MLSCN Probe Summary

SOUTHERN RESEARCH MOLECULAR LIBRARIES SCREENING CENTER
The Drug Discovery Division of Southern Research

Screening Center: Southern Research Molecular Libraries Screening Center

PI of center: Gary A. Piazza, Ph.D.
Project Title: High Throughput Screen for *Mycobacterium tuberculosis* Pantothenate Synthetase
Grant Number: 1R03-MH-076412
Assay Submitter and Institution: E. Lucile White, Southern Research Institute
Assay or Pathway Target: bacterial pantothenic acid biosynthesis
Probe PubChem Compound Identifier (CID): 16740889

Assay Provider Information

Specific Aim: To identify specific probes that inhibit the *Mycobacterium tuberculosis* pantothenate synthetase enzyme for X-ray crystallographic studies

Significance: By the late 1980s, tuberculosis (TB) reappeared as a serious threat to public health in the United States. Several factors contributed to the reemergence of the disease in the U.S., including a steady decline in mandated public health standards concerning TB, increased numbers of patients in long-term care facilities, overcrowding in prisons, increased immigration from countries with a high incidence of TB, and large increases in the inner city homeless population (1-3). The relatively recent epidemic of the human immunodeficiency virus (HIV) and the development of AIDS in a large population have overshadowed these factors. AIDS, and the attendant immunodeficiency characterizing this syndrome, have resulted in an ever-increasing, large population highly susceptible to opportunistic pathogens including the mycobacteria *M. avium* and *M. tuberculosis* (4). TB commonly has a much earlier onset in AIDS patients than other opportunistic pathogens and is oftentimes hard to detect by standard techniques such as a positive tuberculin skin test that relies on a potent immune reaction not possible in the immune compromised individuals (5). In fact, TB is now the single largest killer of AIDS patients (6). Recent advances in the treatment of HIV (highly active antiretroviral therapy) are positively impacting survival and reducing the incidence of opportunistic diseases in AIDS patients (7). It is unclear what the long-term impact on TB incidence in this population will be. Sexual transmission of multiple drug-resistant forms of the virus has been documented (8,9), however, suggesting that the need for effective treatment of opportunistic pathogens, particularly Mtb, should remain a high priority.

Worldwide, TB statistics are even more staggering. It is estimated that one third of the world's population is infected with TB, with about 8 million new cases annually (6,10). Of these, 3.1 million die annually, more deaths than caused by malaria or any other single infectious disease (6,10). TB is the leading killer of youths, women, and AIDS patients in the world (6). It has been estimated that up to 50 million people are infected with drug-resistant forms of TB (2,6). The development of drug resistance in the population has increased concern that TB may once again become an incurable disease. Of particular concern is the development of multi-drug-resistant forms of the disease (MDR-TB), defined as forms resistant to two or more of the front line anti-TB agents (2,6). These forms of the

disease are more often fatal and are difficult and expensive to treat. In industrialized nations, standard TB treatment costs on the order of $2,000 (US) per patient, but escalates more than 100-fold in patients with MDR-TB, to as much as $250,000 (6). Such costs are difficult to bear in industrialized nations, much less in developing countries.

Rationale: Pantothenate is a water soluble vitamin essential in microorganisms and plants as it is the key precursor for the biosynthesis of coenzyme A (CoA) and acyl carrier protein (ACP; 11). Pantothenate synthetase, encoded by the *panC* gene, catalyzes the formation of pantothenate in bacteria, yeast, and plants (12). In mammals it is obtained through the diet. In most plants, bacteria and yeast, pantothenate biosynthesis is essential. The pathway is shown in **Figure 1**.

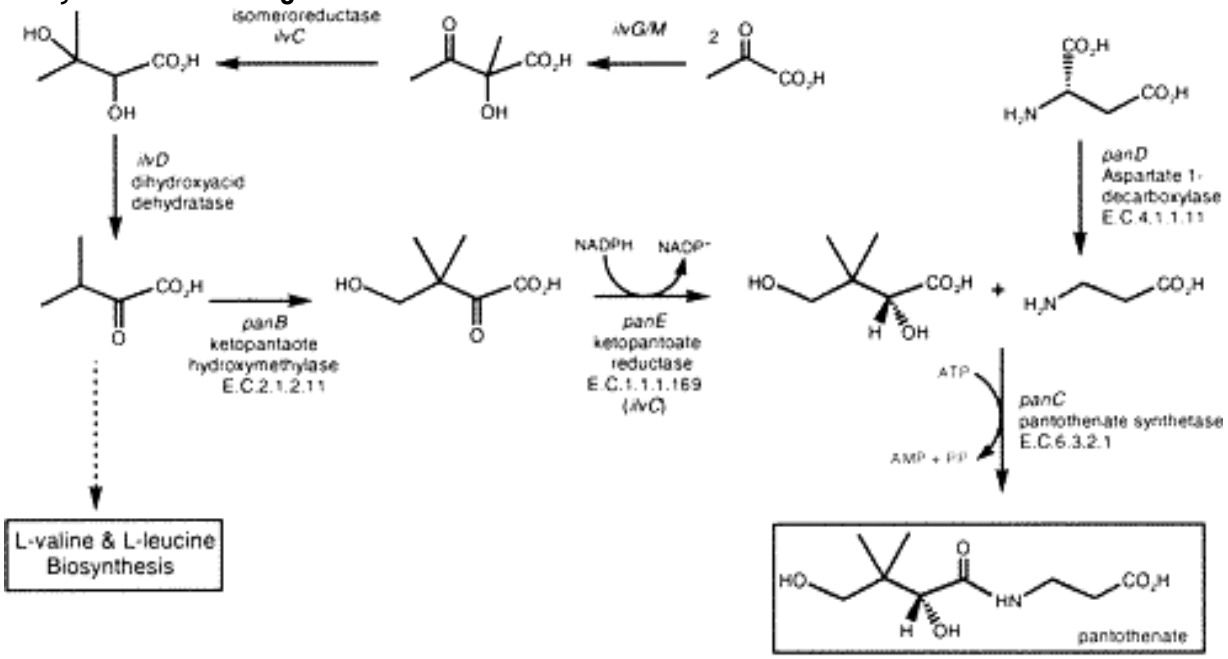


Figure 1 Pantothenate Biosynthesis in Bacteria, Plants, and Yeast.

Recently Jacobs *et al.* (13) reported that an auxotrophic mutant of MTB defective in the *de novo* biosynthesis of pantothenate is highly attenuated in immune compromised mice. This indicates that a functional pantothenate biosynthetic pathway is essential for virulence of MTB. Also Jacobs (14) has been studying *M. tuberculosis* knock-outs of *pan*C/*pan*D and *lys*A in the virulent H37Rv strain of *M. tuberculosis*. The phenotype of the mutant *M. tuberculosis* cells is the same as the one observed when the RD1 region (where RD1 is the biggest deletion region in the vaccine BCG strain compared to the virulent H37Rv strain) is also knocked out of H37Rv *M. tuberculosis* strain. This implicates the pantothenate biosynthetic pathway in the pathogenetic nature of H37Rv *M. tuberculosis* strain.

Screening Center Information

Assay Implementation and Screening

- **PubChem Bioassay Name:** Mycobacterium tuberculosis Pantothenate Synthetase Assay
- **PubChem Bioassay AIDs**: 375, 838
- **Primary Assay Description (PubChem**):
  The activity of pantothenate synthetase (PS; EC 6.3.2.1) was measured spectrophotometrically by coupling the formation of AMP to the reactions of myokinase, pyruvate kinase, and lactate dehydrogenase. The rate of AMP formation was quantitated

by the reduction of absorbance (OD340) due to the oxidation of NADH to NAD by lactate dehydrogenase. A kinetic assay was chosen to minimize interference by compounds that absorbed at 340 nm.

Purified recombinant pantothenate synthase enzyme was provided by Dr. David Eisenberg and Dr. Celia W Goulding at Howard Hughes Medical Institute and UCLA. Subsequently, pantoic acid was synthesized at Southern Research Institute. The reagent mix included a final concentration of 0.4 mM NADH, 5 mM pantoic acid, and 18 units each of myokinase, pyruvate kinase, and lactate dehydrogenase diluted in assay buffer (100 mM HEPES buffer (pH 7.8), 10 mM $MgCl_2$, 5 mM ß-alanine, 10 mM ATP, and 1 mM potassium phosphoenolpyruvate) and was added to assay plates in a volume of 15 µl. Compounds were then added to plates in 1 µl volumes at 25x dose concentration. The reaction was initiated with the addition of 10 µl of pantothenate synthetase, diluted in assay buffer for a final concentration of 20 µg/ml. The test plate was immediately transferred to a Perkin Elmer Envision microplate reader and absorbance was measured at 340 nm every 16 seconds for 160 seconds. Data was analyzed using ActivityBase software, and hits were determined by comparing the assay rates of the compound wells to assay plate controls.

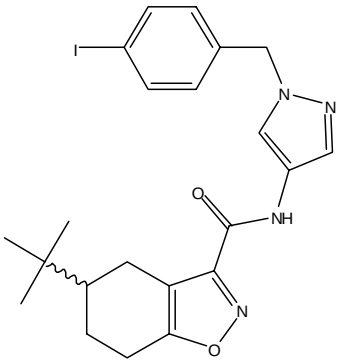
- **Center Summary of Primary Screen:**
  A total of 9,993 compounds were initially screened at a final concentration of 10 uM. Z' scores for this assay were consistently greater than 0.65 demonstrating that this is a very robust assay. The mean inhibition of the compound wells was 3.36% with a standard deviation of 3.99%. Inhibition values of greater than 15.34% were outside the calculated noise of the assay defined by $\bar{x} + 3\sigma$.

  Compounds with more than 30% inhibition were scored as hits. These hits were then tested in dose response at ten concentrations, ranging from 100 uM to 0.196 uM. To confirm that the compounds were specifically inhibiting PS and not one of the other enzymes in the assay, the hits were tested in parallel in an assay that contained only these 'coupling enzymes'. Compounds that demonstrated confirmed activity in dose response and specificity for pantothenate synthetase were selected to enter chemistry.

Probe Optimization

- Chemical name of probe compound:
  5-*tert*-butyl-N-[1-[(4-iodophenyl)methyl]pyrazol-4-yl]-4,5,6,7-tetrahydro-1,2-benzoxazole-3-carboxamide; PubChem CID 16740889

- Chemical Structure (incl stereochem if known):



- Describe mode of action for biological activity of probe:

PubChem CID 16740889 is a 60 nM inhibitor of the mycobacterial enzyme, the most potent in its series. The corresponding *p*-fluoro derivative was slightly less active, and was also found to be unsuitable for X-ray crystallographic analysis.

- Has this compound been provided to the MLSMR:

Vials have been requested to submit samples of the entire series (~30 compounds). We note that normally the MLSMR does not accept samples containing iodine, so this exact compound would be excluded. However, we seek an exception from this arbitrary rule. First, the heavy atom was intentionally introduced after the assay submitter requested it in order to facilitate phasing of the X-ray structure of the bound enzyme-ligand complex. The fluoro counterpart and other similar derivatives had failed for this purpose. Consequently, for probe as opposed to drug design, use of a heavy atom is precisely what this program was designed to do. Secondly, the rule to exclude all phenyl iodides from the repository is not well thought out. These molecules are perfectly stable, and drugs, even natural human hormones, contain the functionality, and the closely related bromo compounds are acceptable to the SMR.

- Description of secondary screens used to optimize probe structure:

In addition to assessing the IC_{90}, noted above, the compounds have been examined for their ability to inhibit *M. tuberculosis* in vitro (they are inactive), the K_i is currently being measured, and samples are undergoing co-crystallization with the bacterial enzyme for X-ray structural analysis by Dr. Celia Goulding, UC Irvine.

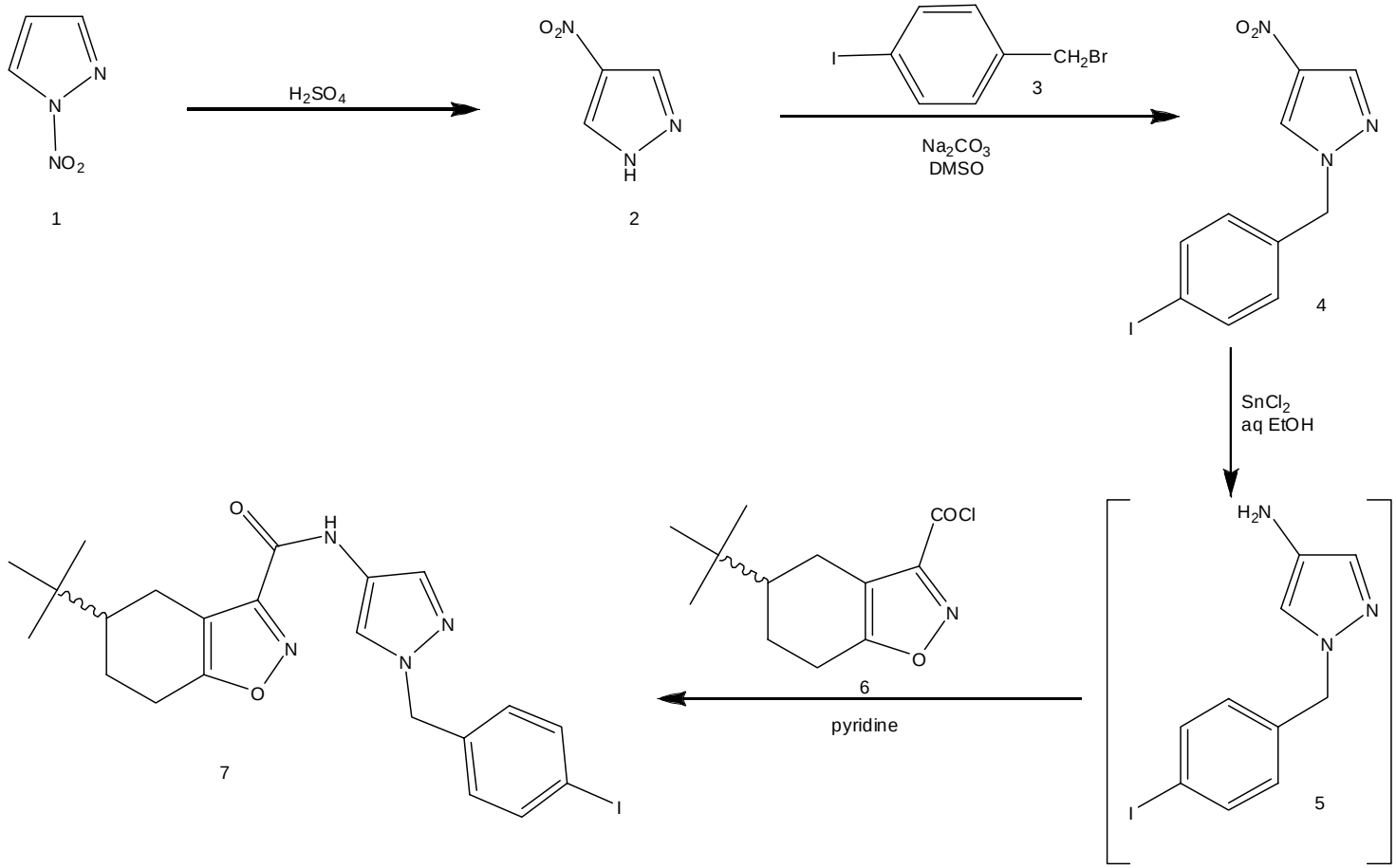
- Center comments on chemistry strategy leading to probe identification:

Chemistry began with the purchase of a series of ~40 commercial analogs of the initial hit to gather preliminary SAR before committing to synthesis. From that analysis, it was clear that modification of the pyrazole benzyl substituent was tolerated by the enzyme and could improve activity. Since the crystallographer had been working with a resupplied sample of the fluoro compound while we were accumulating SAR on the commercial derivatives, by the time we were ready to begin synthetic chemistry, the decision to move to a heavy atom derivative had already been made. Consequently, we were able to generate the 2-, 3-, and 4-iodophenyl analogs quite quickly. Both the 3-iodo and 4-iodo derivatives were more potent the corresponding 4-fluoro compound.

Simultaneously, we pursued a second design strategy that involved aromatization of the cyclohexene ring that is annulated to the oxazole fragment. Aromatization has the effect of removing the chiral center at the site of attachment of the *t*-butyl substituent, which could also improve the quality of the X-ray structure (assuming both of the enantiomers are able to bind to the enzyme in somewhat different modes). Unfortunately, aromatization of the ring resulted in a large decrease in potency, so this approach was abandoned once the iodo probes were identified.

- Detailed synthetic pathway for making probe:

The synthetic scheme used for the synthesis of this series is depicted below, along with the detailed experimental protocol:



Experimental:

1. Synthesis of 4-nitropyrazole (**2**)

1-Nitropyrazole **1** (10g) was slowly added to a round-bottomed flask containing H_2SO_4 (98%, 30 mL) and stirred for 20h at room temperature. The reaction mixture was slowly transferred to a beaker containing 300g of ice with stirring. The solution was extracted with ethyl ether (200 mL x 3). The organic layer was dried with Na_2SO_4 then evaporated to afford **2** as a colorless solid (9.6g, 96%), which can be used directly in next step without further purification. The solid was recrystallized from ether/hexane for analysis.

2. Synthesis 1-[4'-phenylmethyl]-4-nitropyrazole (**4**)

Compound **2** (223 mg), 4-iodobenzylbromide **3** (297 mg and Na_2CO_3 (226 mg) were dissolved in anhydrous DMSO (6 mL). The mixture was stirred at 90°C for 16 h. After cooling to ambient temperature, the mixture was poured into a beaker containing 30g of ice and 5g of NaCl. The solid was collected by filtration, washed with warm water 3 times, and then treated with acetone. The suspension was filtered, and the filtrate evaporated. The residue was purified on a silica column, and then recrystallized from acetone/hexane to afford **4** as a colorless solid (360 mg). Concentration of the mother liquor afforded another 78 mg.

3. Synthesis of probe **7**

Compound **4** (165 mg) was treated with $SnCl_2$ (0.3g) in 95% ethanol. The mixture was stirred at 90°C for 2 h. The solvent was removed and crude amine **5** was purified on a silica gel

column to give a pink solid, which was dried *in vacuo*, then dissolved in pyridine (1 mL). Compound **6** (247 mg), prepared from the corresponding commercially available carboxylic acid by treatment with thionyl chloride, was added to the solution. After 2 h the reaction mixture was evaporated. The residue was purified on a silica column to give probe **7** as a colorless solid (165 mg).

- Center summary of probe properties (solubility, absorbance, fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:

Compound **7** and its analogs display good solubility in organic solvents including DMSO. Dissolution in DMSO followed by aqueous dilution provides satisfactory solubility for in vitro and biochemical testing. There are no issues with storage, handling, or reactivity. There is no detailed toxicological profile available for this compound, since that data were not relevant to the requirements desired for a crystallographic tool, but no unusual toxicity was noted during *in vitro* testing against mycobacteria. The compounds do not display significant fluorescence.

Appendices:

Comparative data on probe, similar compound structures and prior probes:

Prior to this study, there were no known potent inhibitors of bacterial pantothenate synthetase.

Comparative data showing probe specificity for target:

We do not currently have access to pantothenate synthetase from other bacteria to assess specificity. However, the hits in this series were selected for probe development because they had not been identified as hits in any other assay reported on PubChem, nor were they known from the scientific or patent literature to possess significant biological activity.

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