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## EDITED BY

Adam Offenbacher,  
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## REVIEWED BY

Steven Gary Van Lanen,  
University of Kentucky, United States  
Wei Yan,  
Nanjing Agricultural University, China

## \*CORRESPONDENCE

Nigel G. J. Richards,  
✉ nrichards@ffame.org

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# The chemistry of Formycin biosynthesis

Nigel G. J. Richards<sup>1,2\*</sup> and James H. Naismith<sup>3</sup>

<sup>1</sup>School of Chemistry, Cardiff University, Cardiff, United Kingdom, <sup>2</sup>Foundation for Applied Molecular Evolution, Alachua, FL, United States, <sup>3</sup>Division of Structural Biology, University of Oxford, Oxford, United Kingdom

Remarkable progress has been made to elucidate the structural and mechanistic enzymology of the biosynthetic pathways that give rise to naturally occurring C-nucleosides. These compounds are generally cytotoxic and exhibit interesting antiviral, antibiotic and anti-parasitic activity. Here we review current knowledge concerning formycin biosynthesis and highlight deficiencies in our understanding of key chemical transformations in the pathway.

## KEYWORDS

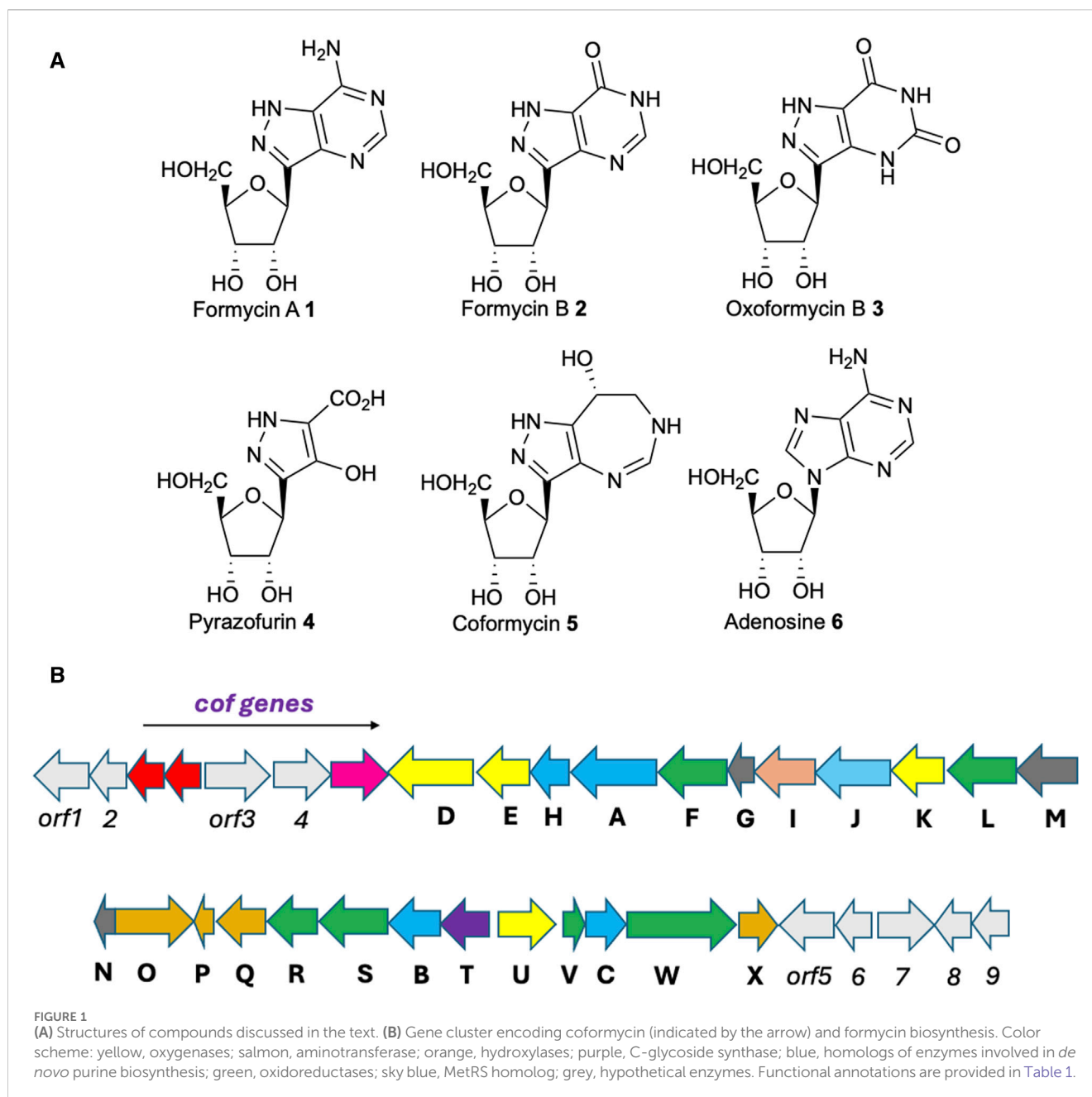
formycin, C-nucleoside, biosynthesis, gene cluster, enzymology

## 1 Introduction

There is renewed interest in the synthesis and clinical application of modified nucleosides and nucleotides (Duffy et al., 2020; Schramm, 2018; Seley-Radtke and Yates, 2018; Yadav et al., 2019). These compounds represent lead structures for antiviral drug discovery (De Clercq, 2016), and are finding use as components of expanded genetic alphabets (Hoshika et al., 2019; Pfeiffer and Nidetzky, 2020), diagnostic reagents (Sefah et al., 2014), and drug delivery systems (Zhang L. et al., 2020). Notably, the effectiveness of remdesivir in treating SARS-CoV-2 infections (Mackman, 2022) has prompted a re-evaluation of the clinical utility of C-nucleosides; compounds in which the nucleobase is connected to the sugar by a C-C rather than the more labile C-N bond. In nature, C-nucleoside linkages are present in numerous microbial secondary metabolites that exhibit interesting antibiotic and anti-viral activities, and the past decade has seen considerable progress in understanding the biosynthetic origins of these natural products (Shiraishi and Kuzuyama, 2019; Sosio et al., 2018; Zhang et al., 2022). In this mini-review, we discuss current knowledge about the pathway leading to the formycins 1-3 (Figure 1A), which are pyrazole-containing bioactive compounds (Santos et al., 2020), and identify gaps in our understanding that remain to be closed.

## 2 Structure, chemistry and biological properties of the formycins

Formycins A 1 and B 2 (Figure 1A) were first isolated from *Nocardia interformata* approximately 60 years ago (Hori et al., 1964; Koyama and Umezawa, 1965), and subsequently in cultures of *Streptomyces kaniharaensis* SF-557 (Zhu et al., 2020), *Streptomyces lavendulae* (Aizawa et al., 1965) and *Streptomyces resistomycificus* NRLL 2290 (Zhang M. et al., 2020). There have been numerous studies into their structure, chemical properties, and biological activity (Robins et al., 1966; Ishizuka et al., 1968; Prusiner et al., 1973; Chenon et al., 1976). As well as being C-nucleosides, the pyrazolopyrimidine ring features an N-N bond, a functional group that is relatively



uncommon in natural products (Blair and Sperry, 2013; Waldman et al., 2017).  $^1\text{H}$  NMR spectroscopy was used to establish the tautomeric preferences of the pyrazolopyrimidine in solution, with the major species being protonated at N7 (Krugh, 1973) consistent with quantum chemical calculations (Orozco and Luque, 1995).

After cellular phosphorylation at C-5' (Mehta and Gupta, 1985), formycins A 1 and B 2 become nonhydrolyzable analogues of AMP and GMP, respectively exhibiting anti-tumor, anti-viral (Dapp et al., 2014), antibiotic and anti-parasitic activity (Bzowska, 2008). The compounds likely exert their biological effects via a number of possible molecular mechanisms, including inhibition of the enzymes AMP nucleosidase (Ehrlich and Schramm, 1994) and purine nucleoside phosphorylase (PNP) (Kierdaszuk et al., 2000). Indeed, the biological properties of formycin A have inspired the synthesis of analogs that exhibit picomolar affinity for human PNP (Ho et al., 2010) and suppress T-cell activity by

interfering with purine recycling (Cohen et al., 1978; Markert, 1991). The incorporation of formycin A into RNA, presumably via the triphosphate, is also thought to be the molecular mechanism underlying its ability to kill *Leishmania* parasites (Rainey and Santi, 1983). Given these findings, it is perhaps surprising that neither formycins nor their derivatives have found clinical use.

### 3 Formycin biosynthesis

Although little was known about the molecular details of formycin biosynthesis until recently, early work established that formycin B 2 is a precursor of formycin A 1 in *N. interforma* (Sawa et al., 1968). Subsequent feeding experiments using  $^{13}\text{C}$  and  $^{15}\text{N}$ -labeled compounds then revealed that i) lysine provides (at least) one of the

TABLE 1 Actual and putative (red or purple) functional annotations of proteins encoded by the 24 ORFs in the formycin BGC found in *Streptomyces kaniharaensis* SF-557.

Protein	No. of residues	Functional annotation
ForD	449	FAD-dependent oxidoreductase
ForE	342	FAD-dependent oxidoreductase
ForH	196	Phosphoribosylaminoimidazolecarboxamide formyltransferase
ForA	423	Adenylosuccinate synthetase
ForF	333	NAD-dependent hydroxyacid dehydrogenase
ForG	210	RNA polymerase $\sigma$ -70 factor
ForI	423	PLP-dependent aminotransferase
ForJ	677	MetRS homolog (N-N bond formation)
ForK	443	L-lysine 6-monooxygenase
ForL	379	L-lysine dehydrogenase
ForM	420	Phosphoribosylglycinamide synthetase
ForN	127	Hypothetical protein
ForO	645	Acylpeptide hydrolase
ForP	188	Nudix hydrolase
ForQ	396	Amidohydrolase
ForR	374	FAD-dependent oxidoreductase
ForS	538	FAD-dependent oxidoreductase
ForB	474	Adenylosuccinate lyase
ForT	341	C-glycoside synthase
ForU	408	Phenylpropionate dioxygenase
ForV	185	FMN reductase
ForC	333	SAICAR synthetase
ForW	936	Fe-S protein, lactate dehydrogenase homolog
ForX	295	Ribokinase

nitrogen atoms in the pyrazole moiety (Ochi et al., 1976), and ii) four carbon atoms in the pyrazolopyrimidine ring are obtained from glutamate (or a related metabolite such as 2-oxoglutarate) (Ochi et al., 1979).  $^{14}\text{C}$ -incorporation studies also demonstrated that 5'-phosphoribosyl-1'-pyrophosphate (PRPP) is the source of the ribose ring in the formycins (Kunimoto et al., 1971). Glutamate is a biosynthetic precursor of other C-nucleoside natural products (Elstner and Suhadolnik, 1972; Isono and Suhadolnik, 1977), including pyrazofurin A 4 (Figure 1A) (Buchanan et al., 1980; Suhadolnik and Reichenbach, 1981); the latter finding has subsequently proved essential for efforts to understand the chemistry of formycin biosynthesis (*vide infra*) (Ren et al., 2019; Zhang L. et al., 2020).

### 3.1 The gene cluster encoding enzymes that mediate formycin biosynthesis

After approximately 35 years of little progress towards elucidating the metabolic pathway leading to the formycins,

whole genome sequencing of *S. kaniharaensis* SF-557 allowed both our group (Zhu et al., 2020), and (independently) Liu and his co-workers (Ko et al., 2017; Wang et al., 2019), to identify the biosynthetic gene cluster (BGC) encoding the enzymes that mediate formycin synthesis (Figure 1B; Table 1). In addition to exploiting comparative bioinformatics analysis (Watanabe et al., 2018), identification of the BGC relied on the presence of genes encoding homologs to both lysine *N*-hydroxylating monooxygenase (Franceschini et al., 2012) and 4-( $\beta$ -D-ribofuranosyl)aminobenzene 5'-phosphate (RFA-P) synthase, which forms a C-glycoside from PRPP and *para*-aminobenzoic acid (Rasche and White, 1998; Dumitru and Ragsdale, 2004; White, 2011; Bechard et al., 2019) during the biosynthesis of the modified folate methanopterin (White, 1996). Direct experimental evidence was provided, however, by Liu and co-workers (Wang et al., 2019). Thus, using the donor strain ET12567/pUSZ8002 (Paranthaman and Dharmalingam, 2003) to introduce *Streptomyces-Escherichia coli* shuttle vectors (Sun et al., 2009) into *S. kaniharaensis* SF-557, mutant strains were obtained

containing in-frame deletions of the *forC*, *forF*, *forH*, *forL*, *forT*, and *forU* genes. Formycin A production in these deletion mutants was either abolished or significantly diminished, with the exception of the  $\Delta$  *forC* and  $\Delta$  *forL* mutants (Wang et al., 2019). Presumably, other enzymes in the organism can perform the transformations associated with ForC and ForL. Similar experiments to show that ForW and ForX are required in the biosynthetic pathway, however, have not yet been reported. Remarkably, the formycin BGC therefore consists of 24 ORFs, three of which encode two enzymes and a transporter involved in the biosynthesis of coformycin 5, an evolutionarily unrelated nucleoside (Figure 1A) (Ren et al., 2020). Formycin and coformycin (Nakamura et al., 1974), which is a potent inhibitor of adenosine deaminase (ADA) (Frieden et al., 1980), are therefore produced by *S. kaniharaensis* SF-557 at the same time. This co-production of adenosine-like, non-canonical nucleosides and ADA inhibitors is frequently observed, and may represent a general mechanism to prevent ADA-catalyzed deamination of nucleoside-containing natural products (Xu et al., 2018).

The large number of open reading frames (ORFs) in the formycin BGC was unexpected given the similarity of this natural product to adenosine 6 (Figure 1A), which is formed by microorganisms from  $\alpha$ -D-ribose-5'-phosphate in only eleven enzyme-catalyzed steps (Buchanan and Hartman, 1959; Kappock et al., 2000). The enzymes ForA, ForB, ForC, and ForH are indeed homologs of those that build the six-membered ring in purine nucleobases (Ko et al., 2017). Moreover, some of the enzymes encoded in the formycin BGC are homologous to those that mediate pyrazofurin A biosynthesis, suggesting that the pathways to formycin and pyrazofurin A (Figure 1A) proceed via a series of common intermediates (Ren et al., 2019; M; Zhang et al., 2020b; Zhao et al., 2020). Due to some confusion in the literature, in this review we use the naming convention for enzymes in the pyrazofurin BGC devised by Ryan and Du (Zhao et al., 2020). Thus, genes encoding a lysine  $N^6$ -hydroxylating monooxygenase are named ForK/PyrM and a methionyl-tRNA synthase homolog are denoted ForJ/PyrN (M. Zhang L. et al., 2020; Zhao et al., 2020). The function of these two enzymes, which are found in other microbial pathways leading to natural products possessing an N-N bond (He et al., 2022; Matsuda et al., 2022a; Twigg et al., 2019; Zheng et al., 2024), is to couple lysine to glutamate in the early stages of formycin and pyrazofurin 4 (Figure 1A) (Zhao et al., 2021). Similarly, the genes ForT and PyrE encode homologous enzymes that catalyze C-C bond formation (Gao et al., 2020; Ren et al., 2019; M; Zhang et al., 2020b).

### 3.2 Early steps in the pathway: Building the N-N chemical bond

The deduced primary structures of ForK, and the homologous enzyme PyrM, identify these enzymes as lysine  $N$ -hydroxylating monooxygenases, similar to the well-characterized enzyme used in kutzneride biosynthesis (Neumann et al., 2012; Setser et al., 2014). ForK seems likely to use dioxygen to hydroxylate the  $\epsilon$ -amino group of lysine 7 (Supplementary Figure S1A) in an interesting reaction that likely proceeds via homolytic O-O cleavage in a flavin hydroperoxide intermediate (Badiyan et al., 2015). Work in our

group has confirmed that ForK is an FAD-dependent enzyme for which NAD(P)H is the reducing agent. As observed for other microbial FAD-dependent  $N$ -hydroxylating monooxygenases (Mügge et al., 2020), ForK is a tetramer at high concentration but becomes dimeric as the protein concentration is decreased.

Given that the hydroxy substituent is a leaving group, especially if activated by acetylation or phosphorylation, the chemical logic of hydroxylating the side chain nitrogen is to activate it for nucleophilic attack by an amine to form an N-N bond. This transformation is likely accomplished by ForJ, based on recent studies of the catalytic activity of the homologous enzyme PyrN in pyrazofurin A biosynthesis, which mediates the coupling of  $N^6$ -hydroxylysine 8 and glutamate 9 via an N-N bond (Supplementary Figure S1B) (Zhao et al., 2021). As is the case for ForK, PyrN is composed of two domains, one of which is homologous to methionyl-tRNA synthetase (MetRS) (Mechulam et al., 1999). The other, smaller domain binds one  $Zn^{2+}$  ion and is a member of the cupin superfamily of enzymes (Dunwell et al., 2000). Elegant experiments using truncated PyrN variants showed that the MetRS-like domain mediates formation of a reactive ester 10 (Supplementary Figure S1B), which undergoes acyl-transfer to form an N-hydroxyamide 11 in the absence of the cupin domain (Zhao et al., 2021). Full-length PyrN, however, yields the hydrazine-containing intermediate 12 when incubated with ATP, glutamic acid and  $N^6$ -hydroxylysine (Supplementary Figure S1B) (Zhao et al., 2021). Thus, the cupin domain plays an essential role in promoting N-N bond formation. Although PyrN could not be crystallized, genome mining identified a cupin protein from *Rhodococcus jostii* RHA1 (also called RHS1) that catalyzes N-N bond formation (Zhao et al., 2021) and for which an X-ray crystal structure is available showing the presence of a  $Zn^{2+}$ -binding site (PDB: 5UQP). QM/MM calculations support a mechanism in which N-N bond formation takes place by reaction of the ester formed by the MetRS-like domain and a second glutamate molecule, which are both coordinated to the  $Zn^{2+}$  center (Zhao et al., 2021). The N-N bond formation in the proposed mechanism, however, proceeds via a formal 6-endo-tet  $S_N2$  reaction, which is technically disfavored by Baldwin's rules (Baldwin, 1976; Gilmore et al., 2016). The calculated barrier for this PyrN-catalyzed step, which is 13.3 kcal/mol, remains to be validated by kinetic experiments.

Based on their sequence homology to a monooxygenase encoded by the Spb39 gene (Matsuda et al., 2017), which forms hydrazinoacetic acid in the pathway leading to a dipeptide natural product containing an N-N bond (Matsuda et al., 2017), ForL and PyrL are probably FAD-dependent monooxygenases, which carry out C-H abstraction on 12 to give an intermediate that spontaneously breaks down to give the aldehyde 13 and hydrazinoglutamate 14 (Supplementary Figure S1B). The detailed kinetic and structural characterization of these enzymes, however, has not yet been reported. This machinery for making N-N bonds appears to be conserved in bacteria to make a wide variety of natural products in addition to pyrazofurin A and the formycins (Matsuda et al., 2022b; Matsuda and Wakimoto, 2024).

### 3.3 Assembling the C-nucleoside

ForT is a C-glycoside synthase that couples PRPP 15 with 4-amino-1*H*-pyrazole-3,5-dicarboxylate (APDA) 16 (Supplementary

Figure S2A) (Ren et al., 2019). Remarkably, the very closely related molecule 4-hydroxy-1*H*-pyrazole-3,5-dicarboxylate (HPDA) **17** is not a substrate, despite the homologous enzyme, PyrE, using both pyrazoles (M. Zhang L. et al., 2020). As a result, HPDA **17** is almost certainly converted to the APDA precursor **19** in the preceding step of the pathway by the PLP-dependent enzyme ForI using L-aspartate as a nitrogen source (Supplementary Figure S2B) (Gao et al., 2019). From an evolutionary perspective, ForT is structurally homologous to homoserine kinase, thereby placing it within the GHMP kinase superfamily of enzymes (Zhou et al., 2000). Using a combination of X-ray crystallography and kinetic studies of a series of ForT variants (Gao et al., 2020; Li et al., 2023), we have been able to obtain evidence that C-C bond formation proceeds via electrophilic substitution to form a tetrahedral intermediate. Subsequent decarboxylation then yields the C-nucleoside **18** (Supplementary Figure S2A), which can be elaborated to a pyrazolopyrimidine (Supplementary Figure S3). The crystal structure of the T138V ForT variant (in which Thr-138 is replaced by valine) bound to APDA **16** and MgATP reveals that the pyrazole substrate forms very few interactions with the enzyme, being held in place by hydrogen bonds to the carboxylate that is retained in the product, thereby correctly positioning APDA relative to the reactive C-1' carbon of PRPP **15** (Li et al., 2023). The only other APDA/ForT interaction, which is mediated by a water molecule, involves a pyrazole nitrogen and the side chain carboxylate of Glu-221. It is therefore possible that Glu-221 acts as a general base to activate the pyrazole for nucleophilic attack, and replacing this residue with alanine does indeed yield an inactive ForT variant (Li et al., 2023). Given that ForT does not make hydrogen bonds with the amino substituent of the pyrazole, elucidating the mechanism by which it can discriminate between APDA **16** and HPDA **17** will likely require computational strategies (Zhang et al., 2023).

The lack of direct APDA/ForT interactions, however, raises the question of whether the enzyme can couple alternate substrates to PRPP (Gong et al., 2021; Pfeiffer and Nidetzky, 2023A; Pfeiffer and Nidetzky, 2023B). To date, we have shown that ForT can accept an isomeric pyrazole, but the product appears to be the N-nucleoside rather than a C-nucleoside (Li et al., 2023). It is possible that pyrroles may also be substrates for the coupling reaction, in the light of recent work on the biosynthesis of showdomycin (Ren et al., 2021), another C-nucleoside with interesting biological activity (De Clercq, 2016).

### 3.4 The final steps of the pathway

Elaboration of the pyrazole-containing C-nucleoside **18** to formycin B 5'-monophosphate **21** is accomplished by an identical series of steps to those used in the *de novo* formation of inosine 5'-monophosphate (IMP) (Supplementary Figure S3) (Ko et al., 2017). The enzymes ForC, ForB, ForH and ForA, which carry out these steps, are therefore homologs of those used in purine biosynthesis (Kappock et al., 2000). In a surprising observation, however, ForH is a truncated form of PurH, the enzyme that forms the pyrimidine ring during purine biosynthesis (Kappock et al., 2000). As ForH is composed only of a cyclohydrolase domain similar to that present in PurH (Wang et al., 2019), it does not exhibit any formylase activity and cannot convert the pyrazole amide **20** into the N-formylated intermediate

**21** that must be cyclized to yield the pyrazolopyrimidine ring in formycin B 5'-monophosphate **22** (Supplementary Figure S3). As shown in a series of elegant experiments by Liu and co-workers, the conversion of **20** into **21** is catalyzed by PurH. On the other hand, although PurH catalyzes the synthesis of the formylated intermediate **21**, it cannot use this compound as a substrate. ForH must therefore catalyze the cyclization of **21** to yield formycin B 5'-monophosphate **22** (Wang et al., 2019). Thus, formycin production depends on the presence of PurH, an enzyme required in purine biosynthesis. The enzymes ForA and ForB then convert formycin B 5'-monophosphate **22** into formycin A 5'-monophosphate **23** using identical amination chemistry used in the synthesis of AMP from IMP in purine biosynthesis (Kappock et al., 2000). Formycin B 5'-monophosphate **22** is also the precursor of oxoformycin B **3** (Figure 1A) (Sawa et al., 1968) although the enzyme that oxidizes the pyrazolopyrimidine ring has not yet been identified. Presumably, it is homologous to IMP dehydrogenase (Hedstrom, 2009). Formycin A, formycin B and oxoformycin B are isolated as the nucleosides, however, meaning the 5'-phosphates must be removed by phosphatases prior to release of the C-nucleosides into the environment. The apparent absence of a gene encoding a phosphatase in the formycin BGC implies that these dephosphorylation reactions are catalyzed by one, or more, phosphatases already present in the cell.

## 4 Discussion

Despite the progress made in understanding the metabolic origins of the N-N and C-nucleoside bonds in the formycins, functions for only nine of the 24 proteins encoded in the formycin BGC have been elucidated. Even excluding the gene for a putative transporter that mediates excretion of these C-nucleoside natural products, this leaves a remarkable number of enzymes seemingly required for the conversion of hydrazinoglutamate **14** into HPDA **17**. As a result, there is little consensus regarding the reactions needed to bridge these two intermediates in the biosynthetic pathway. The key chemical problems are to form a new C-N bond at C-4 and to hydroxylate C-3 of hydrazinoglutamate **14**. In principle, both reactions can be accomplished by oxidizing **14** to obtain an intermediate containing a double bond between C-3 and C-4 but no such desaturase has been identified. The latest proposal therefore postulates a dehydropyridine intermediate, which is easily oxidized to the pyridine **24** (Supplementary Figure S4). Enzyme-catalyzed ring-opening followed by C-N then yields the target pyrazole skeleton (Ren et al., 2019). Although this "simple route" appears consistent with annotated oxygenases and hydroxylases encoded in the cluster (Table 1), C-N bond formation to give the pyrazole proceeds via an energetically disfavored five-endo-trig reaction (Baldwin, 1976; Gilmore et al., 2016). An approach based on in-frame deletion or modification of specific genes in the cluster (Alberti and Corre, 2019), as described for *S. kaniharaensis* by Liu and co-workers (Wang et al., 2019), coupled to identifying intermediates seems the most promising strategy to resolve the issue (Caesar et al., 2021). Whatever the outcome of such studies, the chemistry used by microorganisms to make and manipulate heterocyclic ring systems will continue to surprise and fascinate natural product chemists (McCarty et al., 2009; Palmu et al., 2017; Kong et al., 2019; Ren et al., 2022).

## Author contributions

NR: Writing—original draft, Writing—review and editing. JN: Writing—review and editing.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchbi.2024.1428646/full#supplementary-material>

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