Solution Conformations of Two Shikimate 3-Phosphates: Determination by NMR and Molecular Mechanics Calculations

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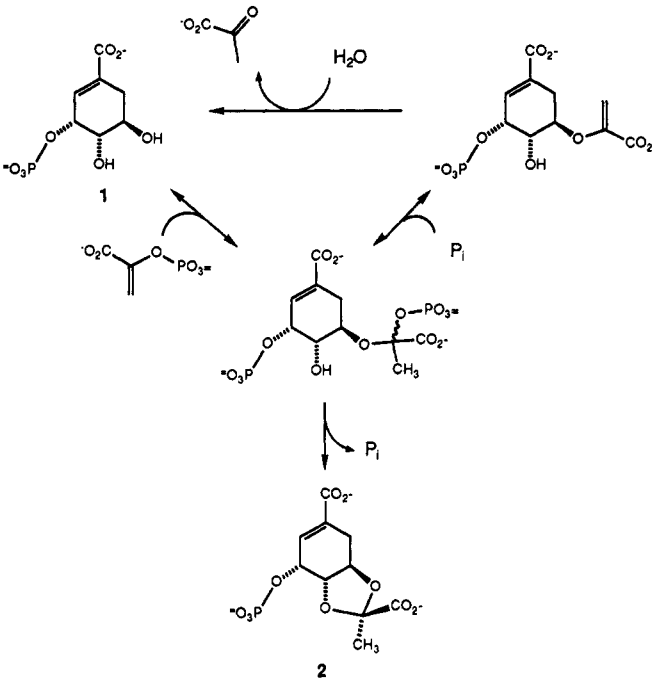
The solution conformation of two substituted cyclohexenes: shikimate 3-phosphate (S3P), 1, and a conformationally constrained derivative, the pyruvoyl ketal of 4,5-shikimate 3-phosphate (EPSP ketal);[1] 2, have been determined by a combination of NMR and molecular mechanics methods. The solution conformations were examined by $^3J_{HH}$ coupling constant analysis, quantified NOE experiments, and molecular mechanics calculations. The preferred conformation of S3P in the pH range 5–9, which brackets the second ionization of the phosphate group, is best described as a half-chair in which the phosphate group occupies an axial position. The proton–proton coupling constants suggest dihedral angle averaging in the ring. Populations of the alternative half-chair with the phosphate group in the equatorial position or twist-boat conformations are not observed. The experimental conformation of the EPSP ketal, a bicyclic analogue, was also determined by coupling constant analysis and NOE measurements. The EPSP ketal retains the half-chain conformation with the axial phosphate. The experimentally derived conformation of 2 reveals less deviation from the energy-minimized structure than was observed for S3P due to the rigid nature of this bicyclic system.

Shikimate 3-phosphate (S3P), 1, is the substrate for 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS) [EC 2.5.1.19], the sixth enzyme in de novo aromatic amino acid biosynthesis.[2] 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 I). The chemical mechanism of this reaction has recently been elucidated by the isolation of a tetrahedral intermediate formed in the normal reaction pathway.[3] The EPSP ketal, 2, is produced when the enzyme is incubated at equilibrium and is the product of a direct catalytic event off the normal reaction pathway.[1,3c,d] This enzyme, found only in plants and microorganisms, is the target for the broad spectrum herbicide, *N*-(phosphonomethyl)glycine (glyphosate).[4] The determination of the three-dimensional solution structure of S3P is an important first step in understanding the conformation of S3P bound in the enzyme catalytic site. The structure of the EPSP ketal was sought to help explain its formation and to serve as a rigid analogue of S3P.

We considered four possible conformations of S3P as reasonable starting geometries: two half-chair conformations differing in the position of the phosphate group, either axial or equatorial, and alternatively, two twist-boat conformations (Figure 1).[5] The dominant solution conformations are expected to reflect the steric and strain energies associated with each conformation. The presence of polar groups and ionizable protons provides additional structural features that must be addressed with regard to the preferred solution conformation.

One experimental approach to determining the three-dimensional structures of 1 and 2 is through vicinal H–H coupling constant analysis ($^3J_{H-H}$). These coupling constants have been shown by Karplus[6] and others[7] to be transformable into dihedral angles. Coupling constant analysis can be especially effective for determining the solution conformation of six-membered rings due to the large differences between axial–axial and axial–equatorial or equatorial–equatorial coupling constants and dihedral angles. This methodology becomes intractable in systems

Scheme I



where there is extensive averaging of various conformer populations. This has been a limitation in determining

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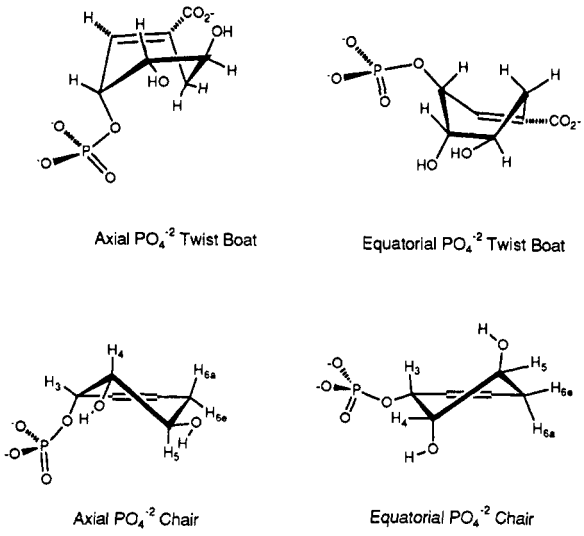

Figure 1.

the solution conformation of shikimic acid in previous reports.[8] Coupling constant analysis was used by Copley and Knowles[9] to study the conformational equilibrium of chorismate, a cyclohexa-1,5-diene that occurs later in the aromatic biosynthetic pathway.

The nuclear Overhauser effect (NOE) is an additional experimental tool for conformational studies. This technique identifies proton pairs that are spatially close to one another. The quantitative application of NOE measurements, especially for small molecules, is generally useful for systems where the proton–proton internuclear distances differ greatly between the various conformations. An expeditious NOE methodology has been advanced by Andersen[10] using truncated recovery times.

Computational methods, such as force field calculations, can be utilized in establishing starting geometries and a qualitative view of the potential energy surface. In addition, molecular graphics allows a careful comparison to be made between structures derived from experiment and calculation. Energy-minimized structures can also be utilized to calculate the expected internuclear distances and coupling constants for a given conformation.

We report the application of coupling constant analysis, quantitative NOE experiments, and molecular mechanics calculations for conformational characterization. We show that the combination of experimental and computational techniques can be successfully employed in detailing the solution conformation and conformational mobility of S3P and its bicyclic analogue **2**.

Results and Discussion

The conformational geometries were determined for the S3P species bearing a −3 charge using MACROMODEL (Figure 1).[11] The lowest energy conformation is best described as the half-chair with the phosphate substituent in the axial position (axial chair conformation). This is 2.49

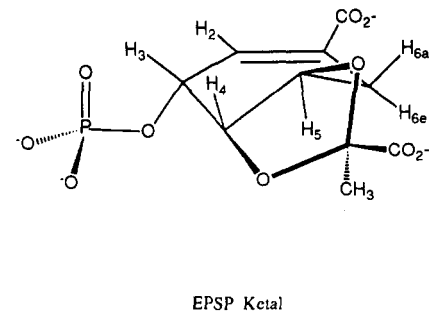

Figure 2.

kcal/mol lower in energy than the corresponding pseudochair conformation with the phosphate in the equatorial position (equatorial chair conformation). According to the force field calculations, the two twist-boat conformations are energetically unfavorable and are only located through the addition of dihedral angle constraints to maintain the proper ring geometry. Removal of these constraints results in the relaxation of the structure to either of the half-chair conformations upon minimization.

The electrostatic term, in the calculations, dominates the energetics as a whole, contributing to the overall stability of the axial chair over the corresponding equatorial chair conformation. The limitations of applying molecular mechanics calculations, which are derived from gas-phase behavior, to describe solution phenomena, are recognized. In the case of S3P, these calculated energy differences between the two half-chair conformations are too small to provide reliable predictions with regard to the preferred solution conformations. Despite this limitation, the calculations do provide initial starting geometries, including all of the internuclear H–H distances and vicinal coupling constants, for each conformation. In addition, the calculations predict no significant change in conformation as a function of charge on the phosphate moiety.

The EPSP ketal **2** is a bicyclic structure in which the oxygens at C_4 and C_5 form a five-membered ring ketal (Scheme I). The stereochemistry at the quaternary ketal carbon was determined elsewhere.[1] The fused rings of **2** provide a rigid framework ideal for NMR structural studies since fewer conformations are expected to be energetically accessible. The energy-minimized conformation for **2** is represented in Figure 2. The spatial orientation of the substituents around the six-membered ring is conserved between the axial chair conformation of S3P and the ketal structure. Attempts to locate the corresponding equatorial 3-phosphate conformation were not successful.

The ability to relate dihedral angles to vicinal coupling constants through Karplus-type equations[6,7] is an extremely valuable structural tool. The large dihedral angle between axial–axial protons (~180°) in six-membered rings generally gives rise to coupling constants between 8 and 10 Hz (range of 6–14 Hz), while the disposition between axial–equatorial or equatorial–equatorial protons (~60°) produces coupling constants of about 2–3 Hz (range of 0–5 Hz).[12] The utility of determining a conformation based on coupling constants can be limited by the extent of internal motion and/or population averaging as well as perturbations due to the electronegativity of substituents, hybridization, bond angles, and lengths.[7] The chair conformation of cyclohexene, in contrast to cyclohexane, contains "axial" and "equatorial" protons only at C_4 and C_5, while substituents at C_3 and C_6 are slightly

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Table I. Coupling Constant Analysis of S3P with Varying pH

H–H	pH 5.04 (Hz)	pH 7.00 (Hz)	pH 9.25 (Hz)	axial PO_4 chair[a] (Hz)	equatorial PO_4 chair[a] (Hz)
2–3	3.8	4.0	4.3	5.0	3.1
3–4	4.1	4.1	4.1	3.2	4.4
4–5	7.8	8.4	8.5	9.5	4.3
5–6a	6.0	6.8	7.0	9.9	3.1
5–6e	5.2	5.3	5.4	6.5	3.0

[a] Calculated coupling constants.

perturbed due to the presence of the double bond.[5] Analysis of the vicinal coupling constants for compounds 1 and 2 was aided by Altona's[7] modified Karplus relationship, which takes into account the electronegativities of the substituents. It was anticipated that the vicinal coupling constants would provide significant insight into the characterization of the conformational preferences of 1 and 2.

Vicinal coupling constants for S3P were experimentally determined at pH 5.04, 7.00, and 9.25 and are reported in Table I with the predicted coupling constants for the chair conformations derived from the molecular mechanics calculations.[13] At pH 9.25, S3P is fully ionized and bears a −3 charge. The experimental coupling constants at pH 9.25 provides a reasonable match with the calculated values for the axial chair conformation. The differences between the calculated and experimental H_4–H_5 and H_5–H_{6a} coupling constants could be attributed to motional averaging between the half-chair and a "sofalike" conformation,[5] which would translate into a flattening of the ring. This points out the limitations in precision when employing either molecular mechanics calculations or vicinal coupling constants alone in defining solution conformations.

At pH 7.00, the experimental coupling constants for S3P have decreased in magnitude relative to those determined at pH 9.25. These values are still consistent with the axial chair conformation, though the fit is less satisfactory. Note that at pH 7.00 the solution consists of an 80:20 mixture of the −3 and the −2 species of S3P. At pH 5.04, S3P is largely in the −2 form with only one of the phosphate oxygens ionized. The experimental coupling constant values at pH 5.04 are slightly smaller in magnitude than the corresponding couplings observed for both pH 9.25 and 7.00. While the experimental coupling constants at pH 5.04 fall within the possible range of the axial chair conformation, the values could also be interpreted to indicate an averaging of populations between the two possible half-chair species (Table I). The ^1H chemical shifts would also reflect this population averaging as a function of pH, since the interconversion between the two half-chair conformations would be occurring rapidly on the NMR time scale.[14] However, there is virtually no change in ^1H chemical shifts for S3P at the three pH's. Therefore, conformational averaging between the two half-chairs can be ruled out.

One possible explanation that would account for the trend in vicinal coupling constants is based on small dihedral angle variations as a function of electrostatic effects. At pH 9.25, S3P has two negative charges on the phosphate moiety in addition to the carboxylate anion. This renders the conformation relatively free of small dihedral angle librations due to the minimization of electrostatic charge interactions and/or accommodation of counter ions. In this case, the vicinal coupling constants demonstrate good agreement with the Karplus relationship predictions for the axial chair conformation. At pH 5.04, the 3-phosphate is largely monoanionic, and thus, the electrostatic interactions are reduced permitting greater dihedral angle variation. The corresponding vicinal coupling constants reflect this motional averaging and, therefore, do not match the calculated coupling constants for the axial chair conformation as well. Another explanation might be differential hydration effects.

Table II. Vicinal Coupling Constants for EPSP Ketal

H–H	observed (Hz)	calcd[a] (Hz)
2–3	5.2	4.8
3–4	4.0	3.2
4–5	10.1	9.7
5–6e	5.3	6.3
5–6a	10.4	10.2

[a] Calculated based on energy-minimized structure.

On the basis of vicinal coupling constant analysis alone, it is difficult to unambiguously establish the solution conformation of S3P in the pH range examined. However, the data strongly suggest that the half-chair conformation with an axial phosphate is the preferred solution conformation. The variation in the coupling constants observed could be attributed to small dihedral angle librations, which occur rapidly on the NMR time scale. The coupling constant data do not support twist-boat conformations. These findings are consistent with previous proton–proton coupling constant analyses of shikimic acid.[8]

Excellent agreement is observed between the calculated (trans fused; diequatorial oxygens, Figure 2) and experimental vicinal coupling constants for the ketal 2 at pH 7.00 (Table II). This observation suggests that there is very little motional averaging occurring in the bicyclic structure. In addition to defining the conformation of the ketal, this result also supports the position that the variation between calculated vicinal coupling constants for the axial chair conformation of S3P and the experimentally determined values may be attributed to small dihedral angle librations occurring as a function of the ionization state.

A complementary experimental technique, to vicinal coupling constant analysis, for elucidating the solution conformation is the quantification of internuclear distances from NOE experiments. Quantified NOE measurements are employed extensively in the structure elucidation of biomolecules.[15] However, the application to "small" molecules is often limited due to the experimental difficulties associated with these measurements in the fast correlation time regime.[10,16]

On the basis of the distances from the force field calculations, the four conformations (Figure 1) should be experimentally distinguishable. Of the 14 possible internuclear H–H distances in the S3P molecule, only five distances can be used to establish the preferred solution conformation. These distances are H_2–H_4, H_4–H_5, H_4–H_{6a}, H_4–H_{6e}, and H_5–H_{6a}. The criteria for choosing these values

(13) The full list of observed H–H couling constants for S3P (pH 9.25) is as follows: H_2 = observe dt(ddd) J = 4.34 and 1.82 (average) Hz, H_3 = 12 lines (nonfirst order), H_4 = dd, J = 8.47 and 4.14 Hz, H_5 = ddd, J = 8.47, 7.00, and 5.35 Hz, H_{6e} = observed dt(dddd), J = 18.0, 5.35, and 1.48 (average) Hz, and H_{6a} = dddd, J = 18.00, 7.00, 2.24, and 1.31 Hz.

(14) This assumes that the ^1H chemical shifts of the two conformations are distinguishable. This is a reasonable assumption in the case of S3P since there is a large chemical shift difference between the two C_6 protons ($\Delta\delta$ 0.5 ppm).

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Table III. NOE H-H Distances for S3P[a]

H-H	pH 5.04 (Å)	pH 7.00 (Å)	pH 7.00[b] (Å)	pH 9.25 (Å)	ax chair[c] (Å)	eq chair[c] (Å)
2-3			2.3		2.4	2.5
2-4	3.1	3.1		3.3	3.7	4.4
2-5	4.1				4.1	4.4
2-6a	4.2				4.1	4.0
2-6e	3.8				4.2	4.2
3-4	2.6	2.4	2.3	2.3	2.4	2.4
3-5	3.4				3.8	3.9
3-6a	3.7				4.3	4.3
3-6e	4.6				4.9	4.4
4-5		2.6	2.9	3.1	3.1	2.5
4-6a	2.8	2.7	2.7	2.7	2.6	3.8
4-6e	3.6	3.6	4.1	3.9	3.7	4.3
5-6a	2.6	3.0	2.7	2.7	3.1	2.5
5-6e	2.3	2.2	2.2	2.4	2.4	2.5

[a] 1D SIRNOE data. Distances based on ratios in which H_{6a}–H_{6e} is taken to be equal to 1.8 Å. The experimental precision in determining distances is taken to be ±10% (see Experimental Section). [b] Values derived from 2D NOESY data at 400 MHz. [c] Distances from energy-minimized structures.

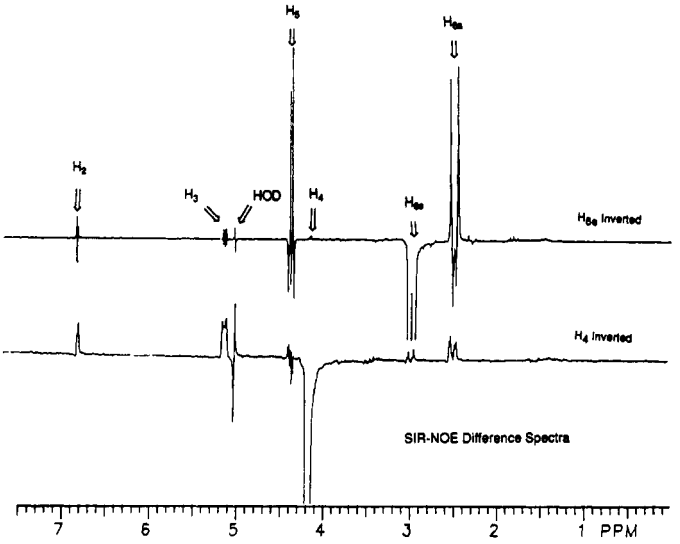

Figure 3.

are based on the differences between the internuclear distances in the molecular mechanics derived conformations, the magnitude of the vectors, and the inherent limitations of NOE measurements (±10% and $r \leq 3.5$ Å). It is worth noting that only four vectors can be used to distinguish the two chair conformations: H_2–H_4, H_4–H_5, H_4–H_{6a}, and H_5–H_{6a}.

Representative SIRNOE difference spectra for S3P are shown in Figure 3. In some of the difference spectra, resonances that are spin coupled to the selectively inverted resonance show selective population transfer (SPT)[17] modulation. The SPT is due to pulse drop-off over the selective band width, resulting in a power differential over the excitation window. The presence of SPT effects does not interfere with the NOE enhancements or quantification, since the net integral of SPT resonances alone is zero. Compiled in Table III are the experimental H-H distances and the distances from the molecular mechanics derived chair conformations.

In the case of S3P at pH 5.04, the SIRNOE data are within ±15% of calculated values for 12 of the possible 14 distances. The data sets collected at pH 7.00 and 9.25 contain all of the key distances but fewer of the longer internuclear H-H distances (≥3.5 Å), since fewer transients were acquired. Some cross-relaxation rates, σ_{i-j} were

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Table IV. NOE Distances for EPSP Ketal[a]

H-H	pH 7.00[b] (Å)	pH 10.00[c] (Å)	calcd[d] (Å)
2-3		2.4	2.5
2-6a		4.5	4.1
4-3	2.5	2.4	2.4
4-6a	2.5	2.4	2.6
4-5		2.9	3.1
5-6a	3.2	2.9	3.1
5-6e	2.3	2.4	2.4
5-CH_3		3.4	3.4[e]

[a] Distances are based on ratios in which H_{6a}–H_{6e} is taken to be 1.80 Å. [b] 1D SIRNOE data at 300 MHz. [c] 2D NOESY data at 400 MHz. [d] Distances based on energy-minimized structure. [e] Estimate of average H–CH_3 distance.

difficult to measure (by SIRNOE at 300 MHz) for the following reasons: σ_{3j}, due to tailing effects of the solvent peak, and σ_{4-5}, because of the small difference in chemical shift between H_4 and H_5. The 2D NOESY data for S3P at pH 7.00 were acquired so a comparison with the SIRNOE methodology could be made. Distances calculated from the 2D experiments were consistent with those determined by the 1D SIRNOE methodology.

Over the pH range examined, the data in Table III are most consistent with the axial chair conformation for S3P. This conformational preference appears independent of pH in the cases examined. One must be cautious in the comparison of the calculated distances and the experimental values. The calculated distances given in Table III are for energy-minimized *rigid* structures derived from gas-phase data and should only be viewed as representing reasonable starting geometries since solvent and charge effects may not be accurately accounted for. The NOE distances reflect the weighted averages, on the NMR time scale, of all contributing conformations.

Table IV lists the calculated distances for the energy-minimized conformation of 2 and the SIRNOE and NOESY data at pH 7.00 and 10.00. There is good agreement between the experimental and calculated distances at both pH's. This observation is consistent with the idea that a motionally constrained or rigid structure is likely to have a steep potential energy well. Computationally, it is easier to locate the energy-minimized conformations using force field calculations for these structures. The precision in determining internuclear H-H distances from NOE experiments is higher since losses in cross-relaxation due to motional averaging are reduced and only a small number of populated conformations are averaged on the NMR time scale. The net result is good agreement between experimental and calculated conformations.

Conclusions

The solution conformation of S3P in the pH range of 5.04–9.25, which brackets the second ionization of the phosphate, is best described as a half-chair with the phosphate moiety in the axial position. The conformation was determined by measuring internuclear H–H distances through the application of NOE techniques and vicinal coupling constant analysis. At all three pH's examined, only minor perturbations of torsion angles, relative to the molecular mechanics energy-minimized structure, are observed. Within experimental precision, there is no consistent trend to the torsional distortions as a function of ionization state. There is no evidence for the interconversion between chair or twist-boat forms associated with the second phosphate ionization of S3P.

The vicinal coupling constants observed as a function of pH for S3P fall in the observed "range" expected for the half-chair conformation with the phosphate group in the axial position. The observed decrease in vicinal coupling constants as a function of decreasing pH and lack of any associated change in chemical shifts of the S3P resonances is noteworthy. These observations suggest that the axial phosphate half-chair conformation of S3P undergoes rapid small torsion angle librations with the second ionization of the phosphate group and/or the addition of a third counterion. The experimental precision of ±10% in the NOE measurements makes detection of any trends in the torsion angle variations difficult. Hence, while the NOE data is the most useful in determing the overall solution conformation of S3P, the vicinal coupling constant data provide a picture of the dynamic nature of this structure.

Examination of the structurally related ketal 2 demonstrates excellent agreement between the energy-minimized conformation determined by force field calculation, vicinal coupling constants, and NOE data. This is attributed to the rigid bicyclic backbone of this compound, which does not energetically allow significant conformational variation. The solution conformation of the ketal is akin to the S3P conformation, an axial phosphate pseudochair.

In general, the determination of solution conformations by coupling constant analysis, NOE experiments, or computational methods have limitations and experimental uncertainties associated with each methodology. The combined use of all three techniques provides a means of circumventing the limitations of the individual methods. Determining the preferred solution conformation of S3P is the first step in understanding substrate recognition by the enzyme EPSP synthase. Efforts are underway to further examine the relationships between conformations of "free" and bound substrates.

Experimental Section

All calculations were completed using the MACROMODEL Version 1.5 program[11] operating on a Vax network. S3P structures were constructed by means of the interactive graphics input and then subjected to the MM2[18] minimization within MACROMODEL. The MACROMODEL implementation of MM2 employs partial atomic charges as opposed to dipole–dipole charge interactions and is also set for distance-dependent dielectric effects. Block diagonal Newton Raphson minimization was implemented with terminal atom movement in all calculations. The two half-chair conformations were found as global minima after repeated conformational searches. The minimized structures had a first derivative RMS of less than 0.008 kJ/A. The twist-boat conformations were located using dihedral angle constraints followed by minimization. The removal of these constraints resulted in relaxation back to the corresponding chairs upon minimization. Vicinal coupling constants were calculated using the Altona[7a] modification of the Karplus equation, which takes into account the electronegativity of substituents.

Shikimate 3-phosphate was prepared by enzymatic phosphorylation of shikimic acid using shikimate kinase and ATP.[19] A crude extract of *E. coli* shikimate kinase was used. The reaction mixture used catalytic amounts of ATP to prevent product inhibition of shikimate kinase by ADP. Excess PEP was used to drive the reaction, and a catalytic quantity of pyruvate kinase was used to regenerate ATP. The overall reaction mixture (in 600 mL) contained 100 mM HEPES, pH 7.0, 50 mM KCl, 10% glycerol, 50 mL of crude shikimate kinase homogenate supernatant (31 IU/mL), 57.8 mmol of shikimic acid (Sigma), 70 mmol of $Mg(OAc)_2$, 65 mmol of PEP, 0.5 mmol of ADP, 2500 IU of pyruvate kinase (Sigma P7768), 80 mg of Na_2WO_4, 5 mM β-mercaptoethanol, and 50 mg of bacitracin. The tungstanate was used to inhibit any phosphatase in the crude homogenate.[20] The formation of S3P was followed by HPLC analysis at 210 nm using a Mono-Q 5/5 column (Pharmacia) eluting with a linear 0–300 mM KCl gradient in 20 min in 25 mM Tris, pH 7.5, at 1.0 mL/min. Retention times were as follows: shikimate (1.65 min), pyruvate (5.50 min), S3P (14.00 min), ADP (14.35 min), PEP (15.3 min), and ATP (18.35 min). The reaction was complete in 6 h and stood overnight at 13 °C. Purification was accomplished by passing the mixture over two ion exchange chromatography columns. The first column (6.5 × 80 cm DEAE cellulose) was eluted with 2.0 L of 0.25 M triethylammonium bicarbonate (TEAB). The major ionic species eluted directly in fractions 10–130 (22 mL each). There was some overlap with the minor ionic species (EPSP), which eluted in fractions 131–170. The EPSP was formed from EPSP synthase in the homogenate. The buffer was removed by rotary evaporation. The sample was diluted to 2.6 L with water at pH 7.6 to give an ionic strength less than 0.2 M TEAB. The sample was loaded onto the second column (6.5 × 90 cm DEAE cellulose) and eluted with a 20 L linear gradient of 0.2–0.8 M TEAB. Analyzing at 226 nm, pure S3P eluted at 0.5 M TEAB. The buffer was removed as before and the yield of pure S3P was 27.3 mmol (47%). The remainder of the material was isolated as S3P contaminated with nucleotides or as EPSP.

EPSP ketal was synthesized enzymatically by incubating EPSPS at equilibrium for 10 days.[1] The apparent side reaction (not involved in the normal reaction course equilibrium) was fueled by the addition of PEP to maintain "internal equilibrium" conditions. The initial reaction mixture contained in 225 mL; 0.204 mM EPSPS (pMON 6001 *E. coli*), purified according to the procedure described previously,[21] 0.1 M KP_i, pH 6.15, 14.69 mM S3P, 25.5 mM PEP (1.552 g, cyclohexylammonium salt, Sigma), 9.4% glycerol, 46.8 mM KCl, 9.4 mM β-mercaptoethanol, 0.05 mM EDTA, 10 mg of Na_2WO_4, 10 mg of bacitracin (Sigma), 10 mg of ampicillin (Sigma), and 25 mg of trypsin inhibitor (Ovomucoid Type II, Sigma). The reaction was maintained at 30 °C and followed by ^{31}P NMR by diluting 0.75-mL reaction-mixture aliquots with 0.5 mL of D_2O. Chemical shift assignments for the reaction mixture are as follows: S3P (2.49 ppm), EPSP (2.31 ppm), EPSP ketal (1.16 ppm), P_i (0.81 ppm), and PEP (−1.65 ppm).[1] The reaction required a total of 28 mmol (7.565 g) of PEP to force all S3P (3.3 mmol) to 2. The reaction was diluted to 325 mL after 5.5 g of PEP had been added. Upon reaching complete conversion to EPSP ketal, the mixture was chromatographed on DEAE Sephadex A-25 column, 7 × 100 cm, equilibrated with 0.2 M TEAB. The reaction mixture was diluted to 2 L and loaded at 12 mL/min and then washed with 1 L of 0.2 M TEAB. The column was then eluted with a 20-L linear gradient of 0.3–1.0 M TEAB. The elutant was monitored at 226 nm while 50-mL fractions were collected. The EPSP ketal was base-line separated in fractions 243–283 (approximately 0.75 M TEAB). The TEAB buffer was removed by rotary evaporation and the solid converted to Na^+ form by passing the resuspended material over a Dowex 50 Na^+ column. After rotary evaporation and lyophilization, 1.1 g (70%) of solid 2 was obtained as the hydrated tetrasodium salt, mp 287–90 °C dec. This off-white solid was shown conclusively to be the EPSP ketal by ^1H, ^{13}C, and ^{31}P NMR and mass spectral

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analyses, as reported previously.[1] Anal. Calcd for $C_{10}H_9O_{10}P_1Na_4{\cdot}4H_2O$: C, 24.80; H, 3.54; P, 6.40. Found: C, 24.52; H, 3.60; P, 6.38.

S3P was prepared for NMR studies by passing the sample over Dowex 50 (Na^+ form). The samples were pH adjusted, concentrated in vacuo, and exchanged (3×) with 2-mL aliquots of demineralized D_2O (Merck MSD). Final samples were at least 50 mM in S3P and 1 μM EDTA. The pK_a of the second ionization of the phosphate group was previously determined to be 6.41.[21] The EPSP ketal NMR samples were prepared similarly to the S3P samples. Solutions were subjected to three freeze–thaw cycles and then sealed in the NMR tubes. All pH's reported are adjusted for D_2O activity. Proton spectra are referenced to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt, TSP, (0.0 ppm) in D_2O as a secondary standard.

^1H NMR spectra were recorded at 300 or 400 MHz. Both spectrometers were equipped with 15-bit analogue to digital converters. 1D spectra were acquired using the selective inversion recovery experiment with on-resonance and off-resonance acquisitions interleaved. For all resonances except H_4 and H_5, selective 180° pulses (49 ms) were employed in the selective inversions. Due to the smaller chemical shift difference between H_4 and H_5, 100° pulses (27.2 ms) were used for selective inversions. The total recycle time employed was 5 s, comprised of a 4-s preparatory delay and a 1-s acquisition time. Mixing times of intermediate values, 200–600 ms, were employed with the maximum number of transients per block set to 1000. In general, a minimum of three mixing times were employed for each proton examined. NOESY spectra were acquired using the standard three-pulse experiment in the phase-sensitive mode.[22] Suppression of the HOD resonance was not used for S3P due to the close proximity (2–10 Hz) of H_3 to HOD. The 2D data sets were zero filled to 2K by 2K and weighted in both dimensions by 75° shifted sinebell functions. Base-line correction was used in ω_1 and drift correction in ω_2. Volume integrations were used to quantitate the observed NOE's.

T_1 values for S3P were determined by the inversion-recovery method and at 300 MHz: H_2 (2.78 s), H_3 (1.07 s), H_4 (1.82 s), H_5 (1.51 s), H_{6e} (0.68 s), and H_{6a} (0.64 s). On the basis of the ratio of T_1's at 300 and 400 MHz the overall correlation time, τ_c,[23] was approximated to be 110 ps. The EPSP ketal T_1 values at 400 MHz are: H_2 (2.12 s), H_3 (1.17 s), H_4 (1.45 s), H_5 (1.25 s), H_{6e} (0.40 s), H_{6a} (0.36 s), and CH_3 (0.56 s).

NOE distances were obtained by averaging the experimental cross-relaxation rates for each H–H interaction. The diastereotopic geminal protons at C_6 provide an internal standard for determining H–H distances, and for these geminal protons it is take to be 1.8 Å and is not expected to vary as a function of pH. In general, for the 1D analysis, the average cross-relaxation rate was derived from six data points, three mixing times taken from two different inversion experiments. 2D analysis followed from single data sets taken in the region where the NOE build-up was linear. The magnitude of the analyzed NOE's ranged from 0.5–10%. SIRNOE data sets for S3P at pH 5.04, 7.00, and 9.25 and the EPSP ketal at pH 7.00 were collected at 300 MHz. NOESY spectra were obtained at 400 MHz for the EPSP ketal at pH 10.0 and for S3P at pH 7.0.

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Supplementary Material Available: Complete tables of molecular mechanics energies, calculated H–H distances and coupling constants for conformers of 1 and 2, and ^1H chemical shifts for 1 at varying pH (6 pages). Ordering information is given on any current masthead page.

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Theoretical Studies in Molecular Recognition: Asymmetric Induction of Benzophenone Imine Ester Enolates by the Benzylcinchoninium Ion

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Molecular-recognition techniques are used to examine the complexes formed between a chiral phase-transfer catalyst and enolates that are known to be alkylated enantioselectively. The ion pairs have well-defined, albeit flat, intermolecular potential energy surfaces that show binding regions consistent with a proposed model. The influence of dispersion vs Coulomb forces on these potential energy surfaces is addressed as is the origin of enantiofacial selectivity. An energy-partitioning algorithm that statistically weights intermolecular interaction energies for all orientations of the two molecules in the complex is used to determine which fragments of the cinchoninium ion are most responsible for enolate binding. The roles other molecular fragments play, on both the catalyst and the enolate, for ion pairing are examined.

Introduction

In an earlier paper one of us reported the enantioselective synthesis of α-amino acids by phase-transfer catalysis (PTC).[1] In contrast to most other asymmetric syntheses of amino acids that use stoichiometric amounts of chiral auxiliaries, this method involves using catalytic quantities of the enantiocontrol element. Under the PTC conditions moderately high levels of enantiomeric excess (ee) were obtained using a chiral catalyst and a prochiral protected glycine derivative 1. This prochiral substrate meets two important criteria for successful synthesis of α-amino acids: it undergoes monoalkylation under PTC conditions and, once alkylated, it is not easily racemized during reaction or workup. The catalysts used as asymmetric templates are derived from the cinchona alkaloid family 2.

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