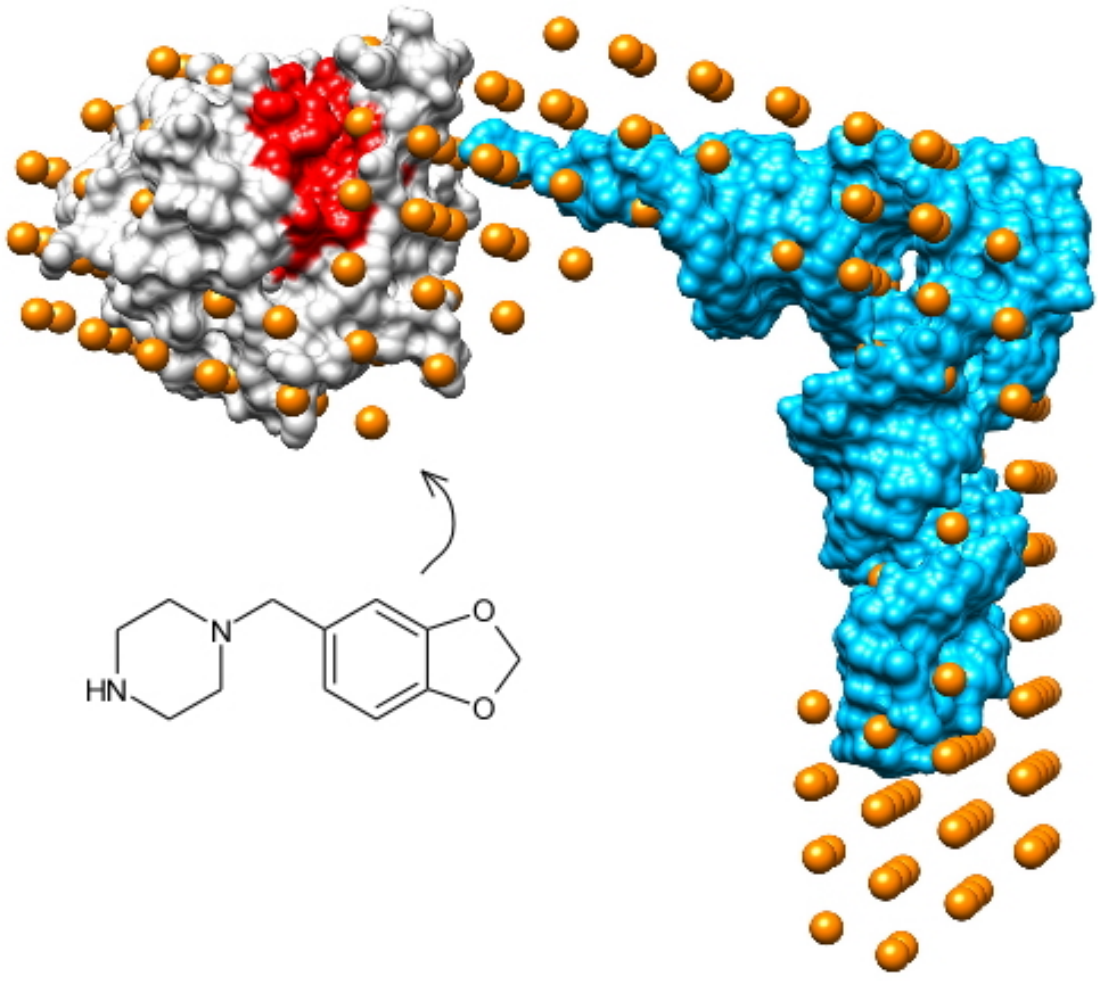
Functional Differentiation of Bacterial Pth1 and Human Pth2: Validation of Pth1 as a Novel Antibiotic Target



Functional Differentiation of Bacterial Pth1 and Human Pth2: Validation of Pth1 as a Novel Antibiotic Target

by

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Abstract

The rise of drug resistant bacteria presents researchers with the serious challenge of developing new antibiotics and new antibiotic targets. Peptidyl-tRNA Hydrolase 1 (Pth1) is a highly conserved and necessary bacterial enzyme that has the potential to become a new and effective target for killing bacteria. However, an enzyme in humans called Pth2 is functionally similar to Pth1. We hypothesize that Pth2 has a distinct pharmacological profile from Pth1. This project begins to differentiate the inhibitory profiles of Pth1 and Pth2 by assaying Pth1 and Pth2 with natural product plant extracts. PTH enzymes were incubated with peptidyl-tRNA with and without inhibitors for a set time. The resulting tRNA and peptidyl-tRNA mixture was analyzed with acid-urea minigels. The inhibitory profile for Pth1 was obtained for twenty extracts, revealing a broad range of inhibition. However, the inhibitory profile for Pth2 was not obtained because Pth2 lost activity due to degradation in solution. Future work will different Pth1 and Pth2's inhibitory and pharmacological characteristics by developing storage protocol for Pth2 and by studying the inhibitory profile for Pth2 using this work's extracts.

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Introduction

The Central Dogma of biochemistry states that genetic information is stored in DNA, which is transcribed to RNA, which is translated to protein. The translation of RNA into proteins occasionally ends prematurely, causing the peptidyl-tRNA to disassociate from the ribosome (Menninger *et al*. 1976). Peptidyl-tRNA is unusable to the cell and traps tRNA in an inert form. The result of tRNA being sequestered in peptidyl-tRNA is the dramatic slowing and eventual blocking of protein synthesis because tRNAs are crucial to translation and are limited in the cell. Cells avoid the build up of peptidyl-tRNA through essential enzymes called Peptidyl-tRNA Hydrolases (PTHs). PTHs recycle tRNA for translation by hydrolyzing the peptide from the peptidyl-tRNA.

A Comparison of Pth1 and Pth2

PTHs have been discovered throughout all forms of life in archaea, bacteria, and eukaryotes (Rosas-Sandoval *et al*. 2002, Richter *et al.* 2010, Dujeancourt *et al*. 2013). Unlike the multiple-member PTH systems in eukaryotes, bacteria are suspected to contain only one class of PTH, Pth1. Some of the earliest research on Pth1 by Menninger *et al.* showed that Pth1 hydrolyzes a diverse range of substrates, including every form of peptidyl-tRNA except aminoacyl-tRNA (AA-tRNA) and the initiator fMet-tRNA^{fMet} (1976). The high level of Pth1 conservation throughout bacteria and eukaryotes is revealed by amino acid sequence analysis of Pth1's from multiple organisms, as seen in Table 1 (Selvaraj *et al*. 2007, De L Vega *et al*. 1996). The structure of Pth1 was discovered by Schmitt *et al.* as a mixed α/β globular protein with a 7 stranded, mixed β-sheet core that is surrounded by 5 α-helices (Schmitt *et al*. 1997). Further research has found that the structures of Pth1 in other strains of bacteria are the same as *E. coli* Pth1, indicating that

Pth1 structure is conserved throughout the bacterial kingdom. Bacteria only have one homolog of PTH that is structurally and sequentially conserved throughout all of the studied species.

Table 1: Amino Acid Sequence Conservation of Pth1

Organism	Sequence Identity to *E. coli* (%)
Saccharomyces cerevisiae	27
Chlamydia trachomatis	33
Mycoplamsa genitalium	34
Borrelia burgdorferi	37
Micobacterium tuberculosis	38
Bacillus subtilis	38
Heamophilus influenzae	62
Salmonella typhi	91

(Selvaraj *et al*. 2007, De L Vega *et al*. 1996)

While bacteria presumably only have one homolog of PTH, eukaryotes have multiple. Eukaryotes, like bacteria, have Pth1 but also have at least three additional functional homologs of PTH, labeled Pth2 through PTH4 (Richter *et al*. 2010, Dujeancourt *et al*. 2013). However, Pth2 is the only eukaryotic homolog of PTH that has received significant study. Pth2 is a smaller protein than Pth1 and is found in archaea as well as eukaryotes (Fromant *et al*. 2003, Ishii et al. 2006, Shimizu et al. 2008, Powers et al. 2005). Pth2 hydrolyses the same substrates as Pth1, except Pth2 does hydrolyze the bacterial initiator, fMet-tRNA^{fMet} (Fromant *et al*. 2003). Interestingly, Pth2 cleaves peptidyl-tRNA at a different site than Pth1. While Pth1 hydrolyzes the ester bond between the peptide and the tRNA, Pth2 actually cleaves the ester bond between the first and second nucleic acids, leaving an additional adenosine nucleotide attached to the peptide (Schulman *et al*. 1975, Gross *et al*. 1992). Pth2 also has a similar core fold as Pth1, a mixed α/β globular structure, but otherwise is structurally distinct from Pth1. In fact, the fold of Pth2 is a novel fold that is not similar to any other known proteins. Pth2 has a four-stranded, mixed β-sheet core

that is surrounded by two α-helices on each side (De Pereda *et al*. 2004). The fact that PTH homologs perform a critical function in all cells and that there are multiple homologs of PTH makes that bacterial homolog, Pth1, a potential antibiotic target.

Characteristics of an Antibiotic Target

There are several factors that make Pth1 a potential antibiotic target: Pth1 is critical to bacteria, Pth1 can be inhibited *in vitro*, and some research suggests Pth1 is not critical to eukaryotes. The first aspect of Pth1 that is important for antibiotic targets is that bacteria cannot survive without Pth1. Bacteria's dependence on Pth1 has been shown repeatedly. Menez *et al.* deleted the gene for Pth1 from *B. subtilus* and replaced it with a *Pth1* gene whose expression could be controlled. The researchers then showed the new strain of *B. subtilus* was dependent of the expression for growth and survival (2002). Another group of researchers, Menninger *et al.*, discovered a strain of *E. coli* that could not survive above 40°C. Investigation showed that this temperature dependent strain of *E. coli* had a mutant *Pth1* gene that translated a mutant Pth1 that is inactive above 40° C, which Menninger *et al*. called temperature-sensitive Pth1 (Ts Pth1). Menninger *et al*.'s continued research demonstrated that the loss of Pth1 function caused the temperature dependent strain of Pth1 to die. Menninger *et al.* demonstrated *E. coli*'s dependence on the function Ts Pth1 by showing that protein synthesis decreased to 20% within 5 minutes of raising the temperature dependent strain to 40°C (1978). Menninger *et al.*'s research shows bacteria's dependence on Pth1 for survival as well as Pth1's critical role in sustaining protein synthesis. Once a potential antibiotic target has been shown to be critical to bacteria, the next question to ask is whether it can be inhibited.

The second characteristic of Pth1 that makes it a potential antibiotic target is inhibition. McFeeters *et al.* have shown that Pth1 can be inhibited *in vitro* by natural product extracts by using acid-urea minigels to determine the degree of hydrolysis of peptidyl-tRNA. McFeeters *et al.* assayed extracts from plant leaves, bark, and vines against Pth1 to find extracts with inhibitory properties. Then, McFeeters *et al.* used enzyme kinetics to determine the IC50s of the inhibitory extracts. Twenty-six inhibitory extracts were found and the IC50s of the inhibitory extracts ranged from .6 to 53.8 μg of extract per 1 U of Pth1 (2012). The same group also modeled the molecular docking of compounds from a particular extract, *Syzygium johnsonii*. Harris *et al.* performed this research and the docking energies of the polyphenolic compounds of the *S. johnsonii* extract ranged from -41.0 to -110.3 kJ/mol (2011). Both McFeeters *et al.* and Harris *et al.*'s studies demonstrate Pth1's susceptibility to inhibition, an important characteristic for potential antibiotic targets.

The final concern about developing Pth1 as a potential antibiotic target is the effects that the antibiotic might have on the host. Eukaryotes have Pth1 as mentioned above, so an inhibitor designed to kill bacteria by impairing the function of Pth1 could have detrimental impacts of the eukaryotic host. However, there is a chance that destroying the function of Pth1 could have little impact on eukaryotes because eukaryotes have multiple functional homologs of PTH (Richter *et al.* 2010, Dujeancourt *et al*. 2013). In fact, research supports the proposition that Pth1 is not critical to eukaryotes. Menez *et al.* have found that deleting the gene for Pth1 from the *S. cervisiae* (yeast) genome is non-lethal, demonstrating that Pth1 is not essential to *S. cervisiae* (2002). Rosas-Sandoval *et al.* have also found that deleting Pth1 is non-lethal to *S. cervisiae.* Additionally, Rosas-Sandoval *et al.* demonstrated

that *S. cerviciae* survived when either Pth2 was deleted from the genome or when both Pth1 and Pth2 were deleted from the genome (2002). These findings suggest Pth1 is not essential in eukaryotes. This research also shows that eukaryotes have more redundant mechanisms than bacteria for recycling peptidyl-tRNA, so removing Pth1's functionality should not dramatically affect eukaryotes.

However, concerns still remain whether inhibiting Pth1 in eukaryotes is safe. Even though Menez *et al.* and Rosas-Sandoval *et al.* showed that Pth1 can be deleted from eukaryotes, inhibition is not the same as deletion. Removing the function of Pth1 through deletion only affects Pth1, but removing the function of Pth1 through inhibition could affect multiple enzymes, particularly functional homologs of Pth1. Even though *S. cervisiea* survives with both Pth1 and Pth2 deleted, an inhibitor of Pth1 could harm eukaryotes by inhibiting multiple homologs of PTH. The binding sites of functional homologs are similar because they bind similar substrates, so an inhibitor that targets one binding site could have similar effects on other enzymes with similar binding sites. Therefore, it is important to show that Pth1 can be inhibited without inhibiting the other PTH homologs to assess Pth1's potential as an antibiotic target. This research assesses the probability of primarily or exclusively inhibiting the bacterial homolog of PTH (Pth1) by comparing the inhibition profiles of Pth1 and Pth2. The hypothesis of this research is that Pth1 and Pth2 have different inhibitory patterns because they differ in structure and origin.

Materials and Methods

Pth1 preparation

The gene for Pth1 from *E. coli* with a hexahistidine tag was cloned into PKQV4 vector, which contains resistance to carbenicilin. This vector was transformed into BL21

competent cells, which were grown on LB media in shake flasks at 37°C. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then used to induce expression of Pth1 when the cell density reached an OD_{600} of 0.8. After 4 h of expression, the cells were harvested through centrifugation and stored at -80°C. Then, the cells were resuspended with 15 mL of Buffer A (50 mM sodium phosphate, 300 mM NaCl, 2mM DTT, pH 7.4). The cells were lysed with lysozyme and mechanical sonication, and then the mixture was centrifuged. The clarified supernatant was separated with a metal chelation column, where Buffer A is as listed above and Buffer B was Buffer A with 150 mM imidazole. The resulting Pth1 fractions, which were <90% pure, were pooled and dialyzed in a buffer of 10 mM Tris acetate, pH 8.0. Next, the dialyzed sample was concentrated, mixed to a final concentration of 30% glycerol, and stored at -80°C until further use.

Pth2 preparation

The gene for *Homo sapiens* Pth2 was cloned into pet28b vector, which contains resistance to kanamycin. This vector was transformed into BL21 competent cells, which were grown on LB media in shake flasks at 37°C. When OD_{600} reached 0.6, the cells were induced with IPTG, the cell culture was cooled, and protein expression continued overnight at 15°C. The cells were centrifuged and stored at -80°C. Then, the cells were resuspended in Buffer A (100 mM Tris acetate, 2 mM DTT, pH 7.5) and lysed with lysozyme and sonication. After centrifugation, the clarified supernatant was separated with an SP column and Pth2 eluted around 50% Buffer B (Buffer A+500 mM NaCl). Next, the fractions containing Pth2 were pooled and dialyzed to the Sizing Buffer (20 mM Tris acetate, 150 mM NaCl, pH 7.5). The sample was then concentrated and further purified using a sizing

column that was equilibrated and run with Sizing Buffer above. Finally, the Pth2 fractions were pooled, concentrated, and stored at 4°C.

Peptidyl-tRNA preparation

Prepared as described by Harris *et al*. Lyophilized peptidyl-tRNA was dissolved to 30 μg/μL in water with diethylpyrocarbonate (DEPC), which is an RNAse inhibitor.

Pth1 extract inhibition assays

Extracts from natural product plant extracts were screened against Pth1 for inhibition analysis. The extracts were prepared by dissolving the dried, solid extract in dimethyl sulfoxide (DMSO) to produce a concentration of 1% (w/w). Inhibition assays were run in sets of 10 reactions due to the method of analysis, and at least three controls were performed with each reaction set, leaving at most 7 reactions for inhibition assays per set. Reaction mixtures were prepared in microtubes by mixing Pth1, peptidyl-tRNA, extracts (when required), and Reaction Buffer B (10 mM Tris acetate, 10 mM magnesium acetate, 20 mM ammonium acetate, pH 8.0). Assays of Pth1 were performed two different ways: either 1.5 μL of Pth1 (150 mM) cleaved for 5 min. or 1 μL of Pth1 (30 mM) cleaved for 30 min. For all reactions, 1.5μL of peptidyl-tRNA (30 μg/μL) was added, the final reaction volume was 20 μL, and Reaction Buffer B was added last to bring the volume to 20 μL. Finally, the volume of inhibitor added to assay reactions was 4 μL.

The positive and negative hydrolysis controls for each reaction set contained no inhibitor. For the solvent control, 4 μL of DMSO was added in place of the inhibitor. Reactions were begun at the same time by adding Pth1 last and placing it on the side of the microtubes, not in the solution. Then, all the microtubes for a reaction set were centrifuged together to add the Pth1 to the reaction mixtures at the same time. After the specified time,

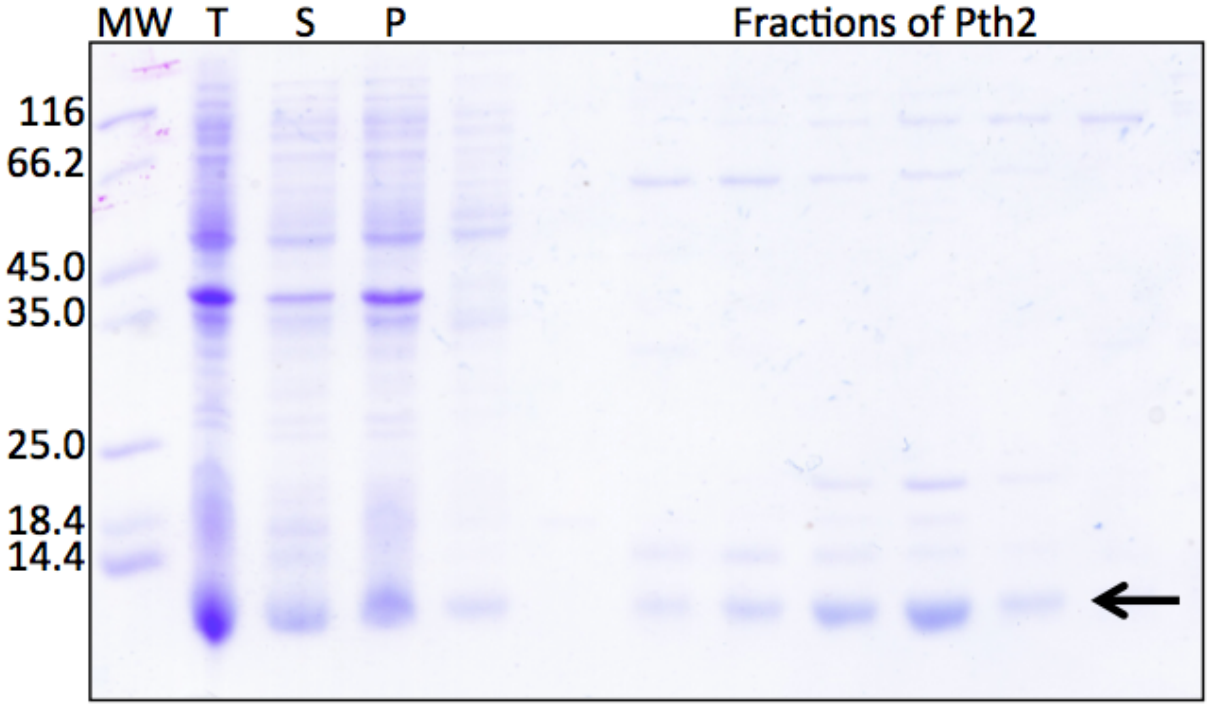
the reactions were quenched by addition of 20 µL of Gel Loading Dye. Then, each reaction set was analyzed on a 7.5 cm, 6.5% acrylamide, acid-urea minigel. After electrophoresis and staining, the gels were scanned and the images were saved. Then, the gel images were analyzed using ImageJ and GnuPlot and the percent cleavage for each reaction was determined.

Pth2 extract inhibition assays

Pth2 assays were performed like the Pth1 assays, except 1.3 µL of Pth2 (150 mM) was added to each reaction and the reaction time was 30 min.

Results

Figure 1: SDS-PAGE Gel of Pth2 SP Chromatography



An example of Pth2 purification using an SP column is shown. MW is the molecular weight marker, T is the total cellular material, and S and P are the supernatant and pellet after lysis. The remaining lanes represent fractions eluted from an SP column with 0-500 mM NaCl gradient. The arrow indicates Pth2.

Figure 2: Example of an Inhibition Assay Gel

Acid-urea minigel after staining with methylene blue. C and U indicate cleaved and uncleaved controls, while DMSO indicates the DMSO solvent control. The unlabeled lanes are different extracts tested for inhibition of Pth1 cleavage.

Figure 3: Image Analysis of Controls

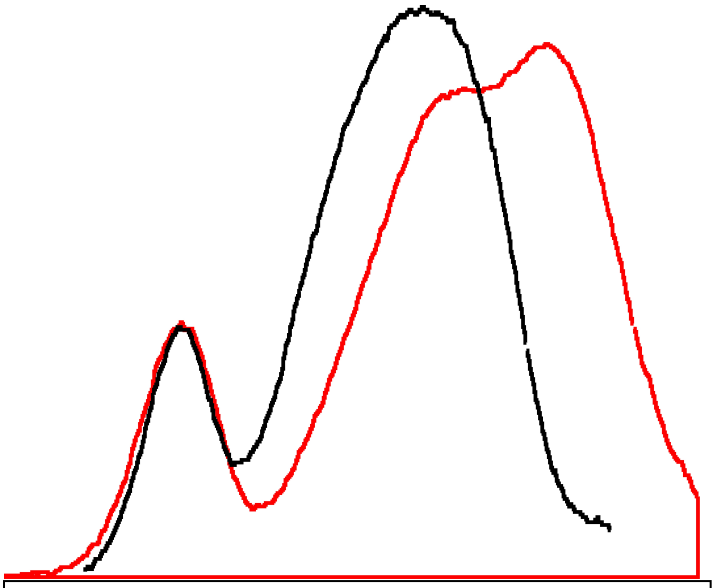
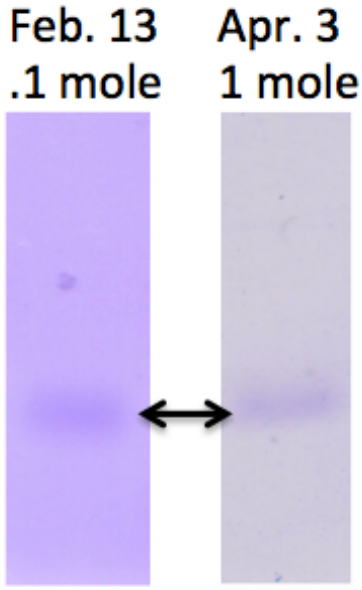


Image analysis of the control lanes from Figure 2 are shown. In black is the uncleaved control and in red is the completely cleaved control.

Figure 4: Pth2 Degredation



SDS-PAGE of Pth2. On the left is an sample of Pth2 run immediately after purification, on the right after 2 months of storage.

Figure 1 is an SDS-PAGE gel of the SP column fractions from purification of Pth2. Pth2 elutes at 250 mM NaCl and is on the order of 80% pure. An example of the acid-urea minigel substrate cleavage assay for analysis of inhibition is shown in Fig. 2 and the image analysis is displayed in Fig. 3. Figure 4 is a comparison of gel samples of Pth2 at different

dates, demonstrating the rapid degradation of Pth2. After only 2 months of being stored in solution at 4°C, a 10-fold decrease in Pth2 is observed. Finally, the results for the extract inhibition assays of Pth1 are shown in Tables 2 and 3.

Table 2: The Inhibition of Pth1 by Plant Extracts (5 min)

Name	Extraction Solvent	Source Matter	Percent Cleavage
Beilschmiedia "chancho blanco"	EtOH	Bark	100
Croton draco	Acetone	Bark	100
Croton monteverdensis	EtOH	Bark	100
Eugenia montecola	Acetone	Bark	40
Inga sierra	MeOH	Bark	0
Mandevillia veraguasensis	MeOH	Bark	20
Ruyschia phylladenia	EtOH	Bark	100
Sasafrass albidum	CHCl3	Bark	0
Trichilia martiana	Acetone	Bark	0

Table 3: The Inhibition of Pth1 by Plant Extracts (30 min)

Name	Extraction Solvent	Source Matter	Percent Cleavage
Apodytes brachystylis	EtOH	Solid	40
Ardisia palmana	Acetone	Bark	0
Cedrela tonduzii	Acetone	Bark	0
Cestrum racemosum	EtOH	Bark	80
Cinnamomum brenesii	Acetone	Bark	0
Diospyros digyna	Acetone	Bark	0
Ficus exasperate	CHCl3		100
Mallotus mollisimus	Mixed	Bark	20
Myrcia splendens	EtOH	Bark	0
Piper aequale	Acetone	Leaf	50
Syncarpia glomulivera	EtOH	Bark	100

Discussion

The purification and isolation of Pth1 and Pth2 was effective and produced active, pure enzymes. The inhibition assays of Pth1 were performed at two concentrations of Pth1 and for two different lengths of time. The information provided is useful and indicative of the inhibition profile of Pth1. To determine the reaction concentrations of enzyme for the

30 min. inhibition assays, the lowest concentration of Pth1 and Pth2 that cleaved practically all of the peptidyl-tRNA was found (i.e. most sensitive point for detecting inhibition). The final reaction concentration was set ~50% higher than lowest full cleavage concentration. The concentration of enzyme just greater than the minimum required for complete substrate cleavage balances consistent, complete hydrolysis of peptidyl-tRNA in the controls with sensitivity of PTH to the inhibitory extracts.

Each control in the extract inhibition procedure is important. The positive control demonstrates that PTH is still active at the time of experiment and sets the limit of complete (100%) cleavage. The negative control demonstrates that peptidyl-tRNA is not spontaneously hydrolyzing in the reaction conditions and provides an example of uncleaved peptidyl-tRNA. Finally, the solvent control, also called the DMSO control, demonstrates that the extracts' solvent (DMSO) does not affect or inhibit PTH and serves as the lower limit cleavage point (0%). It should be noted that this gave the same cleavage (0%) as the negative control. The visual appearance of the different controls can be seen in Fig 2. Also, the right positive control of Fig. 2 does not show cleavage of peptidyl-tRNA. Two sets of cleaved and uncleaved controls are often run on the same gel for error analysis. The failure of one of the cleaved controls indicates human error, also supporting the use of redundant controls. Image analysis of the assay controls are shown in Fig. 3, plotting the image intensity for the cleaved and uncleaved samples in red and black, respectively. The initial peak in Fig. 3 is due to top band in Fig. 2, which is a constant for every sample. The shift of the cleaved sample away from the first peak relative to the distance between the peaks in the uncleaved sample is due to peptidyl-tRNA losing the peptide and migrating through the gel faster (because it is smaller) than uncleaved peptidyl-tRNA.

The activity of Pth2 was determined as the optimal reaction concentration was studied. However, the long delay between initial cleavage and the follow-up Pth2 inhibition assay lead to no Pth2 activity, even in the cleaved control sample. In assessing why Pth2 lost activity, the protein gels in Fig. 4 were created. The gels show approximately equal amounts of Pth2, but ten times more sample was loaded in the later gel. Fig. 4 shows that Pth2 degrades in the storage buffer, likely due to the presence of proteases, indicating the need to develop better storage protocols for Pth2. For this reason, the inhibitory profile of Pth2 could not be studied at this time.

In contrast, the inhibition assays of Pth1 were successful, showing a range of extract inhibitions and discovering several inhibitors that prevented any noticeable cleavage. The inhibitory profile of Pth1 is displayed in Tables 2 and 3. Both reaction protocols revealed a range of extract inhibitions, indicating that both concentrations of Pth1 were in the sensitive range for the reaction time. It is interesting to note the relationship between the extraction solvent and the inhibitory effects of extracts. Five out of the eight samples extracted with acetone completely inhibited Pth1 under the reaction conditions, while only one out of seven ethanol extracts were complete inhibitors. Acetone is less polar the ethanol and therefore tends to extract less polar compounds. It may be that less polar compounds are more likely to inhibit Pth1. The inhibitory profile for Pth1 continues to demonstrate that Pth1 can be inhibited, suggesting Pth1's potential as an antibiotic target.

Future Work

The development of Pth1 as a novel antibiotic target requires extensive future research. Effective protocol for the storage of Pth2 needs to be developed so that the

inhibitory profile of Pth2 can be obtained accurately and reliably. The inhibitory profile of Pth2 is needed for the comparison of Pth1 and Pth2's inhibitory profiles and is therefore necessary for the assessment of whether inhibition of Pth1 is safe for eukaryotes. Next, extracts that inhibit Pth1 without affecting Pth2 can be researched to obtain more detailed information on their inhibitory potential through IC50s, and then most potent extracts need to be studied to find the active substance. Once substances are discovered that differential inhibit Pth1 over Pth2, their effects can be studied *in vivo* to discover if the inhibitors are actually lethal to bacteria. Finally, promising inhibitors will enter clinical trials and hopefully become the next generation of novel antibiotics.

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