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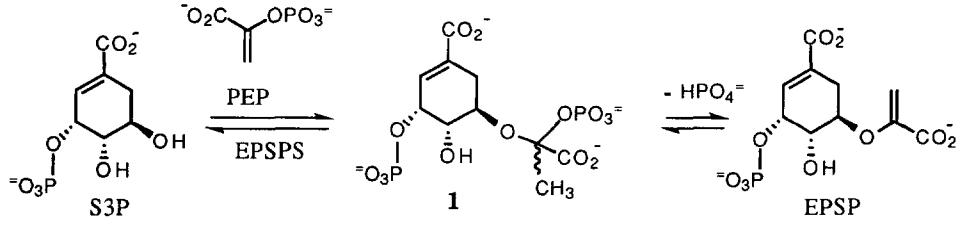
NEW SIMPLIFIED INHIBITORS OF EPSP SYNTHASE: THE IMPORTANCE OF RING SIZE FOR RECOGNITION AT THE SHIKIMATE 3-PHOSPHATE SITE

Mark L. Peterson,‡[1a] Susan D. Corey,‡ Jose L. Font,‡[1b] Mark C. Walker,‡ and James A. Sikorski§*

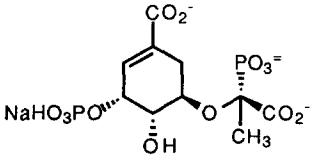
‡*Ceregen and* §*Monsanto Corporate Research, Units of Monsanto Company; 700 Chesterfield Parkway North, St. Louis, Missouri 63198*

Abstract: The simple 4-[(α-hydroxy)phosphonomethyl]-1H-pyrrole-2-carboxylate **4** is a surprisingly good S3P-analog inhibitor of EPSP synthase (competitive with S3P, $K_{i(app)}$ = 14.5 ± 0.7 μM). The affinity of **4** for free enzyme is nearly comparable to that of S3P (K_d = 7 ± 1.2 μM). Compound **4** thus represents the first successful attempt to replace the highly substituted shikimate ring in S3P with a readily accessible heterocyclic scaffold.

The enzyme EPSP (5-enolpyruvoylshikimate 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 carboxyvinyl portion of phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of shikimate 3-phosphate (S3P) forming EPSP and inorganic phosphate (P_i).[3] This enzyme exhibits a random kinetic mechanism[4] through a single, kinetically competent,[5] tightly bound ($K_{d(est)}$ ≈ 50-250 pM),[6] tetrahedral intermediate **1**.

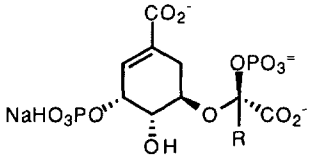


Several shikimate-based analogs of **1**, such as **2** and **3a-c**, have been reported as highly potent EPSPS bisubstrate inhibitors.[7-9] The potency of these inhibitors is thought to be a direct consequence of their ability to occupy simultaneously both the S3P and PEP subsites. Our efforts have recently been directed toward identifying simplified scaffolds with which to replace the complex shikimate ring in such systems. Here we report that a suitably substituted pyrrole **4** represents a readily accessible surrogate with surprisingly high affinity for the S3P subsite.



R-**2**

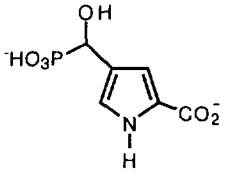
$K_{i(app)}$ = 5 ± 1 nM



3a R = CH_2F $K_{d(est)}$ ≈ 0.6 nM

R-**3b** R = CHF_2 $K_{i(app)}$ = 4 ± 0.3 nM

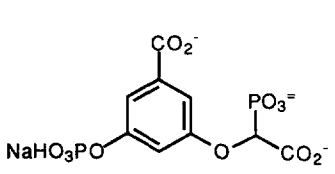
R-**3c** R = CF_3 $K_{i(app)}$ = 32 ± 3 nM



4

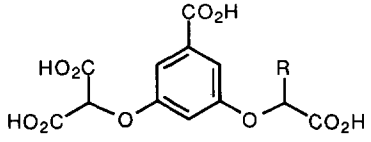
Spectroscopic investigations conducted in these laboratories have determined that the shikimate ring adopts an unusually flattened conformation in both enzyme-bound S3P and EPSP.[10] As a result, the aromatic tetrahedral intermediate mimic **5** has recently been identified as a potent EPSPS inhibitor, where the simplified benzene ring functions as a suitable substitute for the more highly functionalized shikimate ring.[11] Several analogs of **5** have also been prepared in which the hydrolytically labile 3-phosphate functionality has been replaced with either a malonate ether group such as in **6** and **7**,[12,13] or the shortened hydroxymalonate moiety as in **8**.[14] The increased potency observed for **6-8** versus their corresponding phenol analogs **9-11** is directly attributable to the charged phosphonoacetate moiety at the 5-position which demonstrated the importance of accessing the PEP binding site in these systems. While **6-8** were effective low micromolar inhibitors of EPSPS, their potencies failed to match the low nanomolar levels frequently observed with 3-phosphate-based inhibitors, such as **2**, **3a-c**, and **5**. Consequently, we sought an alternative approach to improve the potency of these inhibitors.

Table 1. Aromatic Inhibitors of *E. coli* EPSP Synthase.[15]



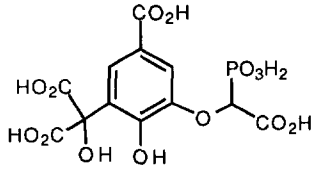
5

$K_{i(app)} = 0.16 \pm 0.04$ μM



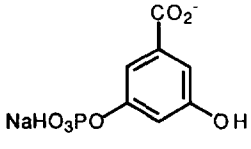
6 R = CO_2H $K_{i(app)} = 2.5 \pm 0.4$ μM

7 R = PO_3H_2 $K_{i(app)} = 1.3 \pm 0.2$ μM



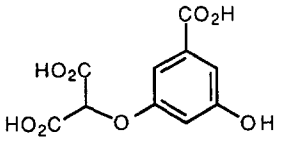
8

$K_{i(app)} = 0.52 \pm 0.06$ μM



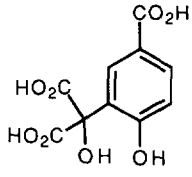
9

$IC_{50} = 7.9$ mM



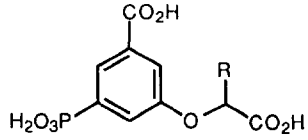
10

$IC_{50} = 10$ mM



11

$IC_{50} = 20$ mM



12a R = PO_3H_2

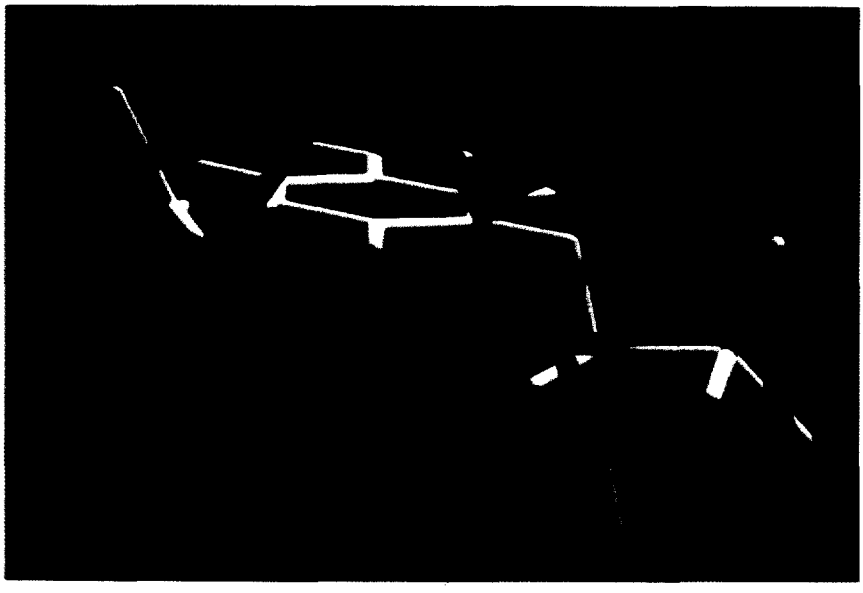
12b R = H

The corresponding phenolic S3P analogs **9-11** displayed relatively weak affinity for enzyme compared to S3P ($K_d = 7 \pm 1.2$ μM).[16] Consequently, the lack of potency in these aromatic tetrahedral intermediate mimics **5-8** (Table 1), compared to the shikimate-based systems, might be due to a less than optimal fit within the S3P subsite. A recently reported molecular modeling comparison of **5** versus **1** suggests that maximizing overlap between the two 3-phosphate moieties, that are known to be critical for effective inhibitor binding, may prevent optimum overlap between these ring systems.[14] As depicted in Figure 1, the C-O-P bond angle in **5** differs significantly from the axial 3-phosphate in **1**. As a result, the benzene ring orientation in **5** becomes sharply skewed relative to the shikimate ring as overlap between the 3-phosphate groups is maximized. The strain introduced by this twisted ring orientation might partially account for the lack of potency in these aromatic systems, since the overlap with the other critical anionic recognition sites appears to be largely maintained.

A smaller distortion in ring orientation is observed with the shortened aromatic phosphonate analog **12a**. This result suggests that aromatic inhibitors incorporating slightly shortened 3-phosphate mimics might be more

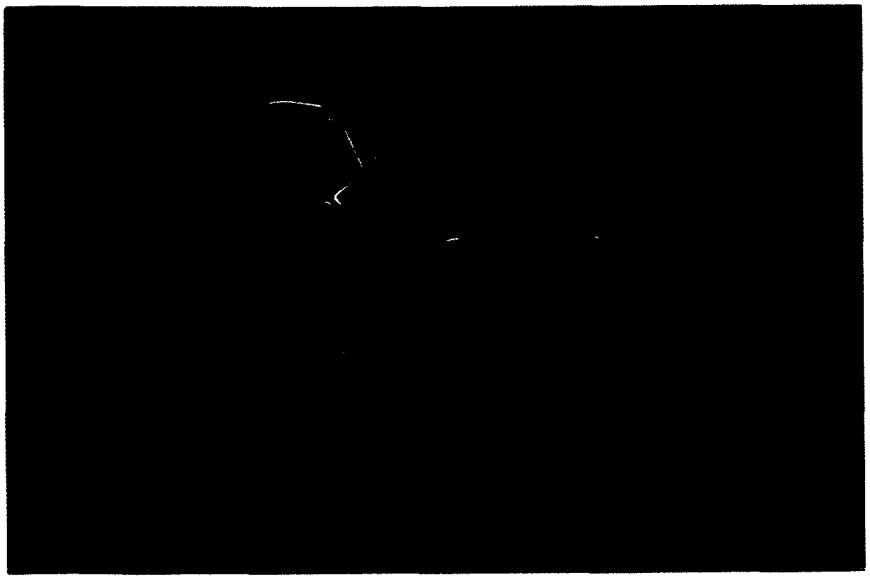
effective EPSPS inhibitors. However, a previous kinetic evaluation of **12b** demonstrated that this shortened phosphonate analog had little significant interaction with enzyme (IC_{50} = 10 mM).[12] This suggests that the bridging oxygen in the 3-phosphate group plays a critical role in enzyme recognition, as observed previously with a series of 4,5-dideoxy shikimate 3-phosphate analogs.[17] This smaller distortion in ring orientation was also observed when the shortened aromatic 3-hydroxymalonate **8** was modeled versus **1**. In this case, analog **8** achieved almost the same ring orientation as observed previously with the shortened phosphonate **12a**. Access to this less distorted conformation may at least partially account for the potency of **8**.

Figure 1. A three-dimensional representation of the single conformation developed for compound **1** (red) at the top versus the corresponding aromatic 3-phosphate **5** (green) at the bottom and the shortened aromatic phosphonate **12a** (white) in the middle.[14]

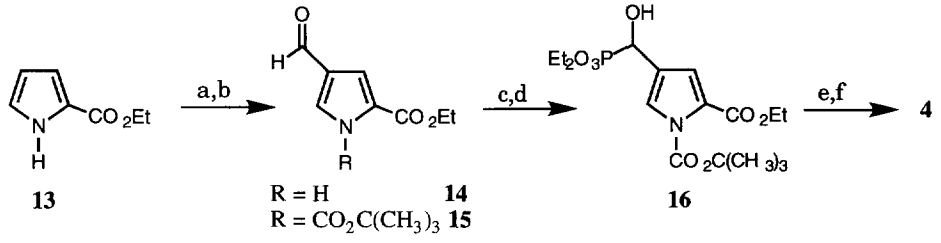


All attempts to identify other planar six-membered ring structures that would more completely alleviate this conformational distortion and still permit maximal overlap with the 3-phosphate center in **1** were unsuccessful. However, we were pleasantly surprised to discover that much, if not all, of this distortion could be eliminated when a planar 5-membered ring system was compared with **1**. As shown in Figure 2, **4** provides excellent overlap with the known enzyme-bound S3P ring conformation. Several 5-membered heterocyclic systems were considered to test this hypothesis. Those systems that would be protonated under physiological conditions were eliminated to maintain a neutral ring system and thus minimize any charge repulsion effects within the EPSPS active site. The pyrrole scaffold in **4** was ultimately selected because of its ease of synthesis and the low basicity of the pyrrole ring. Finally, a 3-(α-hydroxymethyl)phosphonate appeared from modeling studies to provide good access to the S3P 3-phosphate subsite. Since EPSPS is better able to recognize aromatic inhibitors containing 3-(α-hydroxymethyl)phosphonates than those with simple 3-methylphosphonates,[12] we incorporated this moiety into **4** as a representative 3-phosphate mimic to improve stability at this center.

Figure 2. A three-dimensional representation of the bound S3P ring conformation (cyan) as determined by NMR versus the pyrrole **4** (magenta).



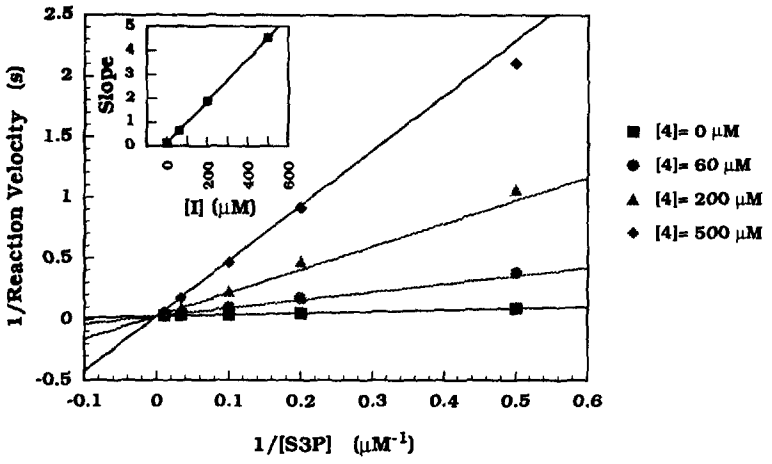
Racemic **4** was readily synthesized from commercially available ethyl pyrrole 2-carboxylate **13** via the known 4-formyl derivative **14**.[18] Following N-protection of **14** to give the N-BOC intermediate **15**, phosphite addition produced the requisite α-hydroxymethylphosphonate triester **16**, after cleaving the trimethylsilyl ether with *p*-tosic acid. Subsequent treatment with trimethylsilyl bromide removed the phosphonate ester groups, and saponification with base produced **4** as a mixture of triethylammonium salts after ion-exchange chromatography.



Reagents and conditions: (a) Cl_2CHOCH_3, $AlCl_3$, CH_3NO_2, CH_2Cl_2, 0 °C, (90%); (b) $[(CH_3)_3CO]_2O$, Et_3N, DMAP, CH_2Cl_2, 89%; (c) $(EtO)_2POSi(CH_3)_3$, THF, reflux; (d) p-TsOH, MeOH, H_2O, reflux, 72% (overall); (e) $(CH_3)_3SiBr$, Et_3N, CH_3CN; (f) MeOH, Et_3N, NaOH, 27%.

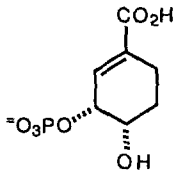
The pyrrole **4** was evaluated for its ability to function as an EPSPS inhibitor using a standard kinetic assay for the EPSPS forward reaction.[4] Compound **4** was found to be a surprisingly good EPSPS inhibitor, competitive with S3P (Figure 3), with a $K_{i(app)}$ of 14.5 ± 0.7 μM. As such, **4** compares quite favorably with either 5-deoxy-S3P[19] **17** or S3P[16] in its ability to bind at the S3P subsite (Table 2). Moreover, **4** displays considerably greater potency than 5-amino-S3P **18**,[19] or many other cyclic phosphonate derivatives **19-22** (Table 2).[2c] These results

Figure 3. Competitive Inhibition Kinetics of **4** Versus S3P with PEP Fixed at 100 μM.[15]

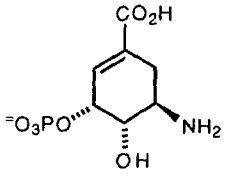


clearly indicate that the highly functionalized shikimate ring in S3P may be effectively replaced with an achiral surrogate. The potency of **4** is also orders of magnitude better than the phenolic S3P analogs **9**-**11**. This suggests that very potent EPSPS bisubstrate inhibitors might be possible by extending **4** into the PEP binding site. Efforts are ongoing to identify EPSPS bisubstrate inhibitors using this simple heterocyclic scaffold as a starting point.

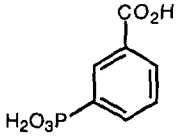
Table 2. S3P Analog Inhibitors of *E. coli* EPSP Synthase.[15]



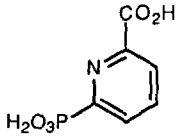
17
$K_{i(app)} = 13 \pm 1.1$ μM



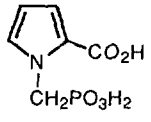
18
$K_{i(app)} = 90 \pm 2$ μM



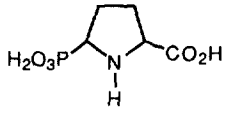
19
$IC_{50} > 50$ mM



20
$IC_{50} > 50$ mM



21
$IC_{50} > 50$ mM



22
$IC_{50} > 50$ mM

References and Notes

1. (a) Current address: Advanced ChemTech, 5609 Fern Valley Road, Louisville, KY 40228. (b) Current address: Astra Arcus USA, Inc. P.O. Box 20890, Rochester, NY 14602.

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15. 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. Apparent K_i's versus S3P were determined under similar conditions using a fixed PEP concentration of 100 μM, and the data were fit to a model for competitive behavior using *GraFit* software: Leatherbarrow, R. J. *GraFit*, Version 2.0, Erithacus Software Ltd,, Staines, U.K., 1990.

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