transition and main transition. It is also interesting to note that while T_{pt} and T_m are affected to a substantial extent by the deuteriation of the acyl chains, the T_s is independent of such deuteriation.[31] This implies that while the mobility and packing of the acyl chains change greatly in the subtransition, it could be controlled by a factor other than the acyl chains. On the basis of ^{31}P NMR and X-ray studies, Fuldner[32] suggested that during the subtransitions a conformational change in the head-group region could possibly accompany the parallel changes in the lateral acyl chain packing and the rate of reorientation of the head group. The importance of such a conformational change (i.e., a change in the orientation of the O-P-O group and therefore the whole head group) is strongly supported by the large configurational effect on the subtransition properties of DPPsC.

Chiral Discrimination in DPPsC Membranes. From the stereochemical point of view, chiral discrimination is expected to be most important in the more condensed phases with crystalline character. The large configurational effect on the subtransition not only supports the tight packing in the subphase but also suggests that the packing may involve a stereospecific interaction between the phosphate group and the neighboring choline group. Such an intermolecular interaction has been well documented by nuclear Overhauser effects in ^{31}P NMR for small unilamellar vesicles of DPPC,[33] DPPsC,[18] and other phospholipids.[34,35] Our results could lead to a hypothesis that in the subphase the quaternary ammonium ion interacts more favorably with one of the two diastereotopic oxygen atoms at the phosphate group of DPPC. Such a possibility warrants detailed investigation by ^{31}P NMR, ^{2}H NMR, FT-IR, X-ray diffraction, etc., on the isomers of DPPsC. Several of these studies have already demonstrated discernible differences between isomers of DPPsC in the liquid crystalline phase.[18-20] The results on the subphase should provide a structural basis for the DSC results presented above.

Experimental Section

Materials. DPPC was purchased from Avanti and was used without further purification. (R_P + S_P)-DPPsC was synthesized chemically and separated into R_P and S_P isomers based on the stereospecific hydrolysis of (R_P)-DPPsC by bee venom phospholipase A_2 as described previously.[14,15] In order to obtain high diastereomeric purity of (R_P)-DPPsC, the phospholipase A_2 reaction was quenched with EDTA before 80% of (R_P)-DPPsC was hydrolyzed, and the resulting lyso-DPPsC was reacylated. The unreacted DPPsC from the phospholipase A_2 reaction was further digested exhaustively with phospholipase A_2 to give pure (unreacted) (S_P)-DPPsC. The diastereomeric purity was then determined by ^{31}P NMR in CD_3OD on a Bruker WM-300 NMR spectrometer. Both isomers obtained by the above procedure were considered >99% in diastereomeric purity since no contaminating isomer was detectable when the signal/noise ratio was >100. The chemical purity of lipid samples was monitored by ^{1}H NMR at 200 MHz on a Bruker WP-200 NMR spectrometer and by TLC on silica gel (EM Science, silica gel 60 F-254) with the solvent system $CHCl_3/CH_3OH/H_2O$, 66:33:4, with visualization by phosphomolybdic acid or I_2 vapor. The R_f values of DPPsC and DPPC were 0.5 and 0.25, respectively. Final purification of DPPsC was accomplished by six to seven precipitations from acetone/ethanol (ca. 10:1, v/v).

DSC Studies. DSC traces were obtained on a MicroCal scanning microcalorimeter Model MC-1 (Amherst, MA). Phospholipid samples dried in vacuo (12 h, 50 °C) were weighed and transferred to the calorimeter cell with chloroform. The chloroform was then removed in vacuo overnight at 50 °C, and the phospholipid was suspended in 20 mM Pipes buffer, pH 7.4, by incubation at 50-60 °C for 10 min with occasional shaking. This means of sample preparation gave reproducible DSC traces. All samples consisted of 2-6 mg of lipid in triply distilled water (5% wt/wt). Scanning rates for the pretransitions and main transitions were approximately 17 °C/h. Phase-transition enthalpies were determined by cutting and weighing the papers and were estimated to be accurate to ±10%.

In subtransition studies, samples prepared as described above were incubated at 0 °C by storage on ice in a refrigerator for the specified time periods. Scanning rates were 27-29 °C/h for subtransition studies.

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Synthesis of "Iso-EPSP" and Evaluation of Its Interaction with Chorismate Synthase

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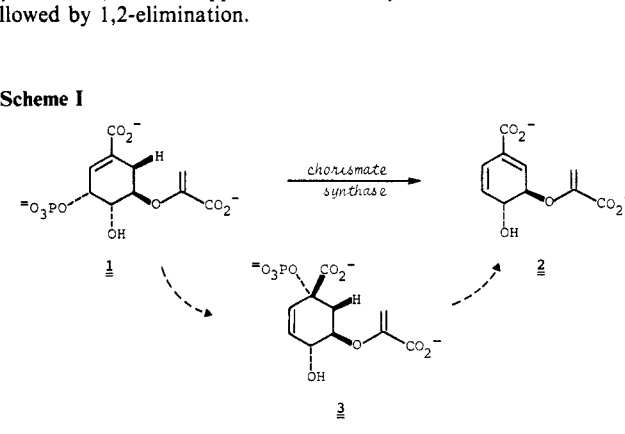
Abstract: A synthesis of "iso-EPSP" (**3**), the allylic phosphate isomer of EPSP (**1**), has been developed, starting with (-)-quinic acid. A key intermediate is the differentially protected triol **7**. Iso-EPSP is not an alternative substrate for chorismate synthase isolated from *Neurospora crassa*, although it is a good inhibitor (K_i = 8.7 μM). It thus appears that the enzymatic conversion of EPSP to chorismate does not involve allylic rearrangement followed by 1,2-elimination.

The shikimate-chorismate biosynthetic pathway is mediated by a number of enzymes which catalyze unique or unusual transformations.[1] One of these enzymes, chorismate synthase, catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP, **1**) to chorismate (**2**) in a process which is formally a trans-1,4-elimination (Scheme I).[2,3,4] In view of the preference which such transformations frequently show for the cis-1,4-stereochemistry,[5] a number of mechanisms other than direct

Scheme I



elimination have been suggested for the enzymatic process.[1,3] An intriguing possibility that has been proposed by Ganem involves

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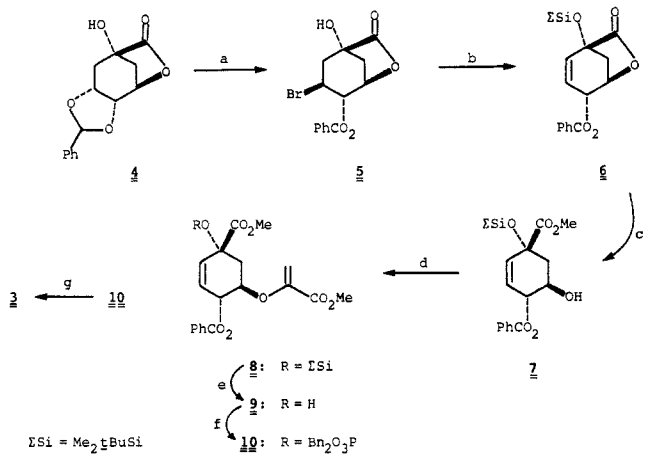
Scheme II[a]



[a] (a) NBS, AIBN, benzene, Δ, 86%. (b) DBU, ΣSiCl, acetonitrile, Δ, 79%. (c) *p*-TsOH, MeOH, 90%. (d) 1, $MeO_2CCN_2CO_2Me$, $Rh_2(OAc)_4$, benzene, Δ; 2, CH_2NMe_2I, Et_3N; 3, MeI, acetonitrile, Δ, 91% overall from **6**. (e) Bu_4NF, THF, 74%. (f) LDA, $Bn_2O_3POPO_3Bn_2$, THF. (g) Me_3SiBr, then aqueous NaOH, 43% overall from **9**.

initial suprafacial 3,3-rearrangement to the allylic isomer **3**, followed by trans-1,2-elimination.[1] Such allylic rearrangements have in fact been shown to intervene in enzymatic cyclizations of geranyl and farnesyl pyrophosphates.[6] We now report a synthesis of iso-EPSP, starting with quinic acid, and an investigation of this compound as an alternative substrate for the chorismate synthase isolated from *N. crassa*.

The benzylidene lactone **4** derived from (−)-quinic acid[7] is converted to the bromobenzoate **5** with *N*-bromosuccinimide (Scheme II).[8] Dehydrobromination of this material is most efficiently carried out with concomitant silylation, to provide the allylic benzoate **6**. Although we were unable to open the lactone selectively under basic conditions, treatment of **6** with *p*-toluenesulfonic acid in methanol at 50 °C provides the desired, differentially protected triol **7**. Introduction of the enolpyruvyl ether is accomplished according to Ganem's protocol to give **8**,[9] and the silyl group is removed to provide tertiary alcohol **9** in 39% overall yield from quinic acid. This hindered hydroxyl group is phosphorylated by reaction of the lithium alkoxide with tetrabenzyl pyrophosphate.[10] The phosphate triester **10** is sensitive to solvolysis and difficult to purify; hence it is deprotected immediately, first by cleavage of the phosphate benzyl esters with trimethylsilyl bromide[11] and then by hydrolysis of the carboxylate esters with sodium hydroxide. The resulting product can be purified by chromatography on DEAE Sephadex A-25 (eluting with a gradient of $Et_3NH^+HCO_3^-$, pH 8.2) and ion exchange on Dowex AG1-X8 (Na^+ form) to give the tetrasodium salt of iso-EPSP, **3**. Final purification by reverse-phase HPLC afforded the analytically pure trihydrate, $[\alpha]_D$ −86°.

To explore the possibility that iso-EPSP lies along the reaction path catalyzed by chorismate synthase, this material was incubated with the purified enzyme isolated from *N. crassa*.[12] Using

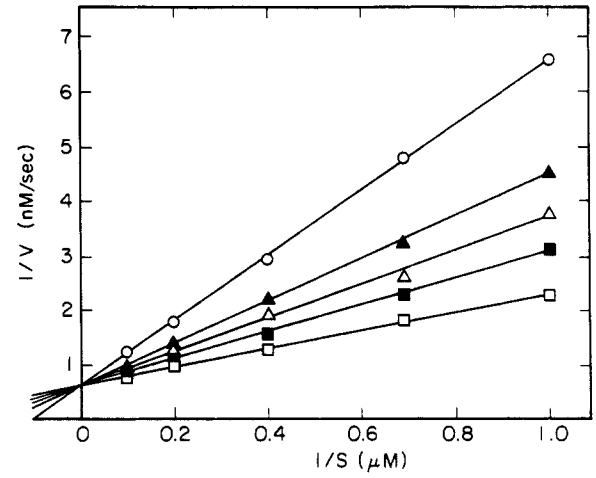


Figure 1. Inhibition of chorismate synthase by iso-EPSP, **3**. Assays were conducted at 29.6 °C, pH 7.8 (Tris·HCl), and included in a final volume of 2.0 mL 3.14 pkat of chorismate synthase, 440 pkat of anthranilate synthase, 10 μM FMN, 44 μM NADPH, 5 mM glutamine, 50 mM Tris buffer, 50 mM KCl, and 2.5 mM $MgCl_2$, in addition to substrate EPSP (**1**) and iso-EPSP (**3**); reaction velocity was monitored by the increase in fluorescence (excitation at 313 nm, emission at 390 nm).[12] Inhibitor concentrations: (□) no inhibitor, (■) 1 μM, (△) 2.5 μM, (▲) 5 μM, and (○) 10 μM.

conditions under which a rate of conversion 0.5% that of EPSP itself would have been observed, we were unable to detect any synthesis of chorismate from the allylic isomer. This material is not excluded from the active site of the enzyme, however, since it is a competitive inhibitor with an affinity comparable to that of the substrate: K_i(iso-EPSP) = 8.7, K_m(EPSP) = 2.7 μM (Figure 1). The most straightforward interpretation of these results is that iso-EPSP does not lie along the enzymatic reaction pathway, although the possibility remains that productive binding of iso-EPSP cannot occur from the exterior of the protein. Whether the enzymatic transformation involves a direct 1,4-elimination or an alternative two-step process via an enzyme-linked intermediate[3] will have to be determined by additional experiments.

Experimental Section

General. All reactions involving moisture-sensitive reagents were performed under a dry nitrogen atmosphere. THF, hexane, and diethyl ether were distilled from sodium/benzophenone, diisopropylamine was distilled under nitrogen from KOH, and acetonitrile and CH_2Cl_2 were distilled from CaH_2.

Unless otherwise indicated, NMR and IR data were obtained in $CDCl_3$ and $CHCl_3$ solutions, respectively. ^1H NMR data (200 or 250 MHz) are reported as follows: chemical shift on the δ scale (multiplicity, number of hydrogens, coupling constant(s) in hertz). The ^1H NMR and ^{13}C NMR references in D_2O solution were residual HOD as δ 4.63 and Me_2SO as δ 42.50, respectively. ^{13}C NMR (50 MHz) chemical shifts are reported relative to solvent $CDCl_3$ as δ 77.0; ^{31}P NMR (81.8 MHz) chemical shifts are reported relative to trimethyl phosphate (internal capillary) as δ 3.086 (downfield positive).

In general, reaction workups culminated in drying the organic phase over $MgSO_4$, filtering, and removing the solvent on a rotary evaporator under reduced pressure. Column chromatography was performed according to the method of Still[13] using the adsorbant and eluting solvent indicated.

1(*R*)-4-*exo*-(Benzoyloxy)-3-*endo*-bromo-1-hydroxy-6-oxabicyclo-[3.2.1]octan-7-one (5). A solution of 2.00 g (7.63 mmol) of acetal **4**,[7] 1.40 g (7.87 mmol) of *N*-bromosuccinimide, and 10 mg (0.061 mmol) of AIBN in 125 mL of anhydrous benzene was heated at reflux for 1 h. The mixture was cooled to 21 °C, diluted with CH_2Cl_2, and washed with 20% aqueous $NaHSO_3$ and saturated Na_2CO_3. Evaporation of the solvent afforded 2.52 g of crude product that was purified by chromatography (9:1 CH_2Cl_2/Et_2O) to give 2.23 g (86% yield) of bromobenzoate **5**: mp 144–146 °C; $[\alpha]^{21}_D$ +54.2° (*c* 1.00, CH_2Cl_2); IR 1095, 1110, 1130, 1265, 1733, 1800, 3560 cm^{-1}; ^1H NMR δ 2.56 (m, 3), 2.80 (dd, 1, *J* = 7.1, 15), 3.10 (br s, 1), 4.48 (d, 1, *J* = 6.9), 5.03 (dd, 1, *J* = 4.5, 4.2), 5.67 (d,

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1, J = 3.9), 7.49 (dd, 2, J = 7.8, 7.8), 7.65 (dd, 1, J = 7.5, 7.5), 8.02 (d, 2, J = 7.2); ^{13}C NMR δ 37.54, 40.65, 41.77, 70.65, 71.31, 74.75, 128.4, 128.7, 129.8, 134.1, 164.5, 177.9. Anal. Calcd for $C_{14}H_{13}O_5Br$: C, 49.29; H, 3.84; Br, 23.42. Found: C, 49.28; H, 3.77; Br, 23.21.

1(*R*)-4-*exo*-(Benzoyloxy)-1-[[(1,1-dimethylethyl)dimethylsilyl]oxy]-6-oxabicyclo[3.2.1]oct-2-en-7-one (6). To a solution of 2.11 g (6.19 mmol) of bromobenzoate **5** and 1.21 g (8.05 mmol) of *tert*-butyldimethylsilyl chloride in 50 mL of CH_3CN was added 2.41 mL (16.1 mmol) of diazabicycloundecene (DBU). The mixture was heated at reflux for 16 h, cooled to 21 °C, and diluted with CH_2Cl_2. After the mixture was washed with H_2O and saturated $NaHCO_3$, the solvent was removed to afford 2.89 g of crude product as a yellow solid. Chromatography (1:1 CH_2Cl_2/hexanes) of this material gave 1.82 g (79% yield) of the olefin **6** as a white solid: mp 74–75 °C; $[\alpha]^{21}_D$ −258° (c 5.7, CH_2Cl_2); IR 850, 1100, 1110, 1140, 1265, 1725, 1800, 2940, 2960 cm^{-1}; ^{1}H NMR δ 0.183 (s, 3), 0.220 (s, 3), 0.939 (s, 9), 2.41 (d, 1, J = 11), 2.52 (ddd, 1, J = 1.7, 5.7, 11), 4.83 (ddd, 1, J = 2.7, 2.7, 5.4), 5.51 (dd, 1, J = 3.2, 3.2), 5.85 (ddd, 1, J = 2.1, 3.2, 9.7), 6.28 (d, 1, J = 10), 7.50 (dd, 2, J = 7.8, 7.8), 7.61 (dd, 7.4), 8.04 (d, 2, J = 8.0); ^{13}C NMR δ −3.141, 17.91, 25.48, 38.07, 66.19, 73.24, 74.82, 123.3, 128.5, 129.0, 129.7, 133.5, 140.1, 165.1, 174.9. Anal. Calcd for $C_{20}H_{26}O_5Si$: C, 64.14; H, 6.99; Si, 7.49. Found: C, 64.08; H, 7.07.

Methyl [1(*R*)-(1α,4α,5β)]-4-(Benzoyloxy)-1-[[(1,1-dimethylethyl)dimethylsilyl]oxy]-5-hydroxy-2-cyclohexene-1-carboxylate (7). A solution of 1.27 g (3.39 mmol) of lactone **6** and 64 mg (0.34 mmol) of *p*-toluenesulfonic acid in 80 mL of anhydrous methanol was heated at 53 °C for 6 h. The mixture was partitioned between CH_2Cl_2 and saturated $NaHCO_3$, and the aqueous phase was extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and evaporated to give 1.38 g of product that was purified by chromatography (1.5:1 hexanes/ether) to afford 1.23 g (90% yield) of methyl ester **7**: $[\alpha]^{21}_D$ −78.7° (c 10.2, CH_2Cl_2); IR (film) 840, 1030, 1050, 1070, 1100, 1110, 1260, 1320, 1720, 2940, 2960, 3500 cm^{-1}; ^{1}H NMR δ 0.090 (s, 3), 0.130 (s, 3), 0.890 (s, 9), 2.16 (dd, 1, J = 11, 13), 2.32 (ddd, 1, J = 1.1, 3.9, 14), 2.97 (d, 1, J = 3.9), 3.77 (s, 3), 4.29 (ddd, 1, J = 3.9, 7.7, 15), 5.48 (ddd, 1, J = 2.1, 2.1, 7.6), 5.86 (dd, 1, J = 2.2, 10), 5.99 (d, 1, J = 10), 7.46 (dd, 2, J = 7.2, 7.2), 7.59 (dd, 1, J = 7.2, 7.2), 8.06 (d, 2, J = 7.2); ^{13}C NMR δ −3.316, −3.058, 18.16, 25.56, 40.68, 52.35, 67.45, 74.96, 75.91, 128.3, 128.4, 129.6, 129.7, 130.8, 132.2, 166.9, 173.7. Anal. Calcd for $C_{21}H_{30}O_6Si$: C, 62.04; H, 7.43; Si, 6.90. Found: C, 61.69; H, 7.47.

Methyl [1(*R*)-(1α,4α,5β)]-4-(Benzoyloxy)-1-[[(1,1-dimethylethyl)dimethylsilyl]oxy]-5-[bis(methoxycarbonyl)methoxy]-2-cyclohexene-1-carboxylate. A solution of 963 mg (2.37 mmol) of alcohol **7**, 449 mg (2.84 mmol) of dimethyl diazomalonate, and 10 mg (0.024 mmol) of rhodium acetate in 65 mL of anhydrous benzene was heated at reflux for 2.5 h. The solvent was removed, and the crude product was purified by chromatography (1.5:1 hexanes/ether) to give 956 mg (75% yield) of the alkoxymalonate: $[\alpha]^{21}_D$ −95.3° (c 2.05, CH_2Cl_2); IR (film) 1030, 1120, 1140, 1210, 1270, 1750, 2980 cm^{-1}; ^{1}H NMR δ 0.086 (s, 3), 0.130 (s, 3), 0.900 (s, 9), 2.25 (dd, 1, J = 12, 13), 2.43 (m, 1), 3.76 (s, 3), 3.79 (s, 3), 3.83 (s, 3), 4.20 (ddd, 1, J = 3.8, 8.0, 12), 4.76 (s, 1), 5.69 (ddd, 1, J = 1.9, 1.9, 8.0), 5.84 (dd, 1, J = 2.1), 5.96 (d, 1, J = 9.9), 7.47 (dd, 2, J = 7.3, 7.3), 7.59 (dd, 1, J = 7.4, 7.4), 8.04 (d, 2, J = 7.3); ^{13}C NMR δ −3.384, −2.926, 18.24, 25.61, 38.92, 52.47, 52.57, 52.89, 74.37, 75.11, 77.07, 79.07, 128.4, 129.6, 129.7, 130.5, 133.2, 165.7, 166.6, 166.9, 173.1. Anal. Calcd for $C_{26}H_{36}O_{10}Si$: C, 58.19; H, 6.76; Si, 5.23. Found: C, 57.99; H, 6.83.

Methyl [1(*R*)-(1α,4α,5β)]-4-(Benzoyloxy)-1-[[(1,1-dimethylethyl)dimethylsilyl]oxy]-5-[[1-(methoxycarbonyl)ethenyl]oxy]-2-cyclohexene-1-carboxylate (8). To a solution of 845 mg (1.57 mmol) of the malonate described above and 0.29 mL (2.05 mmol) of triethylamine in 50 mL of CH_2Cl_2 was added 350 mg (1.89 mmol) of solid *N*,*N*-dimethylmethyleneammonium iodide.[14] The yellow mixture was stirred at 21 °C for 21 h, diluted with CH_2Cl_2, and washed with H_2O and saturated Na_2CO_3. The solution was dried and evaporated to afford 895 mg (96% yield) of the Mannich base that was of sufficient purity to be carried on to the next step: IR (film) 840, 1040, 1070, 1100, 1140, 1200, 1265, 1320, 1440, 1460, 1735, 1745, 1775, 2880, 2970 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.087 (s, 3), 0.123 (s, 3), 0.901 (s, 9), 2.20 (s, 6), 2.27 (m, 1), 2.49 (dd, 1, J = 3.2, 13), 2.82 (d, 1, J = 4.5), 2.92 (d, 1, J = 14), 3.61 (s, 3), 3.75 (s, 3), 3.76 (s, 3), 4.65 (ddd, 1, J = 3.3, 6.6, 13), 5.73 (ddd, 1, J = 1.2, 2.6, 7.9), 5.86 (dd, 1, J = 2.9, 10), 6.01 (d, 1, J = 9.9), 7.45 (dd, 1, J = 7.3, 7.3), 7.57 (dd, 1, J = 7.2, 7.2), 8.07 (d, 2, J = 7.0).

A solution of the Mannich base and iodomethane (0.94 mL, 1.51 mmol) in 50 mL of CH_2Cl_2 was heated at reflux for 6 h. After removal of the solvent, the crude quaternary ammonium salt was treated with 50 mL of CH_3CN and heated at reflux for 21 h. The mixture was diluted with CH_2Cl_2 and washed with H_2O, and the solvent was evaporated to give 800 mg of crude product. Purification by chromatography (2.3:1 hexanes/ether) gave 702 mg (95% yield) of enol ether **8**: $[\alpha]^{21}_D$ −120° (c 3.75, CH_2Cl_2); IR (film) 845, 1040, 1080, 1115, 1180, 1210, 1270, 1335, 1735, 1745, 2950, 2970 cm^{-1}; ^{1}H NMR δ 0.118 (s, 3), 0.142 (s, 3), 0.924 (s, 9), 2.17 (dd, 1, J = 3.1, 12), 2.50 (ddd, 1, J = 1.4, 3.4, 14), 3.70 (s, 3), 3.77 (s, 3), 4.75 (ddd, 1, J = 3.4, 8.0, 15), 4.93 (d, 1, J = 2.6), 5.48 (d, 1, J = 2.5), 5.82 (ddd, 1, J = 1.9, 1.9, 8.0), 5.91 (dd, 1, J = 2.1, 9.8), 6.03 (d, 1, J = 9.9), 7.44 (dd, 1, J = 7.2, 7.2), 7.57 (dd, 1, J = 7.3, 7.3), 8.02 (d, 2, J = 7.3); ^{13}C NMR δ −3.419, −2.916, 18.16, 25.56, 37.19, 52.14, 52.40, 72.56, 73.80, 74.96, 96.90, 128.3, 129.6, 129.7, 130.5, 133.0, 149.9, 163.3, 165.7, 172.8. Anal. Calcd for $C_{25}H_{34}O_8Si$: C, 61.20; H, 6.98; Si, 5.72. Found: C, 61.26; H, 7.01.

Methyl [1(*R*)-(1α,4α,5β)]-4-(Benzoyloxy)-1-hydroxy-5-[[1-(methoxycarbonyl)ethenyl]oxy]-2-cyclohexene-1-carboxylate (9). A solution of 23.0 g (46.9 mmol) of silyl ether **8** in 500 mL of THF was cooled to 0 °C and treated with 49.3 mL (49.3 mmol) of a 1 M solution of tetrabutylammonium fluoride in THF for 1 h. After the addition of 200 mL of saturated NH_4Cl, the mixture was partitioned between ether and water. The aqueous phase was extracted with three 200-mL portions of Et_2O, and the combined organic layers were dried and evaporated to give 23.2 g of crude alcohol. Purification of this material by chromatography (19:1 CH_2Cl_2/ether) afforded 13.1 g (74% yield) of the allylic alcohol **9**: mp 104–106 °C; $[\alpha]^{21}_D$ −190° (c 3.30, CH_2Cl_2); IR (film) 1070, 1110, 1170, 1200, 1270, 1330, 1625, 1740, 3500 cm^{-1}; ^{1}H NMR δ 2.33 (m, 2), 3.45 (br s, 1), 3.70 (s, 3), 3.85 (s, 3), 4.77 (ddd, 1, J = 5.1, 8.3, 11), 4.97 (d, 1, J = 2.7), 5.48 (d, 1, J = 2.7), 5.76 (ddd, 1, J = 2.0, 2.0, 8.3), 5.85 (ddd, 1, J = 2.0, 2.0, 8.3), 5.95 (dd, 1, J = 2.0, 10), 7.44 (dd, 2, J = 7.7, 7.7), 7.57 (dd, 1, J = 7.3, 7.3), 8.02 (d, 2, J = 7.0); ^{13}C NMR δ 36.25, 52.25, 53.53, 72.82, 73.00, 73.58, 97.58, 128.3, 128.7, 129.9, 133.1, 149.8, 163.4, 165.9, 174.9. Anal. Calcd for $C_{19}H_{20}O_8$: C, 60.63; H, 5.35. Found: C, 60.17; H, 5.47.

Methyl [1(*R*)-(1α,4α,5β)]-4-(Benzoyloxy)-1-[[bis(benzyloxy)phosphinyl]oxy]-5-[[1-(methoxycarbonyl)ethenyl]oxy]-2-cyclohexene-1-carboxylate (10). To a solution of 136 mg (0.361 mmol) of tertiary alcohol **9** in 8 mL of THF at −78 °C was added a solution of LDA (0.379 mmol) in 3 mL of THF precooled to 0 °C. After 15 min, a solution of tetrabenzyl pyrophosphate[10] (195 mg, 0.361 mmol) in 3 mL of THF at 0 °C was added dropwise over 15 min. The reaction mixture was stirred at −78 °C for 0.5 h and warmed to 0 °C over the course of 2 h, and the reaction was quenched with 10 mL of saturated NH_4Cl. The mixture was partitioned between H_2O and CH_2Cl_2, and the organic layer was dried and evaporated to afford 230 mg (100% yield) of labile phosphate **10**. Analysis of this material by ^{1}H NMR indicated that **10** was contaminated with 25% unreacted **9**. IR (film) 1000, 1170, 1200, 1260, 1320, 1440, 1450, 1620, 1730, 1740 cm^{-1}; ^{1}H NMR δ 2.32 (m, 1), 2.72 (ddd, 1, J = 1.2, 3.3, 14), 3.67 (s, 3), 3.78 (s, 3), 4.71 (ddd, 1, J = 3.6, 8.0, 12), 4.82 (d, 1, J = 2.8), 5.06 (d, 2, J = 7.9), 5.16 (dd, 2, J = 4.5, 7.8), 5.42 (d, 1, J = 2.8), 5.81 (ddd, 1, J = 2.1, 2.1, 7.9), 6.03 (dd, 1, J = 2.2, 10), 6.30 (d, 1, J = 10), 7.24–7.46 (m, 12), 7.55 (m, 1), 7.95 (m, 2); ^{31}P NMR δ −3.69.

Iso-EPSP Tetrasodium Salt (3). A solution of 230 mg (0.361 mmol) of tetraester **10** (contaminated with 25 mol% **9**) in 8 mL of CH_2Cl_2 at 0 °C was treated with a precooled solution of bromotrimethylsilane (200 μL, 1.52 mmol) and pyridine (162 μL, 1.99 mmol) in 3 mL of CH_2Cl_2 for 1 h. After the addition of H_2O, the aqueous layer was separated and cooled to 0 °C, and the methyl esters were saponified with 3.6 mL (3.61 mmol) of 1 N NaOH for 6 h. The mixture was extracted with $CHCl_3$, acidified to pH 8 with 3 N HCl, and lyophilized. The crude, yellow product was applied to an anion-exchange column (DEAE Sephadex A-25, HCO_3^- form) and eluted with a linear gradient of triethylammonium bicarbonate (0.0–0.5 M, pH 8.2). The fractions absorbing at 240 nm were combined and lyophilized to give 155 mg (59% yield) of the tetrakis triethylammonium salt of iso-EPSP. Cation-exchange chromatography (Dowex AG1-X8, Na^+ form) afforded 83 mg (56% yield from alcohol **9**) of the tetrasodium salt of iso-EPSP: $[\alpha]^{21}_D$ −106° (c 1.25, pH 6.5, H_2O); ^{1}H NMR δ 1.54 (m, 1), 2.33 (d, 1, J = 13), 4.23 (m, 2), 5.00 (d, 1, J = 2.2), 5.69 (d, 1, J = 10), 6.02 (d, 1, J = 10); ^{13}C NMR δ 39.77 (d, J = 7.8), 74.33, 79.80, 82.82 (d, J = 5.8), 96.16, 133.0, 135.2, 157.2, 174.8; ^{31}P NMR δ −1.51.

Final purification of the product was achieved by reverse-phase HPLC. A Whatman Partisil 10 ODS-3 column (0.9 × 50 cm) was employed using 1.6% (v/v) of methanol in 50 mM triethylammonium bicarbonate (pH 7.3) as the mobile phase at a flow rate of 3 mL/min. The retention time was ca. 15 min. A total of 42 mg was purified (5 mg/injection) to give after cation exchange (see above) 32.4 mg (43% overall yield from alcohol **9**) of the tetrasodium salt of iso-EPSP: $[\alpha]^{21}_D$ −86° (c 0.046, pH 7.0, H_2O); ϵ_{240} (pH 8.0) 2300 M^{-1} cm^{-1}. Anal. Calcd for $C_{10}H_9O_{10}PNa_4(H_2O)_3$: C, 25.77; H, 3.24; P, 6.62. Found: C, 26.17; H, 3.48; P, 6.60.

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Enzymatic Analysis. All enzyme assays were performed at 29.6 °C in an SLM Instruments fluorescence spectrometer. Excitation and emission wavelengths were 313 and 390 nm, respectively (both with an 8-nm bandwidth). All buffers contained 50 mM Tris·HCl, 50 mM KCl, and 2.5 mM $MgCl_2$, pH 7.80. Stock solutions were prepared as follows: FMN, 700 μM in buffer; NADPH, 880 μM in buffer; L-glutamine, 200 mM in water; substrate and inhibitor, 1.0 mM in water; anthranilate synthase, 8.8 nkat/mL in 20 mM potassium phosphate buffer (pH 7.1) containing 0.5 mM EDTA and 0.1 mM 2-mercaptoethanol; and chorismate synthase, 3.9 nkat/mL in 50 mM Tris·HCl (pH 7.5) and 0.5 mM DTT in 50% glycerol (v/v). For use in the assay, the chorismate synthase solution was diluted 25-fold with the buffer described above.

Standard curves of anthranilate concentration vs. fluorescence intensity were prepared. A full-scale deflection (25.4 cm) usually represented 0.5–5 nmol of chorismate. For the assay of chorismate synthase, the reaction mixture contained the following: chorismate synthase, 3.14 pkat; anthranilate synthase, 440 pkat; FMN, 10 μM; NADPH, 44 μM; L-glutamine, 5 mM; Tris·HCl (pH 7.80), 50 mM; KCl, 50 mM; and $MgCl_2$, 2.5 mM. For inhibition studies, iso-EPSP was also included. This mixture was preincubated at 29.6 °C for 5 min, and the reaction was started by the addition of 50 μL of a substrate solution of appropriate concentration. The total volume of the reaction mixture was always 2.00 mL. The substrate concentrations were 1.0, 1.45, 2.5, 5, and 10 μM; inhibitor concentrations were 2.5, 5, 10, and 20 μM. The initial velocities were calculated manually from the plot of fluorescence intensity vs. time. Each point was the mean of two independent assays. The analysis of the data was carried out by using the HYPER program.[15] K_m(EPSP) and K_i(iso-EPSP) were calculated to be 2.7 and 8.7 μM, respectively.

Anthranilate synthase was assayed under the conditions of the chorismate synthase assay, except that chorismate synthase was omitted and the reaction was initiated by the addition of chorismate (15 μM).

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Origin of Oxygen Atoms in Cantharidin Biosynthesized by Beetles

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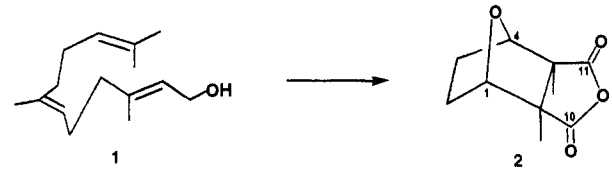
Abstract: Biosynthesis by blister beetles (Coleoptera:Meloidae) of the defensive substance cantharidin (**2**), an apparent monoterpene, proceeds by unprecedented degradation of farnesol (**1**), a sesquiterpenoid precursor. To obtain chemical insight into this transformation, we examined the origin of the four oxygen atoms in **2**. Labeling studies used adult male *Epicauta pestifera* that were exposed to either ^{18}O-enriched O_2 or H_2O. Analysis of the mass spectrometric data of the resulting **2** indicated that the tetrahydrofuranyl oxygen atom and two, but not three, of the anhydride oxygen atoms are derived from O_2, whereas the third anhydride oxygen atom comes from H_2O. Examination of maximally labeled **2** using mass spectrometry–mass spectrometry, which obviated complications owing to isotope dilution, revealed that the H_2O-derived oxygen atom is located in the anhydride ring in some molecules and in a carbonyl group in others, implicating intramolecular oxygen scrambling. Results indicate that O_2-derived incorporated oxygen atoms undergo no appreciable exchange with the medium. The possibility that **2** is a juvenile hormone metabolite is suggested.

Insects not only are the largest and most diverse group of organisms on earth but also are evolutionarily distant from vertebrates, plants, and bacteria. For these reasons, the study of insect metabolism promises to reveal novel biological chemistry. For example, use by some insects of homomevalonate to synthesize their juvenile hormone,[1] a homosesquiterpenoid, is unique among all types of organisms studied. An understanding of the unique metabolic features of insects may reveal insights regarding biological chemistry and could provide the foundation for new approaches for the control of pest insects.

Cantharidin (**2**), an apparent monoterpene that serves as a defensive substance in blister beetles,[2] is formed by an unprecedented degradation of the C_{15} farnesyl skeleton. Following early experiments,[3] an extensive series of ^{14}C radiolabeling studies[4] by Schmid and his collaborators demonstrated that the carbon atoms in **2** are derived from farnesol (**1**), which itself is derived from mevalonate in the normal manner. Intriguingly, the two terminal methyl groups of **1** are almost completely randomized during its conversion into **2**.[3,4d] Tritium labeling experiments demonstrated that all of the hydrogen atoms in **2**, with the exception of one attached to C-6, are derived from mevalonate.[3b,5] Unfortunately, knowledge of the origins of the carbon and hydrogen atoms in **2** fails to unveil chemical details regarding its biosynthesis beyond **1**.



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Information regarding the origins of the oxygen atoms in **2**, in contrast, may provide chemical insight. Utilization of molecular oxygen implicates oxygen introduction during direct oxidation, such as that typical of C–H oxidation and of olefin epoxidation. Incorporation of oxygen from water is suggestive of introduction during alternative, nonoxidative processes, such as that typical of addition of water to an olefinic functionality and of nucleophilic displacement. Also, the extent of oxygen exchange with the medium and of intramolecular oxygen scrambling may be detected by ^{18}O studies, offering evidence about the involvement or absence

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