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EPSP SYNTHASE INHIBITOR DESIGN II. THE IMPORTANCE OF THE 3-PHOSPHATE GROUP FOR LIGAND BINDING AT THE SHIKIMATE-3-PHOSPHATE SITE & THE IDENTIFICATION OF 3-MALONATE ETHERS AS NOVEL 3-PHOSPHATE MIMICS.

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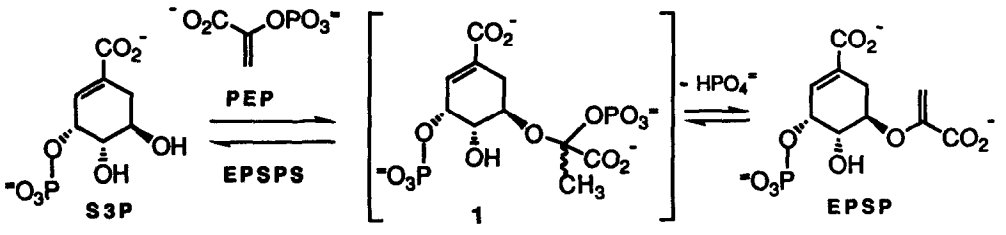
Abstract: Studies using alternate substrates, inhibitor product mimics and new derivatives of 4,5-dideoxy-shikimate-3-phosphate (ddS3P) are reported which indicate that the 3-phosphate group contributes significantly to substrate and inhibitor recognition at the shikimate 3-phosphate (S3P) site and that 3-malonate ethers will function as suitable 3-phosphate replacements for substrate and inhibitor binding to the S3P site of this enzyme.

The enzyme EPSP (5-enolpyruvoyl-shikimate-3-phosphate) synthase (EPSPS, E.C. 2.5.1.19) has generated considerable interest as a target for new inhibitor design since it functions as the biological target for the commercially successful herbicide, glyphosate.[2] EPSPS catalyzes an unusual transfer reaction of the carboxy-vinyl portion of phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of S3P forming EPSP and inorganic phosphate (P_i).[3] The enzyme exhibits a random kinetic mechanism[4] through a single, kinetically competent,[5] tightly bound,[6] tetrahedral intermediate, 1 (Scheme I). Several shikimate analogs of **1** have been prepared which function as highly potent EPSPS inhibitors.[7,8,9] In a search for a simplified inhibitor class that would be more synthetically accessible and have greater stability, it was important to define which of the various functional groups in **1** contributed to its extremely tight interaction with enzyme. Secondly, we sought to define what, if any, functional group replacements would be tolerated to maintain these favorable binding interactions. Here we report the results of our efforts to characterize the contributions of the 3-phosphate group to binding in S3P. A series of ddS3P analogs are also reported which identify a 3-malonate ether as a novel replacement for this 3-phosphate. Based on these results, a 3-malonate ether analog of S3P was prepared which demonstrated enzymatic turnover to an EPSP-like product.

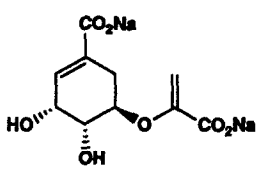
Scheme I



The unique chemical transformation catalyzed by EPSPS suggests that this enzyme has evolved to recognize multiply charged anions very specifically. Presumably, catalysis occurs with solvent exclusion to prevent the facile hydrolysis of PEP to pyruvate.[10,11] Ligand binding experiments using fluorescence[12] and microcalorimetry[13] indicate that significant molecular reorganization of the active site occurs during catalysis and inhibitor binding. The specific recognition of substrates must provide the energy required for reorganization of this protein. Each of the 4- and 5-hydroxyl groups in S3P contribute about 1-2 Kcal/mol in binding energy to EPSPS,[14] suggesting that each hydroxyl group contributes at least one hydrogen bond to the enzyme active site. The nascent ability of phosphates to form a double salt bridge with active site residues implies that recognition of the S3P 3-phosphate center should contribute significantly more binding energy.[15] Recent x-ray crystallographic analyses of enzyme-bound phosphate-containing substrates demonstrate that phosphates often also contribute multiple hydrogen bonds to their substrate recognition sites.[16]

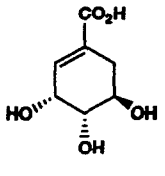
In order to investigate the contribution of the 3-phosphate group to the binding energy of S3P, we prepared several shikimate analogs for direct comparison with their 3-phosphate counterparts (Table 1). The EPSP analog, EPS **2**, was readily synthesized from commercially available shikimic acid **3** using literature methods.[17] The shikimate 5-glycolate derivatives **4,5** were also synthesized and were evaluated for their EPSPS inhibition properties using standard enzyme kinetic assays.[4] As summarized in Table 1, the glycolate **4** inhibited EPSPS competitively with respect to S3P with a K_i of 1.5 ± 0.3 μM, in good agreement with the previously measured K_d of EPSP (1.0 ± 0.01 μM).[12] This suggests that a hybridization change from sp^2 to sp^3 at the carboxyvinyl center has only a slight effect on the overall recognition at the S3P site. However, while EPSP binding to enzyme

Table 1. Inhibition of *E. coli* EPSP Synthase by Shikimates.[a]



2

IC_{50} > 90 mM



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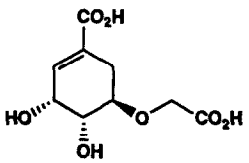
IC_{50} > 90 mM



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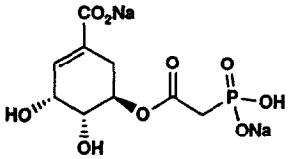
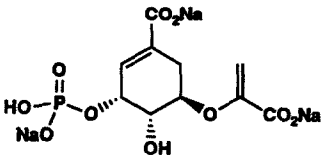
IC_{50} > 90 mM



6

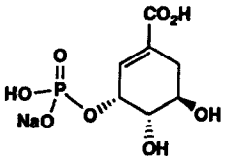
IC_{50} > 90 mM



EPSP

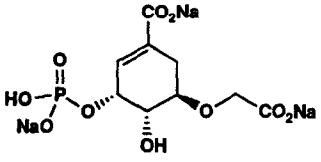
IC_{50} = 0.18 mM

K_d = 1.0 μM



S3P

K_d = 7.0 μM



4

IC_{50} = 0.10 mM

K_i = 1.5 μM

[a] IC_{50} = the concentration of inhibitor required to provide 50% inhibition with S3P and PEP concentrations fixed at 100 μM in 100 mM HEPES/KOH, 50 mM KCl, pH 7.0 at 30 °C.

induces a clear fluorescence change,[12] no comparable change in fluorescence was detected using **4** alone with enzyme. Therefore, this subtle change in hybridization may prevent access to some key interaction with the EPSPS active site.

None of the compounds lacking the 3-phosphate displayed any significant inhibition of the *E. coli* enzyme using a kinetic assay with an IC_{50} detection limit of 90 mM. Comparison of analogs **2**,**3**,**5** with their phosphorylated counterparts consistently demonstrates that the 3-phosphate enhances shikimate binding by at least three orders of magnitude. Moreover, the presence of the 5-glycolate functionality in **5** does not compensate for loss of this 3-phosphate group, nor will the addition of more extended anionic groups at this 5-position, as seen in **6**. These results indicate that the 3-phosphate group is exceedingly important for shikimate recognition and provides several Kcal/mol in binding energy to the system. Consequently, this 3-phosphate may have more than just a simple double salt bridge interaction with EPSPS at the S3P binding site.

While shikimic acid **3** displays a relatively weak affinity for enzyme, it will function as an EPSPS substrate exhibiting slow turnover to **2**, although very high enzyme concentrations are required. A separate species with a shorter retention time than either S3P or PEP but a longer retention time compared to **3** is readily detected by anion exchange chromatography (Figure 1). Labeling with either [1-^{14}C]PEP or [U-^{14}C]-shikimic acid demonstrates that all ten carbon atoms are present in this new species. Interestingly, no comparable turnover of either quinic acid, methyl shikimate[18] or dehydroshikimic acid[19] with [1-^{14}C]PEP occurs under these conditions. Sufficient EPS product can be easily isolated using micromolar enzyme concentrations. This material is essentially identical

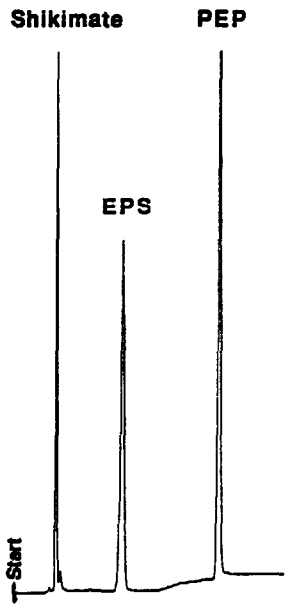


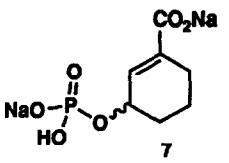
Figure 1: Elution profile of shikimate (3.8 min), EPS (10.0 min) and PEP (18.9 min) using anion-exchange chromatography on an AX-100 column eluting with a phosphate gradient buffer a) 50 mM potassium phosphate at pH 6.5 for 5 min then b) linear gradient from 5 to 11 min until 0.4 M potassium phosphate is reached. UV detection is used at 220 nm.

by ^{1}H-NMR, ^{13}C-NMR, HPLC and MS to the known synthetic material[17] and by HPLC with the product derived from dephosphorylation of EPSP with alkaline phosphatase.

A detailed kinetic evaluation of this EPS turnover process has recently been completed[4] which provides a more quantitative assessment using k_{cat} and K_m measurements. The K_m for **2** was determined to be 24 mM with a k_{cat} of 0.2 sec^{-1}. The corresponding K_m for S3P using this *E. coli* enzyme[4,20] is 4 μM with a k_{cat} of 57 sec^{-1}. Thus, removal of the 3-phosphate from S3P significantly affects both recognition and turnover rate. The ratio, k_{cat}/K_m, is often used as an estimate of the relative accessibility of the transition state during catalysis. A comparison of the k_{cat}/K_m values for each of these substrates indicates that the 3-phosphate assists shikimate catalysis by nearly a million fold, or more than 8 Kcal/mol! This 3-phosphate moiety is therefore critical for catalysis and access to the optimum transition state leading to **1**. Consequently, any loss of this hydrolytically labile allylic 3-phosphate greatly diminishes the potency of any potential bisubstrate inhibitor which mimics **1**.

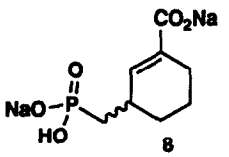
A variety of phosphonate functionalities have been described previously as potential isosteric and/or isoelectronic phosphate mimics.[21] In order to identify suitable 3-phosphate replacement groups with increased stability, a series of new racemic 4,5-dideoxy-S3P analogs **8-13** were prepared to probe the effects of ionization, pK_a and hydrogen bonding on EPSPS recognition as well as the overall spatial requirements for the S3P site. These compounds were again evaluated as EPSPS inhibitors under comparable conditions versus the known racemic 4,5-dideoxy-S3P[11] **7**, as summarized in Table 2. Of the variety of groups introduced at the 3-position, only the 3-malonate ether exhibited EPSPS inhibition comparable to **7**. Simple phosphonate-based replacements were clearly ineffective. The vinyl phosphonates **10,11** displayed essentially no inhibitory activity. Interestingly, neither the carbon linked phosphonate **8** nor the malonate **13** were inhibitors, which suggests that an important enzyme interaction may occur with the bridging oxygen in the 3-phosphate group of S3P.

Table 2. Inhibition of *E. coli* EPSP Synthase by 4,5-Dideoxy-shikimate 3-phosphate Analogs.[a]



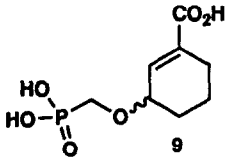
7

IC_{50} = 7.5 mM



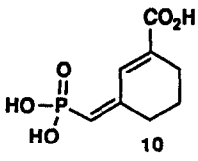
8

IC_{50} > 90 mM



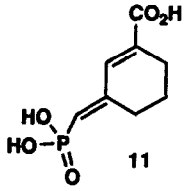
9

IC_{50} > 90 mM



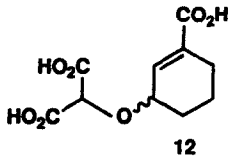
10

IC_{50} > 90 mM



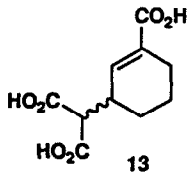
11

IC_{50} > 90 mM



12

IC_{50} = 15 mM



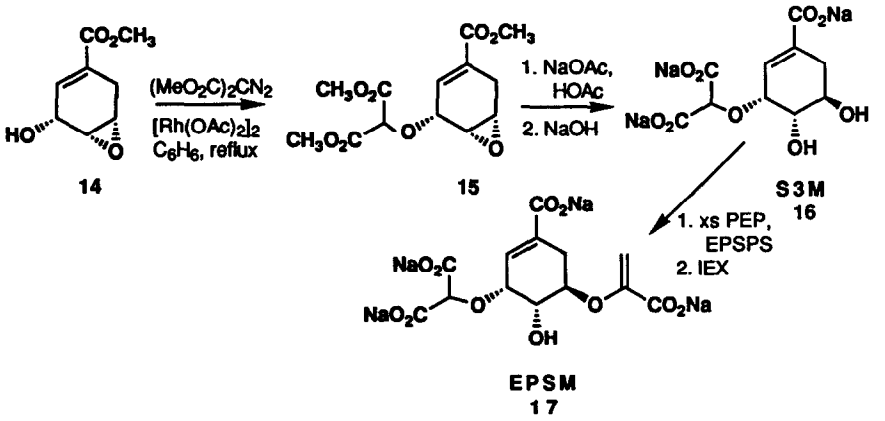
13

IC_{50} > 90 mM

[a] IC_{50} = the concentration of inhibitor required to provide 50% inhibition with S3P and PEP concentrations fixed at 100 μM in 100 mM HEPES/KOH, 50 mM KCl, pH 7.0 at 30 °C.

Presently we know of no other enzyme system where the malonate ether group functions so effectively as a phosphate mimic. This result is surprising given the overall larger spatial requirement for recognition of this group. Modeling experiments[22] suggest that a malonate ether occupies nearly twenty percent more volume than a phosphate group. If this malonate ether group can be recognized at the S3P site, then it should be possible to observe substrate turnover with a comparably substituted S3P derivative. Indeed, the 3-malonate derivative, S3M **16** can be readily prepared from the known[23] shikimate epoxy alcohol **14** *via* intermediate **15** (Scheme II). Isolated S3M has been characterized by ESMS and ^{1}H NMR. The measured pKa's for S3M (pK_{a2} = 4.0; pK_{a3} = 5.3) compare very favorably with those measured for S3P (pK_{a2} = 4.1; pK_{a3} = 6.4). Like **3**, S3M (40 mM) will form the product, EPSM **17** in the presence of [1-^{14}C]PEP (5 μM) and micromolar (50 μM) concentrations of enzyme. Product conversion is again easily monitored by anion exchange chromatography. Sufficient unlabeled material can also be isolated using excess PEP to be fully characterized by ^{1}H NMR. Thus, the 3-malonate substituent will support productive turnover and permit access to an EPSPS transition state during catalysis.

Scheme II.



In this study we have demonstrated that an understanding of the major functionalities contributing to substrate binding can provide unique insight into the design of new inhibitors. For EPSPS, the 3-phosphate group in S3P plays a critical role for substrate recognition and catalysis as well as access to potent mechanism-based inhibitors. Turnover experiments using shikimate as a substrate versus S3P indicate that this 3-phosphate facilitates shikimate catalysis by nearly a million fold. Structural analogs of the tetrahedral intermediate display at best only weak interaction with EPSPS in the absence of the 3-phosphate group. The presence of polar substituents at the 5-position cannot compensate for the loss of this critical 3-phosphate. Thus, access to the enzymatic conformation binding optimally to the tetrahedral intermediate is induced when this 3-phosphate moiety is present. The susceptibility of this crucial 3-phosphate functionality to hydrolytic and/or enzymatic cleavage presents a major limitation for expression of *in vivo* activity for potent, mechanism-based EPSPS inhibitors. A search for more stable 3-phosphate replacements using 4,5-dideoxy-S3P analogs uncovered 3-malonate ethers as a unique phosphate mimic in this system. Efforts are currently underway to further exploit this functionality in more complex EPSPS inhibitor design and synthesis.

References:

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