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Overlooked Potential of EPSP Synthase as an Antimicrobial Target

Matthew Donghoon Han*, Tayler Young*, Godwin Chan*, Kirill Pankov*, Ruilin Wu* & Mohammed Adam*

*Authors contributed to equally to this work.

Abstract
The rise of antibiotic resistance has necessitated a need to discover novel drug candidates. In consequence, an enzymatic protein found in Escherichia coli (E. coli) bacteria called 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) has shown promise as a novel drug target. EPSPS is an enzyme found in the shikimate pathway that forms EPSP and is essential for the downstream synthesis of vital aromatic amino acids: tryptophan, tyrosine, and phenylalanine. Using high-throughput screening (HTS), a library of 1000+ compounds was used to screen against the target of interest to discover inhibitors that significantly limited cellular growth. M9 minimal media was used in HTS to render EPSPS essential for bacteria. This media does not contain the aromatic amino acids, which would otherwise make EPSPS unnecessary for survival. Thus, it was rationalized that minimal media had to be used to avoid cell survival from external sources of amino acids. After conducting HTS and normalizing the data, 8 successful inhibitors of the enzyme were identified. However, these findings cannot say whether or not the compounds were targeting EPSPS or inhibiting cellular growth via another mechanism. Therefore, a series of secondary screens have been proposed to identify the target specificity of these 8 hits towards the shikimate pathway, EPSPS, and S3P binding site found within EPSPS, in the given order.

Introduction

The reduced susceptibility to current antibiotics available today has been a recurring issue and it has necessitated the need for discovery of novel drug candidates (1). One method of discovering novel drug candidates is to perform a screen using a library of compounds against a specific target to extend the range of targets of current antibiotics (2). Thus, the focus of this paper is to investigate an enzymatic drug target called 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase.

EPSP synthase (EPSPS) is the sixth enzyme of the shikimate pathway (Supplemental Figure 4) found in algae, higher plants, fungi, and bacteria (3). EPSPS brings its two substrates, shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to synthesize EPSP. The latter is essential for the synthesis of vital aromatic amino acids: tryptophan, tyrosine, and phenylalanine (3). Without these amino acids, protein synthesis is obstructed. Consequently, bacterial cells are unable to survive due to the lack of amino acids necessary for complete protein synthesis. Additionally, this pathway is not present in humans, which decreases the potential of harmful off-target binding effects during treatment (4). The combination of these factors validates EPSPS as a valuable drug target.

Currently, there is only one known inhibitor of EPSPS in the market: glyphosate. It is an effective inhibitor of EPSPS because it is a transition-state analog of PEP (Supplemental Figure 5). However, glyphosate is not used as an antibiotic due to its carcinogenic properties and its relatively low potency against bacterial cells, which is measured to be about 0.6 mg/mL for its minimum inhibitory concentration (MIC) (5, 6). This is the concentration required for glyphosate to have a potent effect which is relatively high compared to established antibiotics. Thus, the effectiveness of glyphosate as a drug is not practical due to the high amounts needed to see an effect. This is not a problem when glyphosate is used as an herbicide as concentrations are much higher than this MIC reported. Therefore, a key difference between glyphosate use on bacteria versus plants is simply the amount. Additionally, EPSPS is divided into two classes which differ in their sensitivity to glyphosate (15). Plants utilize class I EPSPS which is described as glyphosate-sensitive. However, class II EPSPS is described as glyphosate-tolerant. Pathogenic microorganisms such as Staphylococcus aureus, Staphylococcus pneumoniae, Pseudomonas sp. strain PG2982, and Agrobacterium sp. strain CP4 have all been reported to utilize class II EPSPS and consequently are resistant to glyphosate (15).

With this evidence of differences between plant-based and bacterial EPSPS, it is critical to discover novel inhibitors that are specific to class II EPSPS.

Glyphosate interacts with the PEP binding site, which is the closed conformation of the enzyme (7). This limits the type of compounds that can act as inhibitors: they would need to be relatively small to fit the closed conformation of EPSPS, and are essentially restricted to analogs of the PEP compound. Conversely, there is no known inhibitor of the other substrate binding site, S3P, which provides a foundation for novel discovery (7). Targeting the S3P binding site also targets the open conformation of EPSPS where the active site is more accessible, allowing for greater structural variability of potential

inhibitors. Without a doubt, there is much potential in focusing on the S3P binding site. Thus, high-throughput screening is an advantageous method used to search for these novel inhibitors.

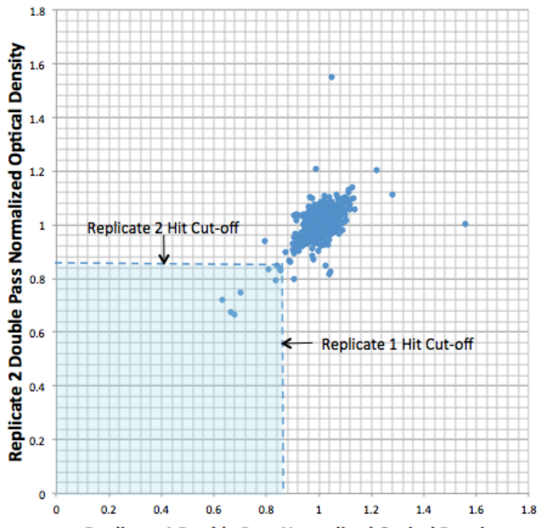

Figure 1: Double-pass normalized replicate plot of HTS results for growth inhibition of E. coli K-12 (AG1) cells. E. coli K-12 (AG1) cultures were screened in duplicate for growth inhibition against a library of compounds from ChemBridge. OD measurements were taken at 600 nm following 12.5 hours of incubation. The data points were normalized using the double- pass method, and each set of replicate plates were plotted on separate axes. The blue shaded area was constructed from the hit cutoff values (3 standard deviations below the mean of each replicate data set), visualized by the blue dotted lines, and the eight data points within the shaded area represent hits that inhibit bacterial growth.

The method for discovering novel inhibitors of the S3P binding site is to begin by identifying general inhibitors of bacterial growth using high throughput screening (HTS). It is a process that takes advantage of robot automation to quickly assess the biochemical activity of thousands of compounds. Most often, these compounds are obtained from chemical libraries which provide a collection of stored chemicals both natural and synthetic *(8)*. An important characteristic of this assay was the use of minimal media *(6)*. Minimal media, compared to the usual lysogeny broth (LB) media used for cell cultures, contains no added nutrients except the essential components for cell growth. On the contrary, LB media provides vital amino acids to the bacterial cells, which would make EPSPS unnecessary for survival. Therefore, it was rationalized that minimal media had to be used to avoid cell survival from external sources of amino acids *(6)*.

The data acquired from the HTS identified 8 successful hits from a compound library that significantly inhibited cellular growth. However, this does not explain whether the compounds were targeting the S3P binding site, or even EPSPS. Future works involve a series of secondary screens to improve the target specificity of these 8 hits towards the shikimate pathway, EPSPS, and S3P binding site. To verify production of EPSPS for these future assays, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on the E. coli K12 (AG1) cell line.

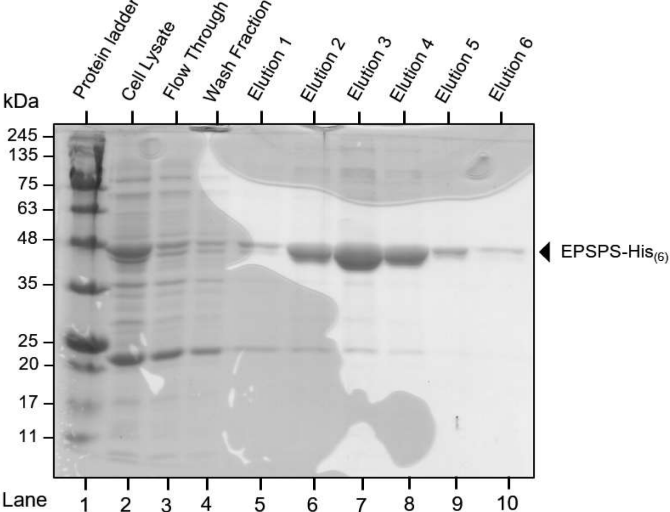

Figure 2: Characterization of purified EPSP synthase (EPSPS)-His(6) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Escherichia coli (E. coli) K-12 (AG1) cells were lysed after isopropyl-β-D-1 thiogalactopyranoside (IPTG) induction for EPSPS-His(6) protein expression. Different samples were then collected after running the cell lysate through a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column. Lane 1 represents the protein ladder. Lane 2 represents the cell lysate. Lane 3 is the flow- through fraction and lane 4 is the wash fraction. Lanes 5-10 represent the six elution fractions eluted using 250 mM of imidazole. The arrowhead indicates the presence of the EPSPS-is(6) protein at around 47 kDa.

Results

Z' Values show High Assay Quality

An assay plate with half positive controls and half negative controls can be set-up to calculate the Z' factor for HTS assays. The Z' factor is a statistical test for assessment of the assay quality. It is a dimensionless parameter used to calculate the signal separation between the highest and lowest assay readouts, which are the negative and positive controls, respectively. The negative control wells contained *E. coli* K12 (AG1) grown in M9 minimal media and produced the highest optical density (OD) readouts. The positive control contained *E. coli* grown in M9 minimal media supplemented with ampicillin, an antibiotic, and produced the lowest OD readouts. The signal separation between the negative and positive control is known as the signal

window as it accounts for both the separation between the signal and background, and the standard deviations of both the positive and negative controls. The Z' factor can be calculated using the standard deviations and means of the OD values from the positive and negative controls:

$$Z' = 1 - \frac{(3\sigma_{(+)} + 3\sigma_{(-)})}{|\mu_{(+)} - \mu_{(-)}|}$$

The ideal Z' factor is a value of 1, but a value of > 0.5 is generally deemed acceptable. Supplemental *Figure* 2 *s*hows the results of the Z' assays for three screens that were conducted. The data from the first and second screens showed Z' values of 0.78 and 0.608, respectively, while the third screen had a Z' value of 0.178. As a result, the first and second screen data demonstrated high assay quality while the third screen data was subpar. Thus, the third screen was omitted from the analysis because the low Z' value indicated that there is not enough variability between the negative and positive controls to determine an appropriate signal window. The poor assay quality thus necessitated that compounds in the third screen could not be included in single-pass and double-pass normalization.

Single-Pass and Double-Pass Normalization of HTS Data

Normalization of primary screen data is necessary because the screening process can span days or weeks. It allows comparison of plates from different days or different incubator positions to a standard.

Single-pass normalization involves the reduction of inter-plate data variation. When comparing the raw versus single-pass normalized plots, the single-pass normalized plot places the rank-ordered plots in a straight line centered at 1. Double-pass normalization involves the reduction of both inter-plate and intra-plate data variation.

Moving forward with the remaining screen data, the two screens were normalized using both the single and double pass methods. Supplemental Figure 3 demonstrates the effect of normalization compared to the raw data. As seen when comparing the graphs, the single-pass normalization corrects inter-plate fluctuations that occurred due to different screening days and environments. Likewise, the double-pass normalization accounts for bias due to the positioning of the plates in the incubators and variations in between the wells of each plate Eight Successful Hits Identified Through Primary HTS.

The primary HTS sought to identify significant inhibitors of cellular growth, or hits, by screening a library of compounds against the E. coli K12 (AG1) cell line. Hits were identified as compounds presenting O.D. values three standard deviations below the mean O.D value of each replicate data set. The actives were identified from a consolidated data set of all screens performed. As shown in Figure 1, eight successful hits were identified that displayed significant inhibition of growth for both replicates. SDS-PAGE Gel Identifies Successful Production of EPSPS at 47 kDa.

The secondary screening process requires functional and isolated EPSPS. Thus, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to verify that E. coli K12 (AG1) cell lines are successful at producing EPSPS. Supplemental Figure 6 illustrates the one letter amino acid sequence of EPSPS from the cell line of interest.

Figure 2 shows the results from the SDS- PAGE. Most notable in this gel is the presence of a thick band corresponding to the theoretical molecular weight of histidine-tagged EPSPS at 47 kDa. The elution lanes additionally contained traces of other sized proteins, notably at around 20 kDa. This band may be due to the expression of wild-type EPSPS by the bacterial strain used for protein expression, as the theoretical molecular weight of wild-type EPSPS without the hexahistidine (His(6)) tag is 20 kDa.

Critical Discussion of Data

The primary HTS sought to identify significant inhibitors of cellular growth, or hits, by screening a library of compounds against the E. coli K12 (AG1) cell line. Hits were designated as compounds presenting optical density (O.D) values below three standard deviations from the mean O.D of each replicate data set (9).

The eight hits were identified from a consolidated data set from all screens performed. Notably, data from the third screen were excluded from this set because of issues arising from the screening conditions. Firstly, the starting concentration of bacterial cells in the third screen was measured at an O.D value of 0.04, whereas the other two screens began with double the O.D. To account for this difference, the third screen was incubated for a longer period of 28 hours, which was inconsistent with the other screening periods of 12.5 hours. Moreover, the Z' value of the third screen was less than the recommended 0.5 cut-off and was relatively low compared to the other screens (9). The low Z' value indicates poor assay quality and a very narrow active window to identify potential hits, thus omitting the third screening data for the purposes of hit identification.

The data were normalized using both the single and double pass methods. A comparison of the graphs shows that single pass normalization corrected for inter-plate fluctuations that occurred due to different screening days and environments, which was necessary because not all the screens were performed on the same day. Likewise, the double pass normalization accounted for bias due to the positioning of the plates in the incubators and variations in between the wells in each plate, minimizing common HTS errors like the edge and stacking effects (10). Overall, each subsequent normalization of data moved the data points closer to the mean value and removed any outliers in the

process. It reduced the biases and errors that may occur during screening.

As shown in Figure 1, the eight identified compounds of interest demonstrated similarly low O.D measurements on both replicates, thereby reducing the likelihood of being false positives. Moreover, the Z' values for these screens were both above 0.5, which indicates a strong active window and establishes the legitimacy of the identified hits.

Figure 2 shows the results from the SDS- PAGE. The thick band patterns at around 48 kDa can be explained by the presence of histidine- tags attached at the ends of EPSPS. These cells were induced using isopropyl β-D-1- thiogalactopyranoside (IPTG) to express the histidine-tagged EPSPS through a T5 promoter system found in the pCA24N plasmid in the E. coli K12 line. These histidine-tagged proteins in the cell lysate have a strong affinity for the nickel resin in the column, allowing for other cellular contents to elute in the flow-through and wash samples while most the enzyme remains in the column. The elution lanes additionally contained traces of other sized proteins. These may correspond to degraded or aggregated protein fragments of EPSPS, which will also remain in the column due to the presence of histidine-tags at the end. To troubleshoot, size exclusion column can be used to separate out the EPSPS enzymes from the other elution fractions (11).

Future Outlook

The purpose of future work is to conduct secondary screens to narrow down the hits from the primary screen to more specific targets. This firstly involves supplementation of media of the hits with the aromatic amino acids: tryptophan, phenylalanine, tyrosine. Normally, the shikimate pathway results in the generation of precursors for the aforementioned compounds (12). Thus, if there is a recovery of cell growth in the primary screen with supplementation, it will indicate that the hits are targeting a member of the shikimate pathway or an entity just downstream of the shikimate pathway.

Subsequently, the hits that target the shikimate pathway will then be used in a malachite green enzymatic assay to see if they specifically target the EPSPS in the pathway. EPSPS normally catalyzes the sixth step in the shikimate pathway, which produces EPSP and an inorganic phosphate by-product. The ammonium molybdate present in the malachite green assay should bind to free inorganic phosphate produced from the EPSPS catalyzed reaction (13). This will result in the assay solution turning green from originally being yellow. However, if the assay is exposed to hits that target EPSPS, the solution should remain yellow because no inorganic phosphate will be produced with EPSPS inhibition. This will provide information on whether EPSPS is being targeted, albeit it will not identify the active site or the conformation of EPSPS that is specifically being targeted.

Thus, leads that target EPSPS will then be subjected to further kinetic analysis to see if they target the open conformation of EPSPS, specifically at the S3P binding site. In this analysis, the hit concentrations remain constant, and S3P concentrations are altered to generate Lineweaver-Burk plots. Based on the slope and intercepts of these plots, we can determine the type of inhibition that is occurring. If the inhibitor targets the S3P binding site, the graph should display a pattern that matches competitive inhibition because it would be directly competing against S3P to bind to the S3P binding site (14). If the inhibitor is occupying the PEP binding site, the graph would display a different plot pattern that matches uncompetitive or non-competitive inhibition because the PEP binding site is an allosteric binding site with respect to the S3P binding site (14). Overall, secondary screens will allow for further identification of the target from the original primary screen.

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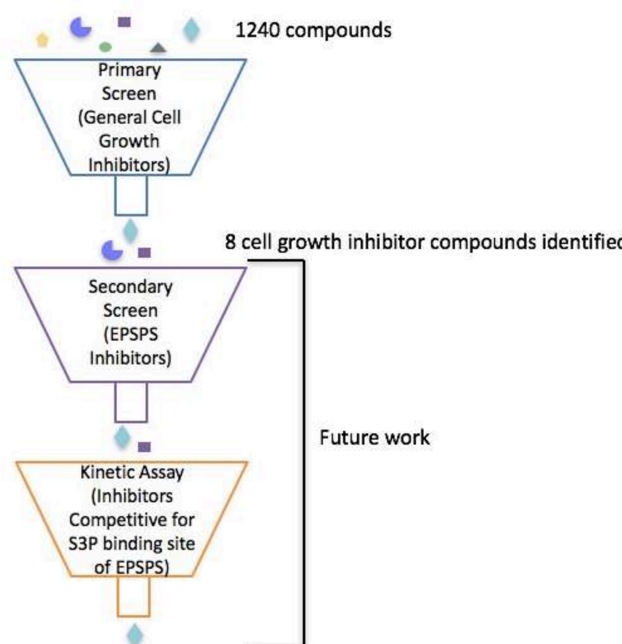
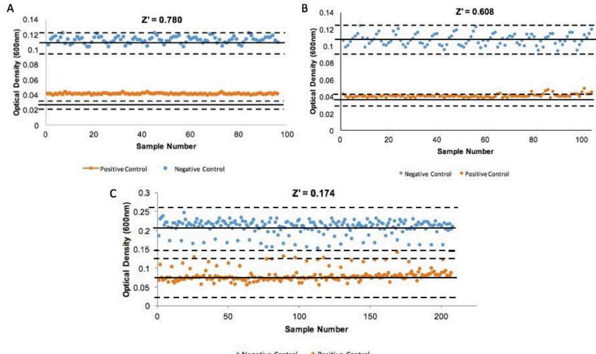
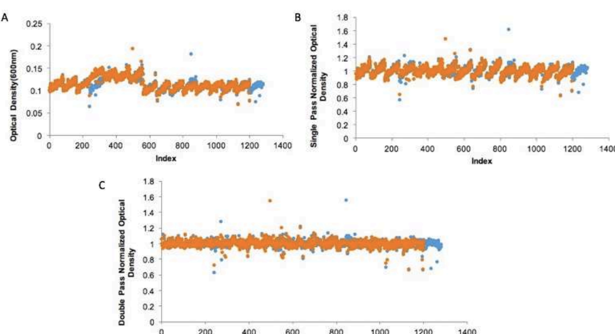
Supplementary Figures:



Supplemental Figure 1: *Flowchart depicting primary, secondary, and kinetic screening process.* Work to date includes a primary high throughput screen which identified 8 cellular growth inhibitors from 1240 compounds. To determine whether these 8 compounds inhibit cellular growth by inhibiting EPSPS, a secondary screen must be performed in the future. Hits from the secondary screen may then be analyzed with the use of a kinetic assay to determine whether the secondary screen hits inhibit the S3P binding site of EPSPS.



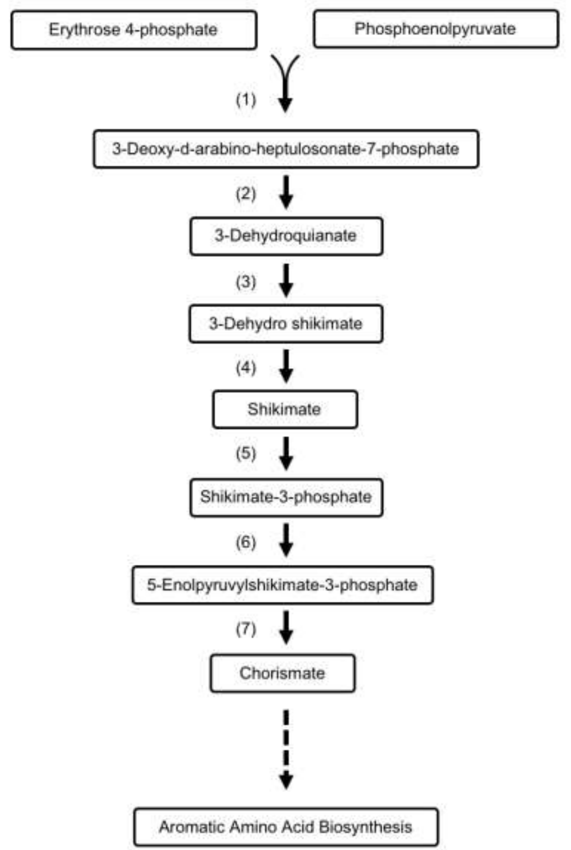
Supplemental Figure 2: Z’ statistical test for ampicillin in M9 minimal media during the primary HTS. OD at 600nm was measured following approximately 28 hours of incubation for **A.** group E3 and 12.5 hours of incubation for **B.** group E1 and **C.** group E2. Sample number is plotted on the x- axis, and the Optical Density at 600 nm is plotted on the y-axis. Positive controls contained ampicillin at a concentration of 4 µg/mL while negative controls contain neat DMSO. The solid line through each data set represents the mean values and the dashed lines above and below the mean values represent three times the standard deviation values. The calculated Z’ value is displayed near the centre of the plot.



Supplemental Figure 3*: Optical Density Plots of Screening data.* **A.** Raw optical density plot of screening data was **B.** single- pass rank-order normalized and then **C.** double-pass rank-order normalized/ More than a 1000 compounds from the ChemBridge library were screened for their ability to inhibit growth of *E. coli* K-12 (AG1) cells in minimal media. Cells were incubated for 12.5 hours and the optical densities (O.D) of the samples were measured at 600 nm. The screen was conducted in duplicates: replicate 1 is shown in blue, replicate 2 in orange.

Supplemental Table 1: *Identified Active Compounds from Primary HTS.* The identified hits from the primary HTS were identified online on the ChemBridge compound database.

Screening Well	Active Compounds	Chemical Structure
54-A02	2-[2-(4-amino-1,2,5-oxadiazol-3-yl)-1H-benzimidazol-1-yl]-N-(3-bromophenyl)acetamide	
55-H07	ethyl 4-methyl-2-{[2-(5H-[1,2,4]triazino[5,6-b]indol-3-ylthio)butanoyl]amino}-1,3-thiazole-5-carboxylate	
57-G11	N~1~-(2,4-dimethylphenyl)-N~2~-(4-methylbenzyl)-N~2~-[(4-methylphenyl)sulfonyl]glycinamide	
59-A09	5-benzyl-2-[(3-bromo-4-methylbenzoyl)amino]-4-methyl-3-thiophenecarboxamide	
64-G08	N-[(5-chloro-8-hydroxy-7-quinolinyl)(2-methoxyphenyl)methyl]propanamide	
66-B02	N-[(4-chlorophenyl)(8-hydroxy-7-quinolinyl)methyl]cyclohexanecarboxamide	
66-H05	3-chloro-4-ethoxy-N-({[4-(4-methoxyphenyl)-1,3-thiazol-2-yl]amino}carbonothioyl)benzamide	
66-H08	N-[(8-hydroxy-7-quinolinyl)(4-methylphenyl)methyl]cyclohexanecarboxamide	



Supplemental Figure 4: *Outline of the shikimate pathway and intermediates.* The conversion of shikimate-3-phosphate to 5- enolpyruvylshikimate-3-phosphate in the sixth step is catalyzed by the enzyme of interest, EPSPS. Thus, inhibition of EPSPS will obstruct the shikimate pathway and ultimately prevent aromatic amino acid biosynthesis.

Glyphosate

Phosphoenolpyruvate (PEP)

Supplemental Figure 5: *Chemical structures of glyphosate and phosphoenolpyruvate (PEP).* Glyphosate has been established as a well-known inhibitor of EPSPS as it competes with PEP for the PEP binding site. Due to its similarities in structure with PEP, glyphosate acts as a transition state analog which explains its success as a potent inhibitor. These similarities include the possession of a carboxylic acid group within both molecules. In addition, glyphosate contains a phosphonate moiety which is analogous to the phosphate group found in PEP.

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>sp|P0A6D3|AROA_ECOLI 3-phosphoshikimate 1-carboxyvinyltransferase OS=Escherichia coli (strain K12)
GN=aroA PE=1 SV=1
MESLTLQPIARVDGTINLPGSKSVSNRALLLAALAHGKTVLTNLLDSDDVRHMLNALTAL
GVSYTLSADRTRCEIIGNGGPLHAEGALELFLGNAGTAMRPLAAALCLGSNDIVLTGEPR
MKERPIGHLVDALRLGGAKITYLEQENYPPLRLQGGFTGGNVDVDGSVSSQFLTALLMTA
PLAPEDTVIRIKGDLVSKPYIDITLNLMKTFGVEIENQHYQQFVVKGGQSYQSPGTYLVE
GDASSASYFLAAAAIKGGTVKVTGIGRNSMQGDIRFADVLEKMGATICWGDDYISCTRGE
LNAIDMDMNHIPDAAMTIATAALFAKGTTTLRNIYNWRVKETDRLFAMATELRKVGAEVE
EGHDYIRITPPEKLNFAEIATYNDHRMAMCFSLVALSDTPVTILDPKCTAKTFPDYFEQL
ARISQAA
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Supplemental Figure 6*: Complete one letter amino acid sequence for EPSPS protein.* A BLAST search of the query gene was used to identify the gene sequence as that of *aroA* from *Escherichia coli* (strain K12), which codes for the protein product 3- phosphoshikimate-1-carboxyvinyltransferase (EPSPS). The image is a screen shot of the one letter amino acid sequence from UniProt.