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Enzyme Characterization of SABATH Family Members

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Characterization of SABATH Family Members

Introduction:

Life is a phenomenon that cannot happen without the use of proteins. Proteins are large complex molecules encoded by DNA that carry out the tasks necessary for an organism to function. Some of these tasks include transporting molecules across a cell membrane, protecting an organism from infection in the form of antibodies and, as is the case with enzymes, act as a catalyst for a chemical reaction. Salicylic Acid Methyl Transferase (SAMT) is one such enzyme that is a member of the Salicylic Acid Benzoic Acid Theobromine synthase (SABATH) family, which is found in many plant lineages. This family of enzymes uses S-adenosyl-L-methionine (SAM) as a methyl donor to methylate organic acids such as salicylic acid (SA), producing methyl salicylate (MeSA) (Ross et al., 1999). MeSA is important for the plant immune system, where it has been shown to act as a signaling molecule that stimulates the pathogen defense response of surrounding plants (Shulaev et al., 1997). MeSA is also an important contributor to floral scent, which can be used to attract moths for pollination (Raguso and Pichersky, 1995). In fact, MeSA has been shown to repel humming birds and ants, suggesting that plants can use this compound to select specific pollinators (Kessler and Baldwin, 2007).

An SAMT in *Clarkia breweri* was the first member of this family to be characterized. It was found that this particular SAMT had higher activity with SA than Benzoic acid (BA) at a ratio of 100:69 (Ross et al., 1999). A study to further characterize this enzyme found an important binding motif consisting of amino acids SSYSLMWLS (Zubieta et al., 2003), which can be seen in Figure 1. In the 6^{th} position there is either a methionine or a glutamine, although other SABATH family members can have histidine in this position. This is important and will be discussed later.

It was determined that the tryptophan, called trp-151, of this motif forms a hydrogen bond with its indole nitrogen to the carboxyl group of SA to orient and stabilize it. Other amino acids responsible for the stabilization of SA in the active site are gln-25, met-150 and met-308. Gln-25 forms a hydrogen bond to the carboxyl group of SA with its side chain amide nitrogen while met-150 and met-308 keep the benzyl ring of SA in place with a thioether group on one side and the other methionine thioether group on the other either side. These stabilization mechanisms will be taken into consideration later in this study when substrates other than SA are fed to the SAMT enzymes.

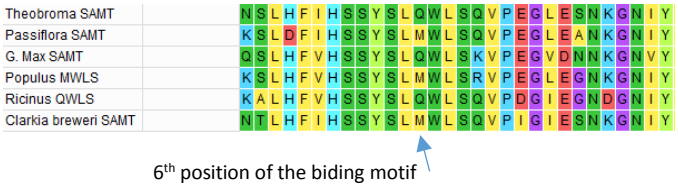

Figure 1: A protein alignment showing the SAMTs in this study as well as the SAMT from *Clarkia breweri*.

The binding motif SSYSMWLS represents a conserved region of SAMT enzymes that is found across plant lineages with very little variation. When studying SABATH family members in species of *Nicotiana*, this same binding motif was found (Hippauf et al., 2010). This is the same for lineages such as *Lilium*, *Cestrum*, *Datura* and *Stephanotis* (Wang et al., 2015; Huang et al., 2012).

There is, however, a variation that can occur in the 6th amino acid of the motif. In this position there can be a methionine, glutamine or histidine. *Nicotiana* has 3 SAMTs with a methionine in this position that all have higher activity with SA than other similar acids (Hippauf et al., 2010). *Solanum lycopersicum*, *Datura wrightii*, *Hoya carnosa*, and *Antirrhinum majus* SAMT enzymes are similar to the *Nicotiana* SAMTs in that they have a methionine in the same position of the binding motif and have shown SA to be the preferred substrate (Tieman et al., 2010; Huang et al., 2012; Negre et al, 2002). The *Glycine max* SAMT is different from the species

mentioned above, in that, it has a glutamine instead of a methionine in the 6th position of the binding motif. Despite the difference in this amino acid, the *Glycine max* SAMT still showed highest activity with SA (Lin et al., 2013). This variation seems to be a key factor in determining substrate preference, that is, depending on which amino acid is present in the 6th position the preference to methylate one substrate over other similar substrates such as SA over BA may be effected.

An experiment to determine the effect of the 6th position of the binding motif was done on the SAMT of *Datura wrightii*. The methionine in this position was mutated to a histidine. Originally the enzyme preferred SA to BA by almost 10 fold. With the mutation, the preference for both acids was almost equal (Barkman et al., 2007). A similar experiment was done in predicted ancestral enzymes of modern SAMTs in the Solanaceae family of plants. Ancestral SAMT enzymes containing a histidine in the 6th position of the binding motif were mutated to a methionine. Originally these SAMTs preferred BA, but with the mutation, the preference changed to SA. Ancestral SAMTs predicted to contain a methionine in the 6th position of the binding motif were also mutated to have a histidine instead. This changed the preference from BA to SA (Huang et a., 2012). This suggests that the variable nature of the 6th amino acid position of the binding motif may be one of the major contributors for substrate preference.

A phylogenetic analysis is a powerful way to determine the relatedness of not only organisms, but enzymes as well. SAMTs that contain methionine in the 6th position of the binding motif tend to form clades with other SAMTs that have a methionine in this position. If other SAMTs in the clade are known to have a certain function, then it is likely that any unstudied SAMTs have that same function. The same can be said for SAMTs that have a glutamine in the 6th

position of the biding motif. In this way, substrate preference can be predicted based on where an unstudied SAMT shows up on a phylogenetic tree containing characterized SAMTs.

This study characterizes 4 different SAMTs from the *Passiflora, Theobroma, Populus,* and *Ricinus* lineages. The *Theobroma* and *Ricinus* SAMTs both have glutamine in the 6th position of the binding motif while the *Passiflora* and *Populus* SAMTs both have a methionine. The substrate preference of these enzymes will be determined and then compared to see if these differences seem to have an effect. The *Glycine max* SAMT, which has a glutamine in the 6th position of the binding motif, will be used as a positive control alongside the *Theobroma* and *Ricinus* SAMTs. These SAMTs will also be assayed with a wide breadth of substrates to elucidate some of the properties of their active sites.

The goal of this study is to show that SAMTs containing a methionine in the 6th position of the binding motif or a glutamine, will both prefer salicylic acid over benzoic acid.

Methods and Materials:

Bioinformatics:

All SAMT sequences were obtained by using the *Clarkia breweri* SAMT sequence (Ross et al., 1999) to BLAST data bases found at NCBI, 1KP and Phytozome.

Phylogenetic Analysis:

The SAMT sequences were aligned using MUSCLE with the program MEGA7. This alignment was used to construct a Maximum Likelihood Tree using the same program.

RNA Extraction:

In order to extract RNA, the RNeasy® Plant mini kit and Quick-Start protocol was used (Qiagen). Plant material was weight out to 0.1g and crushed after being frozen with liquid nitrogen. 450 µL RLT buffer was added to the crushed tissue and vortexed. The solution was placed in a QIA shredder spin column and centrifuged at full speed for 2 minutes. The supernatant was collected and 0.5 volumes of 200 proof ethanol was added, mixed and transferred to an RNeasy mini spin column. The column was centrifuged at 10,000 RPM for 15 seconds and the flow-through was discarded. A solution containing 10 µL DNase stock solution and 70 µL RDD was added to the spin column and allowed to incubate for 15 minutes. The column was spun down and the flow through discarded. 350 µL of buffer RW1 was added to the spin column and centrifuged at 10,000 RPM for 15 seconds. The flow through was discarded and 500 µL of buffer RPE was added to the column and centrifuged again. A second wash with buffer RPE was done in the same fashion, but centrifuged for 2 minutes. The flow through was discarded and the column was then centrifuged for 1 minute to dry the membrane. In a new collection tube, 30 µL of H_20 was added to the spin column and centrifuged at 10,000 RPM for 1 minute. The flow through was placed back in the spin column and centrifuged again. The sample was stored at -80˚C.

RT-PCR:

SuperScript® III One-Step RT-PCR System With Platinum® Taq DNA Polymerase was used to obtain and amplify the first strand cDNA(Invitrogen). To perform reverse-transcriptase polymerase chain reaction (RT-PCR) a 50 µL solution was made containing 25 µL of 2x reaction mix (Invitrogen), 1 µL of 10 µM forward and reverse primer, 500 ng of extracted plant RNA, 2 µL taq polymerase/superscript III mixture (Invitrogen) and H_20 to bring the solution to 50 µL. To obtain double-stranded cDNA, the solution was placed in a thermocycler and the temperature was

set to 55 ˚C for 30 minutes. This was followed by 40 cycles of 15 seconds 94˚C denaturation followed by 30 seconds of annealing at 50˚C, and extension at 72˚C for 1 minute 15 seconds. Finally a single cycle of extension at 72˚C for 10 minutes to ensure all DNA strands were extended to completion. Primer sequence information can be made available upon request.

Gel Electrophoresis:

In order to determine if the RT-PCR process was successful, and the correct length gene had been amplified, gel electrophoresis was performed. A 1.5% agarose gel was made using Sybr Safe® (Thermo Fisher) as a stain to visualize the DNA under UV light. In order to visualize the length of the DNA a 1Kb plus DNA ladder was used.

Cloning:

In order to insert the PCR product into a plasmid the pTrichHis TOPO® kit and protocol was used (Invitrogen). 4 µL of PCR product was mixed with 1 µL of TOPO vector and allowed to incubate for 5 minutes at room temperature. 2 µL of this solution was mixed with a tube of One Shot Top 10 cells (Invitrogen) and mixed gently, taking special care not to pipette up and down. These cells were allowed to sit on ice for 30 minutes, after which they were heat shocked at 42˚C for 30 seconds. The cells were placed back on ice and 250 µL of SOC media was added. The cells were incubated with shaking at 37˚C for 30 minutes. This culture was then spread aseptically on lysogeny broth (LB) plates containing 100 µg/ml ampicillin and 0.5% glucose. The plates were incubated at 37˚C for 16 hours then stored in 4˚C

Colony Screeing:

In order to determine if the colonies contained a plasmid with the desired insert in sense orientation, a colony screening was performed. A solution containing 12.5 µL of 2x reaction master

mix (Qiagen), 0.5 µL of 10 µM pTrchHis forward primer and gene specific reverse primer were added. 0.5 µL of colony, and 6.5 µL of H_20 to make a total of 25 µL of solution. The sample was then subjected to PCR with 40 cycles of 1 minute denaturation at 94˚C, 30 seconds of annealing at 50˚C, extension for 1 minute 15 seconds at 72˚C, and ended with 10 minutes of extension at 72˚C.

Protein Expression:

A colony with a plasmid containing the SAMT gene was grown in 3 mL of LB with 100 µg/mL ampicillin. The culture was incubated at 37˚C with shaking overnight. After 16 hours the overnight growth was added to 47 mL of 100 µg/mL LB and incubated with shaking at 37˚C until a density of 0.6 was reached. IPTG was added to induce the cloned SAMT gene. 250 uL of 50mM salicylic acid and 250 uL of 50 mM benzoic acid was added to the culture and incubated at room temperature with shaking overnight. The culture was centrifuged at 4000 RPM for 20 minutes to pellet the cells. The supernatant was collected and Hexane was added to extract the methyl salicylate and methyl benzoate.

Protein Isolation:

To isolate native protein an overnight culture of 100 µg/mL LB media inoculated with E. coli Top 10 Cells containing the SAMT gene was grown. The overnight culture was added to 50 mL of 100 µg/mL LB media and upscaled to a density of .6 at a wavelength of 600 nm. IPTG was added to reach a final concentration of 1mM and the cells were incubated for 8 hours. The cells were pelleted by centrifuging at 2900 x gravity at 4˚C for 20 minutes. The media was discarded and the pelleted cells were re-suspended in 4 mL of 1X equilibration buffer. Lysozyme was added to reach .75 mg/mL and incubated on ice for 30 minutes. The sample was sonicated on ice with 3

ten second bursts with 30 second rests in-between. The sample was centrifuged at 10,000 x g for 20 minutes at 4˚C to pellet insoluble material. The supernatant contained the total protein and was extracted and stored at -80˚C.

Gas Chromatography and Mass Spectroscopy:

Gas chromatography mass spectrometry (GC-MS) is used to separate volatile compounds and measure their quantity and mass. 1 µL of the Hexane extract from the enzyme assay the GC-MS. The instrument started with a 40˚C hold for 2 minutes and was set to increase by 20˚C every minute until 300˚C where it was set to hold for 10 minutes.

Radioactive Enzyme Assay:

Total protein was extracted from Top 10 E. coli cells as described earlier. 10 µL of 250 mM tris HCL buffer pH 7.5, 8.5 µL of water and 30 µL of total protein isolate was mixed together. 1 µL of 50 mM substrate was added. For negative controls 1 µL of pure ethanol was added. .5 µL of S[methyl-^{14}C]adenosyl-L-methionine was added to the reaction mix and incubated at 25˚C for 1 hour. To stop the reaction 3 µL of HCL was added then 100 µL of ethyl acetate was added to extract the now methylated product. The reaction mix was centrifuged for 1 minute at 10,000 rpm. The organic phase was extracted and placed in a scintillation vial. 2 mL of counting buffer was added and counted in a liquid scintillation analyzer.

Results:

Phylogenetic Tree:

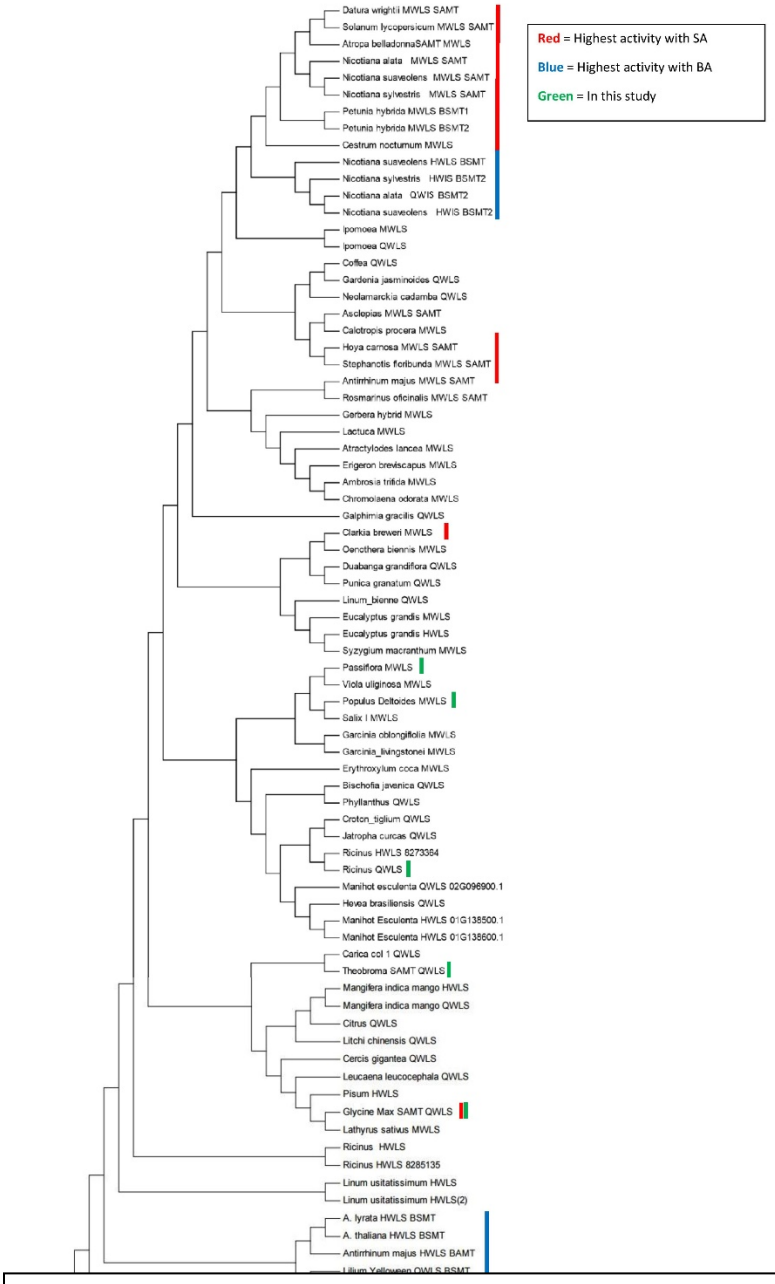

Figure 2: A phylogenetic tree consisting of SAMT sequences found on NCBI, 1KP, and Phytozome. The taxa with red and blue lines represent SAMTs that have been characterized in published studies. Those SAMTs include *Nicotiana alata, suaveolens* and *sylvestris* SAMTs and BSMTs (Hippauf et al., 2010), *Datura wrightii* SAMT (Barkman et al., 2007), *Solaum lycopersicum* SAMT (Tieman et al., 2010) *Atropa belladonna* SAMT (Fukami et al., 2002), *Petunia hybridum* BSMT1 and BSMT2 (Negre et al., 2003), *Cestrum nocturnum, Hoyra carnosa* and *Stephanotis floribunda* SAMTs (Huang et al., 2012), *Antirrhinum majus* SAMT (Negre et al., 2002) *Clarkia breweri* SAMT (Ross et al., 2000), *Glycine max* SAMT (Lin et al., 2013), *Arabadopsis lyrata* and *thaliana* BSMTs (Effmert et al., 2005), *Antirrhinum majus* BAMT (Murfitt et al., 2000), and *Lilium yelloween* BSMT (Wang et al., 2015).

Figure 2 illustrates a phylogenetic tree consisting of SAMTs of the species in this study as well as other SAMT sequences found by using the *Clarkia breweri* SAMT sequence to BLAST various databases on NCBI, the 1KP database and the Phytozome database. Three of the species in this study, *Passiflora*, *Populus*, and *Ricinus*, appear in a group with other Malpighiales. This is to be expected because enzymes from species of the same order share a more recent common ancestor than enzymes from plants of a different order. In the case of the *Theobroma* SAMT, it is on a branch containing SAMTs from different orders. It is placed near the *Carica* and *Mangifera* SAMTs from the orders

Brassicales and Sapindales respectively. This is also expected given that the *Theobroma* SAMT is from the order Malvales, which is closely related to Brassicales and Sapindales. Also noteworthy is the placement of the *Passiflora*, *Populus*, and *Ricinus* SAMTs within their clade. *Populus* and *Passiflora* SAMTs have methionine in the 6th position of the binding motif and are placed near other enzymes that have methionine in this position. Lower on the clade, The *Ricinus* QWLS SAMT is present which has a glutamine in the 6th position of the binding motif. Each of these enzymes in question seem to be placed in small subgroups that are similar in the 6th position of the binding motif and likely have a similar function.

GC-Mass Spec Analysis:

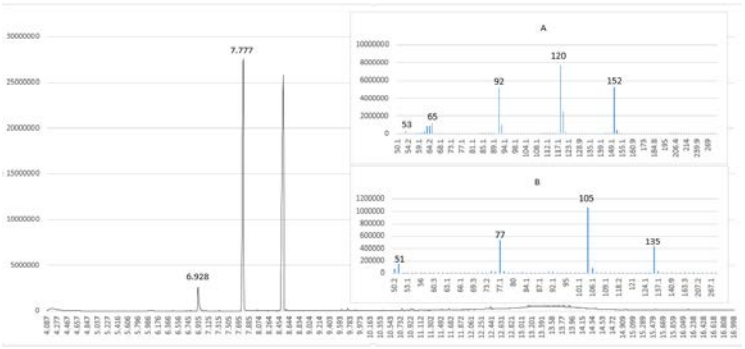

Figure 3: A gas chromatogram of the hexane used to extract MeSA and MeBA from the broth used to assay the *Passiflora* SAMT. The peak at 6.928 minutes represents MeBA and the peak at 7.777 minutes represents MeSA. The unmarked peak is an indole peak and was not produced by the expressed enzyme as is shown by the negative control in figure 5. A) The mass spectrum at 7.77 minutes. B) The mass spectrum at 6.928 minutes.

The hexane used to extract organic molecules from the broth used in the protein assay was analyzed by GC-MS. Figure 3 shows that a MeBA peak appears at 6.928 minutes while a MeSA peak appears at 7.777 minutes. This assay and analysis were done in triplicate and used to calculate the average ratio of MeSA to MeBA based on abundance. This ratio was found to be 100:19 meaning that SA was methylated about 5

times more often than BA. The same thing was done for the *Ricinus*, *Theobroma*, *Populus* and *Glycine max* SAMTs

whose ratio of MeSA to MeBA were found to be 100:0, 100:7, 100:0, and 100:12 respectively. These results can be seen in Table 1 and the analysis for each of these enzymes can be found in Figure 4.

Table 1: The results of the competitive enzyme assay. The activity with the preferred substrate was set to 100. These are the averages of triplicate replication.

	Ricinus SAMT	*Populus* SAMT	*Passiflora* SAMT	*Theobroma* SAMT	*Glycine max* SAMT
SA	100	100	100	100	100
BA	0	0	19	7	12

Figure 5 shows a negative control containing the same plasmid used for the other SAMT genes, but in anti-sense orientation. This means that when IPTG is added, it is extremely likely that a nonfunctional enzyme will be made. This was done to show that native proteins in the E. coli cells used are not responsible for the methylation of SA and BA.

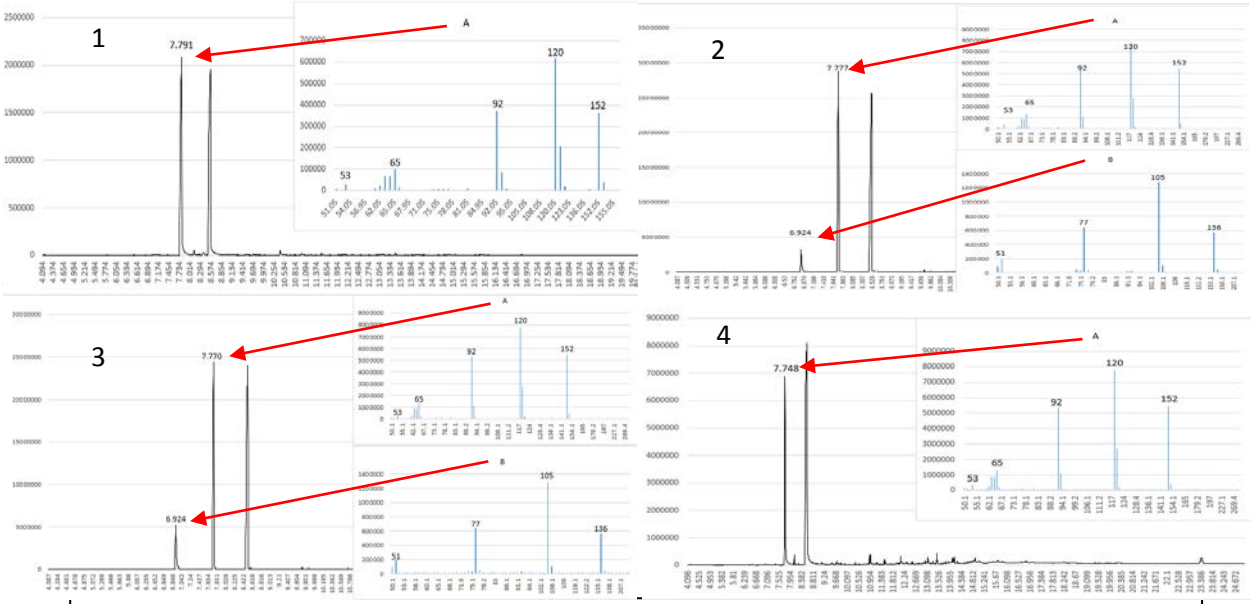

Figure 4: Gas chromatograms showing MeSA peaks ~7.8 minutes and MeBA peaks ~7 minutes. Graph 1 is the *Ricinus* SAMT, 2 is the *Theobroma* SAMT, 3 is the *Passiflora* SAMT and 4 is the *Populus* SAMT. Mass spectra labeled A represent the mass spectrum found at the MeSA peak of their respective gas chromatogram, and mass spectra labeled B represent the mass spectrum at the MeBA peak of their respective gas chromatogram.

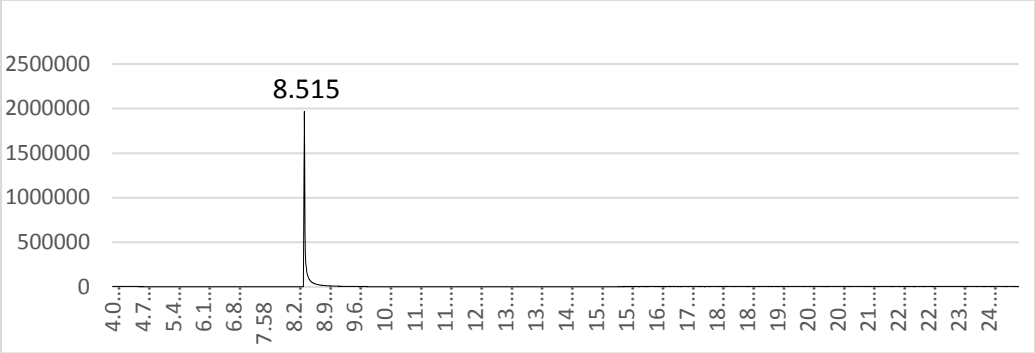

Figure 5: A gas chromatogram of the hexane used to extract from the broth of a negative control cell culture. At 8.515 minutes indole is present and no other peaks are seen.

Radio Active Assay:

Table 2: The relative activities of *Passiflora, Populus deltoides, Theobroma, Glycine max* and *Ricinus* with various substrates.

	Passiflora SAMT	Populus Deltoides SAMT	Theobroma SAMT	G. Max SAMT	Ricinus SAMT
Salicylic Acid	100	100	0	100	0
Benzoic Acid	27	0	0	0	0
Anthranilic Acid	5	0	0	0	0
Cinnamic Acid	0	0	0	0	0
Nicotinic Acid	14	0	0	0	0

Total protein was extracted from E. coli top ten cells that contained the induced SAMT gene. This total protein was assayed with radioactively labeled SAM and 5 nanomoles of substrate. The *Populus* and *Glycine max* SAMTs showed no activity with any of the substrates other than SA. *Theobroma* and *Ricinus* SAMTs showed no activity. The *Passiflora* SAMT showed activity with SA, BA, Anthranilic Acid (AA) and Nicotinic Acid (NA). The ratio of SA

to BA for the *Passiflora* SAMT in the radioactive assay was 100:27, which is near that of the GC-MS analysis of 100:19 SA to BA.

Discussion:

It was shown that the *Ricinus* and Theobroma SAMTs, which have a Q in the 6th position of the binding motif of their SAMTs, both prefer salicylic acid over benzoic acid. The same has been shown for *Passiflora* and *Populus* SAMTs, which have a methionine in the 6th position instead. One would assume this difference would have a drastic effect since it is a change in the active site of the enzyme, but the data provide evidence that an SAMT with a methionine or a glutamine in the binding motif will prefer SA. It does not seem to affect the substrate preference as mutating methionine in this position to a histidine did with the *Dautura* SAMT in the 2007 study done by Barkman et al. It is highly likely that glutamine and methionine could be interchangeable without affecting the substrate preference of the SAMT.

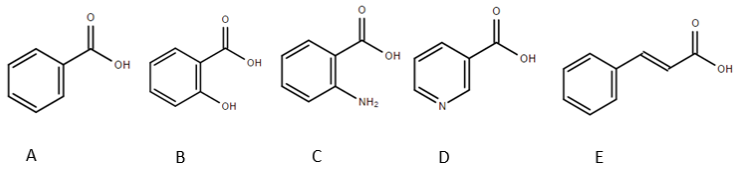

Figure 6: A) Benzoic Acid. B) Salicylic Acid. C) Anthranilic Acid. D) Nicotinic Acid. E) Cinnamic Acid.

Using different substrates in the radioactive assay gives an idea to what the active sight is like. Some substrates may cause more steric hindrance in the active site which would not allow them to fit properly. Zubieta et al. 2003 determined the SAMT structure of *Clarkia breweri* which has a methionine in the 6th position of the binding motif. It was shown how the methionine in the binding motif, called met-150, forms a “clamp” with its side chain and the side chain of met-308 which collectively help hold SA in place. Trp-151 forms a hydrogen bond with the carboxyl group of SA further holding it in place to allow the transfer of the methyl group from SAM to SA. This can be seen in Figures 8 and 9. It is important to note though, that the

SAMT structure in Figures 8 and 9 represent an SAMT with a methionine in the binding motif. Glutamine has a very different side chain than methionine which can be seen in Figure 7. Methionine has a thiol group in its side chain whereas glutamine has an amine group and a carbonyl group. Amine groups and carbonyl groups are much more electro negative than thiols, and one would think that this would have a drastic effect on substrate preference. But the data show that SA is still the preferred substrate. In order to determine exactly what is happening in the active site of an SAMT with a glutamine in the binding motif, it would be necessary to determine the structure as Zubieta et al. did.

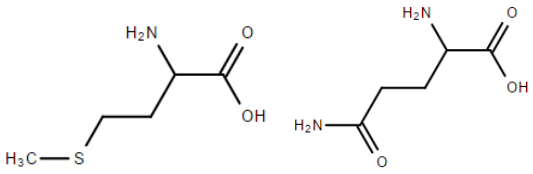

Figure 7: To the left the chemical structure of methionine is shown. To the right the chemical structure of glutamine is shown.

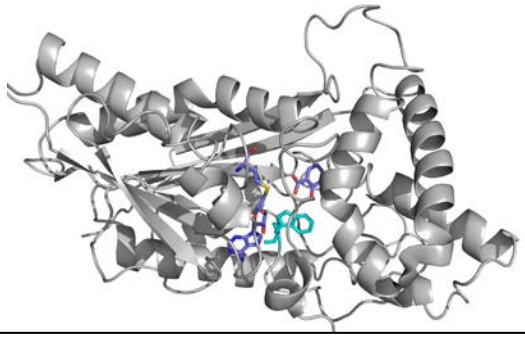

Figure 8: The *Clarkia breweri* SAMT structure as determined by Zubieta et al. in 2003 containing the substrates SA and S-adenosyl-L-homocysteine (SAH) in blue and showing the trp-151 amino acid in turquoise.

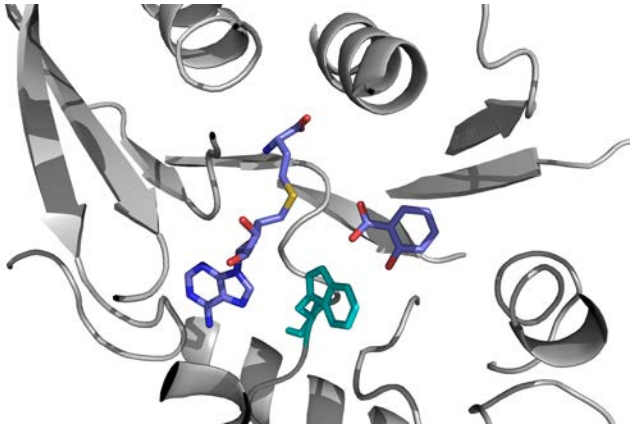

Figure 9: A zoomed in point of view on the active site of the *Clarkia breweri* SAMT structure as determined by Zubieta et al. 2003. The substrates SA and SAH are present in blue with the sulfur atom of SAH in yellow. The trp-151 amino acid is visible in turquoise.

In conclusion, it would seem as though SAMTs with a methionine in the 6th position of the binding motif and SAMTs with a glutamine in the same position will both prefer salicylic acid over benzoic acid. There was also some insight given to the active site of these SAMTs based upon which acids they methylated, showing that certain amino acids are in the perfect position to

stabilize SA the best. To confirm and further solidify these findings it would be necessary to determine the structure of each individual SAMT in this study and to mutate the methionine of an SAMT to a glutamine (or vice versa) and characterize it.

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