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Structure–Activity Relationship for the Picolinamide Antibacterials that Selectively Target *Clostridioides difficile*

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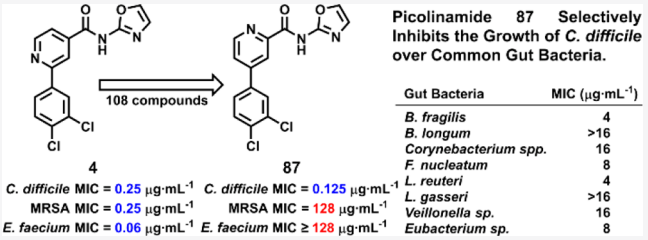
ABSTRACT: *Clostridioides difficile* is a leading health threat. This pathogen initiates intestinal infections during gut microbiota dysbiosis caused by oral administration of antibiotics. *C. difficile* is difficult to eradicate due to its ability to form spores, which are not susceptible to antibiotics. To address the urgent need for treating recurrent *C. difficile* infection, antibiotics that selectively target *C. difficile* over common gut microbiota are needed. We herein describe the class of picolinamide antibacterials which show potent and selective activity against *C. difficile*. The structure–activity relationship of 108 analogues of isonicotinamide **4**, a compound that is equally active against methicillin-resistant *Staphylococcus aureus* and *C. difficile*, was investigated. Introduction of the picolinamide core as exemplified by analogue **87** resulted in exquisite potency and selectivity against *C. difficile*. The ability of the picolinamide class to selectively target *C. difficile* and to prevent gut dysbiosis holds promise for the treatment of recurrent *C. difficile* infection.



KEYWORDS: *Clostridioides difficile, recurrent C. difficile infection, picolinamide, antibacterial, microbiota*

Clostridioides (formerly known as *Clostridium*) *difficile* is a Gram-positive anaerobic bacterium which causes intestinal infections that can lead to diarrhea, sepsis, and life-threatening colitis.[1−3] Since the outbreak of the hypervirulent strain NAP1/027 in 2000,[4,5] the incidence of *C. difficile* infections (CDIs) continues to trend upward.[6] In 2017 alone, CDI resulted in 223 900 hospitalizations and 12 800 deaths in the United States, with nearly $6.3 billion of estimated healthcare costs.[7,8] A challenge in the treatment of CDI is the formation of spores that are not susceptible to antibiotics, resulting in 24−27% recurrence of CDI.[9,10]

CDI is currently treated with metronidazole (MTZ), vancomycin (VAN), and fidaxomicin (FDX), with VAN and FDX recommended for initial CDI and MTZ used only in mild cases where VAN or FDX is unavailable. Furthermore, MTZ is no longer recommended for repeated or prolonged CDI treatment due to neurotoxicity.[11] The failure rates of these antibiotics are 22.4%, 14.2%, and 12%, respectively.[9,12] In addition, treatment of CDI with MTZ and VAN promotes emergence of vancomycin-resistant enterococci.[13] The perturbation of gut microbiota (gut dysbiosis) caused by antibiotics allows for *C. difficile* colonization.[14] While VAN and FDX have similar clinical cure efficacy, patients treated with FDX had lower recurrence (15.4% for FDX compared to 25.3% for VAN), which was attributed to the higher selectivity of fidaxomicin toward *C. difficile* than to gut microbiota.[15] New antibacterial agents that selectively target *C. difficile* are needed to tackle persistent and recurrent CDI.

We discovered the isonicotinamide antibacterial **4** during the structure–activity relationship (SAR) exploration on the cinnamonitrile class of antibiotic adjuvants (compounds **1** and **2**, Figure 1) against methicillin-resistant *Staphylococcus aureus* (MRSA).[16] Cinnamonitrile **1**, a protein kinase(s) inhibitor, showed promise as an adjuvant for β-lactam antibiotics and also exhibited modest antibacterial activity.[17] Since azoles are common among protein kinase inhibitors,[17,18] the oxazole was introduced as a favored moiety (compound **3**, Figure 1). Upon insertion of the oxazole, the resulting series of molecules showed respectable Gram-positive antibacterial activity (minimum inhibitory concentration (MIC) $\leq$ 4 μg·mL^{-1} against MRSA strain NRS70). Further modifications of **3** (amide linker, pyridine core, and removal of the bridging oxygen) led to the potent isonicotinamide antibacterial **4**.

Compound **4** was evaluated against the ESKAPE panel (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumo-*

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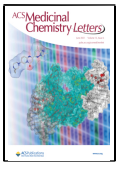


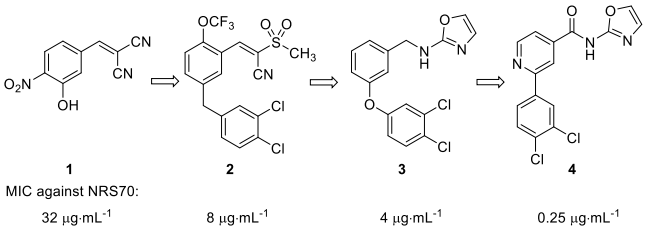


Figure 1. Evolution of the structural template of the cinnamonitrile class of potentiators into the isonicotinamide antibacterial **4**.

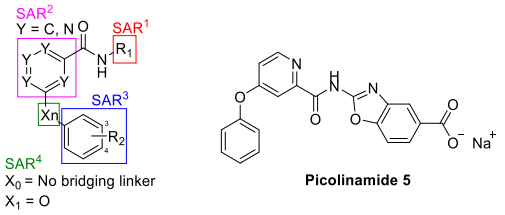
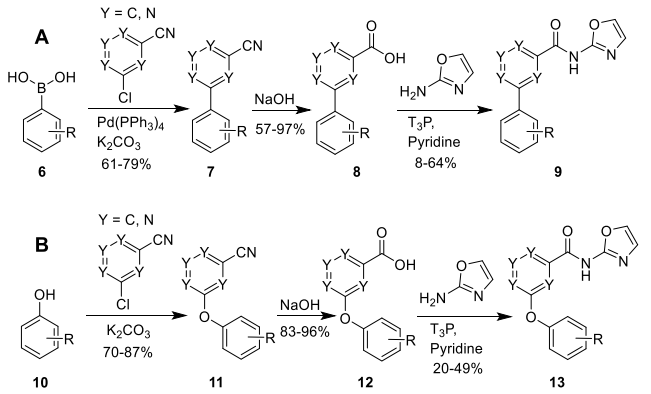


Figure 2. Left, the four sites of structure modification for the SAR study as highlighted by the boxes. Right, the structure of picolinamide **5**.

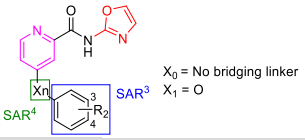
Scheme 1. Synthetic Routes for Accessing (A) Biaryl and (B) Biarylether Derivatives



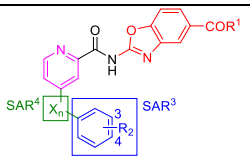
niae, Acinetobacter *baumannii*, Pseudomonas *aeruginosa*, and Enterobacter species). These bacteria account for the majority of human problematic infections.[19,20] The compound was active only against the Gram-positive members of the panel (*E. faecium* and *S. aureus*). We further evaluated **4** against *C. difficile* ATCC 43255, also a Gram-positive bacterium, and obtained an MIC of 0.25 $\mu g \cdot mL^{-1}$. These results provided the impetus for an SAR investigation around this structural template. We report herein the preparation and evaluation of 108 analogues of compound **4** (Figure 2), of which the prototype picolinamide **5** was reported recently.[21] As will be outlined, several of these analogues exhibited exceptional activities against *C. difficile*, both in potency of the antibiotics and in selectivity toward the organism. The aforementioned selectivity in targeting *C. difficile* is extremely important, as it would avoid gut dysbiosis and mitigates recurrent infections, which are at the root of the bad outcome in the clinical setting.

The initial compound of interest, **4**, was prepared in three steps following the general Scheme 1A. The first step was a Suzuki-Miyaura coupling reaction[22] between the chloro-cyanopyridine and arylboronic acid **6**, using $Pd(PPh_3)_4$ as catalyst and potassium carbonate as a base, yielding the cyano-derivative **7**. Alkaline hydrolysis of the nitrile gave the carboxylic acid **8**. A coupling reaction between **8** and 1,3-oxazol-2-amine

Table 1. Activity of Picolinamides Analogues against MRSA and *C. difficile* (SAR^3 and SAR^4)



	SAR^4	SAR^3	MIC ($\mu g \cdot mL^{-1}$) MRSA [a]	*C. difficile* [b]	Selectivity [c]
87	X_0	3,4-Cl	128	0.125	1024
88		4-Cl	128	4	32
89		4-F	64	1	64
90		3,4-F	128	0.50	256
91		3-Cl, 4-F	128	0.25	512
92		4-Cl, 3-F	128	0.25	512
93		3-F	64	0.50	128
94		3,5-F	64	1	64
95		4-OCF_3	128	0.25	512
96		3-OCF_3	≥256	0.50	≥512
97		4-CF_3	128	0.25	512
98		3-CF_3	64	0.50	128
106	X_1	3,4-F	64	1	64
107		3-F	≥256	1	≥256
108		3-OCF_3	64	1	64
109		4-OCF_3	128	1	128
110		4-SCF_3	≥256	1	≥256
111		4-OMe	≥256	1	≥256



	$SAR^{3/4}$	R^1			
5 [d]	X_1, R_2 = 3-OCF_3	ONa	16	0.125	128
114	X_1, R_2 = 3,4-F	OMe	64	0.25	256
115		OH	64	0.50	128
116		ONa	64	0.50	128
118	X_0, R_2 = 4-OCF_3	OH	8	0.03	256
119	X_0, R_2 = 3-OCF_3	OH	16	0.03	512

[a]NRS70 strain, resistant to erythromycin and spectinomycin. [b]ATCC 43255, ribotype 087. [c]Selectivity is defined as $MIC_{MRSA}/MIC_{C.difficile}$. [d]Reported previously[21] and included for comparison.

(or other amines) using propanephosphonic anhydride (T3P)[23] as the coupling reagent led to derivatives **9**. The preparation of compounds possessing the diarylether moiety followed a similar synthetic route, but using an initial nucleophilic aromatic substitution of a chloro-cyanopyridine with a phenol derivative (Scheme 1B). We used the general synthetic route to derivative **13**[21] for syntheses of compounds **112**−**119**.

The syntheses produced 108 analogues corresponding to diversification at four structural regions (Figure 2, left structure with four different colored boxes). A representative *C. difficile* strain (ATCC43255) along with MRSA strain NRS70 were chosen for MIC evaluation. Compounds with MICs of ≤8 $\mu g \cdot mL^{-1}$ against *C. difficile* were considered active.

SAR^1 involved modifications of the oxazole ring in lead compound **4**, a main substructure that defines the antibacterial activity of the isonicotinamides. Compounds **14**−**31** were prepared. The majority of these compounds retained activity against both bacteria, albeit with poor selectivity (Table S1).

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Table 2. XTT IC_{50} of Selected Picolinamides Analogues with HeLa Cells

	IC_{50} ($\mu g \cdot mL^{-1}$)
4	79.9 ± 1.0
5	179.3 ± 3.6[a]
87	95.2 ± 1.0
89	170.2 ± 2.5
90	NT at 64[b]
92	NT at 256[b]
93	NT at 64[b]
94	40.1 ± 1.6
97	135 ± 8.6
98	114.8 ± 2.2
99	37.0 ± 1.0
106	105.9 ± 2.1
107	88.0 ± 2.3
108	93.3 ± 1.7
109	77.4 ± 2.3
110	91.6 ± 1.3
114	53.3 ± 3.6
116	NT at 128[b]
118	79.9 ± 6.3
119	123.6 ± 3.3

[a]Reported previously[21] and included here for comparison. [b]NT, no detectable toxicity at the specified concentration.

The SAR study then focused on optimizing the phenyl ring (SAR^3) with respect to the central pyridine ring (SAR^2), while leaving the oxazole group intact. Most of the compounds containing isonicotinamide (**32**−**74**, Table S2) were active against *C. difficile*, however the selectivity between MRSA and *C. difficile* was <16, except for the nitro-containing analogues (**49**−**50**). The 2,6-substituted picolinamides (**75**−**80**) were inactive.

The most compelling modification was 2,4-substitution of the picolinamide (**87**−**98**). This substitution imparted the desired selectivity toward *C. difficile* compared to MRSA. Picolinamide **87**, a constitutional isomer of compound **4**, was inactive against MRSA (MIC = 128 $\mu g \cdot mL^{-1}$) yet active against *C. difficile* (MIC = 0.125 $\mu g \cdot mL^{-1}$). This exquisite selectivity of 1024-fold was achieved by the mere repositioning of one nitrogen atom.

Table 4. Drug Concentrations in Fecal Samples of Selected Compounds

	MIC, $\mu g \cdot mL^{-1}$[a]	selectivity[b]	dose, $mg \cdot kg^{-1}$	$\mu g\ g^{-1}$ feces	conc. feces/MIC
4	0.25	1	20	2.8	11
5[c]	0.125	128	20	13	108
87	0.125	1024	10	0.79	6.6
107	1	≥256	20	2.4	2.4
108	1	64	20	49	49
116	0.50	128	20	3.7	7.4

[a]*C. difficile* strain, ATCC 43255, ribotype 087. [b]Selectivity is defined as $MIC_{MRSA}/MIC_{C.\ difficile}$. [c]Reported previously; included for ease of comparison.

Moreover, compounds **87**−**98** lacked activity against MSSA and *E. faecium* (Table S3).

Introduction of an ether linkage between the pyridine core and the phenyl ring resulted in MIC values for *C. difficile* of ≤1 $\mu g \cdot mL^{-1}$. The picolinamides (**106**−**111**, Table 1) had significantly higher selectivity for *C. difficile* than the isonicotinamides (**99**−**105**, Table S2). Unfortunately, the introduction of the ether linkage did not increase the antibacterial activity of the picolinamides. These SAR studies led us to introduce a carboxylate, either as a salt or as an ester (compounds **112**-**119**), which improved water solubility of the family while still maintaining potent activity against *C. difficile* (0.12 $\mu g \cdot mL^{-1}$ for **5** and 0.50 $\mu g \cdot mL^{-1}$ for **116**).

The XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-carboxanilide) assay[24] with HeLa cells was performed on representative compounds (Table 2). Most of the compounds tested had IC_{50} values >64 $\mu g \cdot mL^{-1}$ (except for picolinamides **94** and **114**, and isonicotinamide **99**). Compound **87** had an IC_{50} of 95.2 ± 1.0 $\mu g \cdot mL^{-1}$, 760-fold higher than its MIC (0.125 $\mu g \cdot mL^{-1}$).

We evaluated the activity of the picolinamides against the common gut bacteria: *Bacteroides fragilis*, *Bifidobacterium longum*, *Corynebacterium* spp., *Fusobacterium nucleatum*, *Lactobacillus reuteri*, *Lactobacillus gasseri*, *Veillonella* sp., and *Eubacterium* sp.[25−30] An optimum antibacterial will target selectively the pathogenic bacterium and not the natural gut

Table 3. MIC Values ($\mu g \cdot mL^{-1}$) for MTZ, VAN, FDX, and Compounds 4, 5, 87, and 108 against Major Gut Bacteria

	MTZ	VAN	FDX	**4**	**5**[a]	**87**	**108**
C. difficile ATCC43255	0.25	0.5	0.0625	0.25	0.125	0.125	1
Bacteroides fragilis[a]	1	16	>32	1	4	4	8
Bifidobacterium longum[b]	1	0.25	<0.01	>16	16	>16	16
Corynebacterium spp.[c]	>32	0.5	<0.0625	0.5	8	16	>16
Fusobacterium nucleatumi[d]	2	0.25	<0.0625	8	4	8	8
Lactobacillus reuteri[e]	>32	>32	>32	8	4	4	4
Lactobacillus gasseri[f]	>32	1	2	>16	>16	>16	>16
Veillonella sp.[g]	2	>32	8	4	16	16	16
Eubacterium sp.[h]	1	2	16	>16	16	8	8

[a]Strain HM-709, a Gram-negative, anaerobic bacterium that is commensal and critical to host immunity and a minor component of the human gut microflora (<1%). [b]Strain HM-846, an anaerobic, nonsporulating Gram-positive bacterium commonly found in the normal human intestinal microflora isolated from human feces. [c]Strain HM-784, an aerobic or facultatively anaerobic Gram-positive bacterium that occurs in the mucosa and normal skin flora of humans and animals. [d]Strain HM-992, an anaerobic, nonsporulating Gram-negative bacterium commonly found in the gastrointestinal tract. [e]Strain HM-102, an anaerobic Gram-positive bacterium commonly found in the normal human gastrointestinal tract, and used frequently as a probiotic to maintain the balance of gut microbial flora. [f]Strain HM-644, a facultative, anaerobic Gram-positive bacterium commonly found in the normal human gastrointestinal tract, used frequently in yogurt production as a probiotic to suppress *Helicobacter pylori* infections. [g]Strain HM-49, a nonsporulating Gram-negative bacterium commonly found in the intestinal tract of humans and animals. [h]Strain HM-178, an anaerobic, nonsporulating Gram-positive bacterium commonly found in the gastrointestinal flora of humans and animals.

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flora. The selectivity ($MIC_{gutbacteria}/MIC_{C.difficileATCC43255}$) ranged from 4 to 128 for MTZ, 0.5 to 64 for VAN, 1 to 512 for FDX, 2 to 64 for **4**, 32 to 128 for **5**, 32 to 128 for **87**, and 8 to 16 for **108** (Table 3). Picolinamides **5** and **87** were more selective than **4** and more selective than the clinically used antibiotics VAN, MTZ, and FDX. The selectivity for *C. difficile* ATCC43255 with respect to *Bifidobacterium longum*, a major gut bacterium that is reported to help repress CDI,[27,31] was 128 for **87** compared to 4 for MTZ, 0.5 for VAN, and 0.16 for FDX.

Recurrence of CDI for the less selective VAN is 25%, while that for the somewhat more selective FDX is 15%.[15] Picolinamide **87** is >1000-fold selective. As gut dysbiosis contributes to CDI recurrence, the importance of this high selectivity is self-evident. Selectivity in targeting *C. difficile* emerges as an important attribute as mere potency (low MIC) might become less significant given that a compound such as **87** has already a remarkably very low/potent MIC of 0.125 $\mu g \cdot mL^{-1}$ (nanomolar range).

For the treatment of CDI, compounds that are poorly absorbed or not absorbed and achieve high concentrations in the gut are desirable. We selected compounds **4**, **87**, **107**, **108**, and **116** for in vivo PK studies in mice based on their potency, safety profile, solubility, and selectivity against *C. difficile*. Both plasma and feces were collected and analyzed for levels of the compounds and compared to those of compound **5**. While all the compounds showed nonquantifiable concentrations in plasma, levels in feces were higher (Table 4). The selected compounds showed 2- to 50-fold higher concentrations in the feces over the MIC values.

The lack of selective antibiotics for the treatment of CDI contributes to gut dysbiosis and recurrence of CDI. The picolinamide family of antibacterials shows exquisite potency and selectivity in targeting *C. difficile*. Starting with isonicotinamide **4**, a compound that is equally active against MRSA and *C. difficile*, structure optimization gave the new picolinamide **87** with >1000-fold selectivity for *C. difficile* compared to MRSA (strain NRS70). Compound **87** shows exceptional selectivity against *C. difficile* while lacking activity against other, normal Gram-positive and Gram-negative gut microbiota. As gut dysbiosis contributes to CDI recurrence, the picolinamides have the potential for treatment of recurrent CDI.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00135.

General synthetic procedures; synthetic experimental procedures and characterization data; X-ray data collection method; crystal data and structure refinement for **87**; general biological methods; activity of picolinamide analogues against *C. difficile* and MRSA, MSSA (ATCC 29213), and *E. faecium* (NCTC 7171); PK studies; NMR spectra for representative compounds; and molecular formula strings for the synthesized compounds (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors approve the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

C. difficile, *Clostridioides difficile*; CDI, *C. difficile* infection;; ClogP, calculated logP value; FDX, fidaxomicin; MDZ, metronidazole; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*; $Pd(PPh_3)_4$, tetrakis(triphenylphosphine)palladium(0); PK, pharmacokinetics; SAR, structure−activity relationship; T3P, propylphosphonic anhydride; TLC, thin layer chromatography; VAN, vancomycin; XTT, (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-carboxanilide).

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