Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 21, pp. 2605-2608, 1994

0960-894X/94 $7.00+0.00

0960-894X(94)00386-6

EPSP SYNTHASE INHIBITOR DESIGN IV. NEW AROMATIC SUBSTRATE ANALOGS AND SYMMETRICAL INHIBITORS CONTAINING NOVEL 3-PHOSPHATE MIMICS.[1]

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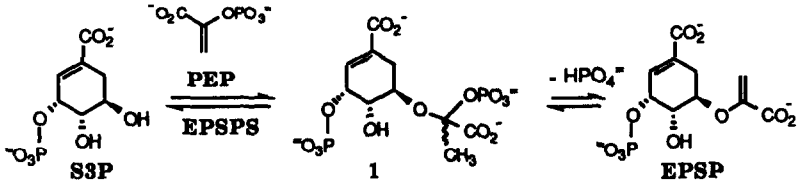
Abstract: New 4-deoxy aromatic analogs of S3P and EPSP have been synthesized as potential substrate-based inhibitors of EPSPS to evaluate various 3-phosphate replacements in combination with the aromatic ring system. These studies identified 3-malonate ethers and α-hydroxymethylphosphonates as suitable 3-phosphate mimics in this series and led to the discovery of two unexpectedly potent symmetrical aromatic inhibitors **6** and **7**.

The enzyme EPSP (5-enolpyruvoyl-shikimate-3-phosphate) synthase (EPSPS, E.C. 2.5.1.19) has generated considerable interest in our group as a target for new inhibitor design since it functions as the biological target for the commercially successful herbicide, glyphosate.[3] EPSPS catalyzes an unusual transfer reaction of the carboxyvinyl portion of phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of S3P forming EPSP and inorganic phosphate (P_i).[4] The enzyme exhibits a random kinetic mechanism[5] through a single, kinetically competent,[6] tightly bound,[7] tetrahedral intermediate, **1**.

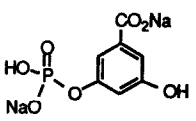


Several shikimate analogs of **1** have been identified as highly potent bisubstrate inhibitors.[8,9,10] However, these shikimate-based inhibitors represent a formidable synthetic challenge. Consequently, we sought another approach to an equally effective inhibitor class that would be more synthetically accessible. NMR studies have previously determined that both S3P and EPSP assume a fairly planar ring conformation when bound to enzyme.[11] This suggested that aromatic substrate analogs of S3P and EPSP might be suitable spatial mimics for this conformational state. Indeed, the aromatic 3-phosphates **2a**, **3a**, **4** (Table 1) have recently been reported as potent EPSPS inhibitors.[12] The relative potency of **4** versus S3P (K_d = 7.0 μM),[13] EPSP (K_d = 1.0 μM),[13] or **5**[9,12] is attributed to the added anionic functionality present at the 5-position and is particularly surprising given the number of chiral centers which have been removed.

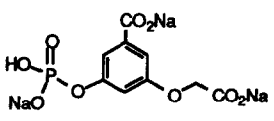
The importance of the 3-phosphate group for substrate turnover and inhibitor binding to EPSPS using shikimate systems is now well established.[1a,5a] In S3P, this 3-phosphate contributes ≥ 8 kcal/mol to active site recognition and catalysis. Thus, inhibitors lacking this critical 3-phosphate group exhibit dramatically reduced potency.[1a] The shikimate allylic 3-phosphate moiety is chemically quite labile and may be easily removed by acid

hydrolysis or phosphatases *in planta*. Unfortunately, the phenolic phosphates **2a**, **3a**, **4** are even more labile. This led us to search for aromatic inhibitors containing suitable 3-phosphate replacement groups with enhanced stability. Here we report our evaluation of various aromatic analogs of S3P and EPSP which identified 3-malonate ethers and α-hydroxymethylphosphonates as novel 3-phosphate mimics. Based on these results, representative symmetrical 3,5-disubstituted benzoate analogs also were prepared and were found to be surprisingly potent EPSPS inhibitors.

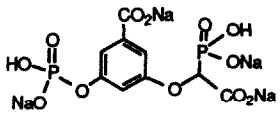
Table 1. Inhibition of *E. coli* EPSP Synthase by 4-Deoxy Aromatic 3-phosphate Analogs.[17]



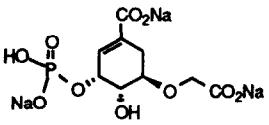
2a
IC_{50} = 7.9 mM



3a
IC_{50} = 1.4 mM



4
IC_{50} = 0.02 mM
K_i = 0.16 ± .04 μM

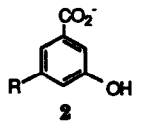


5
IC_{50} = 0.10 mM
K_i = 1.5 ± 0.26 μM

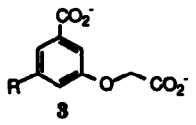
A variety of phosphonate functionalities have been described previously as potential isosteric and/or isoelectronic phosphate mimics.[14] In order to identify suitable 3-phosphate replacement groups with increased stability, a series of new 4-deoxy aromatic S3P analogs **2b-d** were prepared to probe the effects of pK_a and hydrogen bonding on EPSPS recognition, as well as the overall spatial requirements for the S3P site. These compounds were evaluated as EPSPS inhibitors under comparable conditions to those of **2a**, as summarized in Table 2. As observed previously with analogs of 4,5-dideoxy-S3P,[1a] the carbon-linked phosphonate **2d** exhibited little significant interaction with enzyme. This suggests that the bridging oxygen within the phosphate group in **2a** is an important recognition element. This result is also consistent with the recently developed x-ray crystal structure of the S3P binding site in *E. coli* EPSPS.[15] Of the variety of groups introduced at the 3-position, only the α-hydroxymethylphosphonate **2b** and the 3-malonate ether **2c** exhibited EPSPS inhibition comparable to **2a**. However, all of these aromatic surrogates **2a**, **2b**, **2c** were considerably less effective than S3P in their ability to bind to free enzyme.

The increased potency observed for EPSP or **5** versus S3P is attributed to the added carboxylate functionality at the 5-position which may interact favorably with active site residues stabilizing comparable groups in **1** during catalysis. Similar functionality should also increase the potency of the aromatic substrate analogs **2b-d**, if favorable interaction is to occur at the active site. Consequently, the glycolate analogs **3b-e** were prepared and evaluated as EPSPS inhibitors in comparison to **3a**. Just as **3a** exhibits increased potency over **2a**, the glycolates **3b-d** were about 3-8 times better as EPSPS inhibitors than their corresponding phenol derivatives. While the 3-malonate ether **3c** and α-hydroxymethylphosphonate **3b** exhibit inhibition potencies nearly comparable to that of the phosphate **3a**, glycolates **3d** and **3e** containing phosphonate groups, are significantly less effective as EPSPS inhibitors. These results confirm the utility of 3-malonate ethers and α-hydroxymethylphosphonates as suitable 3-phosphate mimics in aromatic EPSPS inhibitors. Again, however, each of the aromatic glycolates **3a**, **3b**, **3c** was considerably less effective than EPSP in binding to free enzyme (Table 2).

Table 2. Inhibition of *E. coli* EPSP Synthase by 4-Deoxy Aromatic S3P and EPSP Analogs.[17]



2

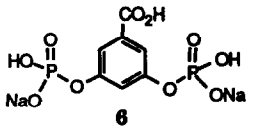


3

(a) R = $-OPO_3H^-$	IC_{50} = 7.9 mM	(a) R = $-OPO_3H^-$	IC_{50} = 1.4 mM
(b) R = $-CH(OH)PO_3H^-$	IC_{50} = 10 mM	(b) R = $-CH(OH)PO_3H^-$	IC_{50} = 3 mM
(c) R = $-OCH(CO_2H)_2$	IC_{50} = 10 mM	(c) R = $-OCH(CO_2H)_2$	IC_{50} = 2 mM
(d) R = $-CH_2PO_3H_2$	IC_{50} ≥ 80 mM	(d) R = $-CH_2PO_3H^-$	IC_{50} = 10 mM
		(e) R = $-PO_3H_2$	IC_{50} = 10 mM

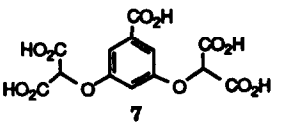
The ability of bisubstrate shikimate inhibitors to interact specifically with the PEP phosphate site is also a key contributor to their potency.[8,9,10] While the utility of 5-phosphonoacetate ether groups has been well established in both shikimate-based[9,10,12] and aromatic inhibitors,[12] we chose instead to simultaneously incorporate the functionalities already present at the 3-position. In so doing, the synthesis of several symmetrical aromatic targets **6-8** was dramatically simplified. As summarized in Table 3, each of these aromatic targets was evaluated for EPSPS inhibition using the standard kinetic assay.[5a,12] To our pleasant surprise, the bis-phosphate **6** and bis-malonate ether **7** were unexpectedly potent EPSPS inhibitors and exhibited clean competitive kinetic behavior versus S3P. Compounds **6** and **7** were clearly superior to the analogous bis-phosphonate **8** (Table 3).

Table 3. Inhibition of *E. coli* EPSP Synthase by Symmetrical 3,5-Disubstituted Benzoate Analogs.[17]



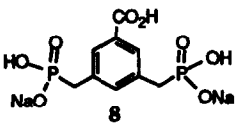
6

IC_{50} = 85 μM
$K_{I(app)}$ = 0.78 ± 0.1 μM



7

IC_{50} = 210 μM
$K_{I(app)}$ = 2.50 ± 0.40 μM



8

IC_{50} = 3 mM

Compounds **6** and **7** apparently fulfill the additional charge requirement at the 5-position quite well and therefore have significantly greater potency than their substrate analogs. The importance of anionic recognition at the EPSPS active site is further reinforced with these highly charged aromatic probes. Given the number of chiral centers present in **1**, it is very surprising that the symmetrical molecules **6** and **7** would exhibit any significant interaction with this system and further exemplifies the utility of probing chiral enzyme active sites with achiral molecules. Furthermore, we know of no other enzyme system where the 3-malonate ether group functions so effectively as a phosphate mimic. This result was unanticipated given the overall larger spatial requirement for recognition of this group. Modeling experiments[16] suggest that a malonate ether occupies nearly twenty percent more volume than a phosphate group. Malonate ethers should have broader applicability to more complex EPSPS inhibitors as well as to inhibitors of other enzymes utilizing phosphate-based substrates.

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17. IC_{50} = the concentration of inhibitor required to provide 50% inhibition with [S3P] and [PEP] 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 at [PEP] fixed at 100 μM. Data were fit to a model for competitive behavior using GraFit (Leatherbarrow, R. J. **1990**, GraFit Version 2.0, Erithracus Software Ltd., Staines, U.K.).

(*Received in USA* 26 *August* 1994; *accepted* 29 *September* 1994)