and PyfQ such that these two enzymes are responsible for *C*-glycosidic bond formation in formycin[33] and pyrazofurin, respectively.

In order to investigate these hypotheses, the putative six-membered pyridazinone[33] intermediate **22** was incubated with ForT and PyfQ, but no *C*-glycosidation product was observed (Scheme 3B and Figure S3). However, when 4-amino-1*H*-pyrazole-3-carboxylic acid (APA, **29**) was prepared (see Supporting Information) and incubated with PRPP (**24**) and ForT for 36 h, two new products were detected (see Figure 3B, trace 4). These products were identified as aminopyrazole ribonucleotide (APR, **30**) and CAPR (**14**) based on MS analysis (see Supporting Information) and HPLC coelution with the synthetic standards. Although the yield was poor (<5%), these results demonstrated that ForT catalyzes a *C*-glycosidation reaction. The production of both APR and CAPR suggested competing electrophilic addition of the putative oxocarbenium intermediate **12** at C3 versus C5 of **29** to yield APR (**30**) versus CAPR (**14**), respectively (see Figure 3A, routes a vs. b). While ForT appeared to promote addition at both C3 and C5, the same experiment with PyfQ demonstrate primarily decarboxylative EAS (route a) at C3 to yield APR (**30**) with essentially no addition at C5 (see Figure 3B, trace 5). Furthermore, APA (**29**) appeared to be a much better substrate for PyfQ compared to ForT showing significantly greater turnover during the incubation time period.

The poor activity of ForT on APA indicated that APA may not be the correct substrate for ForT. The observed decarboxylation of APA (**29**) to APR (**30**) also implied that a C3 carboxylation reaction is required to convert APR to CAPR (**14**) or a derivative thereof in the biosynthesis of both formycins and pyrazofurin (see Figure 3A). However, no genes in either cluster were annotated to encode a carboxylase. Furthermore, neither PurE nor PurK, which catalyze an analogous carboxylation reaction in *de novo* purine biosynthesis, [42,43] led to CAPR (**14**) formation when incubated with APR (**30**) (see Figure S4, S5 in the Supporting Information). Moreover, prior labeling experiments have indicated that both the C6 of formycin A and the amide carbon of pyrazofurin originate from C1 of glutamate

rather than bicarbonate. [44] Taken together, APA (**29**) is unlikely to be a biosynthetic intermediate in the formycin and pyrazofurin pathways.

If ForT and PyfQ are indeed the *C*-glycosidases of formycin and pyrazofurin biosynthesis, then the carboxylate group at C3 carbon of their pyrazole substrates should be retained. Therefore, 4-amino-1*H*-pyrazole-3,5-dicarboxylic acid (APDA, **31**) and 4-hydroxy-1*H*-pyrazole-3,5-dicarboxylic acid (HPDA, **32**) were prepared (see Supporting Information) and tested for activity with ForT and PyfQ (see Figure 4A). Indeed, incubation of APDA (**31**) with PRPP (**24**) in the presence of either ForT or PyfQ led to quantitative conversion of APDA to CAPR (**14**) within 1 h (see Figure 4D). Similarly, PyfQ catalyzes complete conversion of HPDA (**32**) and PRPP (**24**) to CHPR (**19**) within 1 h of incubation (see Figure 4E); however, **32** was not found to be a substrate for ForT. These results strongly support correct assignment of the clusters and indicate that ForT exhibits greater substrate specificity compared to PyfQ. Moreover, no reaction was observed with either ForT or PyfQ when 1*H*-pyrazole-3,5-dicarboxylic acid (PDA, **33**) was supplied as the substrate (Figure 4C and Figure S6). This implies that an electron donating group is required to increase the nucleophilicity of the heterocycle and is consistent with a *C*-glycosidation mechanism that involves EAS.

To further investigate the *pyf* gene cluster, the CHPR (**19**) product of the PyfQ-catalyzed reaction with HPDA (**32**) was investigated as a substrate for the *pyfP*-encoded gene product. PyfP is annotated as a phosphoribosylaminoimidazole-succino-carboxamide (SAICAR) synthetase and thus predicted to catalyze amidation of **19** with aspartate (see Figure 5A). When purified PyfP was incubated with **19**, L-Asp, and ATP, a single product was produced (see Figure 5B, trace 3) and displayed a mass consistent with succinyl-hydroxypyrazole carboxamide ribonucleotide (SHPCAR, **34**) (calcd *m/z* for $C_{13}H_{17}N_3O_{13}P$ [M - H]$^-$ 454.0499; found 454.0529). Furthermore, addition of purified PyfT, a putative adenylsuccinate lyase, to PyfP previously incubated with **19**, L-Asp and ATP, led to consumption of **34** and formation a new compound (Figure 5B, trace 4) having the expected mass 338.0384 of pyrazofurin 5'-phosphate (**23**, calcd *m/z* for $C_9H_{13}N_3O_9P$ [M - H]$^-$ 338.0389). Confirmation of this assignment was obtained when the PyfQ/PyfP/PyfT product from **32** was isolated, treated with calf intestinal phosphatase and found to coelute with a standard of pyrazofurin (**6**) on HPLC (see Figure S7 in the Supporting Information). It was also found that ForC and PurC, which are homologues of PyfP in the formycin and *de novo* purine pathways, also catalyzed the conversion of **19** to **34** (see Figure S8 in the Supporting Information).

In summary, PyfQPT encoded in the *pyf* cluster [45] are able to catalyze the conversion of HPDA (**32**), PRPP (**24**), and L-aspartate to pyrazofurin 5′-phosphate (**23**). Similarly, ForT catalyzes the coupling of APDA (**31**) and PRPP (**24**) to produce CAPR (**14**), which is subsequently converted to formycin A 5′-phosphate (**18**) in reactions catalyzed by ForABCH and PurH. [33,34] In particular, PyfQ as well as ForT are *C*-glycoside synthases [46] and are likely characterized by catalytic cycles that involve EAS reactions. These observations indicate that the *for* and *pyf* clusters are indeed responsible for formycin and pyrazofurin biosynthesis and thus establish the latter stages of both biosynthetic pathways.

Nevertheless, the early stages of these pathways involving the assembly of the pyrazole rings in APDA (**31**) and HPDA (**32**) remain to be determined and may involve a hydrazine-generating process (see Scheme S3 in Supporting Information) analogous to that found in the s56-p1 biosynthetic pathway. [35]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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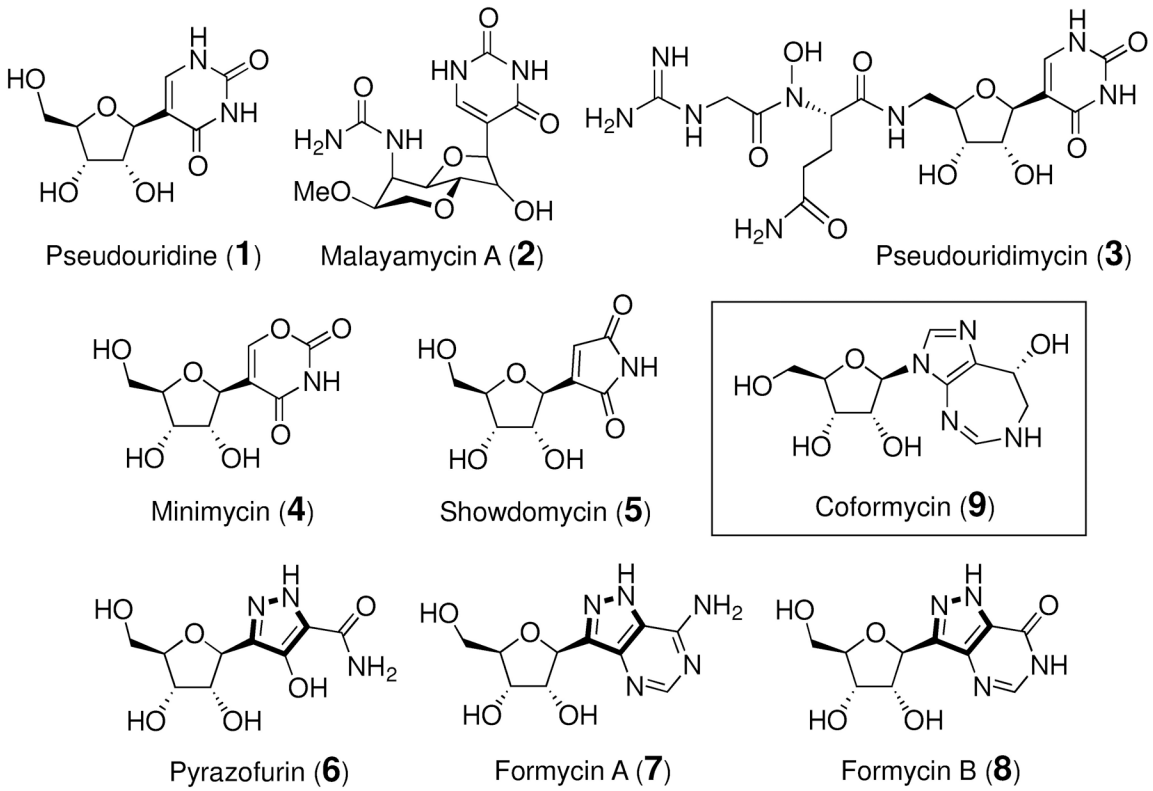


Figure 1.
Representatives of *C*-nucleoside antibiotics (**1**-**8**) and coformycin (**9**).

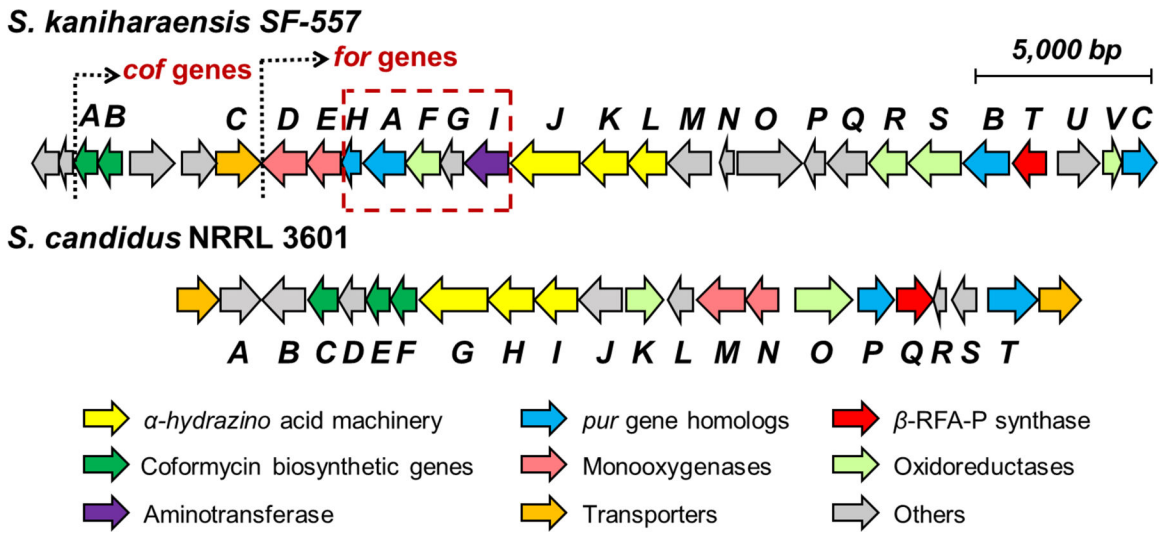


Figure 2.

The pyrazofurin (*pyf*) biosynthetic gene cluster from *S. candidus* in comparison with the formycin (*for*) gene cluster from *S. kaniharaensis*. The coformycin biosynthetic genes (*cof* genes) are found adjacent to the main clusters in both the *pyf* and *for* clusters.

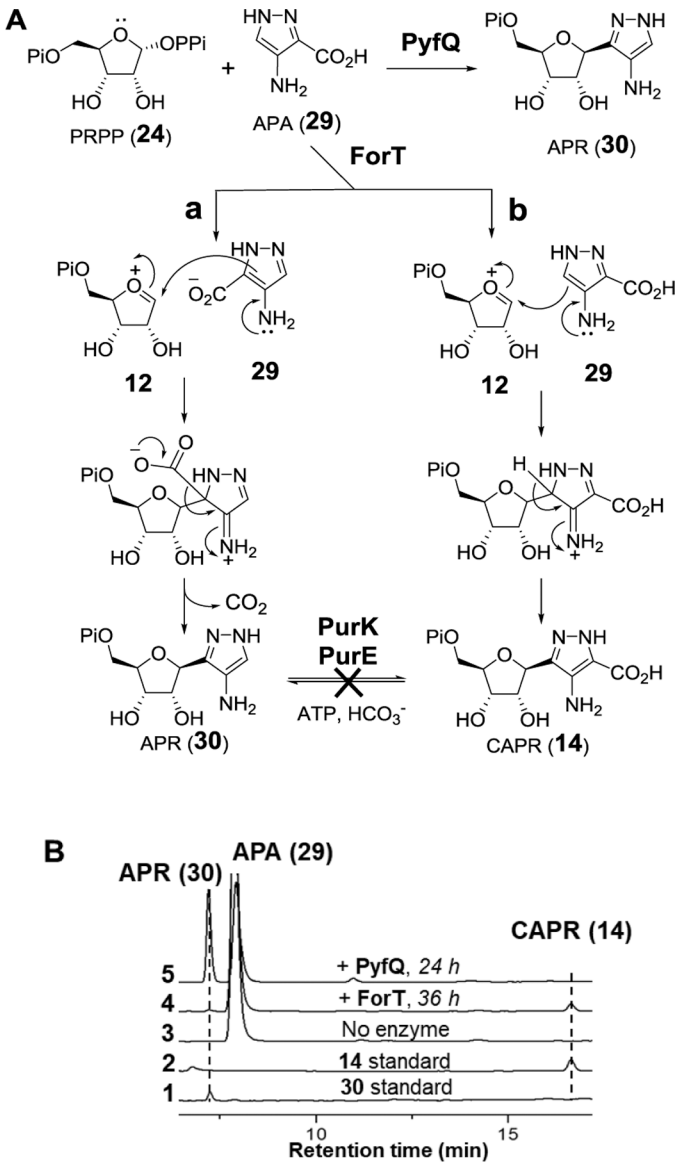


Figure 3.

(A) ForT/PyfQ reactions using APA (**29**) as substrate. (B) HPLC analysis of ForT/PyfQ reactions with APA (**29**).

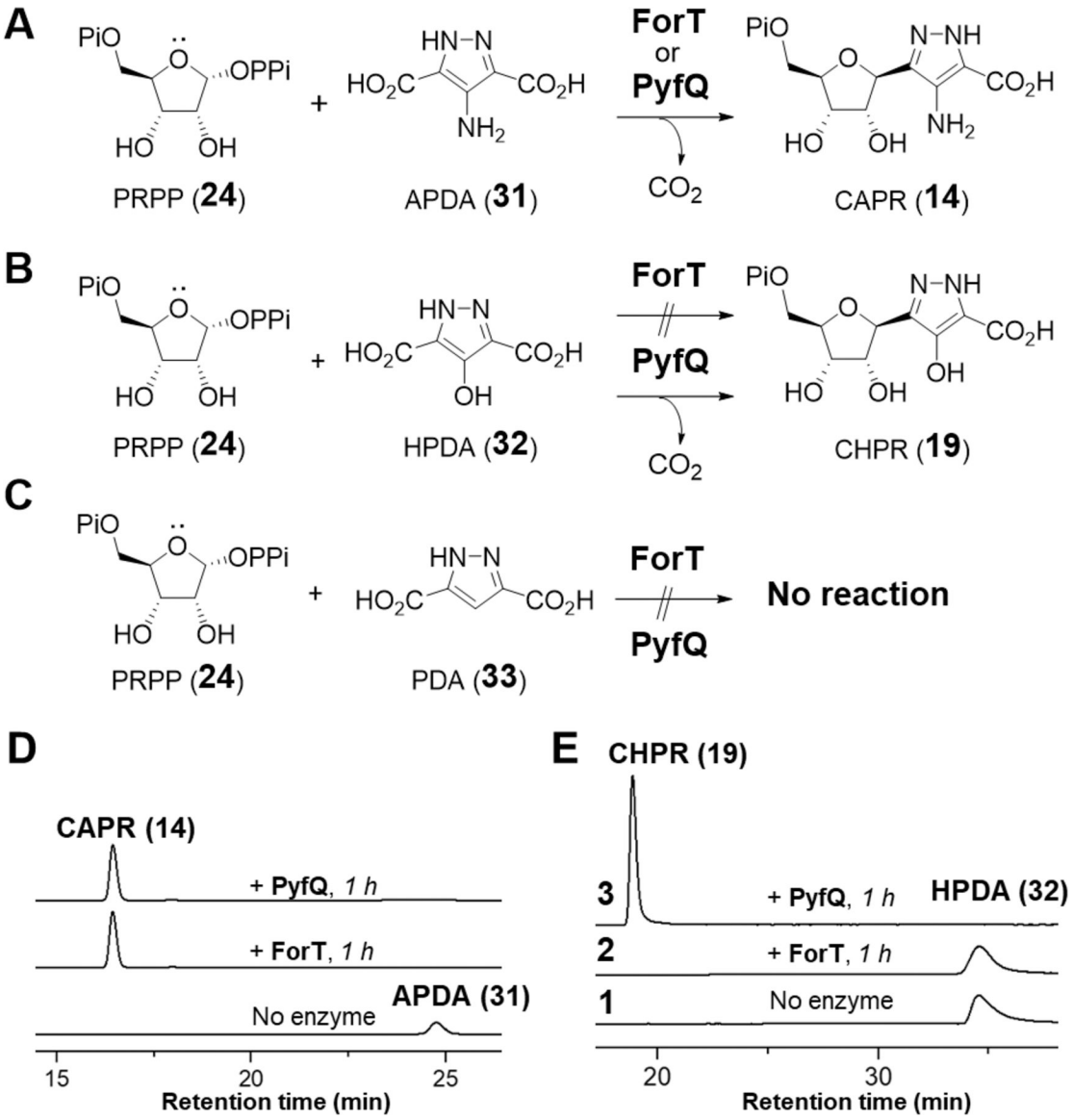


Figure 4.

ForT/PyfQ reactions with (A) APDA (**31**); (B) with HPDA (**32**); (C) with PDA (**33**). (D) HPLC analysis of ForT/PyfQ reactions with APDA (**31**). (E) HPLC analysis of ForT/PyfQ reactions with HPDA (**32**).

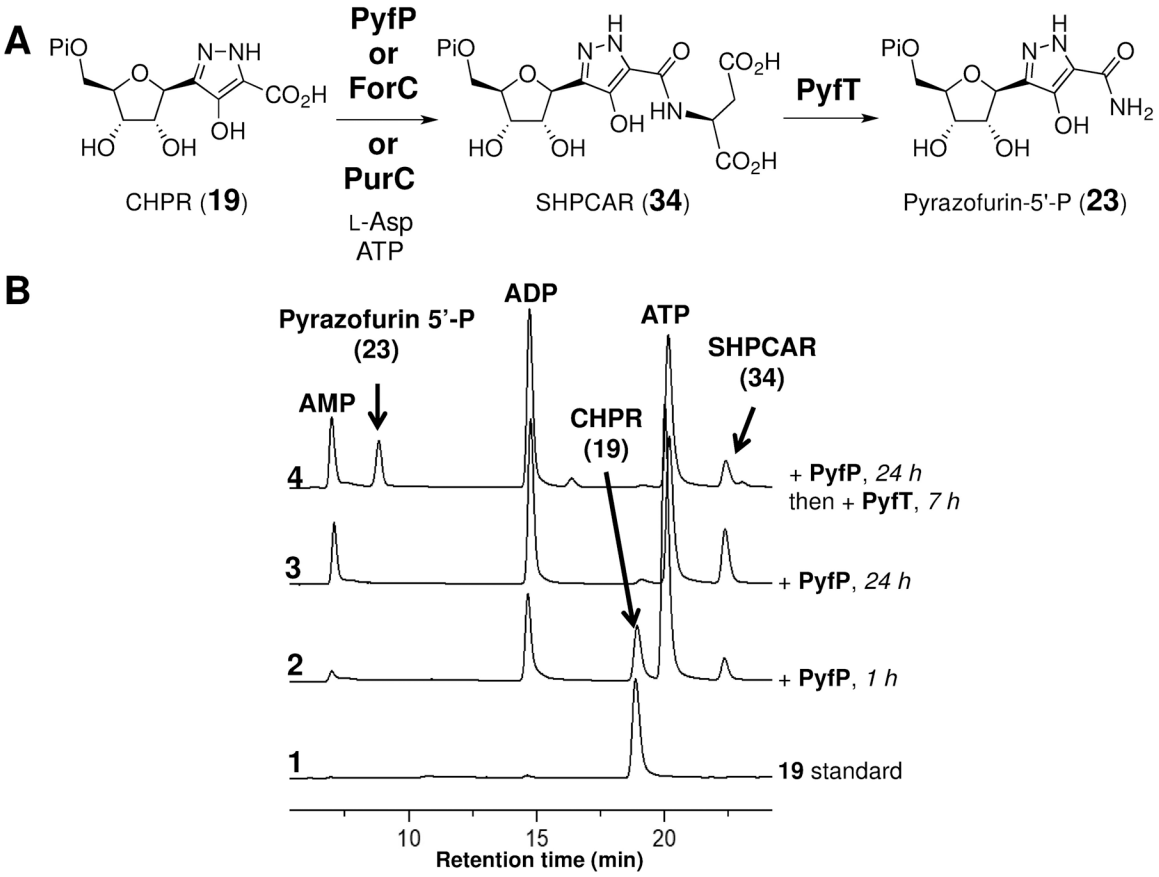
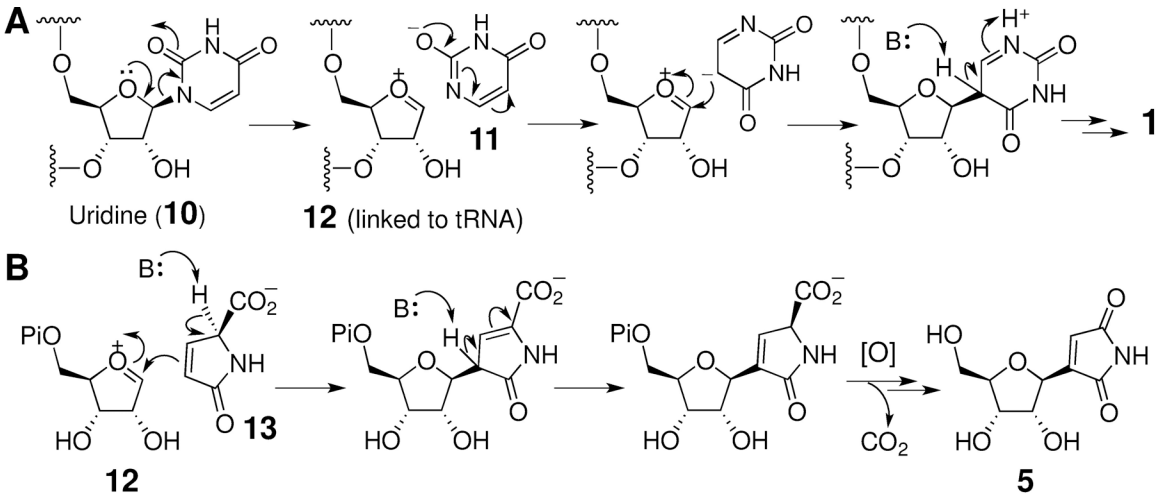


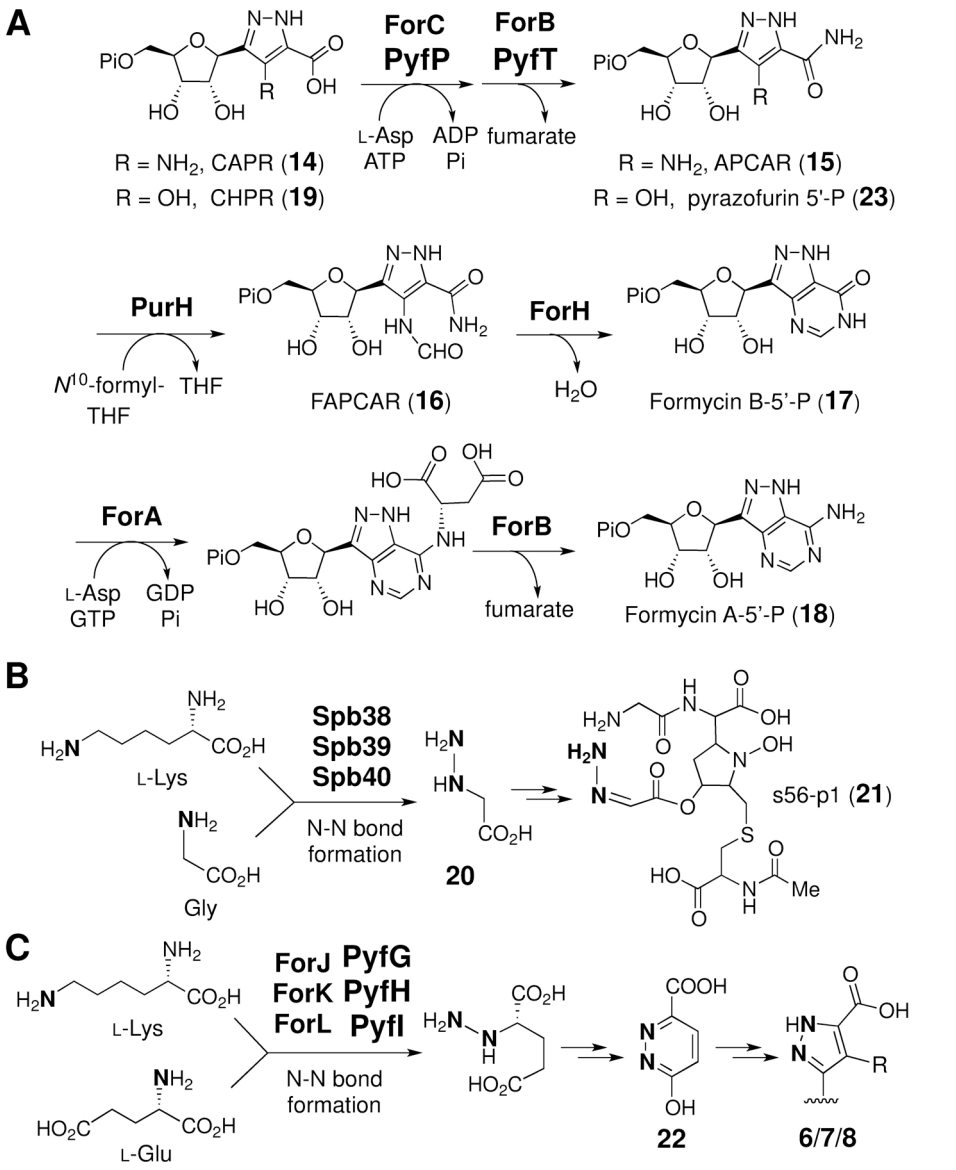
Figure 5.

(A) Reactions of CHPR (**19**) with PyfP and PyfT. (B) HPLC analysis of the PyfP and PyfT reactions.



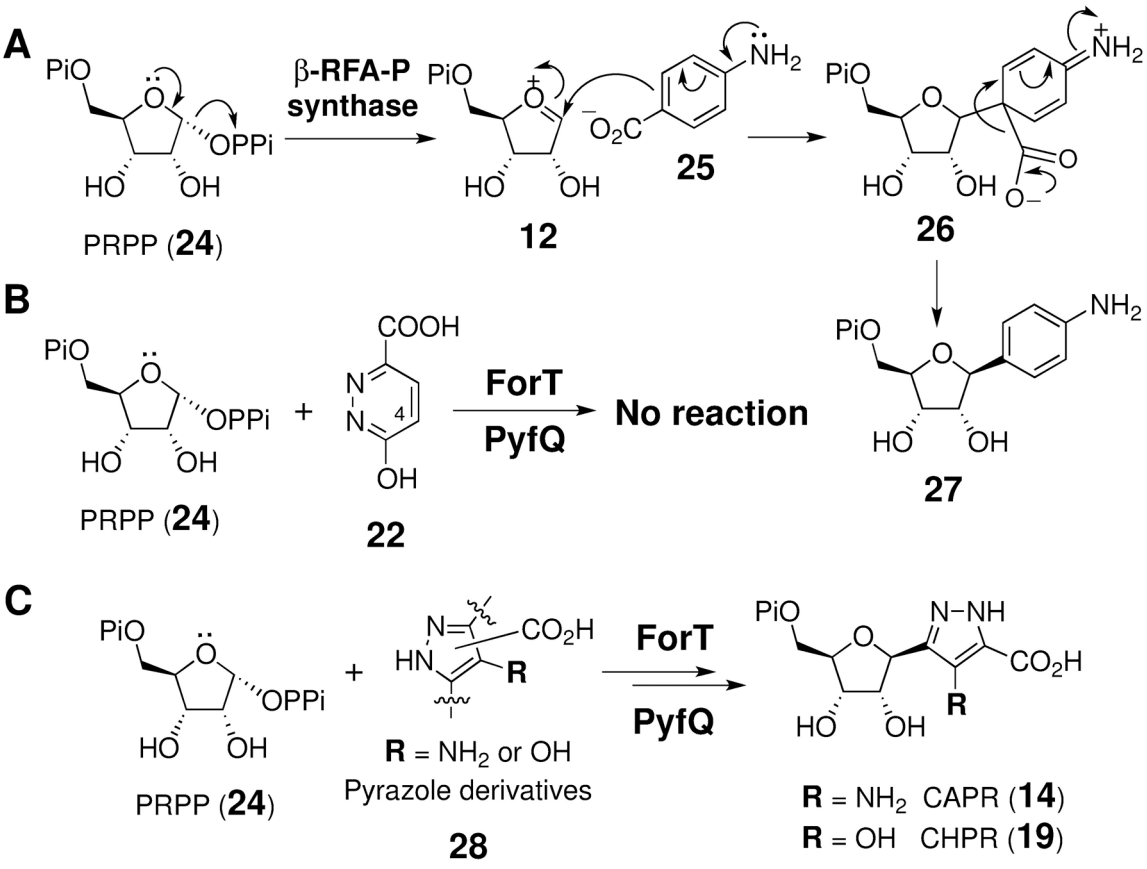
Scheme 1.

Proposed mechanism for pseudouridine synthase. The cosubstrate **13** in SdmA reaction is likely derived from L-glutamine. [11,12]



Scheme 2.

(A) Conversion of CAPR (**14**) to formycin A 5'-phospahte (**18**) in the formycin biosynthetic pathway and the intermediacy of CHPR (**19**) in pyrazofurin pathway. (B) Formation of hydrazino acetic acid (**20**) in the biosynthesis of s56-p1 (**21**).[35] (C) Possible involvement of ForJ/K/L and PyfG/H/I in hydrazine assembly in formycin and pyrazofurin biosynthesis, respectively.



Scheme 3.

(A) Reaction catalyzed by *β*-ribofuranosylaminobenzene 5'-phosphate synthase. (B) and (C) Reactions catalyzed by ForT and PyfQ using PRPP (**24**) and different nitrogen-containing compounds as substrates.