DEFINING THE ACTIVE SITE OF 2-(2'-HYDROXYPHENYL) BENZENESULFINATE DESULFINASE

THESIS

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ABSTRACT

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May 2003

Supervising Professor: Dr. Linette M. Watkins

Diminishing resources of light crude oils containing low sulfur fractions have compelled petroleum refineries to process sour crude oils. Current sulfur removal processes include the use of hydrodesulfurization. Hydrodesulfurization uses an inorganic catalyst, hydrogen, high temperature and pressure to remove sulfur from petroleum leaving the sulfur in the form of hydrogen sulfide, resulting in environmental problems. Since larger volumes of sour crude oils, in the form of middle distillates, are being used in processing, the use of hydrodesulfurization has become costly and inefficient. Additionally, governmental regulation of the allowable sulfur content in refined fossil fuels has become more stringent. These trends have led scientists to research new desulfurization methods.

One such method is biodesulfurization, which utilizes microbes that possess carbon-sulfur bond cleavage capabilities to remove the sulfur. These microbes utilize multiple enzyme pathways to perform the desulfurization of compounds. An example of a carbon-sulfur bond cleaving organism is *Rhodococcus erythropolis* IGTS8. *R. erythropolis* uses a metabolic pathway consisting of four enzymes. This pathway catalyzes the desulfurization of dibenzothiophene to sulfite and hydroxybiphenol. The research conducted for this project has focused on the enzyme found in the final step of the pathway. The enzyme, known as 2-(2'-hydroxyphenyl) benzenesulfinate desulfinase (HPBS desulfinase), is considered the rate limiting step in the pathway. The initial phase of this study included the purification of HPBS desulfinase. The enzyme was purified 260-fold from *R. erythropolis*. Purification of the enzyme was monitored using UV-Vis, spectrofluorimetric assays, and SDS-PAGE. In order to define the active site of HPBS desulfinase, it was necessary to determine what types of compounds could be used as substrates or inhibitors. Twenty-one commercially available analogs of both the substrate (HPBS) and the product (HBP) were tested, along with two synthesized substrate analogs, and none were found to act as substrates. However, several behaved as competent inhibitors, and the two synthesized analogs increased the activity of the enzyme. Analysis of the two synthesized analogs was performed using ^{13}C NMR, ^{1}H NMR, mass spectroscopy, TLC, and pK_a determination. Further studies were conducted using thiourea dioxide and $CuCl_2$, which had both previously been shown to inhibit HPBS desulfinase. The results from the current study agree. In addition, it was determined that $CuCl_2$ binds to the cysteine in the active site and not the sulfonic acid on the substrate. Chemical modification of the cysteine in the active site using DTNB

showed that once modified, the enzyme showed no activity in the presence of substrate. Finally, a fluorescence method of determining inhibitor binding was developed and a K_D of 1.6 μM was calculated for 1, 8-naphthosultam's (an inhibitor) binding to the enzyme.

CHAPTER I

INTRODUCTION

Sulfur content in fossil fuels has become increasingly regulated, decreasing the percentage allowed to remain in refined oils. The current allowable sulfur content is 500 ppm, however, the Environmental Protection Agency has recently suggested a 97% reduction to15 ppm by the year 2006 (1). Declining availability of light crude oils containing lower sulfur contents will force petroleum refining companies to process sour crude oils and invest heavily in building and operating hydrodesulfurization processes (2). These processes include the use of an inorganic catalyst reacting with petroleum fractions and hydrogen in pressurized, high temperature conditions producing the desulfurized petroleum product and hydrogen sulfide (3, 4). One such inorganic catalyst is a binuclear ruthenium complex (4). This complex cleaves carbon-sulfur bonds by forming coordination spheres around each thiophene atom by binding the thiophene to a ruthenium center. This process, though, is very costly (5). Consequently, the increased necessity to use less preferential crude oils is arising. This is increasing the need to utilize middle-distillate fractions, which contain organic sulfur compounds consisting mainly of benzothiophenes and dibenzothiophenes (2). The sulfur bound to these organosulfur compounds is difficult to remove by current processing methods, including

hydrodesulfurization, because these compounds are heteroaromatic (6). Some examples of heteroaromatic compounds are shown in Figure 1:

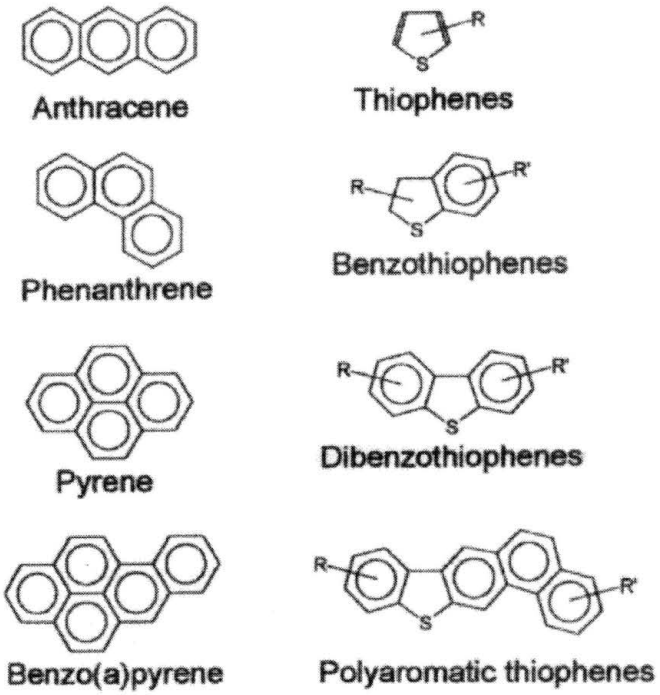

Figure 1. Heteroaromatic and polyaromatic compounds found in fossil fuels (10).

These heteroaromatic conformations cause steric problems for cleavage of the carbon-sulfur bond by hydrodesulfurization. Furthermore, conventional refining processes, such as hydrodesulfurization, release the sulfur in the form of hydrogen sulfide, which must be further processed to avoid release into the environment (2). Thus, it has become critical that new processes be developed in order to efficiently remove sulfur from fossil fuels.

In order to confront these issues, the use of microbes in removing sulfur has become an increasingly viable technique to process fossil fuels without the damaging environmental release of sulfites (15). Even with the advantages of using microbes,

however, a number of issues still must be resolved to make this a viable alternative. These issues involve the large volumes of water necessary for microbial desulfurization, the long reaction time required, and the inability of some of the microbes to metabolize the large numbers of chemical structures existing within the petroleum (6, 7). So, along with the studies of the organisms themselves, current research must include ways to surmount the shortcomings of microbial desulfurization.

One of these bioprocessing systems currently being investigated is the use of *Rhodococcus erythropolis* IGTS8, which is a gram-positive soil bacterium originally found around coal deposits (8). *R. erythropolis* IGTS8 utilizes a pathway which has the ability to cleave carbon-sulfur bonds in thiophenes, sulfides, disulfides, mercaptans, sulfoxides, sulfones, as well as petroleum products. (9). Apparently, the steric problems of heteroaromatic compounds encountered by hydrodesulfurization are not problematic when using the enzyme. Furthermore, this microbe selectively removes sulfur from these organic molecules while leaving intact carbon. The desulfurization metabolic pathway associated with *R. erythropolis* IGTS8 has been previously elucidated by Kilbane (16) and was shown to involve four proteins: DszA, DszB, DszC and DszD which are expressed from four different genes (3, 6, 10-14). Two of these enzymes DszA (dibenzothiophene monooxygenase/DBT-MO) and DszC (dibenzothiophene-5,5-dioxide monooxygenase/$DBTO_2$-MO) are monooxygenases which require an $FMNH_2$ cofactor. Both of these enzymes require the presence of DszD, which is a flavin reductase requiring a NADH cofactor. The DszD enzyme is responsible for the regeneration of $FMNH_2$ (6). The final enzyme in the pathway is the DszB (2-(2'-hydroxyphenyl)

benzenesulfinate desulfinase) enzyme. which functions as a desulfinase. The Dsz enzymes expressed by the four dsz genes catalyze the following reaction scheme:

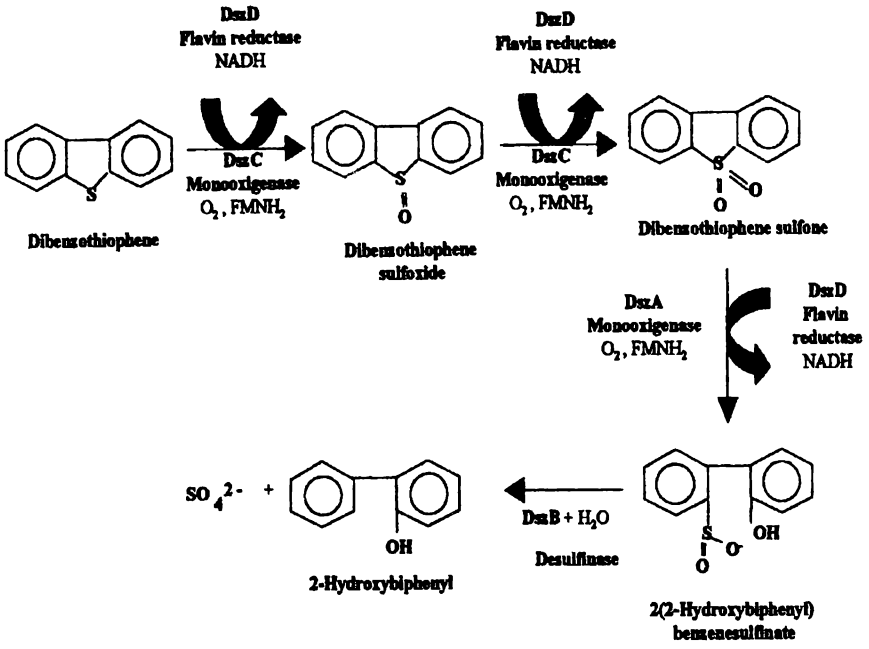

Figure 2. Schematic of the metabolic pathway of dibenzothiophene desulfurization (10).

The first step in the pathway utilizes DszC and converts DBT (dibenzothiophene) to a sulfoxide through electron transfer from $FMNH_2$ to DBT producing FMN and DBTO (dibenzothiophene sulfoxide). This step also requires DszD to regenerate $FMNH_2$ using NADH. DszC also catalyzes the formation of $DBTO_2$ (dibenzothiophene sulfones) from DBTO, again using DszD. The third step of the pathway is the cleavage of one of the carbon-sulfur bonds by electron transfer from $FMNH_2$ to $DBTO_2$ by DszA and DszD to form 2-(2'-hydroxyphenyl) benzenesulfinate. The final step in the pathway is cleavage of the second carbon-sulfur bond releasing the sulfur as sulfite, and liberating the intact hydrocarbon molecule as hydroxybiphenyl (HBP) (10).

Many of the desulfinase systems studied involve the use of DBT, a model compound of sulfur-containing fossil fuels (Figure 3). DBT is used as a model compound because it is found in considerable amounts in oils with high sulfur content and is also the most abundant species found in petroleum distillates and residues (10). Similarly, the experiments performed using the *Rhodococcal* enzymes also utilize DBT as its model starting material.

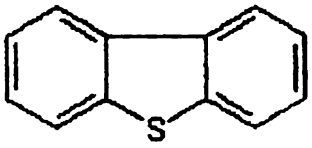

Figure 3. Dibenzothiophene

Through catalytic studies, the rate determining step for the reaction scheme previously shown was proposed to be the conversion of HPBS to HBP by DszB (15). This was proposed because DszB has the slowest catalytic rate in the pathway. Also known as 2-(2'-hydroxyphenyl) benzenesulfinate desulfinase (HPBS desulfinase), this protein is unique. It has no known sequence homology to any other protein and has been considered a new class of carbon-sulfur bond cleavage enzyme. Plus, it has no apparent cofactor requirement which makes direct characterization much more straightforward (6). Also, it is the only aromatic desulfinase and is a monomer. Since no crystal structure for this enzyme is yet available, characterization of the enzyme must be done through the use of other biophysical methods including fluorimetry and chemical modification studies.

Initial characterization of HPBS desulfinase has been accomplished. Kinetic assays were performed and the K_m (0.90 μM) and V_{max} (2.6 μmol hour^{-1} mg^{-1}) were determined.

Inhibition studies were also conducted using HBP and sulfite, and no significant inhibition was observed. Additionally, an optimum reaction pH (7.0) and temperature (35°C) were ascertained. In order to determine the pI of the enzyme, isoelectric focusing was performed and the pI was estimated to be 5.5. Further analogs of HBP were tested to find inhibition. Only 2,2'-biphenyl exhibited inhibition with a K_I of 16.5 μM. Metal binding studies were also run to determine their effects on enzyme kinetics. Only Cu^{2+} and Zn^{2+} behaved as inhibitors, whereas Ca^{2+} enhanced activity. Chelators were also tested. Three chelators acted as inhibitors: 1,10-phenanthroline, 2,2 dipyridol, and 8-hydroxyquinoline. Analogs of these chelators with no chelating ability were also tested. These analogs displayed the same inhibition as their chelating counterparts suggesting that chelation ability is not responsible for the inhibitory effect; it is instigated by the structure. In addition, using chemical modification studies and the genetic code, the active site has been shown to contain two conserved amino acid residues, cysteine-27 and tyrosine-24, and a large number of tryptophan residues. Through further chemical modification studies, the cysteine was shown to be important to catalysis. Current studies are focusing on the role of the tyrosine, and thus far, the tryptophan residues seem to have solely binding properties.

Several other microbial systems have been shown to utilize enzymes known to cleave carbon-sulfur bonds including cystathionine γ-lyase, methyl-CoM reductase, cysteine lyase, dimethylpropiothetin dethiomethylase, etc. (16-17). These enzymes all display a range of efficiencies at removing sulfur; however, they behave as model systems for non-oxidative carbon-sulfur bond cleavage reactions. The enzymatic mechanisms which have been proposed for these models have also been proposed for HPBS desulfinase, making

them of particular interest for study. Many of these species involve a metal cofactor, such as nickel, magnesium, or iron-molybdenum (11, 16, 18-23). One such system is NifS. This enzyme catalyzes the carbon-sulfur bond cleavage from L-cysteine resulting in the formation of L-alanine and elemental sulfur (cysteine desulfurase activity). NifS is utilized in nitrogen fixation pathways, and its suggested role is the biosynthesis of a necessary metallocluster required by nitrogenase (24-26). The desulfurization mechanism of this protein resembles the proposed base catalyzed mechanism of HPBS desulfinase, except that it requires a pyridoxal phosphate cofactor (Figure 4):

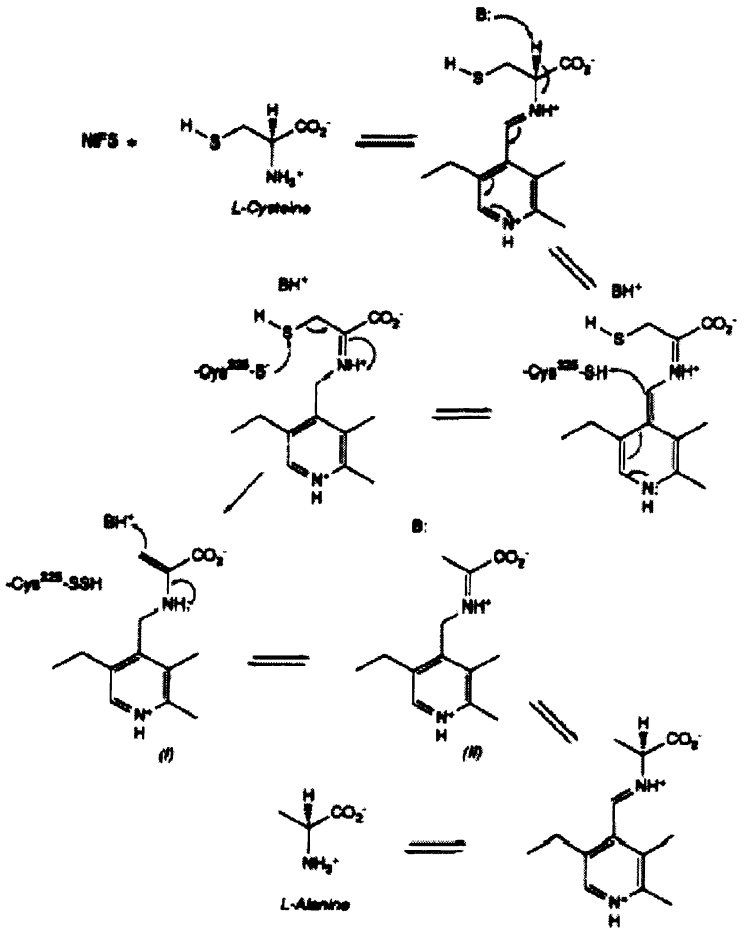

Figure 4. Suggested desulfurization mechanism of NifS (24).

Other organisms contain similar proteins, such as the selenocysteine lyase found in *E. coli* (26). Selenium is homologous to sulfur, and the mechanism which this lyase uses to remove selenium is very similar to NifS removal of sulfur (26). Moreover, enzymes such as tyrosine phenol-lyase are also mechanistically similar to NifS (27-31). Tyrosine phenol-lyase catalyzes carbon-carbon bond cleavage, and this protein depends on pyridoxal phosphate in their reactions like NifS (31). However, despite the importance of these enzyme systems, the research presented in this composition is centered on HPBS desulfinase.

The focus of this thesis is defining the active site of 2-(2'-hydroxyphenyl) benzenesulfinate desulfinase. The studies undertaken to determine the active site morphology concentrated on identifying specific amino acids present at the active site and their role in binding and catalysis. Additionally, the catalytic mechanism of the enzyme was examined by utilizing substrate (HPBS) analogs. Specific studies discussed in this document are chemical modification of the cysteine residue found in the active site, fluorimetry titration studies, and inhibitor studies.

The cysteine chemical modification studies utilized 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) in order to identify cysteine residues found within the active site (32-35). This experiment was performed to determine the cysteine residue's role in catalysis. Fluorimetry assays were utilized to measure enzymatic binding both directly and indirectly, as well as to measure enzyme activity (36). Inhibitors and substrate analogs were used to determine structural and electronic features required for binding and catalysis (37). Most of the analogs and inhibitors tested were commercially available. However, two inhibitors were synthesized, characterized and purified, and these are

currently being evaluated further. Several experiments have been performed with the newly synthesized halogenated substrate analogs along with HPBS desulfinase to determine binding ability and/or inhibition ability. All of the compounds used in the assays performed were modeled after HPBS to determine the requirements of substrates. In addition, the structures of the compounds included multiple ring systems, sulfonic acids, carboxylic acids, hydrogen bond donating and accepting groups, etc. (Figure 5).

For all of the studies, HPBS desulfinase was obtained from cell culture and purified using a multiple column purification strategy: anion exchange, hydrophobic interaction, hydroxyapatite and gel filtration chromatography. Currently, though, a single column purification step is being analyzed (26, 38-42).

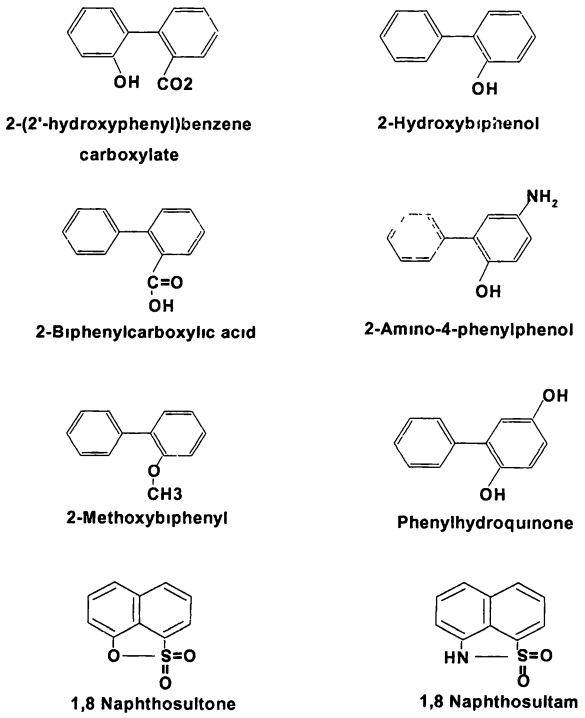

Figure 5. Examples of several substrate and product analogs of HPBS desulfinase.

Studying the active site of this enzyme should illuminate ways to maximize catalysis. HPBS desulfinase could become an economically viable method for fossil fuel

desulfurization at the industrial level if the enzyme's catalytic ability could be increased. Additional resources must be employed to overcome the many remaining problems with microbial desulfurization. However, with diligent research, microbial desulfurization will become an important technique for sulfur removal from petroleum.

CHAPTER II

EXPERIMENTAL PROCEDURES

Substrate Analog Synthesis

The following synthetic scheme applies to both substrate analogs produced: 2-(2'-Hydroxy-4'-bromophenyl) benzenesulfinate and 2-(2'-Hydroxy-4'-chlorophenyl) benzenesulfinate. The stock solvents used in this reaction, which were purchased at Aldrich Chemical Company (Milwaukee, Wisconsin), Fisher Scientific (Pittsburgh, Pennsylvania), or Sigma Chemical Company (St. Louis, Missouri), did not undergo any further purification. A 50 mL Schlenk flask was attached to a Schlenk line. Alternated vacuum and dry argon were pulled on the flask three times. The final time a vacuum was left on the flask. The flask was then clamped into an ice bath, and a glass syringe was used to add 5 mL methylene chloride and 0.64 mL of thionyl chloride to the flask. In a separate flask, 1.36 grams of brominated hydroxybiphenyl was added to 5 mL methylene chloride and swirled to mix contents. This solution was transferred to the flask containing thionyl chloride via syringe. Using a glass syringe, 1.12 mL triethylamine was then added dropwise over 5 minutes. The mixture was stirred for 15 min. at 0 ℃. The ice bath was removed and the mixture was stirred 15 min. The flask's stopcock was closed. Using a Schlenk filter, the contents of the flask were filtered into another Schlenk flask. A small pressure equalized dropping funnel was clamped into place. To an argon-

filled Schlenk flask (repeating the alternating vacuum/argon procedure above) 2.18 g of $AlCl_3$ was added. Next, 0.75 mL methylene chloride was added with a syringe. The mixture was stirred rapidly, and attached to the bottom of the dropping funnel (argon diffused). The filtered solution from a previous step was syringed into the dropping funnel, and added dropwise for 15 min. This solution was stirred for 48 hours at 0 ℃, then for another 48 hours in a H_2O bath. The resulting dark green solution was cooled in an ice bath, and 50 mL of cool H_2O was added to the solution. The solution was filtered using a glass frit into a 100 mL round-bottomed flask. The filtrate was placed into a separatory funnel and washed three times with 10 mL of 3 M HCl. After each wash, the organic (bottom) layer was collected. Anhydrous $MgSO_4$ was added to the organic solution until "snowing" occurred and the mixture was left overnight to dry. The solution was then filtered to remove the magnesium sulfate and the solvents were removed under reduced pressure at 45 ℃ in a rotary evaporator. The resulting reddish oil was distilled using a Kugelrohr apparatus at 128.2 ℃. The final product was a light yellow oil. Recrystallization was performed using 1.5 mL of absolute alcohol. The resulting white precipitate was isolated by removing the ethanol using the rotary evaporator.

A small amount of the chlorinated sultine was placed in a mortar and 2 drops of nujol was added. This mixture was ground for 5 minutes making a fine paste. The resulting oil was spread between NaCl IR discs. The same procedure was followed for the brominated product. Using a Perkin Elmer Spectra One Fourier transform infrared (IR) spectrophotometer, the IR spectrum was measured for both compounds. The NMR spectra were obtained in deuterated acetonitrile. Spectra were obtained using a 400 MHz Varian NMR. The same protocol was followed for the brominated sultine. The resulting

yields were 1.23 g (43.5%) for the brominated sultine and 1.37 g (59.6%) for the chlorinated sultine. The chemical shifts in the ^{1}H and ^{13}C NMR spectra were referenced to tetramethylsilane (TMS). Chlorinated sultine: IR (nujol) 1600.0, 1027.5, 754.5, and 696.7 cm^{-1}; ^{1}H NMR (CD_3CN) δ=1.87 (s, 2H), 6.01 (s, 1H), 7.24-7.26 (m, 2H), and 7.56-7.85 (m, 3H). Brominated sultine: Rf= 2.25 in acetone: hexane (60:40); IR (nujol) 1100-1150.4, 1600.0, and 598.2 cm^{-1}; ^{1}H NMR (CD_3CN) δ=1.72 (s, 2H), 5.65 (s, 1H), 7.24-7.28 (m, 2H), and 7.6-7.8 (m, 3H); ^{13}C NMR (CD_3CN) δ=151.6, 132.6, 131.7, 129.4, 129.0, 128.7, 128.3. The following figure shows the synthetic scheme for the reaction:

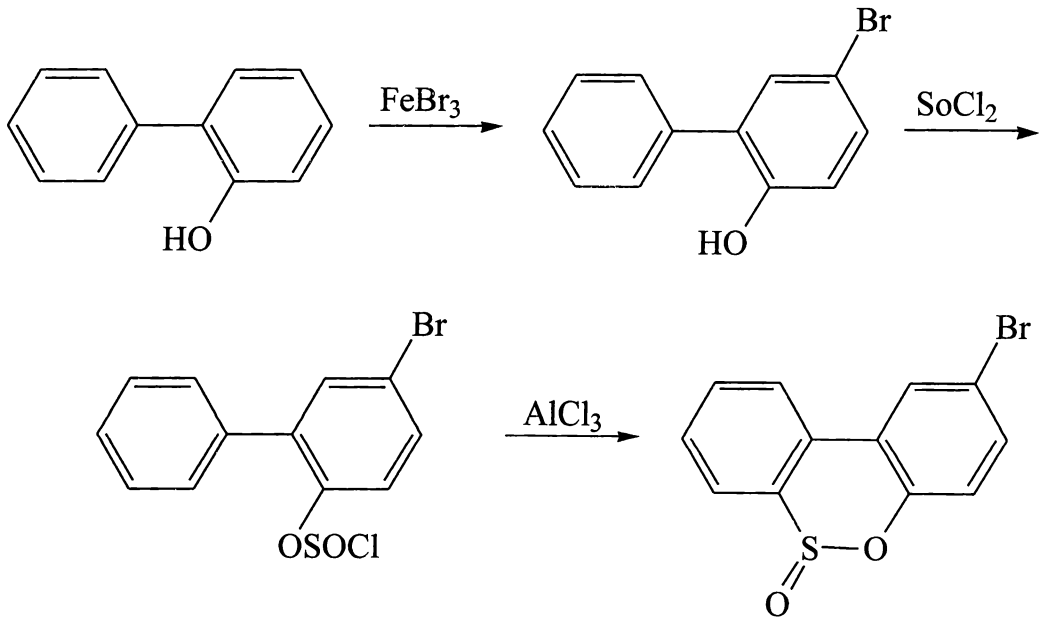

Figure 6. Synthetic scheme for halogenated sultines.

Ring Opening Reaction

A stock solution of 0.044 M NaOH was prepared using 1.76 g NaOH per liter solution. In 10 mL 0.044 M NaOH solution, 0.05 g chlorinated sultine was dissolved. The volume of the solution was then brought up to 20 mL with 10 mL 0.044 M NaOH. The solution was stirred overnight. The same protocol was followed for the brominated sultine with the exception of adding 0.06 g to the NaOH solution. The pH of the solutions was adjusted to 9.8 for the chlorinated product, and 8.6 for the brominated.

Purification and Analysis of Halogenated HPBS Compounds

Charcoal Column Chromatography

Glass wool was placed at the end of two Pasteur pipettes, which had the long, slender tips broken off. A slurry of charcoal (Vulcan XC-72, GP-3137) was made using an acetone: hexane (60:40) solvent. This slurry was pipetted into the columns. Once the column filled slightly over halfway, 0.5 mL HPBS-Br dissolved in the solvent was loaded onto the column. The product was detected in the eluting fractions by thin layer chromatography on silica gel plates (Silica Gel IB2-F) using acetone: hexane (60:40) as the solvent, and the column was washed with the solvent. This procedure was repeated using 0.5 mL HPBS-Cl.

Mass Spectroscopy

Mass spectroscopy was performed by Rene Rodriquez in Dr. Walter Rudzinski's lab. Samples containing 20 mM HPBS-Cl or HPBS-Br were analyzed on a Finnegan ESI/MS. A plot of relative abundance against m/z was produced.

pK_a Determination

A stock solution of 1.047 M standardized HCl was produced using a quantitative analysis protocol. The standardized HCl solution was used to titrate samples of HPBS, 19 mM HPBS-Cl or 12 mM HPBS-Br from a pH range of 9.79-1.84 for the HPBS-Cl and a pH range of 8.79-1.92 for the HPBS-Br. The pK_a values of the compounds were determined using Microsoft Excel.

HPBS Synthesis

Biphenosultine (0.0432g), a gift from Dr. H. L. Holland of Brock University, was suspended in 10 mL of 44 mM NaOH and stirred overnight. The pH of the resulting 20 mM solution was adjusted to 8.5 with the addition of 1 M HCl. The final 20 mM HPBS stock solution was then portioned into 1 mL aliquots and stored in a -20 ℃ freezer. The synthetic method for HPBS was developed by Gregory Mrachko while at Energy Biosystems Corporation (The Woodlands, Texas).

Enzyme Measurements

UV Assays

Protein concentrations were determined using Kalb and Bernlohr's equation: μg/mL protein = 183 (A_{230}) – 75.8 (A_{260}). Solutions of each of the fractions obtained from the protein purification assays were placed in 1 mL quartz cuvettes and absorbances were read at 230 and 260 nm. The measurements were performed using a Beckman DU 7400 UV-Vis spectrophotometer. Once the measurements were performed, the samples were diluted as necessary to ensure measured absorbances were less than 1.000 AU.

Fluorescamine Assay

Protein concentrations were further tested utilizing a fluorescamine assay. This assay requires Bovine Serum Albumin (BSA) to be serially diluted from 0 to 1000 μg/mL. Samples were diluted into a 25 mM sodium-phosphate buffer at pH 7.4. The BSA dilutions were pipetted into a 96 well plate to a volume of 150 mL. Unknown protein samples were placed in separate wells. To all wells, 50 μL of 1.08 μM fluorescamine was added. The plate was placed on a shaker for one minute. The fluorescence was measured at 460 nM (λ_{exc} = 400 nM) using a Perkin Elmer LS50B Luminescence spectrophotometer. A standard plot of intensity against BSA concentration (μg/mL) was constructed using Microsoft Excel. A best fit line was fit to the data and the unknown protein concentration was ascertained using the standard curve.

HBP Standard Curve

Using 0.017 g of HBP dissolved into 10 mL of H_2O, a 10 mM stock solution of HBP was prepared. The solution was heated with stirring to facilitate dissolving. The stock solution was then diluted to 1 mM. Using a 96 well plate, the 1 mM HBP was further diluted in a total volume of 200 μL to the following concentrations: 50, 40, 30, 20, 10, 5, 3.3, 1, and 0 μM. The fluorescence of the dilutions was calculated using a Perkin Elmer LS50B Luminescence spectrophotometer at 414 mM (λ_{exc} = 288 nM). The recorded intensity values were plotted against HBP concentration and a standard curve was calculated using Microsoft Excel. The equation of the best fit line was I = 26.5/ μM HBP. (R^2 = 0.9992)

Fluorescence Assay

A standard assay was developed previously and utilized for all of the enzymatic activity measurements of HPBS desulfinase. In a 96 well plate, water, Buffer A (250 mM NaH_2PO_4, 1 M NaCl, pH 7.4) and 100 μM HPBS were mixed and incubated on a well plate warmer to 34°C. The reaction was initiated upon addition of the substrate HPBS. The reaction volumes ranged from 150-220 μL and HPBS total concentration ranged from 2-100 μM depending on the particular experiment. Product formation was measured over time using a Perkin Elmer LS50B Luminescence spectrophotometer. Increase in fluorescence was monitored at 414 mM (λ_{exc} = 288 nm). A standard plot of HPBS intensity against HBP concentration was constructed using Microsoft Excel. Velocity of reaction was determined using the equation: v = (26.5 I/μM)(slope (I/sec)), where I = intensity, μM= concentration of HBP, and slope = Intensity/Time. The specific

activity (units/mg) of the enzyme was then calculated using the velocity and enzyme concentration. A unit is equal to 1 μmol HBP produced / hour.

HPBS Desulfinase Purification

Cell Lysis

Rhodococcus erythropolis IGTS8 cells were removed from the -70°C freezer and thawed at 4°C. Once the cells were thawed, they were resuspended in ice cold Buffer A. To this suspension, 0.5 mM dithiothreitol (DTT), 0.2 mg/mL DNAse, and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) was added. The resulting cell suspension was placed in a Stansted Cell Disrupter to lyse the cells. The cells were subjected to 16-18,000 psi. The resulting solution of lysed cells was collected on ice then pelleted by centrifugation at 10,000 rpm at 4°C for 90 minutes. In this step, the unwanted cell debris and non-lysed cells were removed. The supernate was transferred to the anion exchange column. The remaining cell debris from the centrifuge was discarded.

FPLC

A Gilson FPLC system was used to perform all of the FPLC procedures at 4°C. The software used was Gilson Unipoint Software version 1.71. The eluents from the columns were continuously monitored using a Gilson 112 UV-Vis detector at 280 nm. A FC203B fraction collector, controlled by the Gilson FPLC system, collected fractions which were then assayed on the Perkin Elmer LS50B Luminescence spectrophotometer at 414 mM (λ_{exc} = 288 nM). The software used for all of the fluorescence assays was

FLWinLab program version 3.00 from Perkin Elmer. The assay used 100 μL of the protein fraction and 91 μM HPBS. Any of the fractions which displayed activity were collected and pooled to place in the next column in the series. From this pooled sample a 1.0 mL sample was stored and refrigerated to await further analysis.

Anion Exchange

The anion exchange column used for this assay was a 300 mL Q-Sepharose Fast Flow column (Pharmacia, Piscataway, New Jersey). Before the supernate was loaded onto the column, the column was equilibrated with 3 L of Buffer A. The cell extract was loaded at a rate of 4.5 mL/min with a Minipuls3 peristaltic pump. Elution was monitored and once the unbound protein fractions washed through the column, the bound protein was eluted using a NaCl gradient from 100 mM to 450 mM. The fraction collector was set to collect fractions every 1.5 minutes after the gradient was applied. To ensure all bound protein was removed, a wash of 1.5 M NaCl was performed. Lastly, the column was re-equilibrated with Buffer A.

Desalting Chromatography

The protein fractions were desalted using a 150 mL P-6DG desalting column (Bio-Rad, Hercules, California), which had been previously equilibrated with 1.5 L of 25 mM sodium phosphate buffer pH 7.4. Q-sepharose fractions which had been collected in the fraction collector were assayed on the fluorimeter as previously described. The fractions exhibiting activity were pooled and loaded on the P-6DG column at a rate of 1.5 mL/minute. The elution buffer used was 25 mM sodium phosphate buffer pH 7.4 and the

fraction collector was set to collect a new fraction every 2 minutes. The protein absorbance on the chromatogram was monitored and when it returned to baseline, the fraction collector was stopped. The column was washed with the 25 mM sodium phosphate buffer pH 7.4 for re-equilibration.

Hydroxyapatite Chromatography

A 25 mL Macro-Prep Type 1 ceramic hydroxyapatite column (Bio-Rad, Hercules, California) was equilibrated using seventy-five mL of 25 mM sodium phosphate buffer pH 7.4. The fractions collected from the desalting column were assayed for activity. The fractions exhibiting activity were pooled and loaded onto the column at 2 mL/minute using a peristaltic pump. Protein fractions were collected every 2 minutes. The chromatogram was monitored, and once it returned to baseline, fraction collection was discontinued. The column was washed with 400 mM potassium phosphate buffer pH 7.4, followed by a wash with 25 mM sodium phosphate buffer pH 7.4 for re-equilibration.

Hydrophobic Interaction Chromatography

A 50 mL Toyopearl-phenyl 650 M column (Supelco, Bellefonte, Pennsylvania) was equilibrated with five hundred mL of 1.7 M $(NH_4)_2SO_4$ buffer pH 6.6. The fractions with activity after the hydroxyapatite column were pooled and 1.7 M $(NH_4)_2SO_4$ was added. This solution was loaded onto the column at 3 mL/minute using a peristaltic pump. The unbound protein was eluted, then a decreasing salt gradient from 1.7 M to 0 M $(NH_4)_2SO_4$ was applied to the column. The fraction collector collected fractions every

minute. Once the gradient was applied, a wash of Buffer A was run through the column. A 1.7 M $(NH_4)_2SO_4$ buffer pH 6.6 was run through the column to re-equilibrate it.

Protein Concentration

The collected fractions from the Toyopearl were placed in Centricon 2 mL YM-10 centrifuge filter devices (Millipore Corp., Bedford, Massachusetts). The samples were centrifuged at 4000 rpm at 4 ̊C for approximately 90 minutes in a HERMLE Z180 M centrifuge (National Labnet Co., Woodbridge, NJ). Once the protein volume was below 3 mL, both the filtrate and the retentate were assayed on the Perkin Elmer LS50B Luminescence spectrophotometer at 414 mM (λ_{exc} = 288 nM). The retentate was saved and applied to the size exclusion column.

Size Exclusion Chromatography

Buffer B (25 mM NaH_2PO_4, 200 mM NaCl pH 7.4) was washed through the 300 mL Superdex 75 gel filtration column (Pharmacia, Piscataway, New Jersey) for equilibration. The retentate from the concentrators was loaded directly onto the top of the resin using a pipette and taking care not to disturb the gel bed. The sample was allowed to settle into the column by gravity. Buffer B was pipetted onto the column using an equal volume as the sample that was loaded. The buffer was also allowed to gravity load. Several milliliters of buffer B were pipetted carefully onto the column, and then loaded using the peristaltic pump at a rate of 2 mL/minute. The pumps were programmed on the FPLC to reduce the flow rate to 0.4 mL/minute stepwise over 30 minutes. Once the program was begun, the fraction collector was set to collect every 2

minutes for 570 minutes. At the end of this time, the column was re-equilibrated with buffer B. The fractions were assayed on the fluorimeter and those demonstrating activity were pooled and equilibrated to 4 ℃. The pooled protein was divided into 0.5 and 1.0 mL aliquots and stored in the -70 ℃ freezer. The sample vials were labeled with the date of preparation, the volume of protein, and the protein concentration.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-Page)

Protein samples of approximately 10 μg were taken from each of the one milliliter pooled fractions taken from the separate columns. These samples were each mixed with reduced lithium dodecylsulfate (LDS) sample buffer, containing 1.09 M glycerol, 141 mM Tris-Base, 106 mM Tris-HCl, 73 mM LDS, 0.51 mM EDTA, 0.22 mM Serva® Blue G-250, and 0.175 mM phenol red, and 2% β-mercaptoethanol. The samples were denatured by placing on a heat block at 90 ℃ for 10 minutes. The samples, along with Novex Mark12™ molecular weight standard were loaded into a pre-cast NuPAGE 4-12% bis-Tris polyacrylamide gel (No. NP0321). The gel was placed in a Novex XCELL-11™ electrophoresis module and filled with MES-SDS running buffer (500 mM 2-(N-morpholino)ethane sulfonic acid, 50 mM Tris-base, 3.5 mM SDS, 1 mM EDTA, pH 7.3). The gel was run for 35 minutes at 200 volts constant voltage. The gel was carefully removed from the cartridge and placed in a fixing solution of 50% methanol, 10% acetic acid and 40% H_2O and shaken for 15 minutes. The fixing solution was discarded and the gel was placed in a staining solution containing 55 mL ddH_2O, 20 mL methanol, and 20 mL Stainer A (Novex SilverXpress Silver Staining kit No. LC6100, Lot #80922) and shaken for 10 minutes. The gel was placed in five milliliters of Stainer B for four hours.

The gel was destained in 200 mL of ddH_2O overnight. The gel was imaged on a Kodak Imager.

Enzyme Activity Studies

Enzyme assays involving HPBS desulfinase used varying concentrations of the substrate HPBS and the synthetic analogs HPBS-Cl and HPBS-Br to determine the enzyme's limitations. All of the assays contained Buffer A, HPBS desulfinase, ddH_2O, and HPBS in a total volume of 220 μL. The total HPBS concentration used in each assay ranged from 2-100 μM. The total HPBS desulfinase used for the assays was 268 μg/mL. The activity was monitored using a Perkin Elmer LS50B Luminescence spectrophotometer at 414 mM (λ_{exc} = 288 nM). Fluorescence was read every 3 minutes for 2 hours. The recorded intensity values were plotted against time and the initial velocity was calculated using Microsoft Excel.

Inhibition Studies

Thiourea Dioxide

A stock solution of 1 mM thiourea dioxide was prepared using 1.08 mg thiourea in 100 mL ddH_2O. The solution was stirred, and 100 μL 1mM thiourea dioxide solution was removed and added to 900 μL of ddH_2O. In a 96 well plate, 22 μL Buffer A, 20 μL enzyme, 10 μL 100 μM HPBS, 10 μL 100 μM thiourea dioxide, and 158 μL ddH_2O were pipetted into 3 wells. In 3 other wells, 22 μL Buffer A, 20 μL enzyme, 0 μL 100 μM HPBS, 10 μL 100 μM thiourea dioxide, and 168 μL ddH_2O were pipetted. In the last

three wells, 22 µL Buffer A, 20 µL enzyme, 10 µL 100 µM HPBS, 0 µL 100 µM thiourea, and 168 µL ddH_2O were pipetted. The first set of wells measured the effect of thiourea dioxide in the presence of substrate. The second set of wells was the negative control, and the third set of wells was the positive control. The recorded intensity values were plotted against time and the velocities in the presence of thiourea dioxide were compared to the velocities measured in the absence of thiourea dioxide.

$CuCl_2$

A stock solution of 100 µM $CuCl_2$ was prepared by diluting 100 mM $CuCl_2$, and a 100 µM HPBS solution was prepared diluting 20 mM HPBS. The assay was set up using a 96 well plate. Wells 1-4 contained 0 µL $CuCl_2$, 20 µL of 100 µM HPBS, 22 µL enzyme, 20 µL Buffer A, and 138 µL ddH_2O. Wells 5-8 contained 20 µL $CuCl_2$, 20 µL of 100 µM HPBS, 22 µL enzyme, 20 µL Buffer A, and 118 µL ddH_2O. Wells 9-12 contained 20 µL $CuCl_2$, 40 µL of 100 µM HPBS, 22 µL enzyme, 20 µL Buffer A, and 98 µL ddH_2O. The first set of wells was the positive control, while the second and third set of wells measured the effect of $CuCl_2$ at different HPBS concentrations. The recorded intensity values were plotted against time in seconds.

Cysteine Chemical Modification Studies

A stock solution of 10 mM DTNB (5,5-dithiobis(2-nitrobenzoic acid)) was prepared with 0.04g of DTNB in 10 mL of ethanol. The stock solution was then diluted with ddH_2O to 6 µM. HPBS desulfinase (70.22 µg/mL) was thawed and concentrated in a Centricon YM-10 centrifugal filter device at 4 ℃ and 4000 rpm in a HERMLE Z180 M

centrifuge. Fifty microliters of the resulting concentrate was mixed with 5 μL H_2O in a reaction tube. This mixture was warmed to 35 ℃. The activity of the mixture over time was measured using a 96 well plate assay. The following reagents were pipetted into 8 separate wells: 20 μL Buffer A, 16 μL of 100 μM HPBS, and 154 μL H_2O. The fluorimeter was set up to run 83 cycles at 30 sec/cycle. Before cycle 1, 10 μL H_2O was added to well 1, and 10 μL of enzyme was added to well 2. Before cycle 11, 11 μL of 6 mM DTNB was added to the reaction tube and mixed. The concentration of DTNB in the reaction tube was 1 μM. Ten microliters of the reaction tube mixture was added to well 3. Before cycle 21, 10 μL of the reaction tube mixture was added to well 4. Before cycle 31, 10 μL of the reaction tube mixture was added to well 5. Before cycle 41, 10 μL of the reaction tube mixture was added to well 6. Before cycle 51, 10 μL of the reaction tube mixture was added to well 7. Before cycle 61, 10 μL of the reaction tube mixture was added to well 8. A plot of intensity against time was constructed on Microsoft Excel and the velocity was determined at each time point. The same assay was repeated for DTNB concentrations of 2-6 μM in the reaction tube mixture. The natural log of residual activity was plotted against time to determine k_{obs} and a plot of k_{obs} against DTNB concentration was used to determine k_I.

Fluorescence Studies

Inhibition Studies

Assays were conducted to determine the fluorescent intensity of HBP in the presence or absence of inhibitors. A 10 mM stock solution of HBP was prepared using

0.017 g HPB in 95% ethanol. Five milliliters of the stock solution was diluted to 100 μM. Stock solutions were also prepared for 14 inhibitors as follows: 2-methoxybiphenyl (0.0184 g/mL), phenylhydroquinone (0.0186 g/mL), xanthene (0.0182 g/mL), 2-biphenyl carboxylic acid (0.0198 g/mL), 2-amino-4-phenylphenol (0.01852 g/mL), 1,8-naphthosultene (0.021 g/mL), 2,2' biphenol (0.0186 g/mL), 2-hydroxydiphenylmethane (0.0184 g/mL), diphenic acid (0.0242 g/mL), 1,8-naphthosultone (0.0206 g/mL), 1,8-naphthosultam (0.0205 g/mL), 1-naphthalene sulfonic acid (0.0230 g/mL), 4-hydroxy-4-biphenyl carboxylic acid (0.0214 g/mL), and 6-hydroxy-2-naphthalene sulfonic acid (0.0246 g/mL) were each added to 10 mL of 95% ethanol except the last three which were dissolved in 10 mL ddH_2O, resulting in final concentrations of 100 μM. All of the stock solutions were diluted to 100 μM concentrations. In a 96 well plate, all of the wells contained 10 μL of 100 μM HBP, 10 μL of 100 μM inhibitor, 160 μL ddH_2O, and 20 μL Buffer A, except 2 wells which contained no inhibitor. The recorded intensity values were measured and the fluorescence intensity of HBP was compared in the presence and absence of inhibitors.

Substrate Studies

Assays were conducted to determine fluorescent intensity of HBP in the presence and absence of substrate. A 10 mM stock solution of HBP was prepared using 0.017 g HPB in 95% ethanol. A 100 μM solution of HPBS was made by diluting a 20 mM stock solution in ddH_2O as described. In a 96 well plate assay, the concentrations of the substrate and HBP were varied using ddH_2O to a final volume of 200 μL. The concentrations of HPBS were 0, 4, and 8 μM, and the concentrations of HBP were 0, 2, 4,

and 6 μM. The recorded intensity values were measured and the fluorescence intensity of HBP was compared in the presence and absence of substrate.

Inhibitor Characterization

Stock solutions were prepared for 14 inhibitors as follows: 2-methoxybiphenyl (0.0184 g/mL), phenylhydroquinone (0.0186 g/mL), xanthene (0.0182 g/mL), 2-biphenyl carboxylic acid (0.0198 g/mL), 2-amino-4-phenylphenol (0.01852 g/mL), 1,8-naphthosultene (0.021 g/mL), 2,2'-biphenol (0.0186 g/mL), 2-hydroxydiphenylmethane (0.0184 g/mL), diphenic acid (0.0242 g/mL), 1,8-naphthosultone (0.0206 g/mL), 1,8-naphthosultam (0.0205 g/mL), 1-naphthalene sulfonic acid (0.0230 g/mL), 4-hydroxy-4-biphenyl carboxylic acid (0.0214 g/mL), and 6-hydroxy-2-naphthalene sulfonic acid (0.0246 g/mL) were each added to 10 mL of 95% ethanol, resulting in final concentrations of 100 mM. All of the stock solutions were diluted to 100 μM concentrations. Quartz cuvets (3/Q/10 Spectrosil) were used for the assays. The cuvets all contained 30 μL inhibitor, 2670 μL ddH_2O, and 300 μL Buffer A. to a total volume of 3 mL. The final concentration of the inhibitors in the cuvets was 10 μM. Emission spectra were performed at an excitation λ of 300 nm on a Perkin Elmer LS50B Luminescence spectrophotometer.

A titration assay was performed using quartz cuvets (3/Q/10 Spectrosil). The cuvet contained 300 μL Buffer A, 2400 μL ddH_2O, and 300 μL of 70.2 μg/mL HPBS desulfinase to a total volume of 3 mL. Five emission scans were achieved with an excitation λ of 280. After each scan, 2 mL of 100 μM biphenol was added. Final concentrations of biphenol after scans 1-5 were: 0, 6.5, 13, 20, and 26 μM.

Determination of K_D

A stock solution of 10 mM 1, 8-naphthosultam was prepared using 0.1025 g in 5 mL of 95% ethanol. The assay was set up using quartz cuvets (3/Q/10 Spectrosil) containing 300 μL of 1 mg/mL Bovine Serum Albumen (BSA) stock solution, 300 μL Buffer A, and 2400 μL ddH_2O. Timed emission scans were performed at an excitation λ of 300 nm for 50 scans. After each scan, 2 μL of 10 mM 1, 8-naphthosultam were added, for a total concentration of 206.7 μM naphthosultam.

1, 8-naphthosultam was also used to titrate HPBS desulfinase solution to determine the K_D for the inhibitor's enzyme binding. The assay was set up on the Perkin Elmer LS50B Luminescence spectrophotometer with the following parameters: 200-900 nm λ range, 300 nm/min scan speed, emission /excitation slit width 10 nm, and excitations at 260 and 280 nm. The quartz cuvets contained either 300 μL of Buffer A and 2400 μL ddH_2O, or 300 μL of Buffer A, 2400 μL ddH_2O, and 100 μL of 90 μg/mL HPBS desulfinase to a final volume of 3 mL. Additions of 2 μL of 1 mM 1, 8-naphthosultam were added after each scan to a final concentration of 3.6 μM. Plots of ΔF (change in fluorescence) against naphthosultam concentration (μM) were constructed on Microsoft Excel. The assay was repeated using an initial 280 μM addition of 1 mM 1, 8-naphthosultam followed by 140 μL additions after each scan. The final concentration of naphthosultam was 285.7 μM. Plots of ΔF (change in fluorescence) against naphthosultam concentration (μM) were constructed on Microsoft Excel.

CHAPTER III

RESULTS

HPBS Protein Purification

The purification procedure described in the Experimental Procedures section was modified from the protocol developed by Gray (43). HPBS desulfinase was purified 355-fold from112 g of *Rhodococcus erythropolis* IGTS8 cells. The overall yield of enzyme was 2.5 mg and 42% of the initial activity was isolated. The specific activity of the purified enzyme was 2.13 U/mg. Table 1 summarizes the purification of HPBS desulfinase. The SDS-PAGE of the purification is shown in Figure 7.

Table 1: Purification Profile

Purification Stage	Volume (mL)	Protein Conc. (mg/mL)	Total Protein (mg)	Activity[a] (U/mL)	Total Units	Specific Activity (U/mg)	Yield (%)	Fold Purification
Cell Lysate	430	4.1	1763	0.023	9.89	0.006		
Q-Sepharose	174	0.85	148	0.072	12.53	0.085	100	14
P-6DG	77	0.76	58	0.063	4.85	0.084	39	14
Hydroxyapatite	114	0.56	63	0.045	5.13	0.081	41	13
Toyopearl	42	0.16	10	0.15	6.38	0.64	51	107
Superdex	28	0.09	2.5	0.19	5.32	2.13	42	355

[a] A Unit (U) of activity is defined as one μmol of HBP formed per hour.

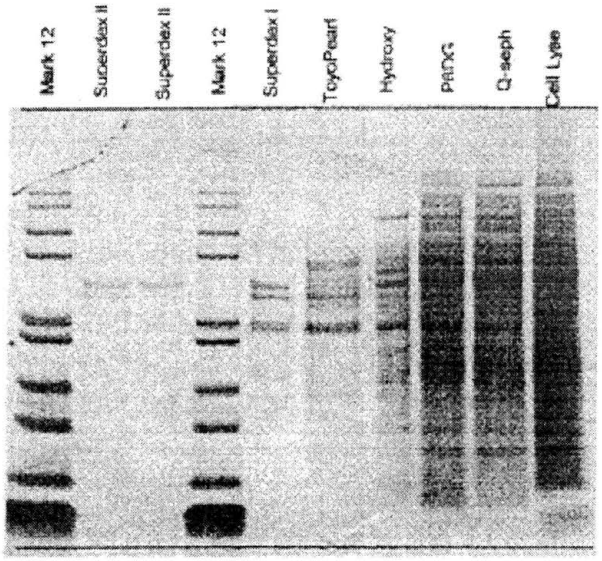

Figure 7. SDS-PAGE gel of fractions collected during the HPBS desulfinase purification. The Superdex column was employed twice to ensure the purity of the enzyme.

The final product from the second Superdex column elution produces one clear band with a molecular mass of 40 kDa. This molecular mass corresponds to that determined from the amino acid sequence previously reported.

Enzyme Activity Studies

Kinetic studies were conducted using the fluorimetric assay described in the Experimental Procedure section. The assays measured enzyme activity at varying concentrations of the substrate (HPBS) and the synthetic analogs HPBS-Cl and HPBS-Br. No product was formed when enzyme was incubated in the presence of HPBS-Cl or HPBS-Br. When enzyme activity was measured in the presence of HPBS and HPBS-Br, the activity increased to 137% of the activity relative to the substrate alone. Similarly,

when enzyme activity was measured with HPBS and HPBS-Cl, the activity increased to 127% of the activity relative to the substrate alone.

Fluorescence Inhibition Studies

Fourteen commercially available analogs were tested for their ability to act as substrates or inhibitors of HPBS desulfinase. None of the fourteen analogs acted as substrates, but six of these showed significant inhibition of the enzyme. The compounds tested are shown in Figure 8. Table 2 exhibits the relative activity of the analogs which behaved as inhibitors.

TABLE 2: Inhibition activity of several HPBS and HBP analogs

ANALOG	RELATIVE ACTIVITY[a]
1-Naphthalene sulfonic acid	43% +/- 8
6-Hydroxy-2-naphthalene-sulfonic acid	81% +/- 9
1,8-Naphthosultam	58% +/- 3
2,2'-Biphenol	37% +/- 9
4'-Hydroxy-4-biphenol carboxylic acid	60% +/- 19
Diphenic acid	81% +/- 9

[a] Activity was measured using the assay described in the Experimental Procedures. The assay measured activity in the presence of 5 μM HPBS, 5 μM inhibitor, and 70.22 μg/mL HPBS desulfinase.

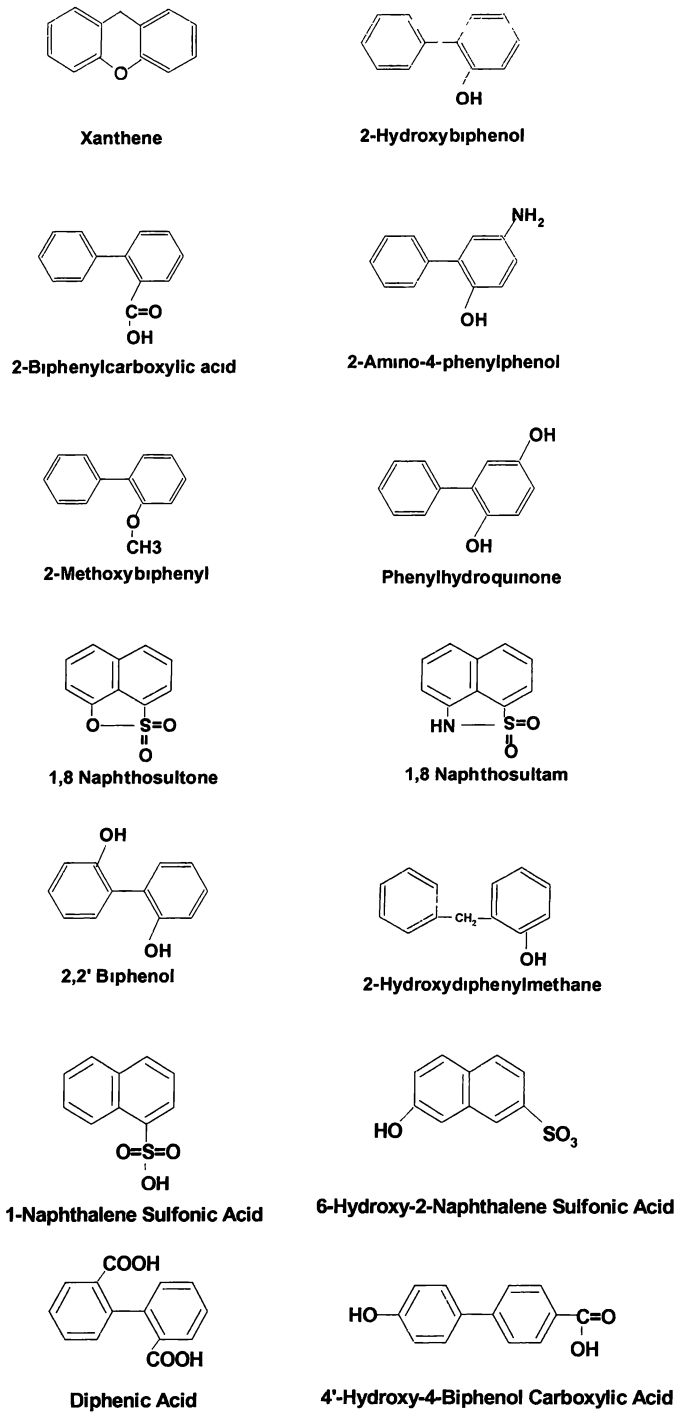

Figure 8. Substrate and product analogs of HPBS desulfinase.

Fluorescence emission spectra were obtained for each of the analogs tested. The experiment was run using 10 μM concentrations of each of the inhibitors in buffer A. Emission spectra of each of the inhibitors were recorded at an excitation λ of 300 nm. Three of the analogs, phenylhydroquinone, 2-amino-4-phenylphenol, and 6-hydroxy-2-naphthalene sulfonic acid had maximum fluorescence intensities greater than 1000. Four others had maximum fluorescence intensities of 70-500, these included: 1-naphthalene sulfonic acid, 2-hydroxybiphenol, 4'-hydroxy-4-biphenyl carboxylic acid, and 2-methoxybiphenol. The remaining six, xanthene, 2-biphenol carboxylic acid, 2-hydroxydiphenylmethane, 1, 8-naphthosultone, 1, 8-naphthosultam, and diphenic acid, had maximum fluorescence intensities of less than 10. The wavelength of maximum fluorescence intensity for all inhibitors was 414 nm.

Several fluorescence titration experiments were also run using 2, 2'-biphenol to measure the K_D for HBP binding to HPBS desulfinase by fluorescence. The excitation λ was 280 nm and emission was monitored between 200 nm and 900 nm. After each scan, 2 μM of 100 μM 2, 2'-biphenol was added up to a final concentration of 26 μM. The fluorescence of HBP was too high to monitor the fluorescence of enzyme, thus, it was not possible to measure a K_D for HBP binding to HPBS desulfinase.

Assays were conducted to determine the fluorescence intensity of product (HBP) in the presence and absence of substrate (HPBS) in order to assess if the fluorescence of increasing substrate concentration quenched the product HBP and interfered with the activity assay. The assay was performed using varying concentrations of substrate (0, 4, and 8 μM) and product (0, 2, 4, and 6 μM). The resulting intensity values were measured and the fluorescence intensity of HBP was compared in the presence and absence of

substrate. In the first plot of HBP fluorescence against HPBS concentration, the intensity of HBP was not significantly affected by increasing HPBS concentration. In the plot of HBP fluorescence as a function of HBP concentration at fixed HPBS concentration, as HBP concentration increased, the intensity of HBP increased proportionately.

Inhibition Studies using Thiourea Dioxide

An assay was performed measuring the effect of thiourea dioxide on HPBS desulfinase activity. Enzyme activity was measured in the presence of thiourea dioxide and 5 μM HPBS. The recorded intensity values were then plotted against time. At 5 μM thiourea dioxide concentration, no inhibition was observed. At 100 μM thiourea dioxide, no enzyme activity was observed.

Inhibition Studies using $CuCl_2$

Another assay was performed to measure the effect of $CuCl_2$ on HPBS desulfinase activity at different HPBS concentrations. The intensity values measured were then plotted against time. As HPBS concentration increased, the inhibitory effect of $CuCl_2$ on HPBS desulfinase was not significantly changed.

Cysteine Chemical Modification

HPBS desulfinase was incubated with 5, 5-dithiobis(2-nitrobenzoic acid), or DTNB, (a cysteine modifier) to determine the role of the cysteine residue presence in HPBS desulfinase. DTNB concentrations of 1-6 μM and incubation times from 0- 40 minutes were used for each experiment. Assays were run as described in the

Experimental Procedures section. Figure 9 shows the reaction of DTNB with a cysteine residue.

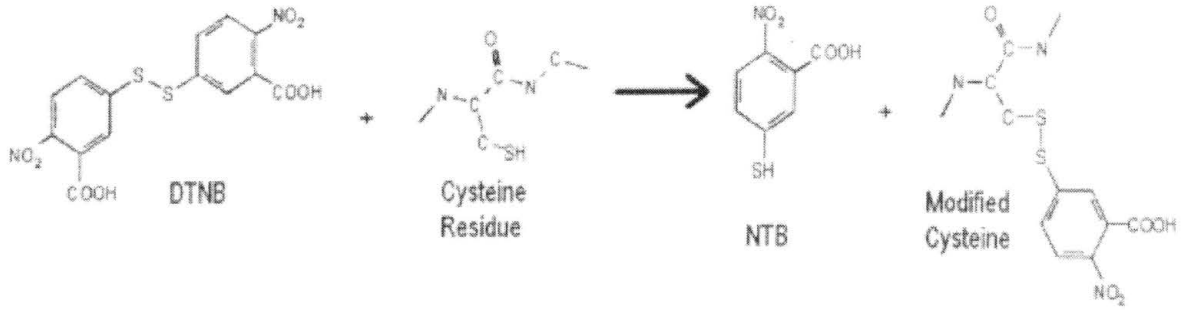

Figure 9. Method of DTNB modification of cysteine residues.

Plots of intensity against time were made using Microsoft Excel. All of the graphs showed immediate loss of HPBS desulfinase activity upon incubation with DTNB. The natural log of residual activity was plotted against time to determine k_{obs} at each DTNB concentration (Figure 10), and a plot of k_{obs} against DTNB concentration was used to determine k_i (Figure 11). The k_{obs} was calculated using the equation: $\ln (E_{at}-E_{a0}) = -k_{obs}t$. Table 3 lists the k_{obs} calculated for each concentration of DTNB, as well as the k_i.

TABLE 3: Calculated k_{obs} for HPBS desulfinase modification with DTNB

[DTNB] (μM)	k_{obs}[a] (min^{-1})
0	1
1	0.0257
2	0.0303
3	0.0963
k_i =0 .0268(min^{-1}*μM^{-1}) R^2 = 0.9545	

[a]k_{obs} was calculated using the equation: $\ln (E_{at}-E_{a0}) = -k_{obs}t$

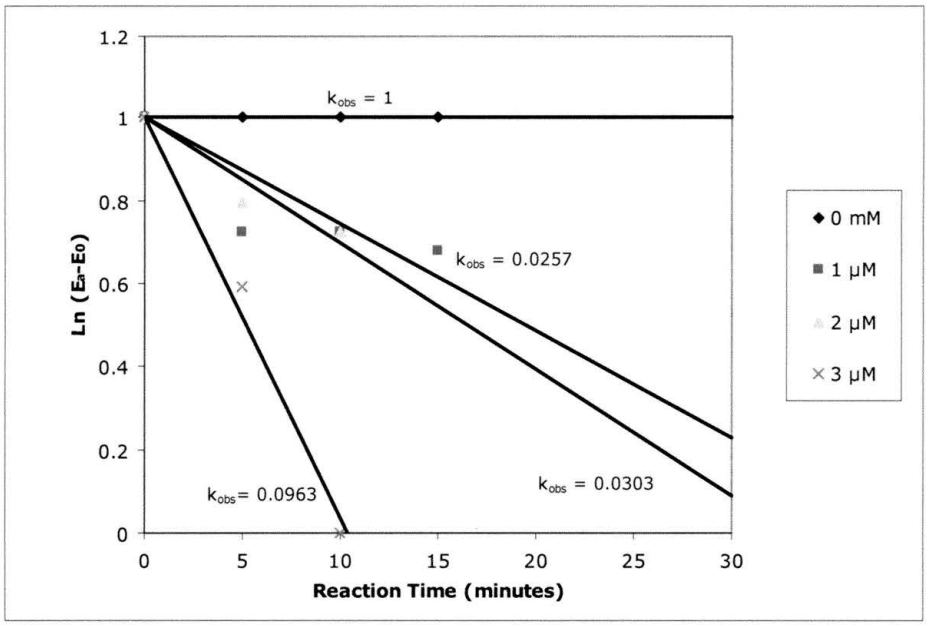

Figure 10. DTNB Cysteine Modification – Plot of residual activity against reaction time. Assay used to observe activity loss for HPBS desulfinase when modified with DTNB from 0 to 30 minutes. The k_{obs} is equal to the negative slope of the line for each DTNB modification reaction. Data were fit to a best fit line using Microsoft Excel.

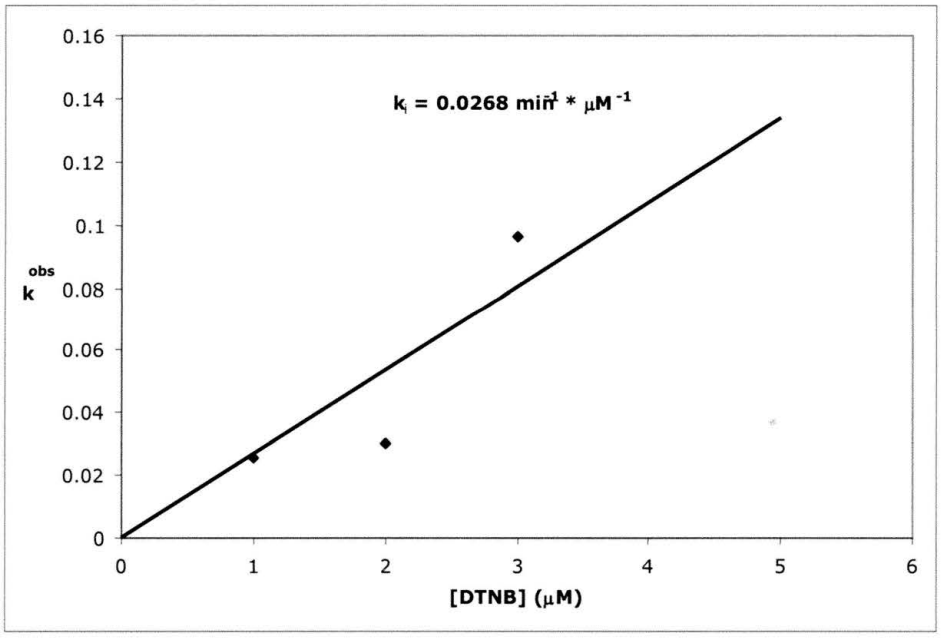

Figure 11. Determination of k_i for DTNB Modification of HPBS desulfinase. Plot of k_{obs} against DTNB concentration (µM). The k_i was calculated using the equation: $k_{obs} = k_i[DTNB]$. Data were fit to a best fit line using Microsoft Excel.

HPBS-Br and HPBS-Cl Synthesis

The synthesis of 2-(2'-hydroxy-4'-bromophenyl) benzenesulfinate and 2-(2'-hydroxy-4'-chlorophenyl) benzenesulfinate was achieved using the Hansen and Kemp reaction for functionalized biphenyls (45). Both of the new compounds were characterized using several analytical techniques. Mass spectroscopy (MS), NMR and IR experiments were used to confirm the structures of the compounds employing the assays described in the Experimental Procedures section. The ^{13}C NMR spectrum of 2-(2'-hydroxy-4'-bromophenyl) benzenesulfinate shows peaks in the 117-135 ppm range. A ^{13}C NMR was not performed on the 2-(2'-hydroxy-4'-chlorophenyl) benzenesulfinate because of solubility problems. Figure 12 shows the ^{13}C NMR spectrum of 2-(2'-hydroxy-4'-bromophenyl) benzenesulfinate. The MS of 2-(2'-hydroxy-4'-chlorophenyl) benzenesulfinate has a molecular ion peak at *m/z* 269. The brominated HPBS mass spectrum has a large peak at *m/z* 279.

HPBS-Br and HPBS-Cl Purification

Assessment of the purity of the two compounds was determined using TLC and ^{1}H NMR (Figures 13 and 14). Both of these methods revealed impurities. Thus, a charcoal column using acetone: hexane (60:40) as the solvent was used to purify both of the compounds. Using TLC, it was determined that both of the compounds eluted from the column were pure as determined by TLC.

Figure 12. ^{13}C NMR spectrum of 2-(2'-hydroxy-4'-bromophenyl) benzenesulfinate.

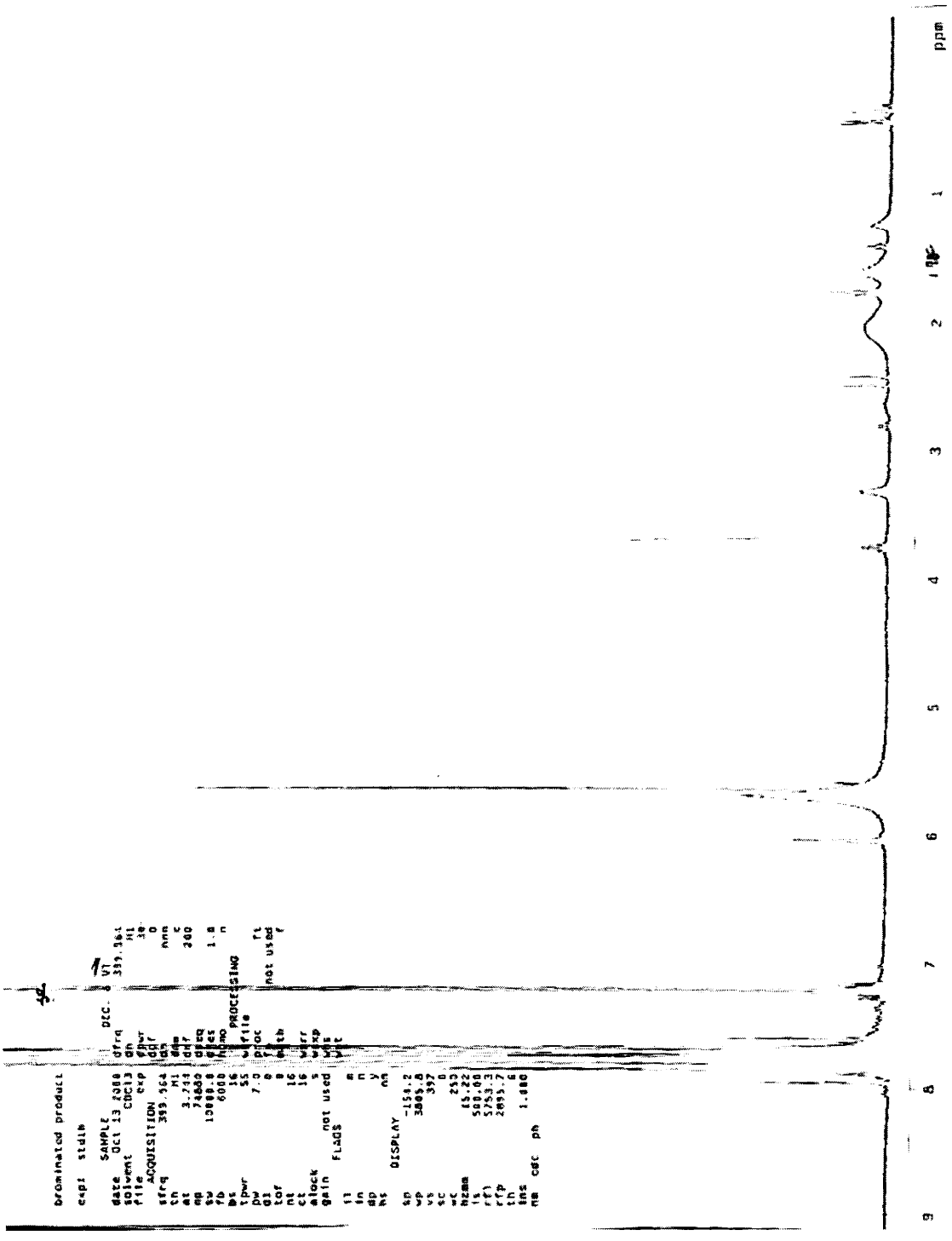

Figure 13. ^{1}H NMR spectrum of 2-(2'-hydroxy-4'-bromophenyl) benzenesulfinate.

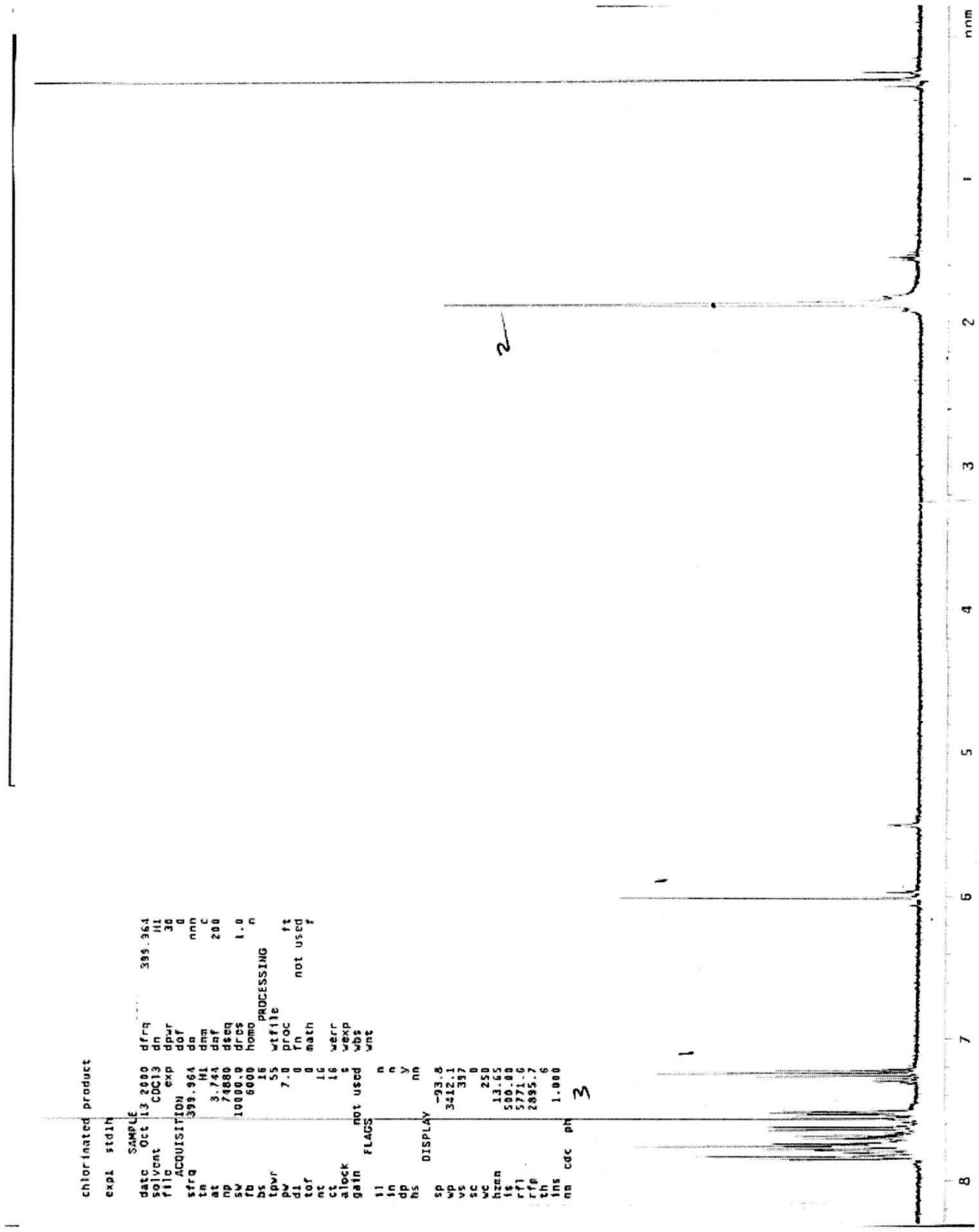

Figure 14. ^{1}H NMR spectrum of 2-(2'-hydroxy-4'-chlorophenyl) benzenesulfinate.

Determination of HPBS, HPBS-Br and HPBS-Cl pK_a Values

The pK_as of the two compounds were then determined by titrating solutions of HPBS, HPBS-Br, and HPBS-Cl with a standardized solution of HCl. Excel was used to do second derivative analysis of the titration curves. The resulting graphs indicate that HPBS-Cl has two pK_as: one at 6.2 and one at 1.9. HPBS-Br has two pK_as: one at 6.9 and one at 2.3, and HPBS has two pK_as: one at 6.9 and one at 3.4.

Determination of K_D

Several fluorimetric assays were performed by titrating a 10 mM solution of 1, 8-naphthosultam into a solution containing HPBS desulfinase. These assays were used to determine the K_D for the inhibitor binding to the enzyme. The concentration range of 1, 8-naphthosultam in the first set of assays was 0-3.6 μM, while the concentration in the second set was 0-285.7 μM. Using Enzfitter (Biosoft, Cambridge, UK) a plot of change in fluorescence (ΔF) against naphthosultam concentration (μM) was constructed for the assay with the 0-285.7 μM concentration of the inhibitor (Figure 15). The K_D was calculated for 1, 8-naphthosultam using the equation $\Delta F = (Q_{max}[I]/K_D + [I])$. $K_D = 1.6$ μM, with a Q_{max} of 85.4 ($R^2 = 0.9710$).

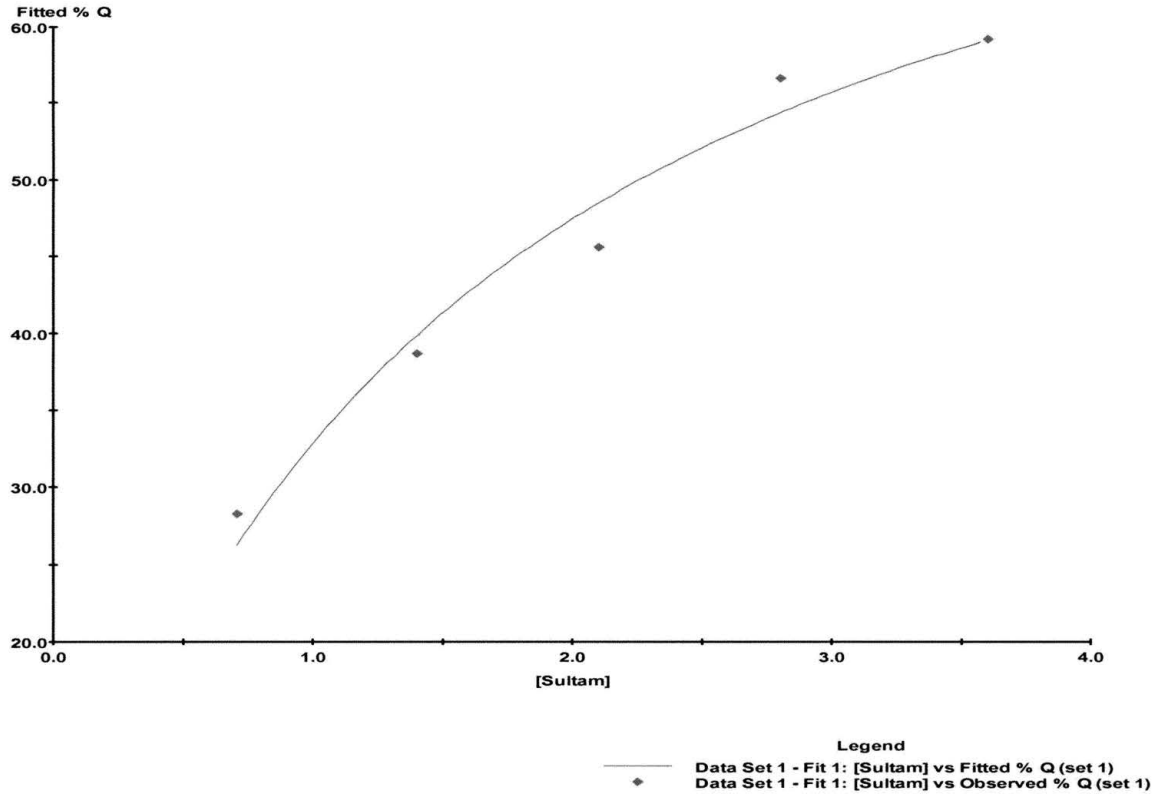

Figure 15. Plot of %Q against 1, 8-naphthosultam concentration (μM). The K_D for the inhibitor was calculated using Enzfitter.

CHAPTER IV

DISCUSSION

Rhodococcus erythropolis IGTS8 has been discussed as a possible tool for the desulfurization of fossil fuels. This organism uses a multi-step metabolic pathway for the desulfurization of organosulfur compounds (10). Some of the substrates degraded include thiophenes, sulfides, disulfides, mercaptans, sulfoxides, and sulfones, which are all found in petroleum products (9). The final enzyme in the pathway used by this organism is 2-(2'-hydroxyphenyl) benzenesulfinate desulfinase (HPBS desulfinase). This unique enzyme catalyzes the cleavage of the final carbon-sulfur bond in a variety of substrates, and is the rate-limiting step of the pathway (Figure 2) (15). In the experiments conducted for this research, HPBS desulfinase is studied in its role in the desulfurization of dibenzothiophene, a major organosulfur compound found in middle petroleum distillates (10).

Carbon-sulfur bond cleavage enzymes are not only important in desulfurization reactions, but also play a role in many other important metabolic processes. As a result, the isolation, purification and characterization of carbon-sulfur bond cleavage enzymes have become increasingly more common; however, the mechanisms associated with these enzymes are still being postulated. Some of the carbon-sulfur bond cleavage enzymes, including cystathionine γ-lyase, methyl-CoM reductase, cysteine lyase and nifS

(16-17, 24-26) are involved in non-oxidative reactions. Most of them require cofactors, and many possess an essential cysteine residue within their active site (11, 16, 18-23, 31). Additionally, the enzymatic mechanisms associated with these systems (Figure 4) are similar to the mechanism proposed for HPBS desulfinase (Figure 16). Although HPBS desulfinase does not require a cofactor, it does possess a cysteine residue which is essential for catalytic activity. Further, this cysteine residue is completely conserved in all known HPBS desulfinase enzymes (Figure 17).

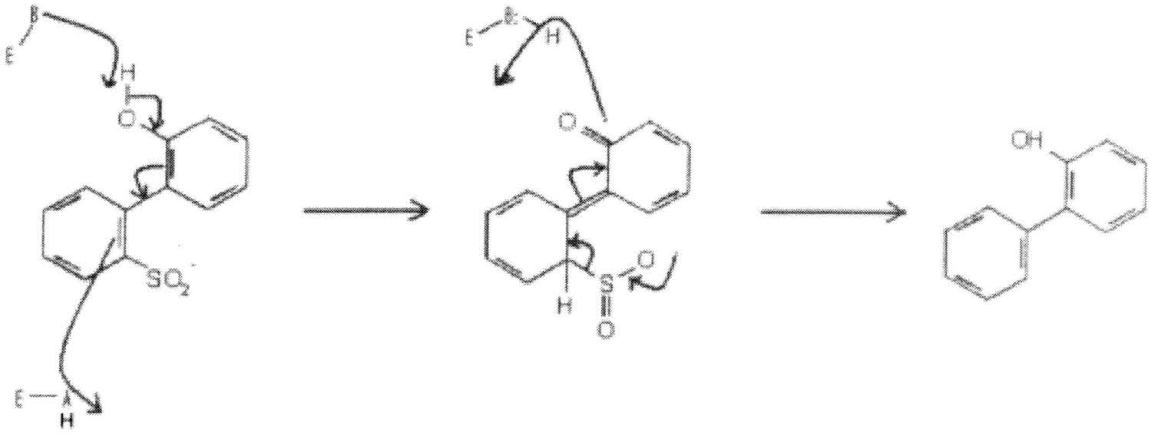

Figure 16. The acid-base mechanism proposed for HPBS desulfinase.

Figure 17. Gene alignments between *Rhodococcus* IGTS8, *A. tumefaciens*, *P. All-2*, and *S. pausimobilis*. (Conserved cysteine residue in blue.)

Purification protocols of many of the enzymes involved in carbon-sulfur bond cleavage reactions have been elucidated. The procedure used in this study was a modification of the procedure developed by Gray (43) to purify all of the enzymes in the IGTS8 desulfurization pathway. The final product from the purification produced one clear band on an SDS-PAGE with a predicted molecular mass of 40 kDa. This molecular mass corresponds to that determined from the amino acid sequence previously reported.

Substrate analogs and inhibitors studies are important for understanding the types of molecules required by the active site for catalysis. A variety of commercially available analogs were previously tested. The initial studies were performed at high concentrations of substrate. Recently, it has been shown that there is a possibility of substrate inhibition at high substrate concentration. So, it was important to test these

concentrations of substrate. Recently, it has been shown that there is a possibility of substrate inhibition at high substrate concentration. So, it was important to test these analogs at lower concentrations of substrate. In addition, earlier studies suggested that the base abstraction of the proton from the phenol ring might be rate-limiting. So, the addition of an electron withdrawing group to the phenol ring might increase the rate of proton abstraction by decreasing the pK_a of the phenolic proton. Two analogs were designed to test this idea. One of the analogs synthesized is 2-(2'-hydroxy-4'-bromophenyl) benzenesulfinate and the other is 2-(2'-hydroxy-4'-chlorophenyl) benzenesulfinate. These two analogs were produced using the synthetic scheme designed by Hansen and Kemp for functionalized biphenyls (Figure 6) (45). Characterization of these two compounds was performed. Mass spectroscopy analysis was run on the synthesized compounds. The results suggest that one chlorine is present on the chlorinated compound, while the spectrum for the brominated species indicates both single and disubstitution. The ^{13}C NMR shows the ring carbons have been de-shielded by the bromine to 128-130 ppm. The ^{1}H NMR and TLC experiments run indicated that an impurity was initially present. So, a column purification was performed using charcoal with acetone:hexane (60:40) as the solvent. The eluent was analyzed using TLC. The TLC showed that the resulting compound was free of the impurity. Further analysis using ^{1}H NMR showed that the impurity noticed initially was absent.

The pK_as of the two compounds were also determined and compared to the pK_as of the substrate HPBS. The desired effect of the halogenation of HPBS was to lower the pK_a of the phenolic proton, and the results of the experiments showed that this goal was achieved. The first and second pK_as for both compounds were lower than that of HPBS.

The pK_as for the two synthesized compounds also behaved as expected with respect to each other, because the pK_as for the brominated compound were higher than those calculated for the chlorinated. This was anticipated due to the differences in the electronegativities of bromine and chlorine.

Using the two purified analogs, and the fourteen commercially available analogs, inhibition studies were conducted using the purified HPBS desulfinase. Fluorescence assays were performed to test for the ability of the analogs to act as a substrate or inhibitor for HPBS desulfinase. It was determined from the data that neither of the synthetic analogs behaved as inhibitors or substrates. However, the data suggests that the enzyme is activated. It is not immediately obvious how a monomeric enzyme can be activated by a substrate analog. There is no data indicating a second binding site on HPBS desulfinase or that monomer association occurs. This unusual behavior, however, does support data indicating there is substrate inhibition at high HPBS concentrations.

The commercially available compounds tested which are functionalized on one of the phenyl rings included xanthene, 2-methoxybiphenyl, 2-hydroxydiphenylmethane, 2-hydroxybiphenyl, 1-naphthalene sulfonic acid, phenylhydroquinone, 2-biphenyl carboxylic acid, and 2-amino-4-phenylphenol. These are all considered product (HBP) analogs. These species ranged from those containing hydrogen bond donors, to those containing hydrogen bond acceptors. Additionally, the functional groups were either charged or neutral. None of these analogs acted as substrates, but 1-naphthalene sulfonic acid showed 57 % inhibition of HPBS desulfinase (Table 2).

The analogs tested with both phenyl rings functionalized included diphenic acid, 4'-hydroxy-4-biphenyl carboxylic acid, and 2, 2'-biphenol. These three compounds are

all considered substrate (HPBS) analogs and performed as inhibitors (Table 2). HPBS desulfinase activity decreased to 81 % and 60 % of the control activity in the presence of diphenic acid and 4'-hydroxy-4-biphenyl carboxylic acid, respectively. Biphenol was the most effective of these inhibitors with the relative activity decreasing to 37 % of the control activity. A K_I for 2, 2'-biphenol was previously estimated to be 17 μM. Three analogs which were functionalized on both rings, with rigid ring structures were tested as well. These analogs included 1, 8 naphthosultone, 1, 8-naphthosultam, and 6-hydroxy-2-naphthalene sulfonic acid. Two of these analogs showed fairly good inhibition: 1, 8-naphthosultam showed 42 % inhibition, and 6-hydroxy-2-naphthalene sulfonic acid showed 63 % inhibition. The other species tested behaved as less effective inhibitors, and did not act as substrates. It was determined from the data and the structures of the analogs tested that effective inhibitors of HPBS desulfinase must contain two benzene rings with a hydrogen bond donating group and a hydrogen bond accepting group, or must contain a sulfonic acid. The calculated percent relative activities of the enzyme after incubation with the inhibitors were the same as those determined by former researchers in experiments done at 100 μM substrate and analog.

Assays were also conducted to determine the fluorescence intensity of the product (HBP) in the presence and absence of substrate (HPBS) in order to assess if HPBS was quenching HBP fluorescence. It had been unclear in previous experiments whether energy transfer might be occurring between the substrate and product resulting in inaccurate measurement of enzyme activity. This could account for earlier results suggesting substrate inhibition. The assay was performed using varying concentrations of substrate (0, 4, and 8 μM) and product (0, 2, 4, and 6 μM). The resulting fluorescence

was measured and the fluorescence intensity of HBP was compared in the presence and absence of substrate. It was determined that no energy transfer between HBP and HPBS was occurring.

Inhibition studies were also run with thiourea dioxide. Previous studies using thiourea dioxide had shown slight inhibition at low concentrations of thiourea dioxide and high concentrations of substrate. So, an assay was run using the same concentrations (5 μM) of both the inhibitor and the substrate. No inhibition was observed. The assay was repeated using high concentration of inhibitor (100 μM) with low substrate concentration (5 μM). This resulted in no enzyme activity. Because of the structure of thiourea dioxide, which contains an $-SO_2$ group, it was determined that it should inhibit HPBS desulfinase. However, the results suggest enzyme inactivation. Further studies should be performed to determine whether dilution of the high concentration thiourea dioxide assay will bring activity back to the enzyme. If thiourea dioxide is modifying the enzyme, then the activity would not be rescued.

A similar set of experiments using $CuCl_2$, was run to determine whether the inhibition previously observed with $CuCl_2$ is a result of metal binding to the enzyme or to the substrate. The assay performed contained constant $CuCl_2$ concentration with varied substrate concentration and enzyme. The results suggest that the substrate concentration does not affect the activity of the enzyme in the presence of $CuCl_2$. Therefore, $CuCl_2$ is binding to the enzyme. Since sulfur behaves as a high affinity ligand for Cu^{2+}, it is proposed that the inhibitor is binding to the cysteine residue within the active site of the enzyme. These results coincide with those ascertained in previous metal binding studies.

The essential role of cysteine in the catalysis of HPBS desulfinase has been previously observed. However, those experiments were performed using less than 1 μM concentrations of the chemical modifier DTNB. The current assay performed used concentrations from 1-5 μM DTNB. Upon incubation with the modifier at a 1 μM concentration, the enzyme was inactivated after 20 minutes. With the incubation of the 2 μM concentration of DTNB, the enzyme was inactivated after 10 minutes. The 3 μM concentration of DTNB inactivated the enzyme after 5 minutes, and the 4 and 5 μM concentrations showed no enzyme activity after 5 minutes. The rate of inactivation at each concentration of DTNB (k_{obs}) was determined for each of the modifier concentrations tested. The second order rate constant (k_i) for DTNB inactivation was determined to be 0.0268 $min^{-1} * \mu M^{-1}$. The results confirmed previous studies showing that the cysteine residue is essential for HPBS desulfinase catalysis. The discrepancies in the k_i values calculated (previous study recorded a k_i of 0.0481 $min^{-1}\mu M^{-1}$) are within experimental error and can also be explained by the differences in the range of DTNB concentrations and the scatter in the previous data (46) as a result of the low sensitivity of the assay.

The low sensitivity of the HPBS desulfinase assay may become a deterrent to measuring accurate K_I values for the inhibitors. The activity of the enzyme is low (1.3 ± 0.07 min^{-1}) and the addition of inhibitors decreases the sensitivity of the assay even further. The inhibitor 1, 8-naphthsultam was used to determine whether the K_D (and thus K_I) for analog binding could be determined by monitoring the change in fluorescence of the enzyme upon analog binding. 1, 8-Naphthsultam was selected for this experiment because it is the analog that shows the most inhibition and least amount of fluorescence

emission. The fluorescence intensity of many of the analogs was so large that it precluding the monitoring of enzyme fluorescence emission. Titration experiments were conducted with final sultam concentrations of 0-3.6 μM. Emission scans (200-900 nm) were performed in the presence and absence of enzyme at an excitation λ of 280 nm. The fluorescence of the enzyme decreases upon addition of 1, 8-naphthosultam. A K_D of 1.6 ± 0.23 μM was then calculated for 1, 8-naphthosultam binding to the enzyme. 1, 8-naphthsultam is the tightest binding inhibitor tested thus far, with a K_I only slightly larger than the K_m of the substrate (1 μM) (43). In addition, these experiments show that this type of experiment can be used to measure binding constants for analogs that have reasonably low inherent fluorescence emission.

The research conducted to define the active site of 2-(2'-hydroxyphenyl) benzenesulfinate desulfinase has been primarily achieved through the use of inhibition studies involving substrate and product analogs. Twenty-one commercially available analogs were tested for their ability to act as substrates for HPBS desulfinase. None acted as substrates. Twenty-one commercially available analogs were tested for their ability to act as inhibitors for HPBS desulfinase. Seven of these showed significant inhibition of the enzyme. From these experiments it was concluded that effective inhibitors of HPBS desulfinase must contain two benzene rings with a hydrogen bond donating group and a hydrogen bond accepting group, or a sulfonic acid. The brominated and chlorinated derivatives of HPBS were synthesized, purified and characterized. The titration data produced to calculate the pK_a of the phenolic proton in the presence of the halogen indicate that the pK_a did not lower as expected. The brominated and chlorinated derivatives of HPBS were not substrates or inhibitors of HPBS desulfinase, but activated

the enzyme. Chemical modification of the cysteine within the active site, using increasing modifier concentration, resulted in complete inactivation of the enzyme. This confirms the necessity of the cysteine residue for HPBS desulfinase catalysis. Fluorescence titration studies were developed and tested using 1, 8-naphthosultam. A K_D of 1.6 μM was measured revealing that this analog binds very tightly to the enzyme and that this is a more sensitive method for measuring the K_I values of many of the analogs.

Although these studies have shown the types of substrates necessary for catalysis within the active site of HPBS desulfinase, and confirmed the importance of the sole cysteine residue, further studies must be done to conclusively determine active site structure. Mutagenesis studies, which are now in the preliminary stages, will progress knowledge about the active site and will be aided by a crystal structure. Additionally, experiments must also be performed to find out why this monomeric enzyme is activated by the synthesized analogs and shows substrate inhibition. Future investigations into the mechanism of carbon-sulfur bond cleavage by HPBS desulfinase will help elucidate this poorly understood system, and improve the potential for this enzyme to play an important role in the desulfurization of fossil fuels.

REFERENCES

1. Fletcher, S. (2000) *O.G. J. Online* **2000**, 1-9

2. Maghsoudi, S., Vossoughi, M., Kheirolomoom, A., Tanaka, E., and Katoh, S. (2001) *Biochem. Eng. J.* **8**, 151-156

3. Schilling, B. M., Alvarez, L. M., Wang, D. I. C., and Cooney, C. L. (2002) *Biotechnol. Prog.* **18**, 1207-1213

4. Vicic, D. A. and Jones, W. D. (1998) *Organometallics* **17**, 3411-3413

5. Jones, W. D., Chin, R. M., and Hoaglin, C. L. (1999) *Organometallics* **18**, 1786-1790

6. Monticello, D. J. (1998) *Chemtech.* **28**, 38-45

7. Cha, M., Shoner, S. C., and Kovacs, J. A. (1993) *Inorg. Chem.* **32**, 1860-1863

8. Kiick, D. M. and Phillips, R. S. (1988) *Biochem.* **27**, 7333-7338

9. Monticello, D. J., and Finnerty, W. R. (1985) *Ann. Rev. Microbiol.* **39**, 371

10. Suske, W. A., Held, M., Schmid, A., Fleischmann, T., Wubbolts, M. G., and Kohler, H-P., E. (1997) *J. Biol. Chem.* **272**, 24257-24265

11. Vasquez-Duhalt, R., Torres, E., Valderrama, B., and Le Borgne, S. (2002) *Ener. Fuel* **16**, 1239-1250

12. Borole, A. P., Kaufman, E. N., Grossman, M. J., Minak-Bernero, V., Bare, R., and Lee, M., K. (2002) *Biotechnol. Prog.* **18**, 88-93

13. Hensen, E. J. M., Vissenberg, M. J., de Beer, V. H. J., van Veen, J. A. R., and van Santen, R. A. (1996) *J Catal.* **163**, 429-435

14. Oldfield, C., Pogrebinsky, O., Simmonds, J., Olson, E. S., and Kulpa, C. F. (1997) *Microbiol.* **143**, 2961-2973

15. Kilbane, J. J., (1995) *Appl. Biochem. Biotechnol.* **54**, 209-221

16. Kilbane, J. J., Jackowski, K. (1992) *Biotechnol. Bioeng.* **40**, 1107

17. Rodgers, R. P., Blumer, E. N.. Emmett, M. R., and Marshall, A. G. (2000) *Environ. Sci. Technol.* **34**, 535-540

18. Agar, J. N., Yuvaniyama, P., Jack, R. F., Cash, V. L., Smith, A. D., Dean, D. R., and Johnson, M. K. (2000) *J Biol. Inorg. Chem.* **5**, 167-177

19. Christiansen, J., Seefeldt, L. C. and Dean, D. R. (2000) *J Biol. Chem.* **275**, 36104-36107

20. Rangaraj, P., Ryle, M. J., Lanzilotta, W. N., Goodwin, P. J., Dean, D. R., Shah, V. K., and Ludden, P. W. (1999) *J Biol. Chem.* **274**, 29413-29419

21. Shriver, Z., Hu, Y., Pojasek, K., and Sasisekharan, R. (1998) *J Biol. Chem.* **273**, 22904-22912

22. Chan, J. M., Christiansen, J., Dean, D. R., and Seefeldt, L. C. (1999) *Biochem.* **38**, 5779-5785

23. Xi, L., Squires, C. H., Monticello, D. J., and Childs, J. D. (1997) *Biochem. Biophys. Res. Commun.* **230**, 73-75

24. Zheng, L., White, R. H., Cash, V. L. and Dean, D. R. (1994) *Biochem.* **33**, 4714-4720

25. Zheng, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993) *Proc. Natl. Acad. Sci.* **90**, 2754-2758

26. Mihara, H., Kurihara, T., Yoshimura, T., Soda, K., and Esaki, N. (1997) *J Biol. Chem.* **272**, 22417-22424

27. Chen, H. Y., Demidkina, T. V., and Phillips, R. S. (1995) *Biochem.* **34**, 12276-12283

28. Sundararaju, B., Antson, A. A., Phillips, R. S., Demidkina, T. V., Barbolina, M. V., Gollnick, P., Dodson, G. G., and Wilson, K. S. (1997) *Biochem.* **36**, 6502-6510

29. Chen, H., Gollnick, P., and Phillips, R. S. (1995) *Eur. J Biochem.* **229**, 540-549

30. Jacobson, M. R., Cash, V. L., Weiss, M. C., Laird, N. F., Newton, W. E. and Dean, D. R. (1989) *Mol. Gen. Genet.* **219**, 49-57

31. Norikazu, N., Toshiyuki, M., Takashi, O., Yuko, M., Yasushi, K., Kenichi, K., Yasuto, H., Masanori, S., Kenji, M., Yoshikazu, I., Ryuichiro, K. (2002) *Biochim. Biophys. Acta.* **1598**, 122-130

32. Naito, M., Kawamoto, T., Fujino, K., Kobayashi, M., Maruhashi, K., and Tanaka, A.(2001) *Appl. Microbiol. Biotechnol.* **55**, 374-378

33. Onaka, T., Okumura, K., and Suzuki, M. (1997) *J Chromatogr. Sci.* **35**, 417-422

34. Smith, A. D., Agar, J. N., Johnson, K. A., Frazzon, J., Amster, I. J., Dean, D. R., and Johnson, M. K. (2001) *J. Am. Chem. Soc.* **123**, 11103-11104

35. Lee, J., Gravel, M., Gao, E., O'Neill, R. C., and Braun, P. E. (2001) *J. Biol. Chem.* **276**, 14804-14813

36. Jung, Y-S., Gao-Sheridan, H. S., Christiansen, J., Dean, D. R., and Burgess, B. K. (1999) *J Biol. Chem.* **274**, 32402-32410

37. Kanaya, S., Kimura, S., Katsuda, C., and Ikehara, M. (1995) in Techniques in Protein Modification (Lundblad, R., Ed) pp.63-83, CRC Press, Inc., Boca Raton, FL

38. Menges, D. A., Ternullo, D. L., Tan-Wilson, A. L., and Gal, S. (1997) *Anal. Biochem.* **254**, 144-147

39. Jayasekera, M. M. K., Shi, W., Farber, G. K., and Viola, R. E. (1997) *Biochem.* **36**, 9145-9150

40. Briganti, F., Pessione, E., Giunta, C., and Scozzafava, A. (1997) *FEBS Lett. 416*, 61-64

41. Lam, W. W. Y., and Bugg, T. D. H. (1997) *Biochem.* **36**, 12242-12251

42. Evans, M. (1998) *Am. Biotechnol. Lab* **16**, 16-17

43. Gray, K. A., Pogrebinsky, O. S., Mrachko, G. T., Xi, Lei, Monticello, D. J., and Squires, C. H. (1996) *Nat. Biotechnol.* **14**, 1705-1709

44. Methods in Enzymology. (1982) **87**, 551

45. Hanson, G. and Kemp. D. S. (1981) *J. Org. Chem.* **46**, 5441-5443

46. Cody, Melissa. (2002) Master's Thesis. Southwest Texas State University

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