Published in final edited form as:

Bioorg Med Chem Lett. 2015 December 15; 25(24): 5699–5704. doi:10.1016/j.bmcl.2015.10.096.

Synthesis and biological evaluation of pyrazolopyrimidines as potential antibacterial agents

Gashaw M. Goshu^a, Debarati Ghose^b, Joy M. Bain^a, Phillip G. Pierce^{c,d}, Darren W. Begley^d, Stephen N. Hewitt^{d,e}, Hannah S. Udell^{d,e}, Peter J. Myler^d, R Meganathan^b, and Timothy J. Hagen^{a,*}

^aDepartment of Chemistry and Biochemistry, Northern Illinois University, 300 Normal Road, DeKalb, IL 60115, USA

^bDepartment of Biological Sciences, Northern Illinois University, 155 Castle Drive, DeKalb, IL 60115, USA

^cBeryllium, 7869 NE Day Road West, BainBridge Island, WA 98110, USA

^dCenter for Infectious Disease Research, formerly Seattle Biomedical Research Institute, 307 Westlake Ave N Ste 500, Seattle, WA 98109, USA

^eCenter for Emerging and Reemerging Infectious Disease, University of Washington, 750 Republican St., Seattle, WA 98109, USA

Abstract

The fragment FOL7185 (compound **17**) was found to be a hit against IspD and IspE enzymes isolated from bacteria, and a series of analogs containing the pyrazolopyrimidine core were synthesized. The majority of these compounds inhibited the growth of *Burkholderia thailandensis* (*Bt*) and *Pseudomonas aeruginosa* (*Pa*) in the Kirby-Bauer disk diffusion susceptibility test. Compound **29** shows inhibitory activity at 0.1 mM (32.2 µg/mL), which is comparable to the control compound kanamycin (48.5 µg/mL). Compound **29** also shows inhibitory activity at 0.5 mM against kanamycin resistant *P. aeruginosa*. Saturation transfer difference NMR (STD-NMR) screening of these compounds against *Bt*IspD and *Bt*IspE indicated that most of these compounds significantly interact with *Bt*IspE, suggesting that the compounds may inhibit the growth of *Bt* by disrupting isoprenoid biosynthesis. Ligand epitope mapping of compound **29** with *Bt*IspE indicated that hydrogens on 2,4-dichlorophenyl group have higher proximity to the surface of the enzyme than hydrogens on the pyrazolopyrimidine ring.

Graphical abstract

*Corresponding author. Tel.: +1 815 753 1463; fax: +1 815 753 4802. thagen@niu.edu (T. J. Hagen).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Pyrazolopyrimidines; *Burkholderia thailandensis* IspE; *Pseudomonas aeruginosa*; Saturation Transfer Difference; STD-NMR; Antibacterial activity; Center for Infectious Disease Research; Seattle Structural Genomics Center for Infectious Disease; SSGCID

In the pre-antibiotic era, minor injuries and common infections could cause life-threatening illnesses. One of the greatest successes in the 20th century was the discovery of antibiotics, which dramatically improved the quality of life and completely revolutionized modern medicine.^{1,2} Most of the currently used antimicrobial agents were discovered from 1945 to 1960, during the “golden era” of antibiotics. Unfortunately, the discovery of new antimicrobial drugs has significantly declined since the early 1960s.³ The rapid decline of new antimicrobial drugs can be related to several factors. New treatments for diseases such as hypertension and cancer are often more profitable than antibiotic drugs, leading to reduced commercial resource investment in the latter.^{2,4,5} The screening methods, chemical composition of materials and other infrastructure developed for drug discovery in other disease areas do not necessarily translate well into antibiotic research.⁶ The ability of pathogenic organisms to develop resistance mechanisms against chemotherapeutics puts an increased burden on the development of new treatments, as well as the general scientific strategy in targeting such organisms.

Although antibiotic resistance is a natural evolutionary process for bacteria adapting to their environment, it has been significantly accelerated by selective pressure employed by the overuse of antibiotics.¹ As a result, antibacterial resistance to drugs has greatly increased worldwide and poses a global health threat. The World Health Organization warns that if appropriate measures are not taken, much of the past success in combating infectious diseases will be reversed.⁷ There is a general consensus that in order to slow down antimicrobial drug resistance, proper use of antibiotics (avoiding underuse or overuse of antibiotics), is crucial. Additionally, there is renewed interest in the discovery and development of new antimicrobial agents. There are new incentives and programs that encourage the discovery and development of new antimicrobials with the ultimate goal to bring new antibiotics more rapidly to patients in need.^{8–10} Investigation of new metabolic pathways that are essential for the survival of pathogenic microorganisms that could lead to the development of new antimicrobial drugs is vital.

One of the promising targets for the development of new antimicrobial agents is the biosynthesis of isoprenoids. Isoprenoids are one of the most diverse sets of compounds that are vital for all living organisms.^{11,12} Isoprenoids and their precursors are synthesized by

either the mevalonic acid (MVA) pathway or the methylerythritol phosphate (MEP) pathway.^{13–15} Several pathogenic bacteria, including *Mycobacterium tuberculosis* (the causative agent of tuberculosis), *B. pseudomallei* (an overlap select agent and causative agent for melioidosis), and *P. aeruginosa* (a causative agent of nosocomial infections particularly in burn victims and an infective agent for cystic fibrosis patients) use the MEP pathway exclusively to synthesize isoprenoids; humans only possess and use the MVA pathway.^{16–19} This metabolic difference can be exploited to develop drugs that selectively disrupt isoprenoid biosynthesis in pathogenic microorganisms with minimal disruption to isoprenoid biosynthesis in humans. In the MEP pathway, seven enzymes are involved in the biosynthesis of two universal isoprenoid precursors: isopentyl pyrophosphate and dimethylallyl pyrophosphate.²⁰

Our group is interested in developing inhibitors towards IspE and other enzymes in the MEP pathway which utilize cytidine nucleotide derivatives in driving reactions forward. IspE is the fourth enzyme in the MEP pathway, and is responsible for transferring a terminal phosphate group from adenosine triphosphate (ATP) to cytidine diphosphate 2C-methyl-D-erythritol (CDP-ME) to form 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate (CDP-ME2P) as shown in Scheme 1²¹.

Previous work by the Seattle Structural Genomics Center for Infectious Disease (SSGCID) demonstrated the utility of saturation transfer difference (STD) NMR-based fragment screening to find chemical starting points for *B. pseudomallei* (*Bp*) IspF.²² In collaboration with the SSGCID, additional NMR-based screening campaigns were conducted against *Mycobacterium paratuberculosis* (*Mp*) IspD, *Mycobacterium abscessus* (*Ma*) IspE and *Bp*IspF with approximately 1000 compounds. From this effort, FOL7185 was identified as a clear fragment binder for both *Mp*IspD and *Ma*IspE, and a very weak binder below the STD-NMR hit threshold for *Bp*IspF (Figure 1). Following up this initial hit, a series of closely related analogs were designed, and synthesized in four steps as shown in Scheme 2.

In the first step, the reaction between phosphorus oxychloride and dimethylformamide generates iminium salt *in situ* which is known as the Vilsmeier-Haack reagent.²³ Reaction of the iminium salt with 4,6-dihydroxypyrimidine followed by hydrolysis afforded the formylating product (**1**). Consequently, reaction of 4,6-dichloropyrimidine-5-carbaldehyde with methyl or tertiary butyl hydrazine gave a monochloro-substituted pyrazolopyrimidine core (**2**), which was then reacted with different primary amines to afford the target compounds (Supplementary information).

Zone of inhibition assays were conducted for each compound against two closely related bacteria, *B. thailandensis* and *P. aeruginosa*, both of which use the MEP pathway to synthesize isoprenoids. Assay data was acquired at 1.0 mM and 0.5 mM, plus 0.1 mM concentration points for each compounds (Table 1). Although the fragment hit FOL7185 did not show inhibition in this assay, the majority of the synthesized analogs (**3–29**) were active against *B. thailandensis* (E264), with fewer compounds demonstrating inhibition with *P. aeruginosa* (ATCC 27853). ATCC 27853 strain is resistant to kanamycin,²⁴ and the smaller number of active compounds is most likely related to the intrinsic resistance development of the bacterium. Fluorobenzyl (**13**, **14** and **16**) and 3,4-dichlorobenzyl (**18**) compounds

showed comparable potency against both *B. thailandensis* and *P. aeruginosa*. Activity was not detected for the 4-hydroxyphenethyl substituent (**25**), suggesting that electron withdrawing groups such as chloro, fluoro and nitro groups are important for biological activity. Moreover, a subtle difference was observed with 2- and 4-fluorophenethyl compounds (**21** and **23**) versus 3-fluorophenethyl (**22**) against *B. thailandensis* and *P. aeruginosa*. This suggested that lipophilic electron withdrawing groups at the 2 and 4 positions on the phenethyl group would result in increased potency. Compound **29** was subsequently designed and synthesized, containing 2,4-dichloro substituents on the phenethyl group. This compound showed inhibitory activity against *B. thailandensis* when tested at 0.1 mM (32.2 µg/mL), comparable to results obtained with kanamycin (48.5 µg/mL).

To help validate target specificity, compounds were tested by STD-NMR for binding to IspD and IspE from *B. thailandensis*, using similar conditions as that of the primary fragment screen. STD-NMR data for these compounds was acquired before and after the addition of competitive binders, to test for binding site specificity on each protein. Data for *BtIspD* was acquired before and after addition of 2 molar equivalents of cytidine diphosphate (CDP) per compound. Data for *BtIspE* was acquired before and after addition of 2 molar equivalents of adenosine diphosphate (ADP), followed by 2× addition of CDP, relative to the compound, to efficiently probe two sites on the kinase.

Most of the compounds tested showed weak (< 10%) STD binding signal against *BtIspD*. The strongest binders to *BtIspD* by STD-NMR include compounds **18**, **25**, **26**, **27**, **29** and the original screening hit FOL7185 (Figure 2). Addition of CDP shows a large decrease in STD binding for compounds **26** and **27**. This is indicative of competitive binding at the same site on *BtIspD*, for compounds with affinities near or below that of CDP. The remainder of compounds with measurable binding by STD showed no significant change upon addition of CDP. It is possible that no change in STD is due to binding in an alternative site on the protein. But given the structural similarities between all compounds tested, it is more likely the case that 2× molar excess of CDP is insufficient to quench STD binding for these compounds at the same site of the protein.

The majority of the pyrazolopyrimidines that were active in the zone of inhibition assay showed significant binding with *BtIspE* by STD-NMR (Figure 3). Overall, there was very little decrease in STD binding signal after addition of ADP, while the subsequent addition of CDP tended to cause a greater decrease. Given the similarity in chemical structure for all compounds tested and their binding behavior with IspD, these results suggest that the compounds are preferentially binding to the substrate site of *BtIspE*, over that of the ATP co-factor. However, it is difficult to deconvolute contributions to STD binding signal which may arise from compounds binding both sites. The %STD binding signal of compound **25** experiences no change upon addition of ADP, but loses all binding signal entirely after the addition of CDP (Figure 3). Compound **25** did not show zone of inhibition activity against *B. thailandensis* and *P. aeruginosa* at the concentrations tested, which correlates with weaker binder by %STD. Hence it is most likely that compounds in this series binding to both *BtIspD* and *BtIspE* at the substrate binding site.

Some compounds that have high %STD values in binding to *BtIspE*, such as **29**, **14**, and **18**, contain methyl groups at the R¹ position, indicating that sterically-demanding groups have unfavorable interactions with the enzyme, or somehow disrupt interactions between the enzyme and the pyrazolopyrimidine core. These compounds also have halo/dihalo benzyl or phenethyl groups at the R² position, suggesting a potential hydrophobic or π - π stacking interactions could enhance binding with *BtIspE*. They may also play an important role by properly orienting the pyrazolopyrimidine core to interact with the cytidine binding pocket. These traits are highlighted by compound **29**, which displays the highest %STD value of any compounds against *BtIspE*, and is the most potent compound tested against *B. thailandensis* in zone of inhibition assays (32.2 μ g/mL).

To determine the relative proximity of the hydrogen atoms of compound **29** to the surface of *BtIspE* when bound, we collected STD-NMR data for the compound in the presence of enzyme across a range of saturation times (0.5 to 5.0 seconds). STD amplification factors were then calculated for each well-resolved proton resonance.^{25–27} STD build-up curves were generated for each resolvable proton, and the slopes of each curve were calculated and normalized to STD-fit values, to estimate the relative proximity of each hydrogen atom to the surface of the enzyme when bound. The binding epitope data indicate that the H6', H3', and H5' hydrogen atoms of compound **29** experience more saturation transfer during the experiment than H3, H6 and methyl hydrogens (Table 2 and Figure 4). This result suggests that the 2,4-dichlorophenyl moiety is the most deeply buried, and/or has the most favorable or complementary contact with the protein when compound **29** is bound.

In summary, FOL 7185 was used as a template to design a number of pyrazolopyrimidine analogs for testing with *BtIspD* and *BtIspE*. Although FOL7185 did not show measurable growth inhibition against *B. thailandensis* and *P. aeruginosa* in the zone of inhibition assay, a number of its analogs did show growth inhibition. Most notably, compound **29** was found to inhibit *B. thailandensis* at 0.1 mM concentration, which is comparable to kanamycin. This compound also showed measurable potency against a multi-drug resistant strain of *P. aeruginosa*, thus demonstrating cross-species inhibition and potential as a starting point for broad-spectrum antibiotic activity. In STD-NMR studies, compound **29** shows significant interactions with the active site of *BtIspE*, suggesting that these analogs may inhibit bacterial growth by disrupting isoprenoid biosynthesis. The high potency of compound **29** appears to be caused by low steric interactions at the R¹ position of the molecule, and is aided by favorable lipophilic interactions with the 2,4-dichlorophenyl group at R² position.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge Northern Illinois University for supporting and funding this work. This project has also been funded in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under grant 1R15AI1136531-01 and Contract Nos. HHSN272200700057C and HHSN272201200025C.

References and notes

1. Davies J, Davies D. Microbiol. Mol. Biol. Rev. 2010; 74:417. [PubMed: 20805405]
2. Wright GD. BMC Biol. 2010; 8:123. [PubMed: 20887638]
3. Wright GD. Nature Reviews Microbiology. 2007; 5:175. [PubMed: 17277795]
4. Davies J. The Canadian Journal of Infectious Diseases & Medical Microbiology. 2006; 17:287. [PubMed: 18382641]
5. Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM, Bartlett JG, Edwards J, America IDSo. Clin. Infect. Dis. 2008; 46:155. [PubMed: 18171244]
6. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. Nature Reviews Drug Discovery. 2007; 6:29. [PubMed: 17159923]
7. World Health Organization (WHO). Antimicrobial Resistance: Global Report on Surveillance. 2014. retrieved from: http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf.
8. FDA. Food and Safety Innovation Act. retrieved from: <http://www.fda.gov/downloads/drugs/developmentapprovalprocess/smallbusinessassistance/ucm361320.pdf>.
9. Brown ED. Can. J. Microbiol. 2013; 59:153. [PubMed: 23540332]
10. Rex JH. Nature Reviews Microbiology. 2014; 12:231.
11. Chang, W-c; Song, H.; Liu, H-w; Liu, P. Curr. Opin. Chem. Biol. 2013; 17:571. [PubMed: 23891475]
12. Cordoba E, Salmi M, León P. J. Exp. Bot. 2009; 60:2933. [PubMed: 19584121]
13. Hunter WN. J. Biol. Chem. 2007; 282:21573. [PubMed: 17442674]
14. Meganathan R. FEMS Microbiol. Lett. 2001; 203:131. [PubMed: 11583838]
15. Meganathan R. Vitam. Horm. 2001; 61:173. [PubMed: 11153266]
16. Eberl M, Hintz M, Reichenberg A, Kollas A-K, Wiesner J, Jomaa H. FEBS Lett. 2003; 544:4. [PubMed: 12782281]
17. Gellatly SL, Hancock RE. Pathogens and disease. 2013; 67:159. [PubMed: 23620179]
18. George AM, Jones PM, Middleton PG. FEMS Microbiol. Lett. 2009; 300:153. [PubMed: 19674113]
19. Rohmer M, Grosdemange-Billiard C, Seemann M, Tritsch D. Current opinion in investigational drugs. 2004; 5:154. [PubMed: 15043389]
20. Masini T, Hirsch AK. J. Med. Chem. 2014; 57:9740. [PubMed: 25210872]
21. Miallau L, Alphey MS, Kemp LE, Leonard GA, McSweeney SM, Hecht S, Bacher A, Eisenreich W, Rohdich F, Hunter WN. Proc. Natl. Acad. Sci. U. S. A. 2003; 100:9173. [PubMed: 12878729]
22. Begley DW, Hartley RC, Davies DR, Edwards TE, Leonard JT, Abendroth J, Burris CA, Bhandari J, Myler PJ, Staker BL. J. Struct. Funct. Genomics. 2011; 12:63. [PubMed: 21359640]
23. Arnold Z, Holý A. Collect. Czech. Chem. Commun. 1962; 27:2886.
24. Hächler H, Santanam P, Kayser FH. Antimicrob. Agents Chemother. 1996; 40:1254. [PubMed: 8723476]
25. Liu J, Begley D, Mitchell DD, Verlinde CL, Varani G, Fan E. Chem. Biol. Drug Des. 2008; 71:408. [PubMed: 18373548]
26. Mayer M, James TL. J. Am. Chem. Soc. 2004; 126:4453. [PubMed: 15053636]
27. Yan J, Kline AD, Mo H, Shapiro MJ, Zartler ER. J. Magn. Reson. 2003; 163:270. [PubMed: 12914842]

Figure 1.
Chemical structure of FOL7185 (**17**).

Figure 2.

Pyrazolopyrimidine compounds with measurable % STD binding against *BtIspD* before and after addition of CDP.

Figure 3. Pyrazolopyrimidine compounds with % STD > 10 against *BtIspE* before and after addition of ADP and CDP.

Figure 4.
Structure of compound **29** and nomenclature of its hydrogens.

Scheme 1.
Proposed mechanism for IspE catalysis.²¹

Scheme 2.
Chemical Synthesis of Pyrazolopyrimidine Derivatives

Table 1

Zone of inhibition assay of pyrazolopyrimidines against *B. thailandensis* and *P. aeruginosa*.

No	R ¹	R ²	Zone of inhibition (diameter, in millimeter)							
			<i>B. thailandensis</i>				<i>P. aeruginosa</i>			
			1.0 mM	0.5 mM	0.1 mM	1.0 mM	0.5 mM	0.1 mM	0.1 mM	0.1 mM
3	-CH ₃		0	0	0	21	15	0		
4	-CH ₃		12	8	0	20	12	0		
5	-CH ₃		12	8	0	13	0	0		
6	-CH ₃		21	13	0	10	0	0		
7	-CH ₃		20	0	0	0	0	0		
8	-C(CH ₃) ₃		14	12	0	12	6	0		
9	-CH ₃		12	0	0	0	0	0		
10	-CH ₃		19	11	0	14	8	0		
11	-C(CH ₃) ₃		18	12	0	0	0	0		

		Zone of inhibition (diameter, in millimeter)									
		<i>B. thailandensis</i>					<i>P. aeruginosa</i>				
No	R ¹	R ²	1.0 mM	0.5 mM	0.1 mM	1.0 mM	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.1 mM
13	-CH ₃		13	8	0	18	12	0	12	0	0
14	-CH ₃		11	6	0	15	10	0	10	0	0
15	-C(CH ₃) ₃		16	12	0	17	13	0	13	0	0
16	-CH ₃		18	9	0	18	12	0	12	0	0
17 (FOL7185)	-CH ₃		0	0	0	0	0	0	0	0	0
18	-CH ₃		18	12	0	20	10	0	10	0	0
19	-C(CH ₃) ₃		20	16	0	16	12	0	12	0	0
20	-CH ₃		20	9	0	0	0	0	0	0	0

		Zone of inhibition (diameter, in millimeter)									
		<i>B. thailandensis</i>					<i>P. aeruginosa</i>				
No	R ¹	R ²	1.0 mM	0.5 mM	0.1 mM	1.0 mM	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.1 mM
21	-CH ₃		16	12	0	16	8	0	8	0	0
22	-CH ₃		14	11	0	8	0	0	0	0	0
23	-CH ₃		22	12	0	17	9	0	9	0	0
24	-CH ₃		13	10	0	10	0	0	0	0	0
25	-CH ₃		0	0	0	0	0	0	0	0	0
26	-CH ₃		21	13	0	19	11	0	11	0	0
27	-C(CH ₃) ₃		16	12	0	0	0	0	0	0	0
28	-C(CH ₃) ₃		18	11	0	0	0	0	0	0	0

		Zone of inhibition (diameter, in millimeter)									
		<i>B. thailandensis</i>					<i>P. aeruginosa</i>				
No	R ¹	R ²	1.0 mM	0.5 mM	0.1 mM	1.0 mM	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.1 mM
29	-CH ₃		18	10	6	18	12	0	0	0	0
31	-CH ₃		14	12	0	0	0	0	0	0	0
Kanamycin (positive control)			30	22	6	0	0	0	0	0	0

Table 2

STD-fit values of compound 29 hydrogens.

Hydrogen	Slope	STD-fit
H6'	1.49	100
H5'	1.33	90
H3'	1.47	99
H3	1.00	67
H6	0.79	53
Methyl	0.55	37