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Antibacterial activity of 2-amino-4-hydroxypyrimidine-5-carboxylates and binding to *Burkholderia pseudomallei* 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase

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ABSTRACT

Enzymes in the methylerythritol phosphate pathway make attractive targets for antibacterial activity due to their importance in isoprenoid biosynthesis and the absence of the pathway in mammals. The fifth enzyme in the pathway, 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase (IspF), contains a catalytically important zinc ion in the active site. A series of *de novo* designed compounds containing a zinc binding group was synthesized and evaluated for antibacterial activity and interaction with IspF from *Burkholderia pseudomallei*, the causative agent of Whitmore's disease. The series demonstrated antibacterial activity as well as protein stabilization in fluorescence-based thermal shift assays. Finally, the binding of one compound to *Burkholderia pseudomallei* IspF was evaluated through group epitope mapping by saturation transfer difference NMR.

Burkholderia pseudomallei is an organism known to cause Whitmore's disease, also known as melioidosis.[1] The organism thrives in tropical regions, being most concentrated in southeast Asia, as well as India, South America, and the Caribbean.[1,2] Melioidosis can be fatal and is the third greatest infectious disease threat to the people of Northeastern Thailand, being surpassed by HIV and tuberculosis.[2] Transmission can occur via ingestion, skin inoculation, and inhalation. To date, no vaccines have progressed to human trials. Vaccines tested in mice have failed to provide long-term immunization.[2] Current treatment requires the injection or intravenous application of ceftazidime or meropenem for as long as twenty weeks. In addition, select strains of *B. pseudomallei* are resistant to ceftazidime.[3] With treatment being tedious and antibiotic resistance developing, new methods of treatment are required.

The methylerythritol phosphate (MEP) pathway is one of two biosynthetic pathways for the precursors of isoprenoids, a large class of compounds numbering over 60,000.[4,5] They serve a variety of essential functions, including protein prenylation, electron transport, peptidoglycan synthesis, aerobic respiration, and membrane stability.[6,7] Isoprenoids are constructed from the five-carbon building block isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP). A separate IPP and DMAPP biosynthetic pathway, called the mevalonate (MVA) pathway, was discovered in yeast in the 1950s.[6,8] The MEP pathway was discovered decades later in the 1990s.[9] Both pathways are linear and do not share common enzymes. The MEP pathway is present in most eubacteria, all Gram-negative bacteria, mycobacteria, apicomplexans, and the plastids of plants.[4,6,10] Meanwhile, archaebacteria, most eukaryotes, and plants (in the cytosol) use the MVA pathway.[6] Although higher plants possess the capacity to perform biosynthesis using both pathways, the compounds synthesized by each pathway serve different functions and thus do not act as redundant pathways. Because the pathways are distinct and linear, an inhibitor of an enzyme in the MEP pathway could effectively cause bacteriostatic or bactericidal activity in one or more organisms that utilize the MEP pathway while leaving organisms that utilize the MVA pathway, such as mammals, unharmed.

2-*C*-Methyl-D-erythritol-2,4-cyclodiphosphate synthase (IspF) is the fifth of seven enzymes in the MEP pathway. It is a homotrimer with three active sites at the interfaces of the monomers.[4,9] Intramolecular cyclization of the substrate, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate, occurs through nucleophilic attack by the 2-phosphate on the 4-phosphate to produce 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) and cytidine monophosphate (Fig. 1).[9] The

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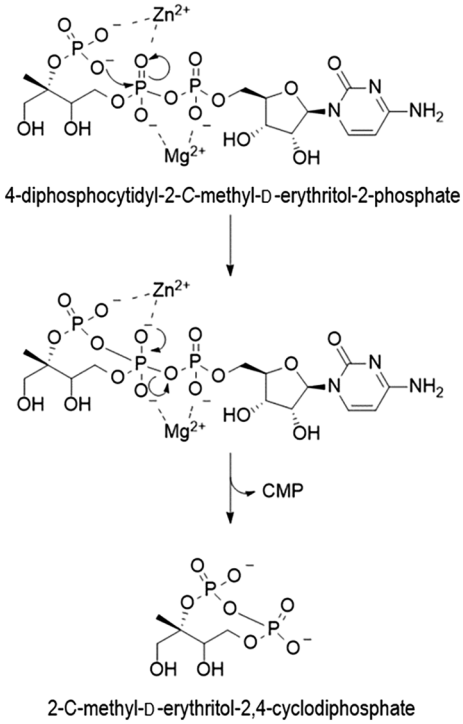


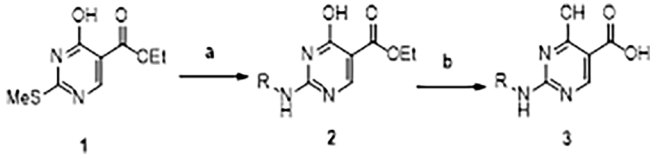
Fig. 1. Proposed mechanism for the MEcPP formation catalyzed by IspF.

active site is composed of four regions: the ribose pocket (Pocket I), the lipophilic flexible loop (Pocket II), the cytidine-binding pocket (Pocket III), and the cation region, where two divalent cations are vital for catalysis. The cations vary but are often Zn^{2+} and Mg^{2+} or Zn^{2+} and Mn^{2+}.[9,11,12] The Zn^{2+} is held in place by an aspartic acid and two histidine residues, while the Mg^{2+} or Mn^{2+} ions interact with the substrate phosphate groups.

Enzyme inhibitors regularly incorporate metal-binding groups (MBGs) that coordinate active site ions. For example, HIV integrase inhibitors target Mg^{2+} in the active site of HIV integrase.[13,14] Here, a *de novo* design of ligands that target the zinc ion in the active site of *Bp*IspF was pursued. Zinc-binding groups (ZBGs) are well-studied based on the numerous important enzymes containing zinc. The benchmark zinc-binding group is the hydroxamic acid, which chelates zinc in either a tetrahedral or trigonal bipyramidal geometry.[15,16] Unfortunately, the majority of hydroxamic acid compounds fail in clinical trials due to issues such as toxicity, selectivity, metabolism by glucuronidation and sulfation, and low oral availability.[4,16] However, cyclic isosteres of hydroxamic acid that target matrix metalloproteinases (MMP), classic zinc-containing enzymes, possessed higher affinity for zinc due to their greater stability.[15] ZBGs have been found to bind to zinc in monodentate, bidentate, and a monodentate-bidentate intermediate in crystal structures.[15–17]

Observing the binding of salicylic acid and methyl salicylate[16] as well as the displacement of cytosine analogs by 2-aminopyridines,[18] a *de novo* design was conceived to bind to Zn^{2+}. The phenyl ring of the salicylic acid was replaced with a pyrimidine isostere. This scaffold consisted of a 2-amino-4-hydroxypyrimidine-5-carboxylate, with the anticipated bidentate binding interaction with IspF's active site zinc ion through the 4-hydroxyl oxygen and the 5-carboxylate carbonyl oxygen, while accumulating additional interactions with the lipophilic flexible loop through substituents on the 2-amino nitrogen. The thioether **1** was reacted with a variety of amines to yield the 2-aminopyrimidine analogs **2,** subsequent ester hydrolysis with NaOH followed by acidification with HCl provided the acids **3** in generally good yields (Scheme 1).

The antibacterial activity of the synthesized compounds was evaluated by Kirby Bauer (KB) disk diffusion assays against nine organisms:



Scheme 1. Reagents and conditions: a) RNH_2, EtOH, reflux, 30–88% yield; b) 1. NaOH, H_2O reflux, 2. HCl 65–99% yield.

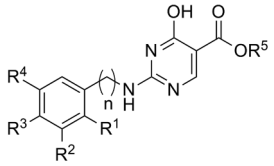
Burkholderia thailandensis (*Bt*), *Pseudomonas aeruginosa* (*Pa*), *Bacillus cereus* (*Bc*), *Escherichia coli* (*Ec*), *Mycobacterium smegmatis* (*Ms*), *Klebsiella pneumoniae* (*Kp*), *Corynebacterium xerosis* (*Cx*), *Corynebacterium pseudodiptheriticum* (*Cp*), and *Micrococcus luteus* (*Ml*). The compounds were tested for binding to IspF using a fluorescence-based thermal shift (FTS) assay of Q151E *Bp*IspF. This mutant retains functional activity similar to the highly stable (T_m > 95 °C) wild-type, yet reduces the T_m to allow for use in the FTS assay. The results are given in Tables 1 and 2.

While not all compounds displayed potent antimicrobial activity through bacteriostatic action, none of the nine organisms were completely immune to every compound. Each organism was susceptible to at least one compound in the series. Also, only one compound failed to produce positive antimicrobial results against any organism, compound **24**.

The compounds with the greatest range of antimicrobial activity were **5**, **22**, and **39**, affecting the growth of seven different organisms. They were all effective to some extent against *B. thailandensis*, *B. cereus*, *E. coli*, *M. smegmatis*, and *K. pneumoniae*, but varied on *P. aeruginosa*, *C. xerosis*, and *C. pseudodiptheriticum*. None of these three compounds displayed activity against *M. luteus*. Structurally, there is no clear pattern that may lead to a broadly effective compound. Compounds **5** and **22** had a carboxylic acid substituent in the 5-position of the pyrimidine, while **39** had an ethyl ester in this position. Compounds **5** and **22** also contained the halogens bromine and chlorine, respectively, but the position of the halogens varied as did the length of the linker between the phenyl ring and the pyrimidine ring. However, bond rotation may make it possible for the two compounds to achieve similar binding if antibacterial activity is proceeding through the same mechanism for both compounds. Compound **39** has a 5-indazole substituent and may be achieving antibacterial activity through a different route.

B. thailandensis, a biosafety level 2 organism (BSL2), was used in place of *B. pseudomallei* (BSL3) and is widely accepted as a surrogate for *B. pseudomallei*. Both were derived from a common ancestor, having 85% gene conservation and highly syntenic genomes.[19] Of the 40 compounds tested, 26 compounds showed antibacterial activity against *B. thailandensis* (Tables 1&2). The most potent of these compounds often contained halogens on a ring attached to the amino group. The position of the halogen on the ring did not make a significant difference, although this is likely due to the unhindered bond rotation in the linker. Likewise, the length of the linker between the nitrogen external to the pyrimidine ring and the phenyl ring varied, the most potent compounds containing all three lengths used: 0, 1, or 2 methylenes. Assuming IspF is successfully being targeted, the hydroxyl and carboxylic acid or ethyl ester substituents on the pyrimidinyl ring are hypothesized to bind the Zn^{2+} in the IspF active site, while the amino substituents extend into and interact with additional active site residues. A carboxylic acid group in the 5-position of the pyrimidine was generally more effective than the ethyl ester, as shown by the comparison between **26** and **27**, **30** and **31**, **32** and **33**, **40** and **41**, and marginally for **22** and **23**. This trend does not hold out for every set of carboxylic acids versus ethyl esters, showing that the amino group is having an impact on these results. While the binding of salicylic acid to zinc in a small molecule crystal structure showed binding to zinc through the carboxylic acid oxygens, methyl salicylate showed binding to zinc through the phenolic oxygen and carbonyl oxygen.[16] Therefore, the interactions or steric

Table 1
Kirby Bauer and thermal shift results of 2-amino-4-hydroxypyrimidine-5-carboxylates against nine organisms and *Bp*IspF Q151E, respectively. The concentration of each compound was 0.5 mM in the Kirby Bauer assays and 100 μM in thermal shift assay.



							Zone of inhibition (diameter, in mm)								ΔT_M (°C)
No	n	R^1	R^2	R^3	R^4	R^5	*Bt*	*Pa*	*Bc*	*Ec*	*Ms*	*Kp*	*Cx*	*Cp*	*Ml*	
4	0	H	H	H	H	H	0	0	0	0	15	0	0	0	0	5.0
5	0	H	H	Br	H	H	13	0	12	15	17	12	16	16	0	3.2
6	0	H	H	I	H	CH_2CH_3	0	0	13	NT	NT	NT	NT	NT	NT	NT
7	0	H	C_6H_5	H	H	CH_2CH_3	0	0	15	0	15	0	0	0	0	4.0
8	0	H	H	C_6H_5	H	H	0	0	12	0	15	15	0	0	0	2.7
9	0	H	H	C_6H_5	H	CH_2CH_3	0	0	11	0	0	0	0	0	0	3.0
10	0	H	H	4-Morpholyl	H	CH_2CH_3	9	11	6	0	14	8	0	0	0	6.0
11	0	H	H	1-Piperdinyl	H	H	11	0	12	11	0	10	0	0	0	5.5
12	0	H	H	1-Piperdinyl	H	CH_2CH_3	14	13	15	19	16	13	0	0	0	4.7
13	0	H	H	4-Pyridyl	H	H	0	0	10	0	0	0	0	0	0	5.5
14	0	H	H	4-Pyridyl	H	CH_2CH_3	0	0	0	0	16	0	0	0	0	2.5
15	0	H	H	CH_2COOH	H	CH_2CH_3	8	0	11	10	0	9	0	0	0	4.5
16	1	H	H	F	H	H	12	0	12	0	0	0	0	0	0	NT
17	1	H	H	F	H	CH_2CH_3	8	0	19	0	0	0	0	0	0	2.0
18	1	H	H	Cl	H	H	11	15	14	14	15	9	0	0	0	2.7
19	1	H	Cl	H	H	CH_2CH_3	11	0	0	0	14	0	0	0	0	2.5
20	1	H	Cl	Cl	H	CH_2CH_3	0	0	12	0	0	0	0	0	0	2.2
21	1	Cl	H	H	H	CH_2CH_3	0	0	10	0	0	0	0	0	15	2.2
22	1	Cl	H	H	Cl	H	10	9	10	11	15	8	0	10	0	NT
23	1	Cl	H	H	Cl	CH_2CH_3	9	0	13	0	0	0	0	0	14	0.7
24	2	H	F	H	H	CH_2CH_3	0	0	0	0	0	0	0	0	0	2.8
25	2	H	H	F	H	CH_2CH_3	9	11	11	0	14	0	0	0	0	0.3
26	2	H	H	Cl	H	H	12	19	NT	0	14	0	0	0	0	NT
27	2	H	H	Cl	H	CH_2CH_3	0	0	7	NT	NT	NT	NT	NT	NT	2.5
28	2	Cl	H	H	H	CH_2CH_3	12	0	9	0	15	0	0	0	0	1.5
29	2	Cl	H	Cl	H	CH_2CH_3	11	0	0	0	0	0	0	0	0	1.5
30	2	H	Cl	Cl	H	H	11	0	13	0	11	8	0	0	0	NT
31	2	H	Cl	Cl	H	CH_2CH_3	0	0	10	0	0	0	0	0	0	2.3

hindrance caused by the 2-amino groups of each compound may change the binding mode in a similar fashion. However, if they are targeting IspF, the pyrimidines may not be targeting IspF alone. One possible alternative method of antibacterial activity is the inhibition of folic acid synthesis, as this is the antitubercular mechanism of action of the related compound,4-aminosalicylic acid (**45**), the antibacterial results of which are included in Table 2.

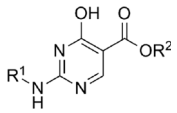
Regarding the results of the FTS assay, there are a few trends that are formed. The highest thermal shifts were often attributed to compounds with two-ring (not fused) amino substituents, for example, **10** (6.0 °C), **11** (5.5 °C), and **13** (5.5 °C). The fact that compounds **10** and **11** do not have aromaticity in the terminal ring indicates that the greater thermal shift is not due to a gained π stacking interaction. Compound **7**, in which the terminal ring is *meta* to the amino group on the internal ring, was found to have a 1 °C higher thermal shift than compound **9**, in which the terminal ring is *para* to the amino group on the internal ring. Comparison of **8** (2.7 °C) with **13** (5.5 °C) and **12** (4.7 °C) with **10** (6.0 °C) shows that having a heteroatom in the 1-position of the terminal heterocycle makes a significant contribution to the thermal shift value.

The thermal shift difference between similar compounds containing either a carboxylic acid or ethyl ester at the 5-position on the pyrimidinyl ring varied between sets of compounds, some having a marginal difference between the two (**8** versus **9**, 2.7 °C and 3.0 °C, respectively) or being the same (**40** versus **41**, 2.5 °C), some favoring the carboxylic acid (**13** versus **14**, 5.5 °C and 2.5 °C, respectively), and some favoring the ethyl ester (**38** versus **39**, 0.2 °C and 3.0 °C, respectively). The binding mode may be different between compounds, as discussed earlier.

Despite the lack of antimicrobial activity, compound **24** did cause a 2.8 °C thermal shift in *Bp*IspF. There are several explanations for discrepancies between the results of the Kirby Bauer assay and the FTS assay. For compounds that gave a positive result in FTS, but not in KB, the analyte may be able to bind to *Bp*IspF, but it is not able to reach its target in the whole-cell organisms, such as lack of cell wall penetration. Alternatively, the compound could be binding IspF, but not inhibiting its activity. It is also possible that it is active against *B. pseudomallei* but not any of the nine organisms tested in the Kirby Bauer assay, but this is unexpected due to the close relation between *B. pseudomallei* and *B. thailandensis*. For compounds that had negligible or weak FTS results, but strong KB results, the compound may be affecting other targets.

Saturation transfer difference (STD) NMR was performed with one of the prominent compounds in the series, compound **13**. Preliminary data by surface plasmon resonance (SPR) demonstrated the binding of compound **13** to wild type *Bp*IspF (*data not shown*). In addition, inhibition of IspF was evaluated using reagents and procedure from Echelon Biosciences.[20] It appeared that compound **13** was weakly inhibiting IspF at micromolar concentrations and partial inhibition was observed. The binding of **13** to IspF was supported by STD NMR, and the results of group epitope mapping are shown in Table 3. The highest saturation transfer was experienced by the proton on the pyrimidine ring, H_E. The saturation received by the other protons on the compound is reported relative to H_E. High saturation transfer for proton H_E supports the zinc-binding as designed. The relative saturation transfer values for protons H_A, H_B, H_C, and H_D are significantly lower. However, observing the decreasing saturation going away from the pyrimidinyl group (H_D to H_C) and increasing toward the pyridyl nitrogen (H_B to H_A) is evidence of an additional interaction occurring with the nitrogen of

Table 2
Kirby Bauer and thermal shift results of synthesized compounds with heterocyclic R^1 substituents against nine organisms and *Bp*IspF, respectively. The concentration of each compound was 0.5 mM in the Kirby Bauer assays and 100 μM in thermal shift assay.



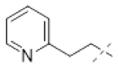
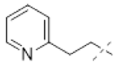
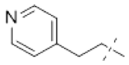
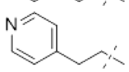
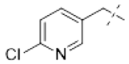
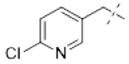
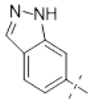
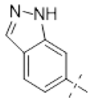
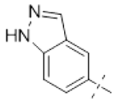
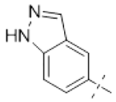
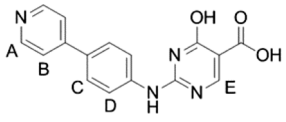
No.	R^1	R^2	Zone of inhibition (diameter, in mm)									ΔT_M (°C)
			Bt	*Pa*	*Bc*	*Ec*	*Ms*	*Kp*	*Cx*	*Cp*	*Ml*	
32		H	10	17	7	0	17	7	0	0	15	NT
33		CH_2CH_3	0	0	8	NT	NT	NT	NT	NT	NT	NT
34		H	10	0	NT	NT	NT	NT	NT	NT	NT	NT
35		CH_2CH_3	4	0	8	NT	NT	NT	NT	NT	NT	NT
36		H	12	10	NT	NT	NT	NT	NT	NT	NT	NT
37		CH_2CH_3	9	0	10	NT	NT	NT	NT	NT	NT	NT
38		H	0	0	9	0	16	9	0	0	0	0.2
39		CH_2CH_3	11	12	10	7	17	12	7	0	0	3.0
40		H	10	0	7	0	14	0	0	0	0	2.5
41		CH_2CH_3	8	10	10	0	10	0	0	0	0	2.5
42		H	7	0	0	0	0	0	0	0	0	0.8
43		CH_2CH_3	11	0	0	0	15	0	0	0	0	1.0
44		CH_2CH_3	NT	NT	NT	NT	NT	NT	NT	NT	NT	2.8
45	4-aminosalicylic acid		12	0	9	11	14	0	0	0	0	NT

Table 3
Relative saturation transfer results for **13** with *Bp*IspF.



Proton	Relative Percent Saturation Transfer
A	35–40%
B	30–35%
C	25–30%
D	35–40%
E	100%

the pyridyl, perhaps as a hydrogen bond acceptor. This is consistent with previous results showing greater interaction with the protein when a heteroatom (O or N) is in this position. Whether that is due to the electronegativity of these heteroatoms or the presence of the lone pairs of electrons they bear is unknown. The dissociation constant of **13** was also calculated by STD NMR and found to be approximately 200 μM. Observed dissociation constants using STD NMR often appear weaker than the true value because of repeated binding by the same molecule to the protein.[21]

In conclusion, the 2-amino-4-hydroxypyrimidine-5-carboxylate series of compounds was designed to target the zinc divalent cation in the active site of IspF using known zinc-binding groups. The pyrimidine ring could then be used to build into new interactions with the rest of the active site using easily varied amino substituents. While no true SAR could be ascertained from the series of compounds, the primary structure-activity relationships were in the functional group located at the 5-position of the pyrimidine as well as the amino substituent. The most potent compounds across the assays contained two-ring cyclic structures, but electron-withdrawing groups could boost potency depending on the placement of the group. The potency against *M. smegmatis* and *B. thailandensis* cannot be ignored. Any compound with significant antibacterial activity against either of these organisms could contribute greatly to antibacterial research, regardless of whether the mechanism

was through IspF. However, analysis by STD NMR with **13** was able to support binding to *Bp*IspF and determine an apparent dissociation constant. One of the most potent compounds could be used as a scaffold in a future series or as part of a fragment merging approach.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.126660.

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