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EPSP SYNTHASE INHIBITOR DESIGN I. CONFORMATIONS OF ENZYME BOUND SHIKIMATE-3-PHOSPHATE AND 5-ENOLPYRUVOYLSHIKIMATE-3-PHOSPHATE USING TRNOE

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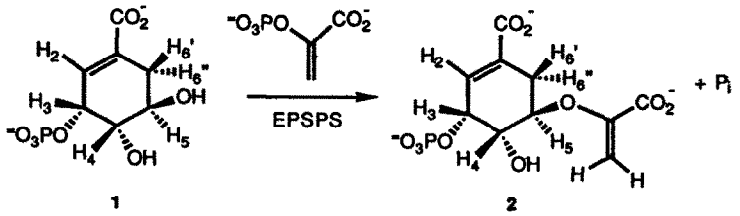
Abstract: The conformations of S3P, **1** and EPSP, **2** bound to *E. Coli* EPSP synthase have been determined using two dimensional transfer NOE (2D TRNOE) measurements. Both S3P and EPSP bound to the enzyme retain the half-chair conformation with the phosphate group in an axial position similar to "free" S3P in solution.[4] The cis enol proton of bound EPSP is in close proximity to H_4 on the ring.

Shikimate-3-phosphate (S3P), **1**, is a substrate for 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS) [EC 2.5.1.19], the sixth enzyme in *de novo* aromatic amino acid biosynthesis. EPSPS catalyzes the transfer of the carboxyvinyl moiety of PEP specifically to the 5-OH of S3P producing inorganic phosphate (P_i) in a rare biochemical reaction (Scheme 1). This enzyme, found only in plants and microorganisms, is the target for the broad spectrum herbicide, N-phosphonomethylglycine (glyphosate). The enzymatic mechanism proceeds through a single[5] kinetically competent[6] tetrahedral intermediate. With an estimated K_d of 50 pM[7], this mechanistic intermediate binds approximately 3000 times tighter than glyphosate to EPSPS. Close structural analogs of this intermediate are potent EPSPS inhibitors.[8] Consequently, spatial mimics of this intermediate offer an attractive target for new inhibitor design. The design of more synthetically accessible inhibitors could be attempted with an experimentally defined three-dimensional model of this intermediate to use as a template. The characterization and comparison of the three-dimensional solution structures of "free" and bound substrates is an important aspect of enhancing our understanding of substrate-enzyme recognition.[9]



1 **2**

Scheme 1

The three-dimensional characterization of EPSPS is not assessable by NMR techniques due to its size, (46.5 kDaltons). Although x-ray data on the enzyme have been reported, no information on bound ligands or substrate sites is available.[10] The exchange rates between the "free" and bound states for S3P and EPSP are much larger than the ^1H spin lattice relaxation rates for the free substrates.[11] These conditions permit us to employ

transfer NOE experiments, (TRNOE) to elucidate the structures of the bound substrates through ^1H-^1H internuclear distances.[12]

A molar ratio of 20:1 for S3P to EPSPS was used to collect the phase sensitive TRNOE data sets.[13] The TRNOE cross peaks of S3P can be distinguished from the protein background by their narrow linewidths and greater intensity. Cross peaks associated with S3P are in phase with the diagonal peaks. This is consistent with cross relaxation effects due to bound S3P as opposed to NOE correlations associated with free S3P. ^1H chemical shifts of free S3P and in the presence of the enzyme are very similar (Table I; Figure 1).[4]

Table II contains the internuclear distances calculated from the experimental data. All long range vectors are listed as ≥3.5Å to indicate that some cross relaxation was observed but the distance could not be quantified accurately in the data sets. A comparison of the bound S3P data with the energy minimized half chair conformation orienting the phosphate group in the axial position and the experimental H-H internuclear distances for free S3P[4] indicate that within experimental error all three structures are identical (Table II; Figure 1).

The binary complex of EPSP*EPSPS was also studied by TRNOE techniques. This experiment is complicated by two enzyme catalyzed side reactions: hydrolysis of EPSP to S3P[14] and the formation of the EPSP ketal.[15] In addition, the EPSP enol protons undergo deuterium exchange in the presence of the enzyme with D_2O as the solvent.[16] These limitations prohibit the collection of quantified TRNOE data. Thus, the cross correlations observed in the 2D TRNOE experiment with EPSP and the enzyme were treated qualitatively using the S3P TRNOE data as a reference. The ^1H chemical shifts for EPSP in the presence of the enzyme are presented in Table I. The most striking features of the TRNOE data for EPSP are the intense H_4-H_6 cross peak which distinguishes the half chair conformation with the axial phosphate and a weak enol-H_4 cross peak which locates the cis enol proton in the proximity of H_4.[17] The low intensity for this latter correlation could be due to deuterium exchange at this enol position.

In summary, the S3P half chair solution conformation with the phosphate group in the axial position is maintained when this substrate is bound to the enzyme as determined by 2D TRNOE data. Presumably the small angle librations observed in the free substrate are more constrained when bound to the enzyme. There are no large conformational perturbations between the free and bound state. Even the ring flip to a conformation which, on the basis of molecular mechanics calculations, is only slightly higher in energy does not occur. Furthermore, the conformation of the ring structure is maintained even after the biochemical transformation of S3P to EPSP is completed.

These data have important implications for EPSPS inhibitor design and have recently been used to construct a three dimensional template model of the tetrahedral intermediate.[18] This experimentally defined model suggests that planar structures should be reasonable EPSPS inhibitors. The first potent aromatic bisubstrate inhibitors have recently been identified.[19]

Acknowledgement: We would like to thank Mr. William C. Hutton (Monsanto Corporate Research) for his assistance in processing the TRNOE data sets.

Table I. ^1H Chemical Shifts

H	Bound S3P (ppm)	Bound EPSP (ppm)
2	6.46	6.51
3	4.82	4.87
4	3.80	4.09
5	4.05	4.47
6e	2.71	2.96
6a	2.19	2.23
enol	-	4.57

Figure 1. S3P Free/Bound Conformation and 2-D TRNOE Data

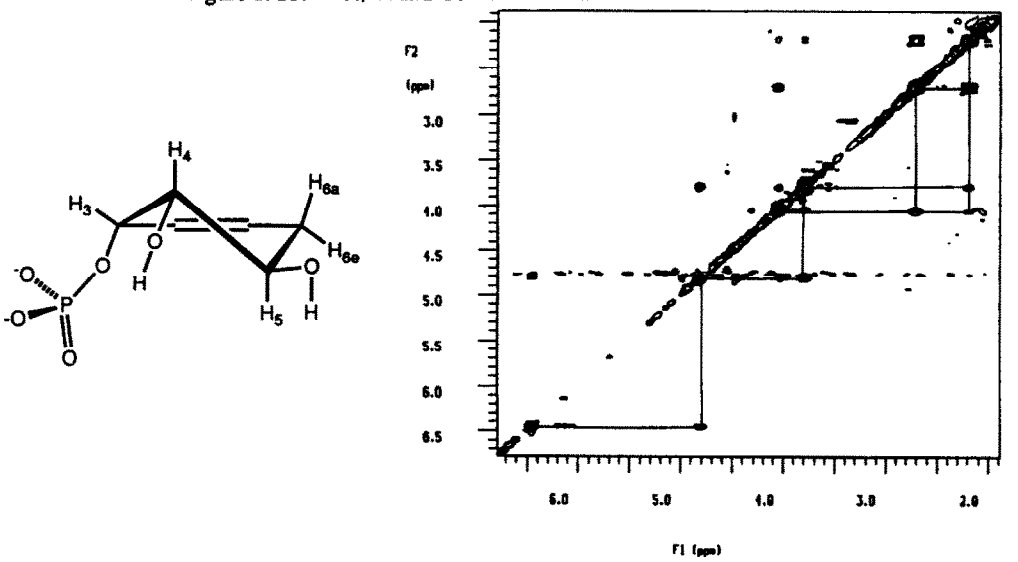


Table II. H-H Distances for S3P

H_{i-j}	Free r_{i-j} (Å)[a]	Bound r_{i-j} (Å)	Calculated r_{i-j} (Å)[b]
2-3	- - -	2.5	2.4
2-4	3.1	≥3.5	3.7
2-5	4.1	≥3.5	4.1
2-6a	4.2	≥3.5	4.1
2-6e	3.8	≥3.5	4.2
3-4	2.6	2.6	2.4
4-5	- - -	2.9	3.1
4-6a	2.8	2.8	2.6
4-6e	3.6	≥3.5	3.7
5-6a	2.6	3.0	3.1
5-6e	2.3	2.4	2.4

[a] NOE data for S3P at pH = 5.04. [4]

[b] Molecular mechanics calculation for the half chair conformation with the axial phosphate group (MM-2).[4]

Footnotes and References:

1. Presented in part at The 12th Enzymes Mechanism Conference, Jan. 4, 1991, San Diego, CA.

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4. Castellino, S.; Leo, G. C.; Sammons, R. D.; Sikorski, J. A. *J. Org. Chem.* **1991**, *56*, 5176.

5. Anderson, K. S.; Johnson, K. A.; Benesi, A. J.; Sikorski, J. A. *J. Am. Chem. Soc.* **1988**, *110*, 6577.

6. Anderson, K. S.; Johnson, K. A.; Sikorski, J. A. *Biochemistry*, **1988**, *27*, 7395.

7. Anderson, K. S.; Johnson, K. A. *J. Biol. Chem.* **1990**, *265*, 5567.

8. Alberg, D. G.; Bartlett, P. A. *J. Am. Chem. Soc.* **1989**, *111*, 2337.

9. For recent studies on the mechanism of chorismate mutase see: a) Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 5008. b) Guilford, W. J.; Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 5013.

10. Stallings, W. C.; Abdel-Meguid, S. S.; Lim, L. W.; Shieh, H. S.; Dayringer, H. E.; Leimgruber, N. K.; Stegeman, R. A.; Anderson, K. S.; Sikorski, J. A.; Padgette, S. R.; Kishore, G. M. *Proc Natl. Acad. Sci. USA*, **1991**, *88*, 5046.

11. Kinetics for EPSPS: S3P, k_{on} = 650 $\mu M^{-1}s^{-1}$, k_{off} = 4500 s^{-1} and EPSP: k_{on} = 200 $\mu M^{-1}s^{-1}$, k_{off} = 200 s^{-1}, see Anderson, K. S.; Sikorski, J. A.; Johnson, K. A. *Biochemistry*, **1988**, *27*, 1604. Spin lattice relaxation rates range between 0.36 and 1.56 s^{-1}. [4]

12. a) Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1982**, *48*, 402. b) Clore, G. M.; Gronenborn, A. M. *J. Magn. Reson.* **1983**, *53*, 423. c) Clore, G. M.; Gronenborn, A. M. *J. Mol. Biol.* **1986**, *190*, 259.

13. TRNOE experiments were conducted at 500 or 400 MHz with a probe temperature of 30 °C. Low power presaturation was used during the delay time to reduce the intensity of the solvent peak (D_2O). S3P-EPSPS data sets consisted of 256 transients per 512 x $2t_1$ increments. EPSP-EPSPS data sets consisted of 48 transients per 512 x $2t_1$ increments. Mixing times of 150 and 200 ms were used for EPSP-EPSPS and S3P-EPSPS, respectively. Only negative NOE cross peaks were observed. Cross peak volumes were extracted and averaged to generate distances using the ratio method. The geminal protons at C_6 served as the internal distance reference of 1.8 Å. 45°and 30° shifted sinebell weighting functions were used to suppress the broader protein signal in t_2 and t_1 respectively.

14. Anderson, K. S.; Sammons, R. D.; Leo, G.C.; Sikorski, J. A.; Benesi, A. J.; Johnson, K. A. *Biochemistry*, **1990**, *29*, 1460.

15. Sammons, R. D.; Leo, G. C.; Sikorski, J. A. *J. Am. Chem. Soc.* **1990**, *112*, 1653.

16. Wibbenmeyer, J.; Brundage, L.; Padgette, S. R.; Likos, J. J.; Kishore, G. M. *Biochem. Biophys Res. Comm.* **1988**, *153*, 760.

17. The unambigious assignments of the two enol protons have not been made however; geometrical considerations restrict the NOE correlations to be between ring ^1H's and the cis enol ^1H.

18. Font, J. L.; Miller, M. J.; Sikorski, J. A. unpublished results.

19. Miller, M. J.; Braccolino, D. S.; Ream, J. E.; Sikorski, J. A. unpublished results.