AWARD NUMBER: W81XWH-12-2-0014

TITLE: Development of Antibacterials Targeting the MEP Pathway of Select Agents

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REPORT DATE: June 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2016	Final	10Feb2014 - 25Mar2016

4. TITLE AND SUBTITLE
Development of Antibacterials Targeting the MEP Pathway of Select Agents

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-2-0014

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Over the duration of this award, five specific aims were pursued: 1) Express, purify, and characterize recombinant Y. pestis IspC and IspD, 2) Optimize HTS assay conditions for IspC and IspD, 3) Provide purified recombinant F. tularensis IspC and IspD for crystallization and structure determination, 4) Provide purified recombinant Y. pestis IspC and IspD protein for crystallization and structure determination, and 5) Evaluate structure-activity relationships of rationally designed inhibitor molecules in enzyme-based assays. Accordingly, we successfully cloned, expressed, purified, and enzymatically characterized the Y. pestis IspC. Expression difficulties with recombinant Y. pestis IspD prompted us to work instead with cloned F. tularensis IspD. We successfully established HTS conditions for both IspC and IspD assays. Pilot scale screening of molecular libraries has identified hit compounds for IspC and IspD, including a novel inhibitor of IspC that binds to an allosteric site on the enzyme. We have provided purified protein on-demand for protein crystallography, and we have thoroughly evaluated structure-activity relationships of several rationally designed inhibitors of IspC and IspD via enzyme-based assays.

15. SUBJECT TERMS
Francisella, Yersinia, MEP pathway, antibiotics, IspC, IspD, MEP synthase, MEP cytidylyltransferase, HTS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	22	19b. TELEPHONE NUMBER *(include area code)*

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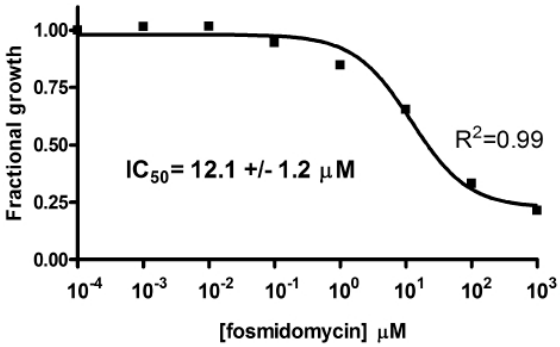
Keywords
Francisella, Yersinia, MEP pathway, antibiotics, IspC, IspD, MEP synthase, MEP cytidylyltransferase, HTS

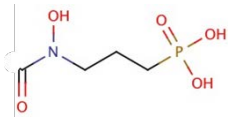
Introduction

The long term objective of this research is to identify and develop a broad spectrum inhibitor of *Francisella tularensis* and *Yersinia pestis*. The methylerythritol phosphate (MEP) biosynthetic pathway of *Francisella tularensis* and *Yersinia pestis* provide multiple enzymes that may be targeted for inhibitor development. This pathway is utilized by bacteria, apicomplexan protozoa, and plants for isoprenoid biosynthesis. Isoprenic compounds are vital for cellular processes such as electron transport, cell wall and membrane biosynthesis, and signal transduction. Despite their structural and functional diversity, all isoprenoids are derived from two building blocks, isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which originate from either the MEP pathway or the mevalonic acid (MVA) pathway depending on the organism. Humans acquire isoprenes through the nonhomologous MVA pathway, making enzymes in the MEP pathway very attractive targets for antimicrobial development.

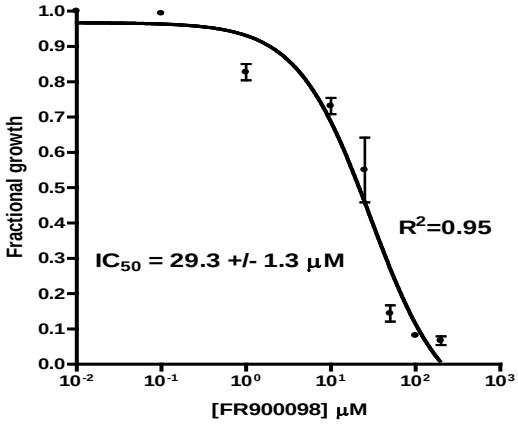
Body

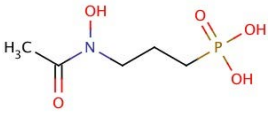
We hypothesize that inhibitors of the MEP pathway in *Francisella tularensis* and *Yersinia pestis* will serve as effective antibiotics by blocking isoprene biosynthesis. In strong support of this hypothesis, we have demonstrated the dose-dependent inhibition of *F. tularensis* and *Y. pestis* growth *in vitro* using the compounds fosmidomycin and FR900098 (Figure 1).





Fosmidomycin





FR900098

Figure 1. Growth inhibition of *F. tularensis* (top) and *Y. pestis* (bottom) by the compounds fosmidomycin and FR900098 (these molecules inhibit IspC, an enzyme in the MEP pathway). The structures of the compounds are shown.

To test this hypothesis, the Couch lab at George Mason University is collaborating with Walter Reed Army Institute of Research (WRAIR) in the screening of compound diversity libraries using enzyme-based assays for lead inhibitor discovery, evaluation of lead inhibitors in microbial growth assays, determining X-ray crystal structures of MEP pathway enzymes IspC and IspD in complex with inhibitors, and using this information to design and synthesize novel broad spectrum antibacterials. Over the duration of this award (Feb 10, 2012 through to Dec 25, 2015), five specific aims were being pursued in the Couch lab. These aims and the corresponding results are detailed below.

Aim 1) Express, purify, and characterize recombinant *Y. pestis* IspC (MEP synthase) and IspD (MEP cytidylyltransferase).

We have successfully cloned the *Y. pestis* CO92 IspC gene into the pET28c protein expression vector and transformed the construct into the *E. coli* BL21(DE3) RIL Codon Plus cell line for expression. [Note: *Y. pestis* CO92 IspC displays 50% identity (71% homology) to the *F. tularensis* IspC, which we have previously characterized [1].] Optimization of both protein expression and purification conditions were subsequently carried out, resulting in an average yield of ~4 mg of purified protein from a 1 L culture. We achieve ~95% purity based on SDS-PAGE and Coomassie staining (see Figure 2, below).

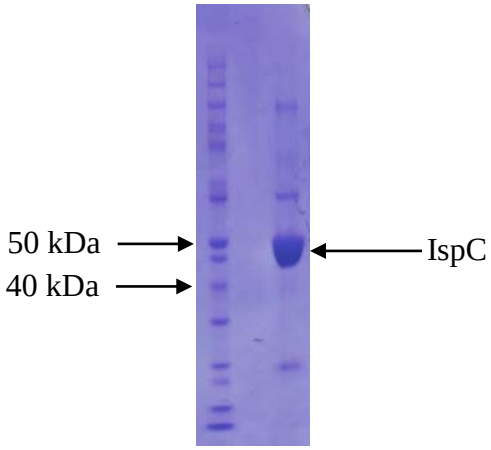


Figure 2. Coomassie stained SDS-PAGE of purified recombinant *Y. pestis* IspC. Molecular weight marker is in the left lane, purified IspC is in the right lane.

We obtain excellent specific activity with the purified protein (Vmax ~15000 µM/min/mg). As demonstrated in the kinetic graphs presented below (Figure 3), we determined the relevant kinetic parameters for the enzyme (apparent K_M^{DXP} = 252 µM, apparent K_M^{NADPH} = 12.7 µM), divalent cation specificity (Mg^{+2} and Mn^{+2} are preferred), and the IC_{50} for the compounds FR900098 (IC_{50} = 249 nM) and fosmidomycin (IC_{50} = 2 µM). The latter was unexpectedly high, however many replicates of the assay with fosmidomycin were performed to instill confidence in the assay results (which are indeed found reproducible, and therefore sound).

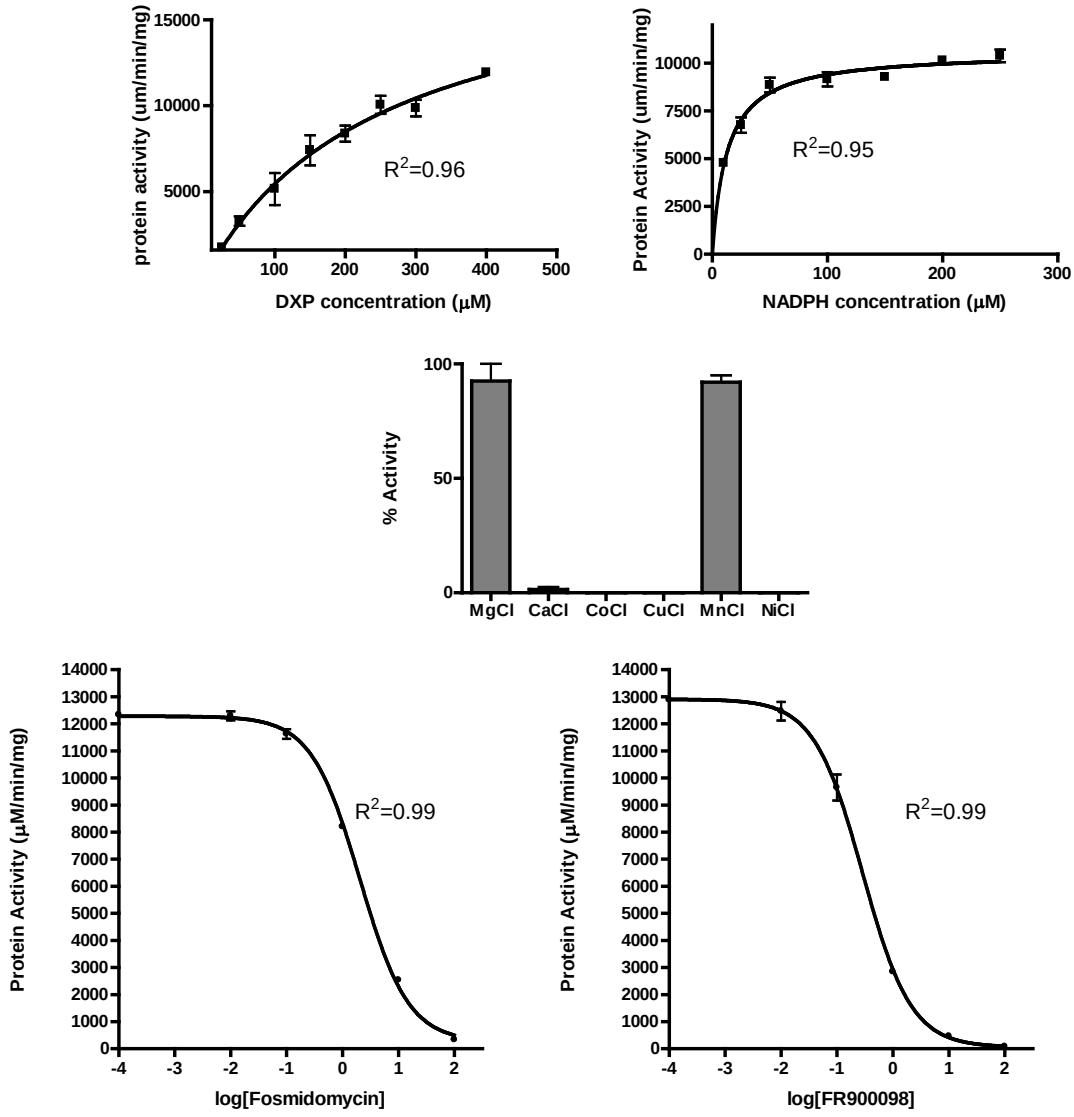


Figure 3. Kinetic assessment of purified *Y. pestis* IspC.

Additionally, we had cloned the *Y. pestis* CO92 IspD gene into the pET28c protein expression vector and transformed the construct into the *E. coli* BL21(DE3) RIL Codon Plus cell line for expression. However, we were unsatisfied with the yield of resulting purified protein. Rational manipulation of the protein expression and purification conditions had not significantly improved the yield, including optimization of codon usage for enhanced expression in *E. coli*. While scale-up of the protein expression could increase the titer of purified protein, budget considerations and our long term objectives suggested that we instead evaluate a different expression construct. We have prior success with expressing *Francisella tularensis* IspD with the pET101D plasmid [2], resulting in yields as high as 80 mg/ml from a 1 L culture of *E. coli* BL21(DE3) RIL

Codon Plus. Hence, we redesigned the *Y. pestis* CO92 IspD construct in the same manner and evaluated protein expression from this plasmid.

We obtained a Yp IspD PCR product of the expected size, cloned the product into a pET101D plasmid for protein expression in *E. coli* BL21(DE3) RIL Codon Plus, and confirmed the acquisition of the desired clone by restriction mapping. Unfortunately, protein expression and purification still resulted in very poor yields of recombinant protein. Furthermore, the purified protein was found to be devoid of any catalytic activity. Hence, we elected to focus our effort on using the *F. tularensis* MEP cytidylyltransferase in lieu of the *Y. pestis* homolog.

Aim 2) Optimize HTS assay conditions for *F. tularensis* IspC (MEP synthase) and IspD (MEP cytidylyltransferase).

The Z-factor

Since purified recombinant *Y. pestis* MEP synthase was found to have significantly greater specific activity than the *F. tularensis* homolog, the *Y. pestis* enzyme is better suited to large scale high-throughput screening (HTS) as less protein is needed per assay, thereby reducing the per-well cost of a screen. The quality and robustness of an enzyme assay are important considerations for the reliable screening of a molecular library, and are typically described in terms of the Z-factor [3]. Ideally, an assay should have a large dynamic range (the difference between the uninhibited and inhibited signals) and small standard deviation across replicates, which corresponds to a Z-factor score near a value of 1 (an assay with a Z-factor score between 0.5 and 1.0 is considered excellent for screening). To determine the Z-factor for the spectrophotometric assay using the *Y. pestis* MEP synthase, we fixed the DXP concentration to the K_M, used a saturating concentration of NADPH (150 mM), and evaluated three separate lots of purified enzyme in a series of assays performed over three consecutive days. FR900098 was used as a positive control for inhibition. The Z-factor was determined to be 0.9, indicative of an assay well suited for library screening. Additionally, the Z-factor for the *F. tularensis* MEP cytidylyltransferase assay was found to be 0.8 [2], also well suited to a HTS campaign.

Assessment of the HTS: MEP synthase (IspC)

Through empirical design, we established IspC assay conditions amenable to HTS. To evaluate our developed assay conditions, we performed a high-throughput screen of MEP synthase using a commercially available molecular library (LOPAC library; Figure 4). The library was provided by Dr. Cynthia Dowd, Professor of Chemistry at George Washington University, whom has an interest in developing inhibitors of the *Mycobacterium tuberculosis* MEP pathway. Due to its wide dynamic range of activity, our developed assay employs a primary screen performed with the *Y. pestis* MEP synthase. To ensure broad spectrum activity, a secondary screen is then performed with purified recombinant *Mycobacterium tuberculosis* MEP synthase (the expression construct was provided to us by the Dowd lab). To eliminate false positives (compounds that have innate absorption at 340 nm, thereby interfering with the NADPH signal (ΔA_{340})), a tertiary screen follows, using an assay coupling the *Y. pestis* MEP synthase with the *F. tularensis* MEP cytidylyltransferase, as illustrated in Figure 5. Through this series of assays, seven molecules demonstrating inhibition were identified in this pilot scale screen, and are considered hit compounds worthy of future examination.

In addition to screening the LOPAC library, we also elected to evaluate our developed IspC HTS platform by screening our in-house, natural product, phytochemical library of molecules. As illustrated in Figure 6, four inhibitor hits were obtained in the screen (demonstrating <25% residual enzyme activity). Follow-on inhibition assays with the 4 hits confirmed the greatest activity is associated with extract 29 (Figure 7). We then performed detailed kinetic evaluation of extract 29 (Figure 8) and have deduced the presence of a new class of IspC inhibitor, functioning by binding to a previously unknown allosteric site on the enzyme (to date, all known inhibitors of IspC are competitive, binding in the active site of the enzyme. This new inhibitor

binds to an allosteric site outside of the active site. An allosteric site has never been described for any known IspC homolog.). This exciting discovery affords the development of a completely new family of antibiotics targeting the IspC enzyme.

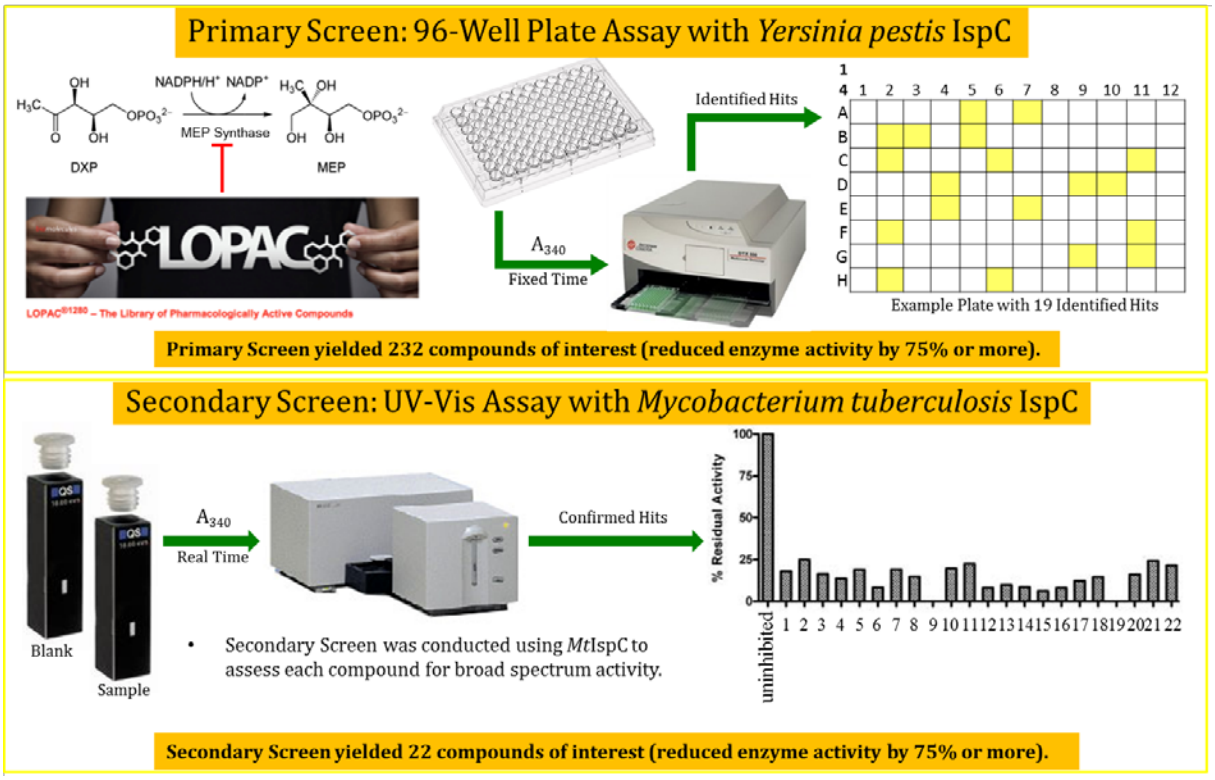


Figure 4. Schematic of the approach to performing the high-throughput screen with IspC. The screen identified 22 hits.

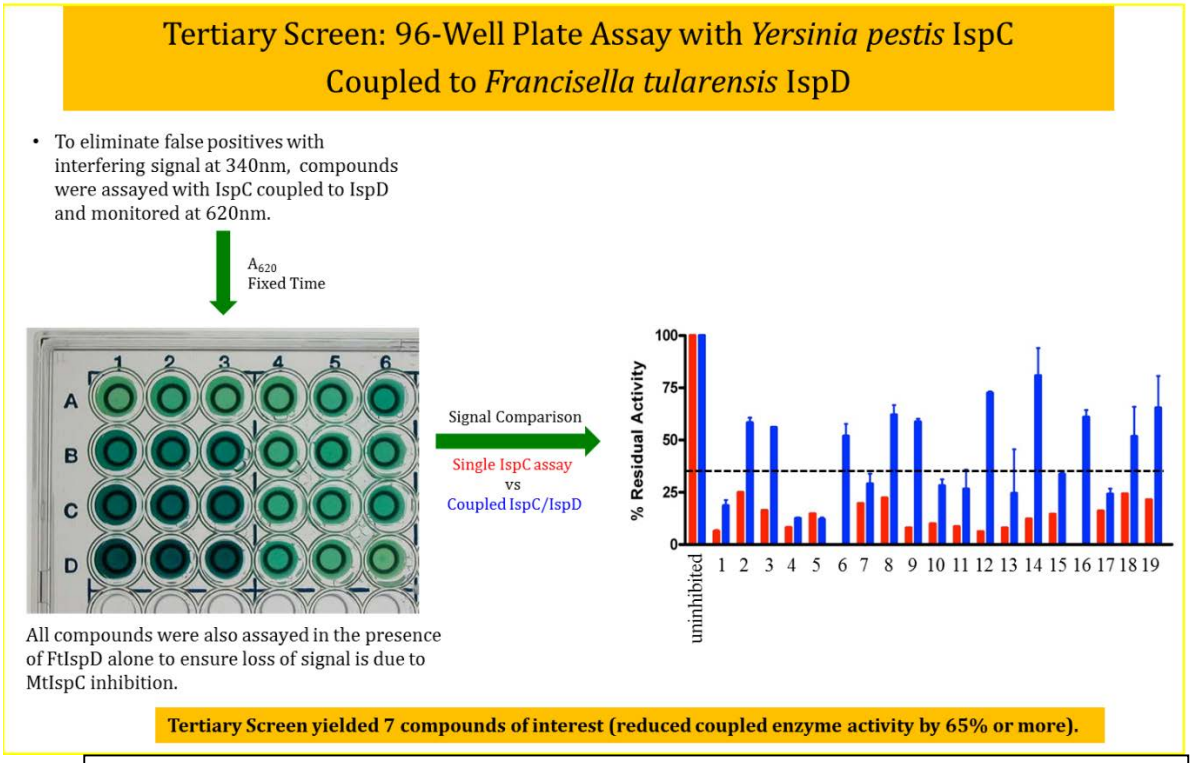


Figure 5. Schematic of the approach to performing the tertiary screen with IspC. Of the 22 hits identified in Figure 4, 19 are readily available commercially. Seven of these compounds resulted in residual enzyme activity <35%, in both the single and coupled assays, and were selected for further assessment.

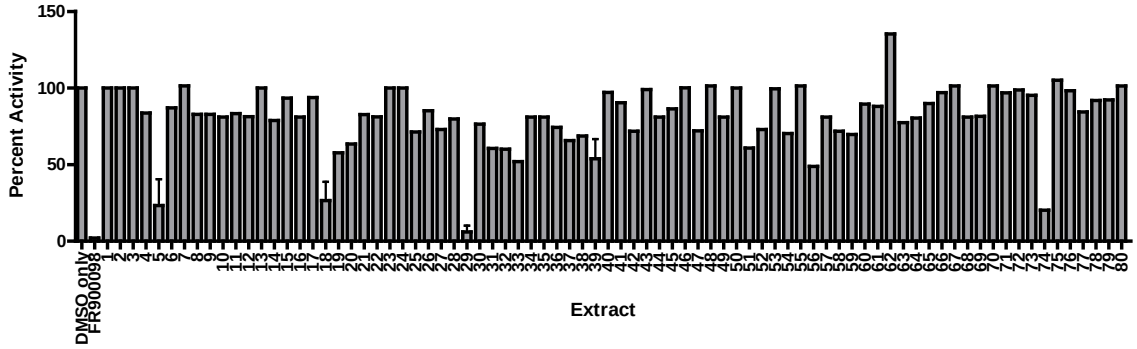


Figure 6. Library screening with purified *Y. pestis* IspC. Enzyme activity, relative to uninhibited enzyme (DMSO only) is shown. The phytochemical library consists of natural product extracts from a variety of domestic plants. Hence, each extract contains multiple compounds.

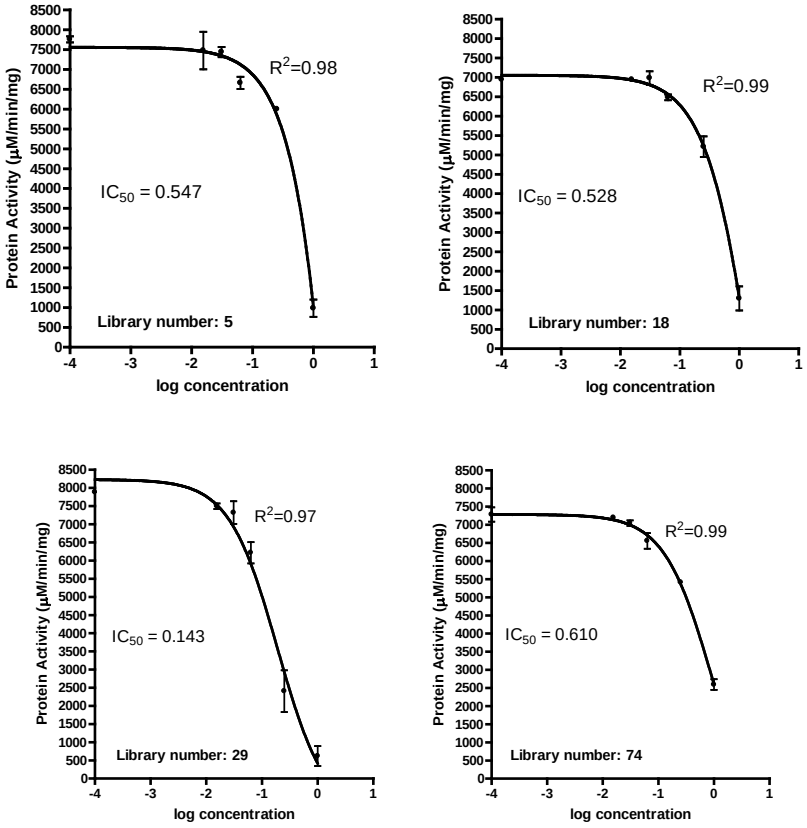
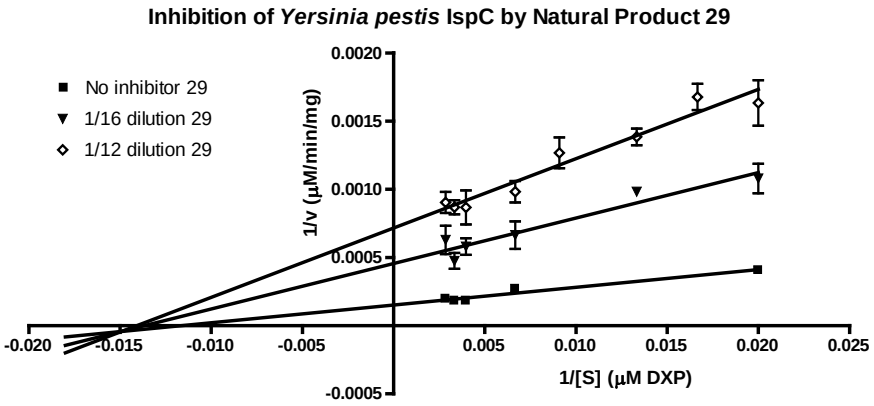


Figure 7. Dose-response plots of the top 4 hits identified in the library screening. Extract 29 demonstrates the greatest relative potency (note: since the extracts are mixtures of several molecules, the IC_{50} values are unitless).



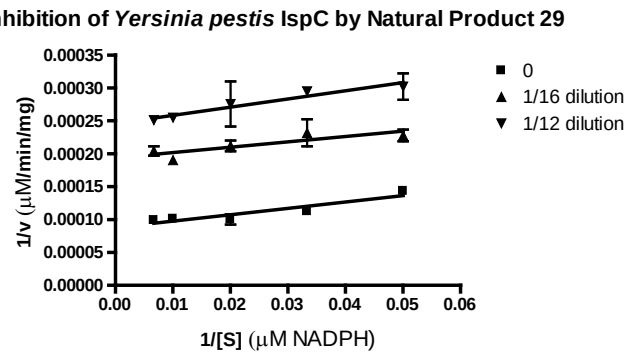


Figure 8. Double reciprocal plots for the diagnostic assessment of mechanism of inhibition. Relative to DXP, the inhibitor in extract 29 is noncompetitive (i.e the inhibitor binds the enzyme at an allosteric site, whether the substrate is bound or not). Relative to NADPH, the inhibitor is uncompetitive (i.e. the inhibitor can only bind the enzyme after NADPH is bound). Collectively, a mechanism is revealed where NADPH binds the enzyme causing a conformational change, creating an allosteric site to which the inhibitor in extract 29 binds, thereby preventing the catalytic conversion of DXP to MEP. This discovery of an allosteric site on IspC is the first of its kind. Hence, the inhibitor in extract 29 represents a new class of inhibitor for IspC, opening the door to the development of a new family of antimicrobials.

Assessment of the HTS: MEP Cytidylyltransferase (IspD)

Through empirical design, we also established IspD HTS assay conditions. To evaluate the assay approach, we performed a high-throughput screen of *F. tularensis* MEP cytidylyltransferase using the commercially available LOPAC library (Figure 9). To ensure broad specificity of inhibitors, a secondary screen is performed with the *E. coli* IspD (which we cloned, expressed, and purified). And since the primary and secondary enzyme assays are each coupled to the activity of pyrophosphatase, a tertiary screen is performed with pyrophosphatase alone to ensure the hit compounds are selective for IspD and not pyrophosphatase (Figure 10). This screening platform identified 5 inhibitor hits within the LOPAC library.

Overall, Aim 2 has been successfully completed as proposed and has led to the identification of inhibitor hits for IspC and IspD. Follow-on work characterizing these hit compounds is being performed, with some results being presented in previous annual progress reports.

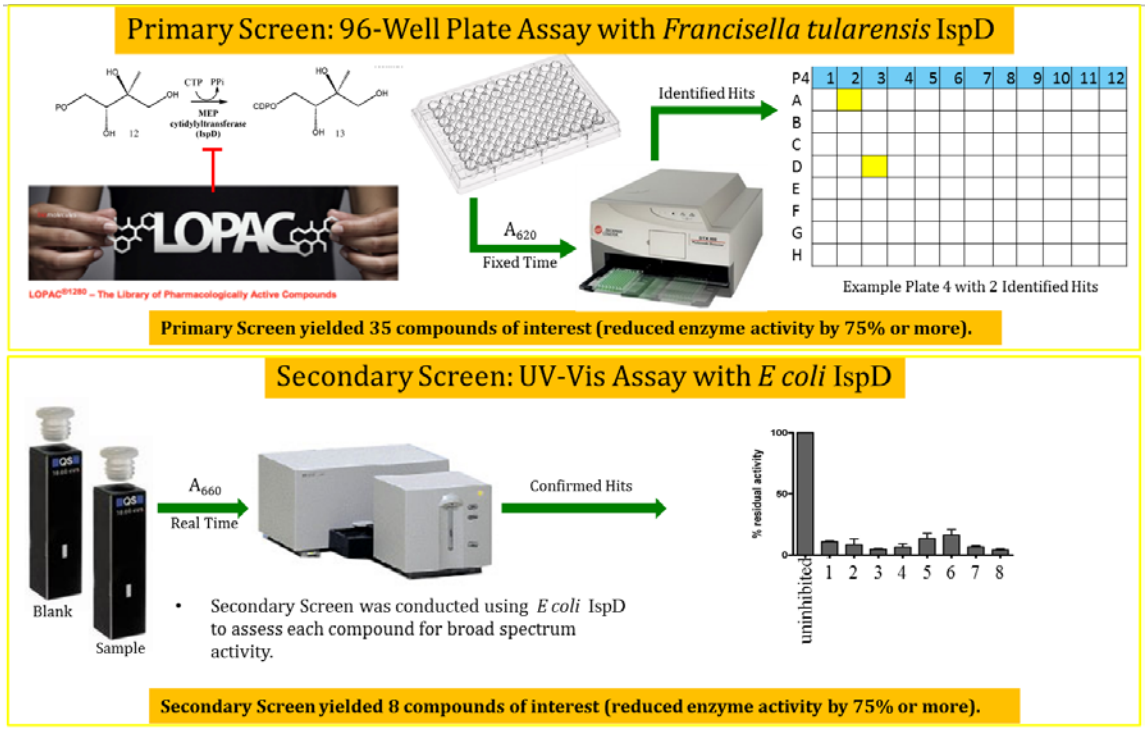


Figure 9. Schematic of the approach to performing the high-throughput screen with IspD.

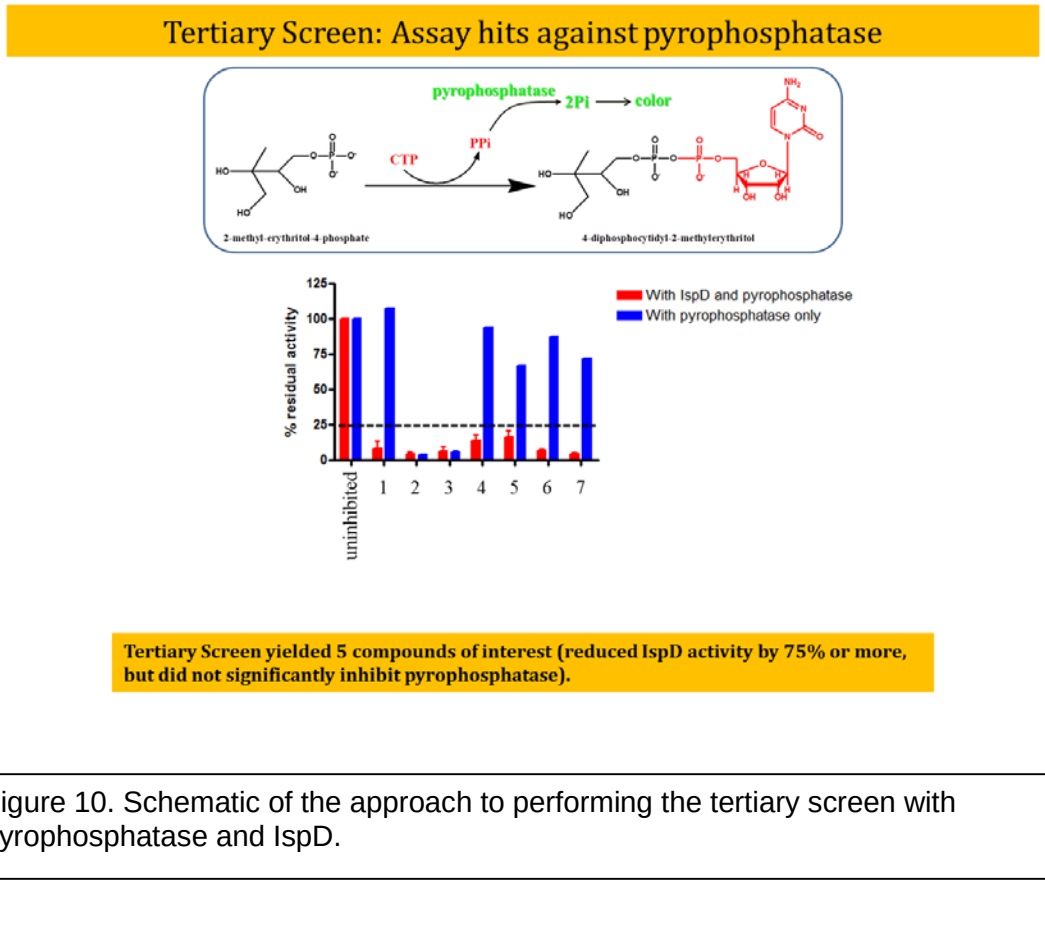


Figure 10. Schematic of the approach to performing the tertiary screen with pyrophosphatase and IspD.

Aim 3) Provide purified recombinant *F. tularensis* IspC and IspD for crystallization and structure determination.

We have provided the purified recombinant *F. tularensis* protein to WRAIR in an on-demand basis.

Aim 4) Provide purified recombinant *Y. pestis* IspC and IspD protein for crystallization and structure determination.

We have provided the purified recombinant *Y. pestis* IspC protein to WRAIR in an on-demand basis. Since catalytically active purified recombinant *Y. pestis* IspD could not be obtained in quantities sufficient for crystallography or for use in HTS, we provided purified recombinant *F. tularensis* IspD instead.

Aim 5) Evaluate structure-activity relationships of rationally designed inhibitor molecules in enzyme-based assays.

MEP synthase (IspC)

To identify additional lead compounds for the development of novel small molecule inhibitors of MEP synthase, we initiated a collaboration with Prof. Cynthia Dowd at George Washington University and screened two molecular libraries for inhibitory activity against the purified *Y. pestis* enzyme. The first library consists of 50 rationally designed synthetic compounds, primarily modeled on the structures of the *M. tuberculosis* MEP synthase in complex with fosmidomycin or FR900098 [4], [5]. As introduced elsewhere [6], the strategy for the synthesis of this library was to create novel compounds with either amide- or O-linked substituents appended to the retrohydroxamate moiety of fosmidomycin/FR900098, thereby targeting the two major binding sites in MEP synthase; the fosmidomycin/DXP site and the NADPH site, bridging these adjacent sites to yield a highly specific ligand. Select structures of the inhibitors are shown in Figure 11. As anticipated, when screening this rational library against purified *Y. pestis* MEP synthase, several of the compounds were found to demonstrate significant inhibitory activity (>75% inhibition), as illustrated in Figure 12. The top five inhibitors were subsequently evaluated in dose-response assays (Figure 13), with compounds **15** and **16** demonstrating the greatest potency. Due to the potential for competitive bisubstrate inhibition, we also evaluated **15** and **16** by preincubating the enzyme with inhibitor prior to the addition of NADPH or DXP (in contrast to the assays depicted in Figure 13, wherein the enzyme was concomitantly exposed to NADPH and the inhibitor). As shown in Figure 14, the resulting IC_{50} values for compounds **15** and **16** improve approximately 3- and 12-fold, respectively, supportive of competitive inhibition relative to both NADPH and DXP. It is particularly noteworthy that the IC_{50} for compound **16** (0.3345 μM) approximates one half of the MEP synthase concentration used in the assay, indicative of a tight-binding inhibitor.

To further explore if compound **16** inhibits by occupying both the DXP and NADPH binding sites, we next performed inhibitor modality assays with the purified *Y. pestis* MEP synthase. Catalysis by MEP synthase involves an ordered bi bi reaction mechanism, wherein NADPH must bind to the enzyme before DXP [7]. This mechanism is indicative of an underlying conformation change accompanying the binding of NADPH, thereby resulting in the formation of the DXP binding site. Accordingly, relative to DXP, fosmidomycin and FR900098 are competitive inhibitors of MEP synthase, while they are uncompetitive with respect to the binding of NADPH [7] [6] (Figure 15). Hence, NADPH must first bind to the enzyme before fosmidomycin/FR900098 can compete with DXP for its binding site.

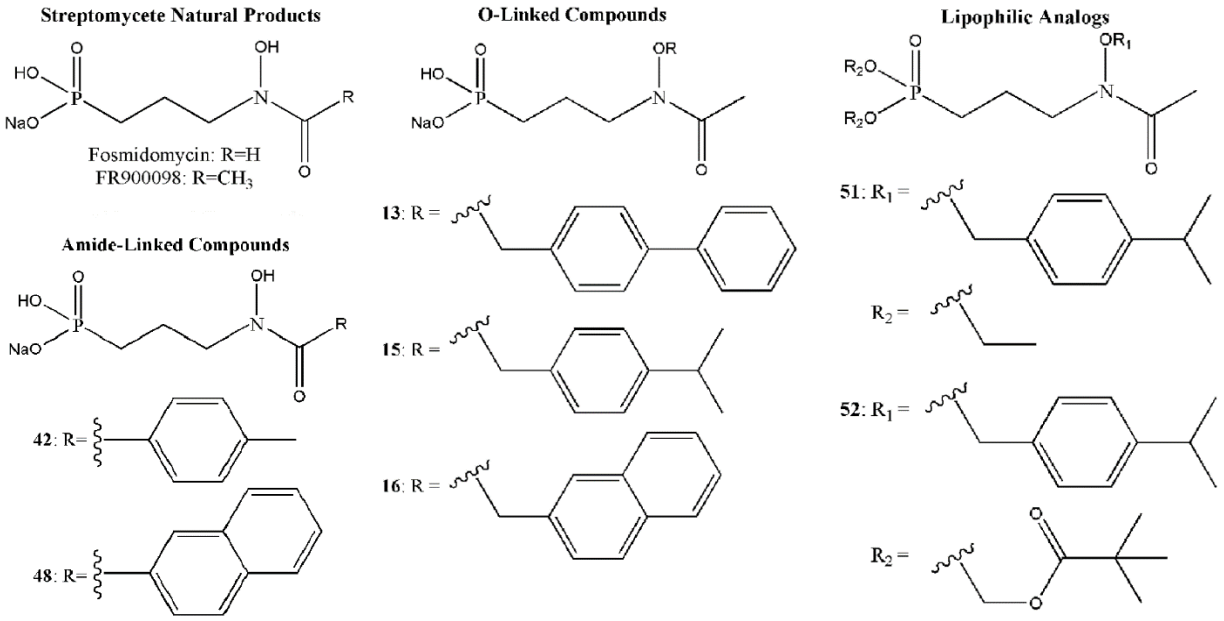


Figure 11. MEP synthase inhibitors. The structures of fosmidomycin, FR900098, and select rationally designed amide-linked and O-linked inhibitors are shown, including lipophilic prodrug analogs of compound **15**.

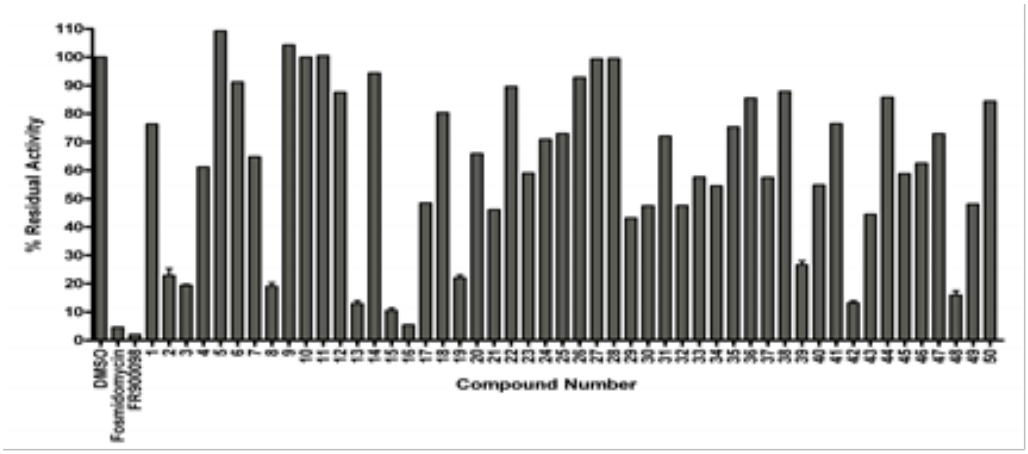


Figure 12. Screening a rationally designed molecular library. The *Y. pestis* MEP synthase was assayed in the presence of 100 µM of the indicated inhibitor. Residual activity is relative to the assay performed with vehicle alone (DMSO). All assays were performed in duplicate. Of those compounds tested, five inhibit the enzymatic activity by >75%, including compounds 13, 15, 16, 42, and 48.

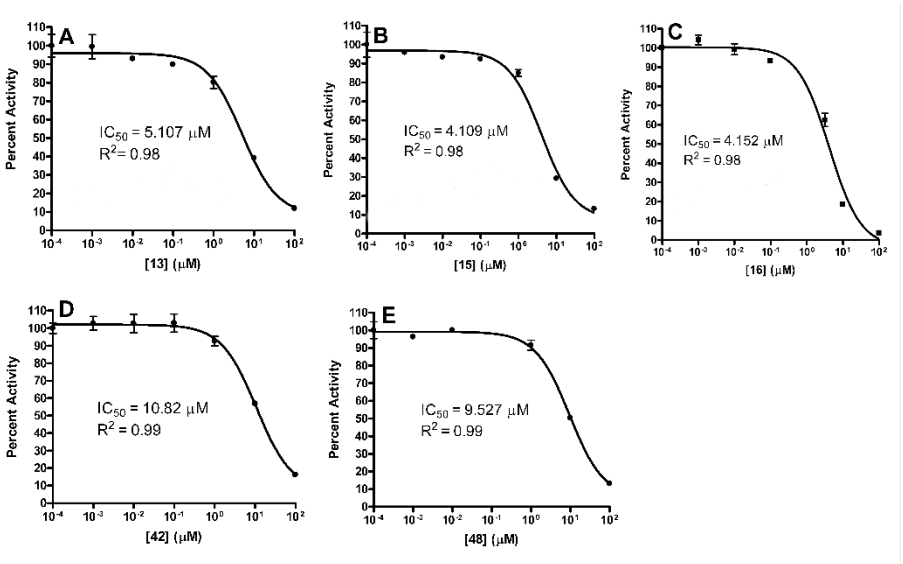


Figure 13. Dose-response plot of *Y. pestis* MEP synthase with the top five rationally designed inhibitors; compounds A) 13, B) 15, C) 16, D) 42 and E) 48. Assays were performed by combining the enzyme with 150 μM NADPH, followed by addition of the inhibitor. After five minute incubation at 37 °C, substrate was added to initiate the reaction. The R^2 value for each plot is indicated. The enzymatic activity is relative to an uninhibited control. All assays were performed in duplicate.

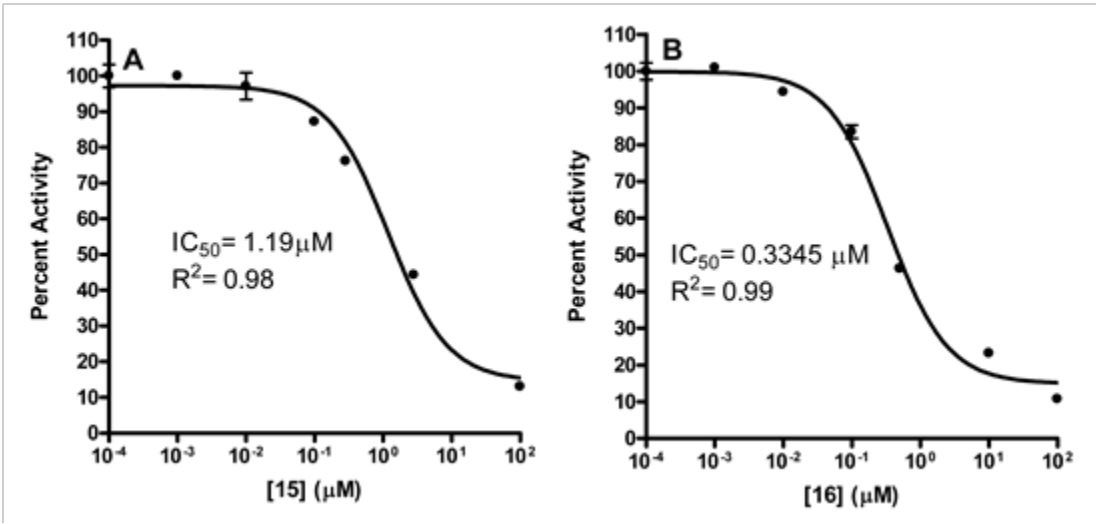


Figure 14. Dose-response plot of the *Y. pestis* MEP synthase when preincubated with the inhibitor. Assays were performed by combining the enzyme with either A) compound 15 or B) compound 16 and preincubating at 37 °C for 10 min before addition of NADPH and DXP. All assays were performed in duplicate. Activity of the enzyme is relative to an uninhibited control.

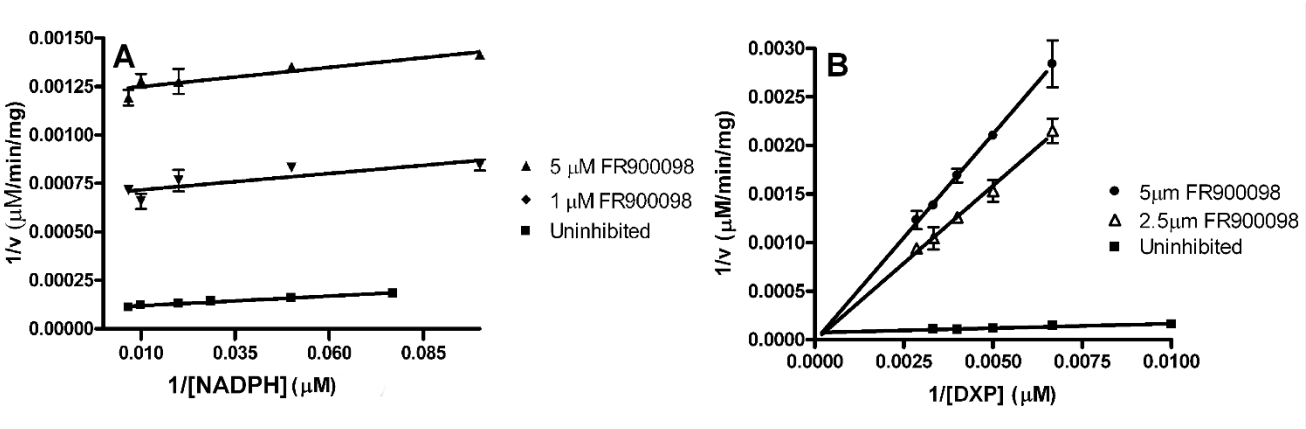


Figure 15. Mode of inhibition by FR900098. The Lineweaver–Burk plots indicate that FR900098 is uncompetitive with respect to NADPH (A), but competitive with respect to DXP (B). All assays were performed in duplicate using purified *Y. pestis* MEP synthase.

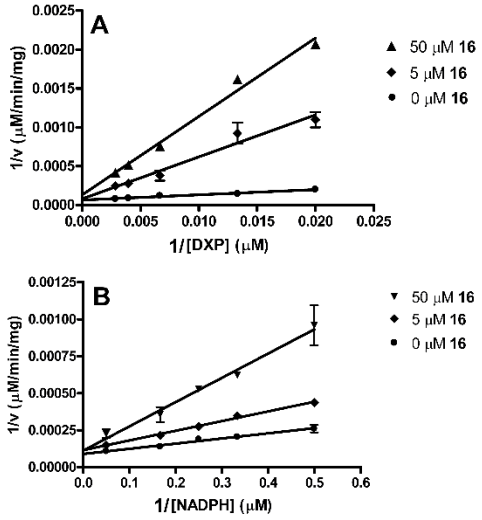


Figure 16. Mode of inhibition by compound **16**. The Lineweaver–Burk plots indicate that compound **16** is competitive with respect to DXP (A) and competitive with respect to NADPH (B). All assays were performed in duplicate using purified *Y. pestis* MEP synthase. The enzyme was not preincubated with compound **16**, in contrast to Figure 17.

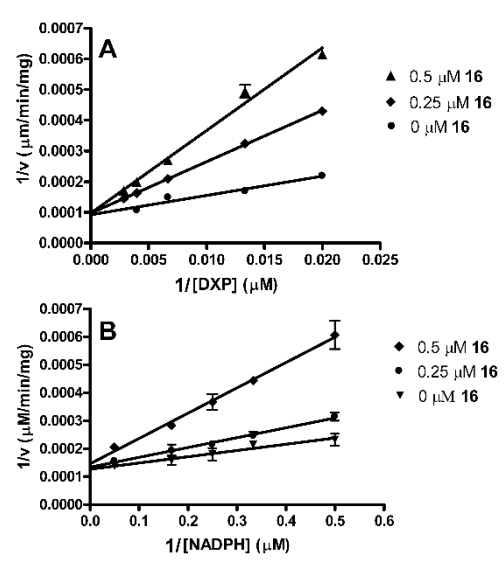


Figure 17. Mode of inhibition with preincubation. When the *Y. pestis* MEP synthase is preincubated with compound **16** (37 °C, 10 min) prior to the addition of NADPH and DXP, the Lineweaver–Burk plots still indicate that compound **16** is competitive with respect to DXP (A) and NADPH (B). All assays were performed in duplicate.

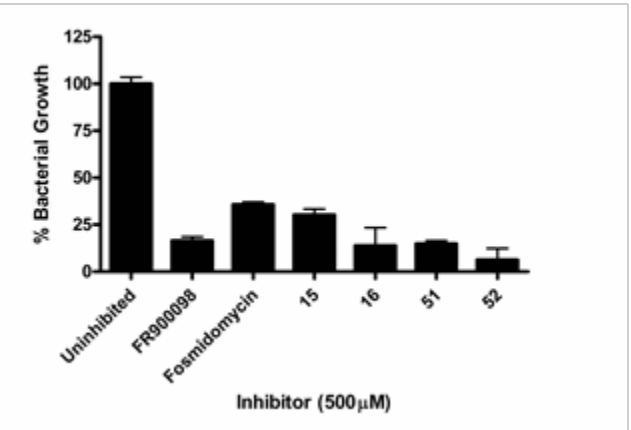


Figure 18. Growth inhibition assay with liquid cultures of *Y. pestis*. *Y. pestis* A1122 was cultured in the presence of 500 μM of the indicated inhibitor. Bacterial growth is relative to an uninhibited culture. All assays were performed in triplicate. Compounds **15**, **16**, **51**, and **52** have inhibitory activity comparable to fosmidomycin and FR900098.

In light of the fosmidomycin and FR900098 mechanism of inhibition, and given the anticipated mechanism for the bisubstrate inhibitor **16**, we performed mode of inhibition assays in each of two ways; the first with **16** added after preincubating the enzyme with NADPH (Figure 16) and the second with compound **16** preincubated with the enzyme prior to the addition of any other substrates (Figure 17). As illustrated in Figures 16 and 17, compound **16** is competitive with respect to DXP and competitive with respect to NADPH, under either of the two assay conditions. Thus, in contrast to fosmidomycin and FR900098, compound **16** does not require the initial binding of NADPH to the enzyme. In fact, as it competes with NADPH for a binding site, its activity is more potent when preincubated with MEP synthase in the absence of NADPH (contrast the concentrations of **16** used in the plots shown in Figures 16 and 17). Due to its ability to bind to the NADPH site, compound **16** appears capable of promoting the same structural change in the enzyme as does NADPH, causing the ensuing formation of the DXP binding site. Consequently, compound **16** behaves as a tightly bound inhibitor, binding to the NADPH site and causing a conformation change that subsequently "locks" the inhibitor into the DXP site. Further exploration of this mechanism is currently underway.

Secondary to the enzyme assays, compounds **15** and **16** were also evaluated in a growth inhibition assay with liquid cultures of *Y. pestis* A1122. As shown in Figure 18, at 500 µM, compounds **15** and **16** demonstrate inhibitory activity comparable to FR900098 and fosmidomycin. Additionally, two lipophilic esters of compound **15** (compounds **51** and **52**) also demonstrate effective growth inhibition. Hence, inhibitors **15** and **16** appear to be excellent lead molecules warranting further development.

MEP cytidylyltransferase (IspD)

As presented in Figure 19, using purified *F. tularensis* MEP cytidylyltransferase, we have evaluated the inhibitory activity of 34 rationally designed inhibitors of MEP cytidylyltransferase (provided by WRAIR). While most compounds demonstrate only modest inhibitory activity, compounds 110039 and 401145 have significant activity (Figure 19). After determination of IC_{50} values with these two compounds and purified cytidylyltransferase (Figure 20), we ascertained the activity of the two compounds in bacterial growth inhibition assays. As shown in Figure 21, both compounds inhibit *Y. pestis* growth *in vitro*, although 110039 demonstrates significantly greater potency. A dose response plot with *Y. pestis* and 110039 indicates activity similar to that of FR900098 (Figure 22). Mechanism of inhibition plots indicate that compound 110039 is competitive with both MEP and CTP (Figure 23), thereby binding in a region occupied by each.

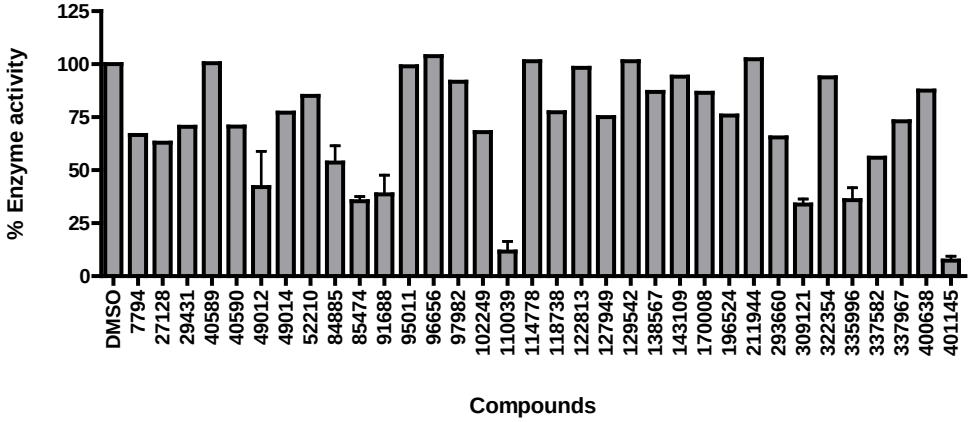


Figure 19. Screening a rationally designed molecular library. The *F. tularensis* MEP cytidylyltransferase was assayed in the presence of 100 µM of the indicated inhibitor. Residual activity is relative to the assay performed with vehicle alone (DMSO). All assays were performed in duplicate. Of those compounds tested, two inhibit the enzymatic activity by >75% (compounds 110039 and 401145).

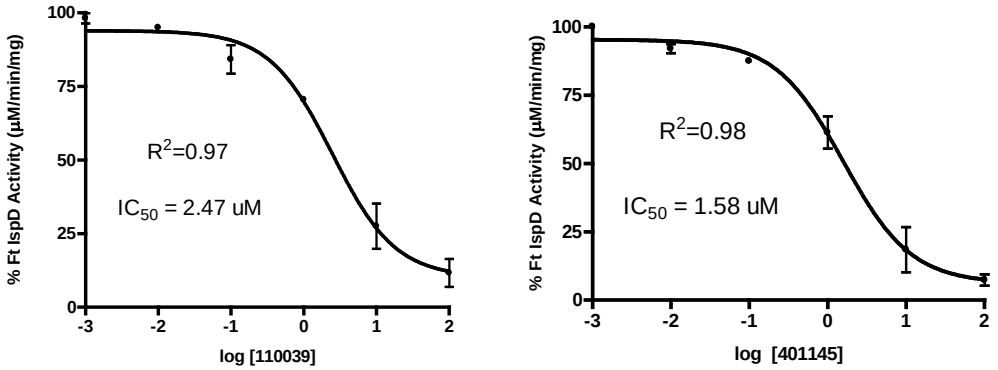


Figure 20. Dose-response plot of *F. tularensis* MEP cytidylyltransferase with the top two rational inhibitors; compounds 110039 (left) and 401145 (right). The R^2 value for each plot is indicated. The enzymatic activity is relative to an uninhibited control. All assays were performed in duplicate.

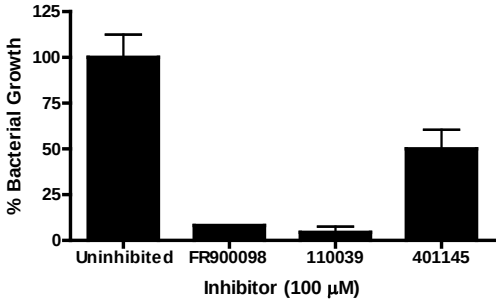


Figure 21. Growth inhibition assay with liquid cultures of *Y. pestis*. *Y. pestis* A1122 was cultured in the presence of 100 µM of the indicated inhibitor. Bacterial growth is relative to an uninhibited culture. All assays were performed in triplicate. Compound 110039 has inhibitory activity comparable to FR900098.

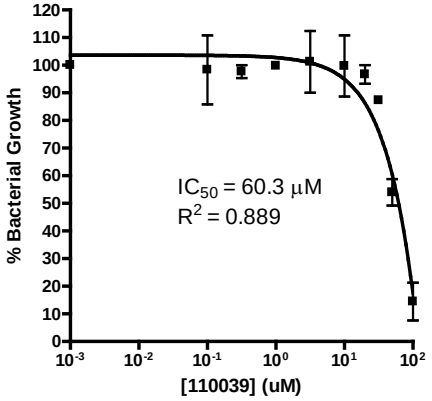


Figure 22. Dose-response plot of *Y. pestis* A1122 cultured in the presence of 110039. Bacterial growth is relative to an uninhibited culture. All assays were performed in triplicate. Compound 110039 has an IC_{50} similar to FR900098.

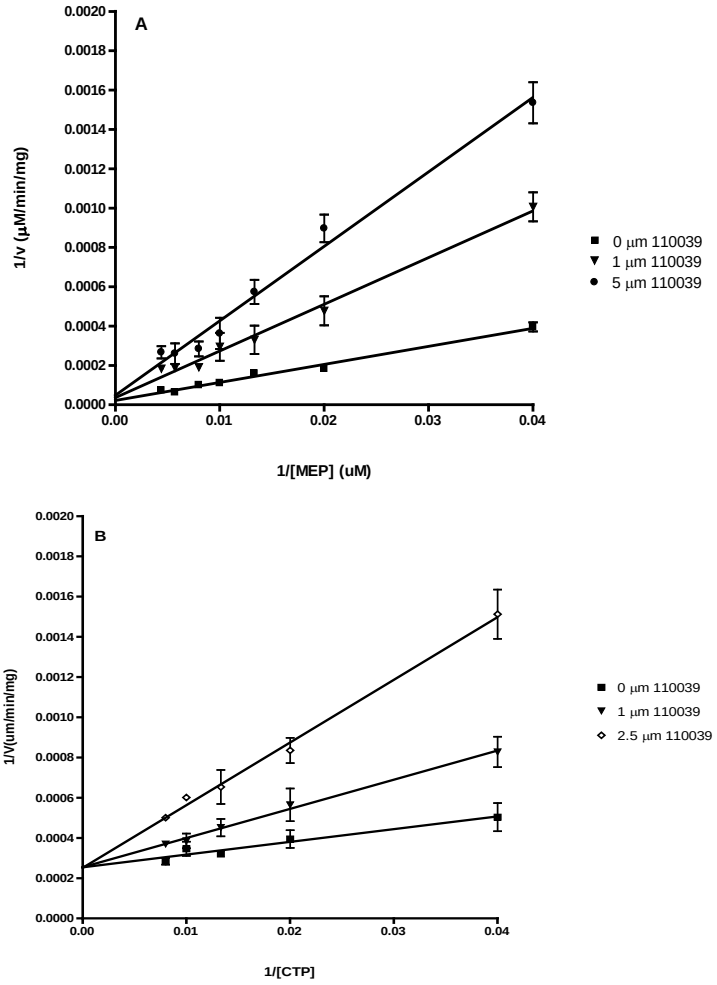


Figure 23. Mode of inhibition. The Lineweaver–Burk plots indicate that compound **110039** is competitive with respect to MEP (A) and CTP (B). All assays were performed in duplicate.

Key Research Accomplishments

- Cloning, expression, purification, and kinetic characterization of recombinant *Y. pestis* IspC.
- Development of HTS assays for the screening of molecular libraries with IspC and IspD.
- Identification of a previously unknown allosteric site on the IspC enzyme.
- Identification of an allosteric inhibitor of IspC, the founding member of a new class of inhibitors.
- Identification of a competitive inhibitor of IspD
- On-demand production and delivery of recombinant proteins to WRAIR for X-ray crystallography.

Reportable Outcomes

- Funds for this project were used to support a lab technician (Ms. Chinchu Johny) and an undergraduate/graduate student (Ms. Amanda Haymond).
- Several publications are the result of this research:

Chofor R, Sooriyaarachchi S, Risseeuw MD, Bergfors T, Pouyez J, Johny C, Haymond A, Everaert A, Dowd CS, Maes L, Coenye T, Alex A, Couch RD, Jones TA, Wouters J, Mowbray SL, Van Calenbergh S. Synthesis and bioactivity of β-substituted fosmidomycin analogues targeting 1-deoxy-D-xylulose-5-phosphate reductoisomerase. Journal of medicinal chemistry. 2015; 58(7):2988-3001.

Haymond A, Johny C, Dowdy T, Schweibenz B, Villarroel K, Young R, Mantooth CJ, Patel T, Bases J, San Jose G, Jackson ER, Dowd CS, Couch RD. Kinetic characterization and allosteric inhibition of the Yersinia pestis 1-deoxy-D-xylulose 5-phosphate reductoisomerase (MEP synthase). PloS one. 2014; 9(8):e106243.

Chofor, R., Risseeuw, M.D., Pouyez, J., Johny, C., Wouters, J., Dowd, C.S., Couch, R.D., Van Calenbergh, S. Synthetic Fosmidomycin Analogues with Altered Chelating Moieties Do Not Inhibit 1-Deoxy-D-xylulose 5-phosphate Reductoisomerase or *Plasmodium falciparum* Growth *In Vitro*. Molecules. 2014; 19(2):2571-2587.

Jackson, E.R., San Jose, G., Brothers, R.C., Edelstein, E.K., Sheldon, Z., Haymond, A., Johny, C., Boshoff, H.I., Couch, R.D., and Dowd, C.S., The effect of chain length and unsaturation on Mtb Dxr inhibition and antitubercular killing activity of FR900098 analogs. Bioorganic & Medicinal Chemistry Letters, 2014 Jan 15;24(2):649-53.

San Jose, G., Jackson, E.R., Uh, E., Johny, C., Haymond, A., Lundberg, L., Pinkham, C., Kehn-Hall, K., Boshoff, H.I., Couch, R.D., and Dowd, C.S., Design of Bisubstrate Inhibitors of 1-Deoxy-D-xylulose 5-Phosphate Reductoisomerase (Dxr) from *Mycobacterium tuberculosis* (Mtb). *Med. Chem. Comm.* 2013 4:1099-1104.

McKenney ES, Sargent M, Khan H, Uh E, Jackson ER, San Jose G, Couch RD, Dowd CS, van Hoek ML. Lipophilic prodrugs of FR900098 are antimicrobial against Francisella novicida in vivo and in vitro and show GlpT independent efficacy. PloS one. 2012; 7(10):e38167.

Conclusion

Over the duration of this award (Feb 10, 2012 through to Dec 25, 2015), five specific aims were being pursued in the Couch lab: 1) Express, purify, and characterize recombinant *Y. pestis* IspC and IspD, 2) Optimize HTS assay conditions for IspC and IspD, 3) Provide purified recombinant *F. tularensis* IspC and IspD for crystallization and structure determination, 4) Provide purified recombinant *Y. pestis* IspC and IspD protein for crystallization and structure determination, and 5) Evaluate structure-activity relationships of rationally designed inhibitor molecules in enzyme-based assays. Accordingly, we have successfully cloned, expressed, purified, and enzymatically characterized the *Y. pestis* IspC. Expression difficulties with recombinant *Y. pestis* IspD prompted us to focus instead on the *F. tularensis* IspD, as we established conditions for its purification in high yield with high specific activity. We have successfully established HTS conditions for both IspC and IspD assays. Pilot scale screening of a commercially available molecular library, as well as our proprietary natural product library, has identified hit compounds for IspC and IspD, including a novel inhibitor of IspC that binds to an allosteric site on the enzyme. This allosteric site has not been previously identified on any homolog of IspC and represents a new site for the rational design of a new class of antimicrobial drugs. We have provided purified protein on-demand for protein crystallography, and we have

thoroughly evaluated structure-activity relationships of several rationally designed inhibitors of IspC and IspD via enzyme-based assays.

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