strongly implies the sense of stereogenicity transfer (1'*S* reactant → 8*S* product) from the origin to the terminus of hydrogen migration. This was confirmed independently (and with some difficulty) in the pyrolysis of the optically active isotopically labeled substrate 1*R*,2*R*,1'*S*-1-(1-methoxyethyl)-2-(2-*d*-1-*E*-propenyl)-cyclobutane (**5b**), which was obtained in 93% ee by the use of optically active (-)-(*S*)-3-butyn-2-ol for the synthesis of lactone **1b** (see Supplementary Material). At 239.4 °C, **5b** gave only about 5% of homodienyl shift products, of which the major component was the 2*E*, 6*Z* isomer (**8**, 90%). Minor components were the 2*Z*, 6*Z* (**9**, 4%) and the 2*E*, 6*E* (**10**, 6%). The 6*Z* products are those derived from an endo receptor orientation, and the observed 23:1 preference for the 2*E*, 6*Z* isomer in this manifold is consistent with an overlap-favored geometry. When hydrolyzed to the ketone and subjected to the same degradation procedure used in the cyclopropane work,[2] the mixed enol ethers **8-10** gave the *S*-2-*d*-propanoate of *R*-methyl mandelate in 81 ± 3% (^{2}H NMR analysis) and 69 ± 5% (^{1}H analysis, corrected for incomplete deuteration) of the diastereomeric excess maximally available from the ee level of the starting material. These again are minimum measures of the true stereospecificity.

At what ring size should stereospecificity of the homodienyl hydrogen shift disappear? If the hypothesis is correct that orbital overlap is the source of specificity here, even cyclopentane would be capable, in principle, of acting as a stereochemical control element, since one of its nearly degenerate HOMOs[7] has the appropriate[2] symmetry. For thermodynamic reasons, this would have to be studied experimentally in the forward ene rather than the reverse ene sense in most cases.

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Supplementary Material Available: Description of and references for synthesis of isotopically labeled optically active reactant **5b** and characterization of substances of Scheme I and earlier synthetic intermediates (26 pages). Ordering information is given on any current masthead page.

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Novel Product from EPSP Synthase at Equilibrium

Gregory C. Leo, James A. Sikorski, and R. Douglas Sammons*

Monsanto Agricultural Company
A Unit of Monsanto Company, Technology Division
700 Chesterfield Village Parkway
Chesterfield, Missouri 63198

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A novel shikimate ketal (**5**) is produced at equilibrium by the sixth enzyme in de novo aromatic amino acid biosynthesis, EPSPS[1] [EC 2.5.1.19].[2] The EPSP ketal is indisputably the species mistakenly labeled as an "enzyme free intermediate" by Barlow et al.[3] While **5** is produced at equilibrium, it clearly is not on the normal catalytic pathway. Understanding the formation of **5** is key to having a complete definition of the EPSPS chemical mechanism (Scheme I).

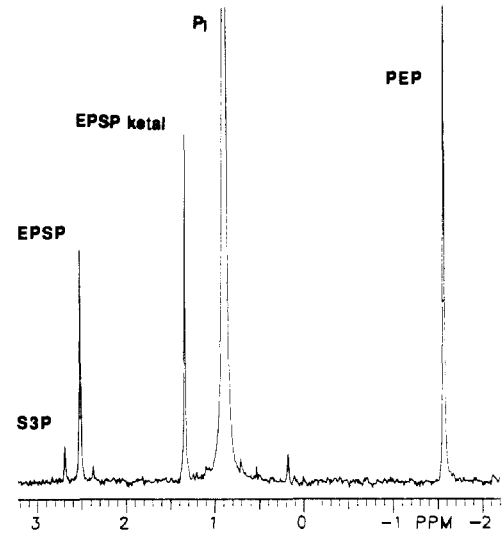
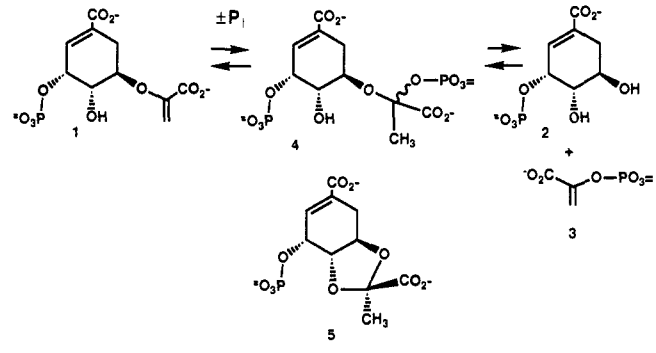


Figure 1. ^{31}P NMR spectrum of an 8-day internal equilibrium reaction with EPSPS set to maximize production of **5**, shown at 60% conversion. This mixture contained, after 8 days, 0.20 mM EPSPS, 0.1 M KP_i, pH 6.15, 14.7 mM **2**, 97.7 mM **3**, 9% glycerol, 47 mM KCl, 9 mM β-mercaptoethanol, 0.05 mM EDTA, 10 mg of Na_2WO_4, 10 mg of bacitracin, 10 mg of ampicillin, and 25 mg of trypsin inhibitor. The PEP was added intermittantly to drive the reaction as **1** was converted to **5** and **2** plus pyruvate. In 10 days, all of **2** was converted to **5**. Purification was carried out as described to remove excess PEP, pyruvate, and P_i to yield 1.1 g of the Na salt.[5] Spectra were taken with 0.75 mL of reaction mixture in 0.5 mL of D_2O on a Varian XL 300 instrument, 5-mm probe, ^{1}H decoupled, referenced to 1 N D_3PO_4.

Scheme I



The EPSPS-bound intermediate (**4**) and **5** are readily apparent by ^{13}C NMR under internal equilibrium conditions.[3,12] When

(1) Abbreviations used: EPSPS, 5-enolpyruvoylshikimate 3-phosphate synthase; EPSP, 5-enolpyruvoylshikimate 3-phosphate; S3P, shikimate 3-phosphate; PEP, phosphoenolpyruvate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); pD, proton activity in D_2O; [U-^{14}C], ^{14}C uniformly labeled; NOE, nuclear Overhauser effect; and P_i, inorganic phosphate.
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(5) Anion exchange on DEAE A-25 Sephadex with a linear gradient of TEAB from 0.4 to 1.0 M with **5** appearing at 0.75 M TEAB.
(6) CH_2N_2 (Arndt, F. *Org. Synth.* **1943**, *2*, 165-167) was added to a 0.5-mL MeOH/H_2O (4:1, v/v) solution of the salts precipitated with *p*-dioxane (5:1, v/v) from the HPLC eluent in footnote 5. The permethylated **1** and **5** (99% ^{13}C labeled at C'-2) were extracted with CH_2Cl_2 after removal of the organic layer with a nitrogen stream.
(7) ^{1}H NMR ($CDCl_3$): δ 6.74 (H-2, dd, *J* = 2.0, 5.1, 1 H), 5.03 (H-3, ddd, *J* = 4.6, 4.6, 7.3, 1 H), 4.125 (H-5, dd, *J* = 5.0, 10.0, 10.2, 1 H), 3.42 (H-4, dd, *J* = 4.2, 10.0, 1 H), 3.00 (H-6e, dd, *J* = 5.3, 16.3, 1 H), 2.385 (H-6a, ddd, *J* = 2.5, 10.6, 16.2, 1 H), 1.681 (CH_3, s, 3 H). ^{13}C NMR in D_2O (referenced to $CDCl_3$): δ 180.55 (q, *J* = 2.4), 178.34 (d, *J* = 5.8), 139.5 (C-1, ddd, *J* = 4.5, 7.4, 7.4), 134.2 (C-3, ddd, *J* = 4.4, 4.6, 164.5), 110.6 (C'-2, q, *J* = 4.5), 82.2 (dm, *J* = 146.6), 74.81 (dm, *J* = 155.4), 68.12 (d, *J* = 152.2), 34.42 (C-6, ddd, *J* = 8.1, 135.2, 135.2), 25.96 (C'-3, q, *J* = 128.5). ^{31}P NMR at pH 9.0 (1 N D_3PO_4/D_2O): δ 4.13 (d, *J* = 6.2).
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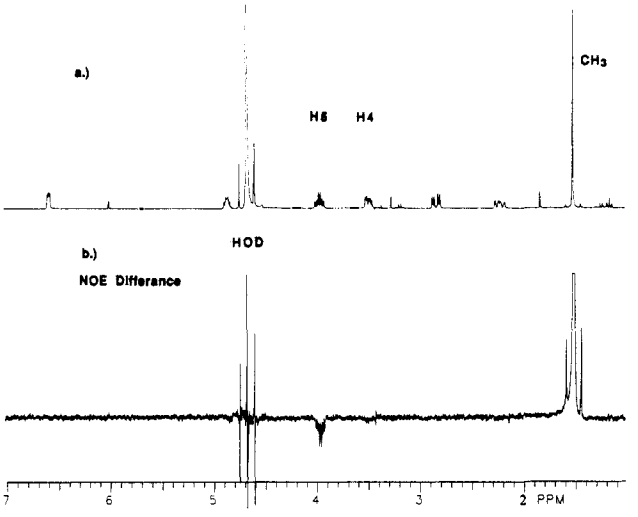

Figure 2. (a) ^{1}H NMR spectrum of EPSP ketal in D_2O at pD 9.0. (b) NOE difference spectrum resulting from irradiation of the methyl signal at 1.68 ppm showing the excitation of the H-5 protons selectively. Spectra were taken on a Varian XL 400 instrument.

the reaction mixture is allowed to stand with excess **3**, the ketal becomes the only detectable shikimate species separable from the mixture without denaturing EPSPS. Maximum production of **5** occurs while **4** is maintained by the enzyme. EPSPS catalytically converts **1** to **5** at $k = 0.2$/h (pH = 6.3, 25 °C), a rate much slower than the reverse reaction.[4] Figure 1 shows a ^{31}P NMR spectrum of an enzymatic synthesis of **5** with the equilibrium mixture containing S3P (2.49 ppm), EPSP (2.31 ppm), EPSP ketal (1.16 ppm), P_i (0.81 ppm), and PEP (−1.65 ppm).

The EPSP ketal is easily isolated from the reaction mixture in Figure 1 by anion-exchange chromatography.[5] A number of corroborative experiments confirm the structure of **5**. Labeling with [U-^{14}C]S3P and [1-^{14}C]PEP indicates that all 10 carbon atoms must be present. Degradation of **5** in dilute acid produces **2** and pyruvate. The permethylated derivative of [2′-^{13}C]-**5** displays a molecular ion at $m/z = 382$ compared to methylated **1** at $m/z = 381$ by CI mass spectrometry.[6] The key structural features of **5** are readily observed by ^{1}H and ^{13}C NMR.[7] The ^{13}C-2′ carbon is quaternary by APT sequence, appears as a quartet in the ^{1}H-coupled ^{13}C spectrum, and is readily seen in the ^{13}C NMR spectrum of the internal equilibrium mixture at 110.6 ppm.[3,12] The ^{1}H spectrum (Figure 2a) contains the C-3′ methyl signal coupled (d, $J = 4.7$ Hz) to the C-2′ carbon (99% ^{13}C). The stereochemistry at the C-2′ position is *R* by 1-D NOE with transfer occurring between the C-3′ (CH_3) signal at 1.68 ppm and the H-5 proton (Figure 2b). The EPSP ketal is stereochemically pure on the basis of this NOE result and the absence of a diastereomeric methyl ^{1}H NMR signal at 1 Hz line width.

The addition of **5** back to EPSPS with or without P_i does not result in conversion to **1** or **2**. A mixture of EPSPS (55 μM), [^{14}C]S3P (32 μM), and PEP (0.5 mM) formed and maintained **5** for 10 months at 4 °C. This nonreversibility suggests that EPSPS is not actually catalyzing the formation of **5** directly, since with time the equilibrium should favor the products with the least energy, S3P and pyruvate.[2a] Formation of **5** is stopped in the presence of glyphosate [*N*-(phosphonomethyl)glycine], which sequesters enzyme with S3P in the herbicidal dead-end complex.[8] Incubation of **1** (50 mM) at pD 4.8 does not produce **5** after 16 days at 25 °C; however, **2** (21%) is observed from hydrolysis.[3,12] Incubation of [^{14}C]S3P (8 μM) with pyruvate (1.7 mM) with or without EPSPS (7 μM) at pH 7.0 for 18 days at 4 °C also does not produce **5**. Therefore, EPSP ketal formation is not an artifact of these incubations.

EPSPS has a cysteine reactive to DTNB at position 408 that is protected from modification by **2** and glyphosate or **1**,[9] but **5** (2 mM) with or without P_i (100 mM) does not protect Cys-408 from modification by DTNB (60 μM). This suggests that productive association of **5** does not occur with the EPSPS active site. Taken together, all of these results preclude **5** from being an intermediate along the catalytic pathway and clearly demonstrate the need for corroborative information when enzyme intermediates are spectroscopically characterized.[12]

EPSPS catalyzes a direct addition–elimination reaction through **4**, which has been isolated and fully characterized.[10] The internal equilibrium conditions maximize the concentration of **4**, facilitating optimum production of **5**. It is tempting to propose that **4** produces **5** from an S_N2 displacement of P_i by the shikimate 4-OH in the active site. However, direct formation at the active site seems unlikely from the protection and reversibility studies discussed above. Nevertheless, EPSPS catalytic activity is required, suggesting that **5** forms in solution from an enzymatically produced species. If the prolonged stabilization of enzyme-bound **4** permits its occasional release, then the solution degradation of **4** must account for the stereospecific formation of **5**. The stereochemistry of **4** and its relationship to **5** is not clear[11] and is the subject of a detailed investigation.[12]

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Stereochemical Course of the Key Ring-Forming Reactions in Clavulanic Acid Biosynthesis

Amit Basak, Scott P. Salowe, and Craig A. Townsend*

Department of Chemistry, The Johns Hopkins University Baltimore, Maryland 21218

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Clavaminate synthase (CS) is an Fe(II)/α-ketoglutarate (α-KG)-dependent oxygenase central to the clavulanic acid (**4**) biosynthetic pathway. It carries out a four-electron oxidative cyclization of proclavaminic acid (**1**) to clavaminic acid (**2**) with concomitant formation of 2 mol of CO_2 and succinic acid[1,2] (Scheme I). An ^{18}O-labeling experiment has established that the 3-OH in **1** gives rise to the oxazolidine oxygen in **2** without detectable loss or exchange of heavy isotope, signaling a significant departure from conventional α-KG-dependent dioxygenase behavior.[3] In this paper, the stereochemical course of this oxidative cyclization at C-4′ of proclavaminic acid (**1**) is determined and a correlation is completed to an earlier configurational analysis.[4] The stereochemical course of each clavulanic acid ring-forming reaction can now be defined and suggests possible mechanisms of β-lactam formation distinct from those proposed for nocardicin (**6**)[5] and penicillin.[6]

Access to the stereospecifically 4′-deuteriated proclavaminates **1a** and **1b** was achieved through (4*R*)-(phenylthio)azetidinone

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