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

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The threat of bioterrorism and the use of biological weapons against both military personnel and civilian populations has become an increasing concern for governments around the world. The 1984 Rajneeshee Salmonella attack, 2001 anthrax letter attacks, 2003 SARS outbreak, 2009 H1N1 swine flu pandemic, and the current US flu epidemic all illustrate our vulnerability to both deliberate and natural outbreaks of infectious disease and underscore the necessity of effective antimicrobial and antiviral therapeutics. The prevalence of antibiotic resistant strains and the ease by which antibiotic resistance can be engineered into bacteria further highlights the need for continued development of novel antibiotics against new bacterial targets. This research project directly addresses this need through the development of a broad spectrum inhibitor of the biothreat agents *Francisella tularensis* and *Yersinia pestis*.

During this period of performance, we have utilized our optimized assays with the *Y. pestis* MEP synthase and the *F. tularensis* MEP cytidylyltransferase to screen molecular libraries and identify effective inhibitors of both MEP synthase and MEP cytidylyltransferase.

**Subject Terms**: Enzymology, MEP pathway, bacteria, isoprene, drug discovery, antibiotics, screening, biothreat, inhibitors
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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).

![Graph showing growth inhibition of F. tularensis and Y. pestis by compounds fosmidomycin and FR900098](image)

Figure 1. Growth inhibition of F. tularensis (top) and Y. pestis (bottom) by the compounds fosmidomycin and FR900098 (these molecules inhibit MEP synthase, 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 the MEP path way enzymes MEP synthase and MEP cytidylyltransferase in complex with inhibitors, and using this information to design and synthesize novel broad spectrum antibacterials.

**MEP Synthase (IspC)**

During this period of effort, we used a combination of affinity column chromatography, mass spectrometry, and enzymology to definitively identify the active component in e29, the natural product inhibitor described in previous reports. A manuscript detailing this research is currently in preparation. As this inhibitor is the first of its kind, able to allosterically inhibit the enzyme, crystal screens with the *Yersinia pestis* MEP Synthase in complex with the inhibitor are presently underway.

As depicted in the Figure below, we have also performed a high-throughput screen of MEP Synthase using a commercially purchased molecular library (LOPAC library). Due to its wide dynamic range of activity, the primary screen was performed with the *Y. pestis* MEP Synthase. To ensure broad spectrum activity, a secondary screen was then performed with purified

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**Primary Screen: 96-Well Plate Assay with *Yersinia pestis* IspC**

Primary Screen yielded 232 compounds of interest (reduced enzyme activity by 75% or more).

**Secondary Screen: UV-Vis Assay with *Mycobacterium tuberculosis* IspC**

- Secondary Screen was conducted using MtIspC to assess each compound for broad spectrum activity.

Secondary Screen yielded 22 compounds of interest (reduced enzyme activity by 75% or more).

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Figure 2. Schematic of the approach to performing the high-throughput screen with IspC.
recombinant *Mycobacterium tuberculosis* MEP Synthase and the 232 hits identified in the primary screen. To eliminate false positives (compounds that have innate absorption at 340 nm, thereby interfering with the NADPH signal (ΔA340)), a tertiary screen was then performed, using an assay coupling MEP Synthase with MEP Cytidylyltransferase (IspD), as illustrated in Figure 3. The seven molecules demonstrating inhibition in both the single and coupled assays are considered hit compounds. Bacterial growth inhibition assays with these top seven compounds are currently underway. Screening of a second commercially available molecular library is also underway.

**Tertiary Screen: 96-Well Plate Assay with *Yersinia pestis* IspC Coupled to *Francisella tularensis* IspD**

- To eliminate false positives with interfering signal at 340 nm, the top 22 compounds were assayed with IspC coupled to IspD and monitored at 620nm (IspD assay).

**Example Hit: Tranilast**

- Very high ΔA340 in plate assay
- Enzyme appears to have no activity in UV/Vis assay
- Or is Tranilast a potent inhibitor, or is high ΔA340 background obscuring the signal?
- Must assay where λ ≠ 340nm to verify

Note: All compounds were also assayed in the presence of FtIspD alone to ensure loss of signal is due to YpIspC inhibition.

**Figure 3. Schematic of the approach to performing the tertiary screen with IspC.**

**MEP Cytidylyltransferase (IspD)**

As illustrated in Figure 4, we have also performed a high-throughput screen with the *Francisella tularensis* MEP Cytidylyltransferase. As the enzyme assay is coupled to the activity of pyrophosphatase, a tertiary screen was performed with the latter enzyme alone to assure the hit compounds are selective for IspD (Figure 5). Bacterial growth inhibition assays with these top five compounds are currently underway. Screening of a second commercially available molecular library is also underway.
Primary Screen: 96-Well Plate Assay with *Francisella tularensis* IsPD

- Identified Hits
- Example Plate 4 with 2 Identified Hits

Primary Screen yielded 35 compounds of interest (reduced enzyme activity by 75% or more).

Secondary Screen: UV-Vis Assay with *E.coli* IsPD

- Secondary Screen was conducted using *E.coli* IsPD to assess each compound for broad spectrum activity.

Secondary Screen yielded 8 compounds of interest (reduced enzyme activity by 75% or more).

**Figure 4.** Schematic of the approach to performing the high-throughput screen with IsPD.

Tertiary Screen: Assay hits against pyrophosphatase

- Tertiary Screen yielded 5 compounds of interest (reduced IsPD activity by 75% or more, but did not significantly inhibit pyrophosphatase).

**Figure 5.** Schematic of the approach to performing the tertiary screen with pyrophosphatase and IsPD.
Key Research Accomplishments

- Optimization of MEP synthase and MEP cytidylyltransferase assays for HTS.
- Screening of molecular libraries for inhibitors of MEP synthase and MEP cytidylyltransferase.
- Identification of a previously unknown allosteric site on the MEP synthase enzyme from *F. tularensis*, *Y. pestis*, and *M. tuberculosis*.
- On-demand production and delivery of recombinant proteins to WRAIR for X-ray crystallography.

Reportable Outcomes


- Funds for this project are used to support a lab technician (Ms. Chinchu Johny) and a graduate student (Ms. Amanda Haymond).

Conclusion

In summary, during this fiscal period, we have optimized assays with the *Y. pestis* MEP synthase and the *F. tularensis* MEP cytidylyltransferase for use in HTS. The screening of a commercially available library has identified several hit molecules for each of these enzymes. We have also identified the natural product allosteric inhibitor of MEP synthase.

During the next fiscal period, we will focus on screening a second large and chemically diverse molecular library for additional inhibitors of MEP synthase and MEP cytidylyltransferase.

References


