# Evaluation of DapE Inhibitors Utilizing the DapE Ninhydrin and Thermal Shift Assays Toward the Discovery of Novel Antibiotics

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An ongoing effort continues against the rising tide of antibiotic resistant bacteria, underscoring the urgent need to discover antibiotics with a novel mechanism of action. To this end, we have focused on the inhibition of the bacterial enzyme *N*-succinyl-L,L-diaminopimelic acid desuccinylase (DapE). Guided by docking with the computational suite Molecular Operating Environment (MOE) and lead molecules obtained through a HiTS (High Throughput Screening) assay, several lead molecules and analogs were identified, synthesized, and optimized by our research group. These potential new inhibitors are tested in our recently described and updated biochemical DapE ninhydrin assay, with $IC_{50}$ data obtained for inhibitors including cyclobutanone, tetrazole, pyrazole, sultam, and indoline sulfonamide analogs. To gain a greater understanding of the thermodynamic effects of these inhibitors on the binding of these synthesized analogs with the DapE enzyme, we have employed a Thermal Shift Assay (TSA).

## Background

Although the world currently faces a major battle against the global COVID-19 pandemic, an ongoing fight continues against an increase in antibiotic resistant bacteria, underscoring the urgent need to discover antibiotics with a new mechanism of action.[1] We are investigating this increase in mortality and morbidity to advance our fight against antibiotic resistance. Our medicinal chemistry research has revealed the possibility of a new class of antibiotics, which targets a previously-unexplored biochemical pathway, utilizing inhibition of the enzymatic activity of *N*-succinyl-L,L-diaminopimelic acid desuccinylase, or DapE, as seen in **Figure 1**.[2] This enzyme is part of a crucial biosynthetic pathway that produces the amino acid lysine and mDAP, both essential to protein synthesis and bacterial peptidoglycan cell wall construction, respectively. Therefore, inhibition of DapE enzymatic activity is very attractive because its inhibition should be selectively lethal to bacteria, while safe for mammals, as lysine is received from our diet, and we do not express the DapE enzyme.

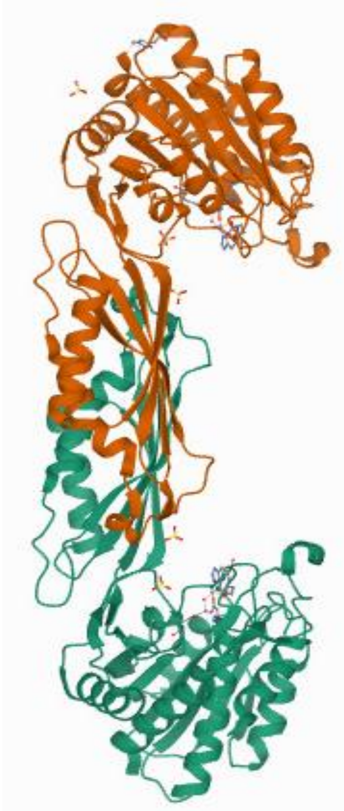


**Figure 1.** DapE X-Ray Crystal Structure (PDB 5UEJ).[2]

## Objectives

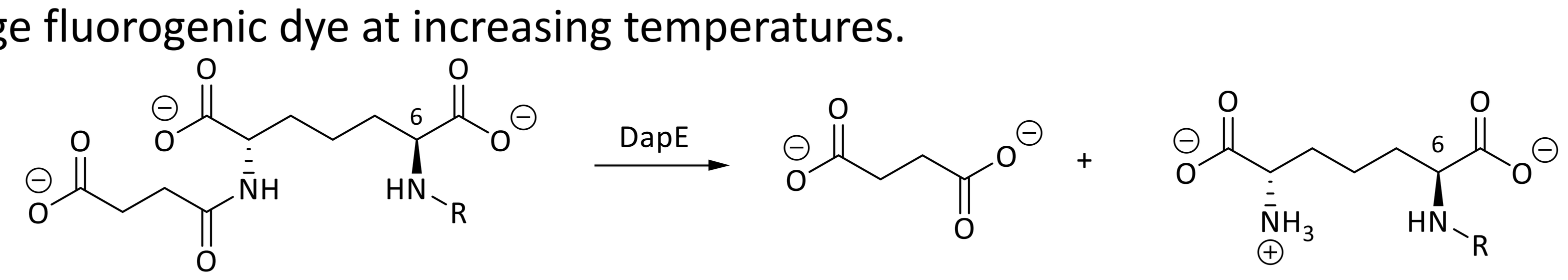
- Work in collaboration with graduate and undergraduate students in our research group
- Utilize the computational suite MOE and the lead molecules obtained from HiTS to discover new inhibitor series for DapE – tetrazoles, pyrazoles, sultams, indoline sulfonamides,[4] and cyclobutanones[5]
- Synthesize analogs of the original hits and obtain inhibition data using the ninhydrin assay
- Analyze the molecular interactions between DapE's structure and the inhibitors to gain greater understanding of the enzyme dynamics involved in the inhibition process
- Use improved understanding to aid in future drug synthesis for the optimization of new inhibitors
- Employ a Thermal Shift Assay (TSA) to further our understanding of the thermodynamic effects of the binding of the inhibitor analogs with the DapE enzyme

## Methodology

Our ninhydrin assay[3] is utilized to demonstrate inhibition of DapE representing potential antibiotic efficacy. The assay consists of incubating the active DapE enzyme, a modified substrate, $N^6$-methylsuccinyl diaminopimelic acid ($N^6$-Me-L,L-SDAP), HEPES buffer, DMSO solvent, and the inhibitor. The potential inhibitors are tested at various concentrations at 30°C for 10 minutes, followed by the quenching of the reaction and deactivation of the enzyme by heating for 1 minute. Ninhydrin is added, reacting with the primary amine of the now-cleaved product, as seen in **Figure 2**, converting the solution to Rhumann's purple. The absorbance at 570 nm is measured in a spectrophotometer using a well-plate reader. The absorbance data obtained represents the amount of substrate converted to product within the assay. These data are exported and analyzed using Excel and PRISM to obtain $IC_{50}$ values.[3]

In medicinal chemistry and drug discovery, $IC_{50}$ refers to half the maximum inhibitory concentration. It is utilized to compare the effect that potential drugs have on specific biological pathways or processes; therefore, it is a common way to report inhibitory effects of small molecule drug candidates. Experimentally obtained $IC_{50}$ data of novel compounds may be compared to that of previously identified DapE inhibitors, such as the thiol-containing ACE inhibitor captopril, to determine their efficacy as potential new antibiotics.

Another means of improving upon our analogs and our overall understanding of the inhibition of the enzyme is through the employment of a Thermal Shift Assay (TSA). This consists of analyzing the thermal denaturation temperature of the DapE enzyme through the utilization of q-PCR to obtain fluorescence data. It involves of the incubation of the active DapE enzyme, the modified substrate, HEPES buffer, DMSO solvent, the inhibitor, and SYPRO Orange fluorogenic dye at increasing temperatures.

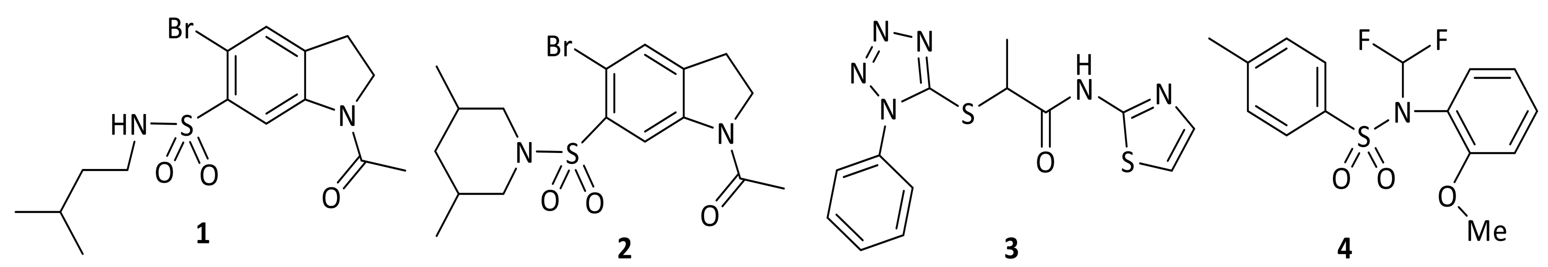



**1a** R = H L,L-SDAP
**1b** R = $CH_3$ $N^6$-Me-L,L-SDAP
succinate **2**
**3a** R = H L,L-DAP
**3b** R = $CH_3$ $N^6$-Me-L,L-DAP

**Figure 2.** Hydrolysis of L,L-SDAP by DapE. L,L-SDAP (**1a**) and assay substrate $N^6$-methyl-L,L-SDAP[6] (**1b**) with formation of hydrolysis products succinate (**2**) and L,L-diaminopimelic acid derivatives **3a** and **3b**, respectively.

## Lead Molecules as DapE Inhibitors

**Figure 3** contains the lead structure from each inhibitor series to demonstrate the wide variety of inhibitors being tested in our critical assays. Some lead molecules are in their beginning stages of synthesis, while we have just published advanced inhibitors based on the indolines **1** and **2**.[4]




| **1** | **2** | **3** | **4** |
|---|---|---|---|
| $IC_{50}$ = 42% @ 200 uM | $IC_{50}$ = 133 uM (nor-Me) | $IC_{50}$ = 89 uM | $IC_{50}$ = 72.9 uM |
| MW = 389 g/mol | MW = 415 g/mol | MW = 332 g/mol | MW = 327 g/mol |
| cLogP = 3.29 | cLogP = 3.5 | cLogP = 1.89 | cLogP = 3.69 |
| PSA = 66.5 Å$^2$ | PSA = 57.6 Å$^2$ | PSA = 81.8 Å$^2$ | PSA = 46.6 Å$^2$ |

**Figure 3.** Lead molecules as DapE inhibitors.

## Results and Conclusions

Inhibition data for a series of indolines has recently published by our group.[4] We have obtained preliminary inhibition data for series **3** and **4** inhibitors, as well as for cyclobutanones, and this data will be published in due course. Furthermore, after performing these reactions and obtaining DapE inhibition data, we will co-crystallize the drug candidates in collaboration with Northwestern CSGID and obtain high-resolution co-crystal structures of the inhibitors with the DapE enzyme to elucidate the binding mode of the inhibitors as well as the distinct enzyme conformations[6] with the different compound moieties bound to the active sites. With this information, we will be able to advance our understanding of the enzyme-drug interactions through an improved knowledge of the structure activity relationship (SAR) between the different classes of inhibitors with the enzyme, which will in turn enhance our effort toward the discovery of new antibiotics.

## Continued Work

Updates and improvements continue to be made to streamline our assay to enable faster throughput and more accurate results. A thermal cycler, an instrument that automatically regulates the temperature of the sample and the incubation time, is now employed in obtaining $C_{50}$ data. This enables the reduction of error, increasing the accuracy of our results, which in turn aids in every project in our group.

To further our understanding of the enzyme dynamics taking place in the ninhydrin assay, we have been conducting kinetic assays to obtain $K_i$ data. With this information, we will have an improved understanding of the effects of the inhibitors on the enzyme.

It is imperative to note our ongoing work to broaden the scope of our research to combat growing antibiotic resistance. We have set up the assay with the DapE enzyme from an additional bacterial species, *A. baumannii* (CSGID), to work toward confirming broad spectrum antibiotic activity. We are working in collaboration with CSGID (Center for Structural Genomics of Infectious Diseases) at Northwestern as well as Argonne National Laboratories to expand the validation of DapE as a target for different types of bacterial infections.

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## Acknowledgements

Members of the Becker Research Lab

Northwestern Center for Structural Genomics of Infectious Diseases

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