Isopentenyl-diphosphate Isomerase: Inactivation of the Enzyme with Active-Site-Directed Irreversible Inhibitors and Transition-State Analogues†

Manfred Muehlbacher‡ and C. Dale Poulter*
Department of Chemistry, University of Utah, Salt Lake City, Utah 84112
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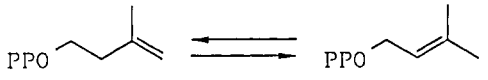
ABSTRACT: Seven analogues of isopentenyl diphosphate (**1**) and dimethylallyl diphosphate (**2**) containing fluorine, epoxy, and ammonium functional groups irreversibly inhibited isopentenyl-diphosphate:dimethylallyl-diphosphate isomerase (EC 5.3.3.2) from the mold *Claviceps purpurea*. Inactivation kinetics, substrate protection studies, and labeling experiments demonstrated that the analogues interacted stoichiometrically with the active site of the enzyme. Radioactive enzyme–inactivator complexes were stable to extended dialysis and treatment with chaotropic reagents. The complexes resulting from inactivation of isomerase by 3-(fluoromethyl)-3-buten-1-yl diphosphate (**3**) and 3,4-epoxy-3-methyl-1-butyl diphosphate (**4**) were also stable to ion-exchange chromatography and gel electrophoresis. Stoichiometric release of fluoride ion occurred during inactivation of isomerase with **3**. This observation is consistent with S_N2 or S_N2' displacement of fluorine by an active-site nucleophile with concomitant covalent attachment of the inactivator to the enzyme. 2-(Dimethylamino)ethyl diphosphate (**9**) formed a stable noncovalent complex with isomerase with $K_{dis} < 1.2 \times 10^{-10}$ M. The enzyme–inhibitor complex was stable in 6 M urea, but the inhibitor was partially released upon treatment with SDS and 2-mercaptoethanol at 37 °C for 1 h. The results indicate that **9** is a transition-state/reactive intermediate analogue where the positively charged ammonium group mimics a tertiary carbocationic species in the enzyme-catalyzed reaction.

Isopentenyl-diphosphate:dimethylallyl-diphosphate isomerase (EC 5.3.3.2) catalyzes the interconversion of isopentenyl diphosphate (**1**) and dimethylallyl diphosphate (**2**) by an anta-



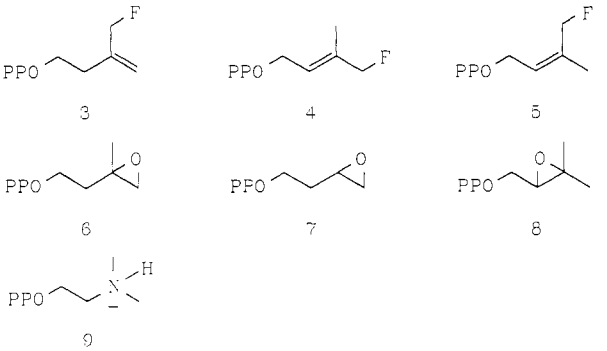
1 2

rafacial [1.3] transposition of hydrogen (Poulter & Rilling, 1981a). During the reaction a proton derived ultimately from water is added to the carbon–carbon double bond, and a carbon-bound proton is lost to water. Under normal metabolic conditions **1** is isomerized to **2**. This is a major activation step in the pathway that transforms the unreactive homoallylic substrate into its highly reactive allylic isomer. Dimethylallyl diphosphate is, in turn, the source of electrophilic isoprenoid residues utilized in all subsequent prenyl-transfer reactions (Poulter & Rilling, 1981b). The stereochemistry of the reaction is consistent with a mechanism involving two bases, one of which is in the conjugate acid form, to assist in introduction and removal of protons (Clifford et al., 1971). Two limiting possibilities for the initial step consistent with solvent isotope exchange experiments are protonation of the double bond to generate a tertiary carbocation or removal of an allylic hydrogen to produce an allylic carbanion (Agranoff et al., 1960; Shah et al., 1965; Stone et al., 1969).

Two mechanistic probes have been used to provide evidence about reactive intermediates generated during catalysis by isoprenoid enzymes. Ammonium and sulfonium analogues of carbocationic intermediates are potent inhibitors of several enzymes in the pathway, including squalene synthetase (Sandifer et al., 1982), bornyl-diphosphate cyclase (Croteau et al., 1986), and sterol methylases (Narula et al., 1981; Rahier et al., 1984, 1985). Recently, we (Muehlbacher & Poulter, 1985) and Reardon and Abeles (1985, 1986) reported that 2-(dimethylamino)ethyl diphosphate, an ammonium analogue of the tertiary carbocation proposed for a cationic mechanism, was a powerful inhibitor of isomerase.

Fluorinated substrate analogues have also been extremely useful in mechanistic studies. Isosteric substitution of hydrogen by electron-withdrawing fluorines exerts a dramatic decrease in the reactivity of substrates by increasing the transition state for formation of carbocations. Linear free energy correlations for farnesyl-diphosphate synthetase (Poulter et al., 1981) and dimethylallyltryptophan synthetase (Woodside, 1987) with appropriate models indicated that these reactions proceeded through carbocationic intermediates. Reardon and Abeles recently reported that 3-(trifluoromethyl)-3-butenyl diphosphate was not a substrate for isomerase as further support for the cationic mechanism in isomerase (Reardon & Abeles, 1986).

We also wanted to study the effect of fluorine substitution on the isomerization and prepared fluoromethyl analogues of **1** and **2** for a linear free energy study. To our surprise, allylic fluorides **3–5** were not substrates for the normal isomerization



but instead were potent active-site-directed irreversible inhibitors of isomerase. We now report a full account of our studies with isomerase from *Claviceps purpurea* and active-site-directed irreversible inhibitors with fluoro and epoxide moieties incorporated into isopentenyl (**3**, **6**, **7**) or dimethylallyl

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‡ University of Utah Graduate Research Fellow, 1984–85.

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(**4**, **5**, **8**) structures and **9**, a transition-state/reactive intermediate analogue.

Experimental Procedures

General. ^{1}H and ^{13}C NMR[1] spectra are reported in parts per million downfield from either internal TMS or DSS, ^{19}F spectra in parts per million upfield from trichlorofluoromethane, and ^{31}P spectra in parts per million downfield from external phosphoric acid. NMR spectra were obtained in either deuteriochloroform or deuterium oxide (Aldrich Chemical Co.). Infrared spectra were calibrated to the 1602-cm^{-1} absorption of polystyrene, and all absorptions were reported in wavenumbers (cm^{-1}). Silica gel flash chromatography was performed on grade 60, 235–400 mesh silica gel (Aldrich), and TLC was on silica gel 60 F-254 glass plates (American Scientific Products). Silica TLC plates were visualized under UV light, by iodine, or by dipping in a 10% solution of phosphomolybdic acid in ethanol followed by heating. Cellulose flash chromatography was performed on Whatman CF-11 fibrous cellulose, and cellulose TLC was on 0.1-mm glass plates (American Scientific Products). Cellulose TLC plates were visualized by iodine or sulfosalycylic acid–ferric chloride stain (Salame, 1964).

All steps in enzyme purifications were at 4 °C except for HPLC chromatography, which was at room temperature. Plastic ware was used exclusively. Dialysis was performed in Spectrapor dialysis bags (25.5 mm, M_r cutoff 6000–8000) or in a Micro-ProDicon Model 115 forced dialysis concentrator using ProDimem PA-10 dialysis membranes (M_r cutoff, 10 000). Concentration of protein solutions was performed in an Amicon stirred ultrafiltration cell (50-mL capacity) using a PM-10 filter (M_r cutoff 10 000). Isomerase–inhibitor complexes were concentrated by spinning material in microconcentrators (Cetricon-10, Amicon Corp., M_r cutoff 10 000) at 5400*g* at 4 °C. SDS–polyacrylamide gel electrophoresis of proteins employed the Laemmli discontinuous buffer system (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R or silver nitrate (Morril et al., 1981).

Reagents. Dowex AG 50W-X8 cation-exchange resin (100–200 mesh) was purchased from Bio-Rad. Microcrystalline DE-52 was from Whatman and Fractogel TSK DEAE 650S from Merck. Reagent grade hexanes were purified by acid and base washes, filtration through neutral alumina, and distillation from glass. Reagent grade anhydrous diethyl ether, tetrahydrofuran, and purified hexanes were dried over lithium aluminum hydride and distilled from sodium metal/benzophenone ketal. Reagent grade acetonitrile and dichloromethane were distilled from anhydrous phorphorus pentoxide. All solvents for chromatography were of reagent grade and glass distilled prior to use, except for acetonitrile and 2-propanol used for cellulose flash chromatography. These solvents were used without purification. Disodium dihydrogen pyrophosphate, ammonium bicarbonate, magnesium chloride, dithiothreitol, 2-mercaptoethanol, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid, phenylmethanesulfonyl fluoride, sodium dodecyl sulfate, acrylamide (monomer), acrylamide (dimer), leupeptin, and proteins for molecular weight markers were purchased from Sigma Chemical Co. *p*-Toluenesulfonyl chloride was purchased from J. T. Baker Co. and recrystallized from cold diethyl ether. Ultrapure ammonium sulfate was purchased from Schwarz/Mann. All other reagents used for synthesis were from Aldrich.

[1-^{14}C]Isopentenyl diphosphate purchased from Amersham was used directly or diluted to a specific activity of 10 μCi/μmol with synthetic material. (*R*)- and (*S*)-2-fluoroisopentenyl diphosphate (**28**) was provided by Dr. V. M. Dixit, University of Utah. [1-^{3}H]-(*E*)-3-(Fluoromethyl)-2-buten-1-yl diphosphate (**4**) and [1-^{3}H]-(*Z*)-3-(fluoromethyl)-2-buten-1-yl diphosphate (**5**) were prepared by Dr. A. Woodside (Woodside, 1987). 3-(Fluoromethyl)-3-buten-1-yl diphosphate (**3**), 4-fluoro-3-methyl-1-butyl diphosphate (**29**), and 2-(dimethylamino)-1-ethyl diphosphate (**9**) were prepared as described previously (Davisson et al., 1986a). INSTAFLUOR and INSTAGEL were obtained from Packard Instrument Co.

Isopentenyl-diphosphate:dimethylallyl-diphosphate isomerase was isolated from the mycelia of *C. purpurea* 26245. The strain was obtained from the American Type Culture Collection (ATCC). Mycelia of *Claviceps* were grown in standing cultures for 6–7 days, harvested, lyophilized, and stored at −70 °C until needed (Lee et al., 1976; Cress et al., 1981).

Synthesis of Inhibitors

4-[(tert-Butyldimethylsilyl)oxy]-1-fluoro-2-butanol (**10**). In a 500-mL Parr hydrogenation flask were placed 0.3 g of palladium/carbon (9%), 250 mL of absolute ethanol, and 20.0 g (103 mmol) of 4-(benzyloxy)-1-fluoro-2-butanol (Muehlbacher & Poulter, 1988). The mixture was shaken under an atmosphere of hydrogen at 40 psi for 3 h. The solution was filtered through Celite and concentrated in vacuo to afford 10.8 g (97%) of a viscous oil. The residue was dissolved in a solution of 15.05 g (100 mmol) of *tert*-butyldimethylsilyl chloride and 7.00 g (250 mmol) of imidazole in 150 mL of dry dimethylformamide. The mixture was stirred at room temperature for 16 h. Water (300 mL) was added, and the resulting solution was extracted with five 100-mL portions of diethyl ether. The combined organic layers were washed with 100 mL of water and 100 mL of brine. The solution was dried over magnesium sulfate, and solvent was removed in vacuo to afford 20.0 g (90%) of a clear liquid: R_f = 0.35 [4:1 (v/v) hexanes:ethyl acetate]; IR (CCl_4) 3600, 3485, 2950, 2925, 2880, 2853, 1465, 1457, 1382, 1355, 1250, 1070, 1015, 826 cm^{-1}; ^{1}H NMR (90 MHz, $CDCl_3$) δ 0.12 (6 H, s, H at $SiCH_3$'s), 0.95 (9 H, s, H at *tert*-butyl CH_3's), 1.72 (2 H, q, $J_{H,H}$ = 5.1 Hz, H at C3), 3.37 (2 H, s(br), OH), 3.73–4.33 (3 H, m, H at C1 and H at C4), 4.00–4.33 (1 H, m, H at C1), 4.63 (1 H, m, H at C4); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ −5.55, 18.13, 25.82, 34.05 (d, $J_{C,F}$ = 5.7 Hz), 61.27, 69.75 (d, $J_{C,F}$ = 20.2 Hz), 86.52 (d, $J_{C,F}$ = 169.2); ^{19}F NMR (282 MHz, $CDCl_3$) δ 228.6 (1 F, dt, $J_{H,F}$ = 18.7 Hz, $J_{H,F}$ = 47.0 Hz); MS (EI), *m/z* (rel intensity) 75 (100.0), 105 (71.5), 145 (8.9), 147 (9.8), 165 (9.5) (M −*tert*-butyl), 185(2.4), 189(4.5) (M − CH_3 − H_2O). Anal. Calcd for $C_{10}H_{23}FO_2Si$: C, 54.01; H, 10.42. Found: C, 53.93; H, 10.43.

4-[(tert-Butyldimethylsilyl)oxy]-1-fluoro-2-butanone (**11**). A solution of methylene chloride (200 mL, dried over calcium hydride) and oxalyl chloride (12.43 g, 98 mmol) was placed in a flask equipped with an overhead mechanical stirrer and two pressure-equalizing dropping funnels, one containing a solution of 15.3 g ('96 mmol) of dimethyl sulfoxide in 50 mL of methylene chloride and the other containing 19.8 g (89 mmol) of 4-[(*tert*-butyldimethylsilyl)oxy]-1-fluoro-2-butanol (**10**) in 70 mL of methylene chloride. Dimethyl sulfoxide was

[1] Abbreviations: NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; MS, mass spectrometry; EI, electron impact; IPP, isopentenyl diphosphate; IR, infrared; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; TMS, tetramethylsilane; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; THP, tetrahydropyran.

[2] ^{1}H decoupled.

added to the stirred oxalyl chloride solution at −50 to −60 °C over a period of 30 min. The alcohol was added slowly over a period of 30 min, and the mixture was stirred at −60 °C for an additional 15 min. Trimethylamine (45.06 g, 445 mmol) was added, the reaction mixture was allowed to warm to room temperature (ca. 2.5 h), and stirring was continued for an additional 2 h. The yellow slurry was diluted with 300 mL of diethyl ether and extracted with three 100-mL portions of diethyl ether. The ether extracts were combined, washed with 100 mL of water, and dried over magnesium sulfate. Solvent was removed in vacuo, and the residual dark brown oil was purified by flash chromatography [4:1 (v/v) hexanes:ethyl acetate] to afford 15.6 g (80%) of a slightly yellow liquid: R_f = 0.35 [4:1 (v/v) hexanes:ethyl acetate]; IR (CCl_4) 2945, 2925, 2880, 2850, 1725, 1464, 1456, 1250, 1091, 1042, 830 cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$) δ 0.06 (6 H, s, H at $SiCH_3$'s), 0.91 (9 H, s, H at *tert*-butyl CH_3's), 2.71 (2 H, dt, $J_{H,F}$ = 3.0 Hz, $J_{H,H}$ = 6.3 Hz, H at C3), 3.95 (2 H, t, $J_{H,H}$ = 6.3 Hz, H at C4), 4.86 (4 H, d, $J_{H,F}$ = 48.0 Hz, H at C1); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ −5.53, 18.21, 25.82, 41.42, 58.41 (d, $J_{C,F}$ = 22.8 Hz), 85.55 (d, $J_{C,F}$ = 184.7 Hz), 205.29 (d, $J_{C,F}$ = 18.1 Hz); ^{19}F NMR (282 MHz, $CDCl_3$) δ 227.7 (1 F, tt, $J_{H,F}$ = 2.4 Hz, $J_{H,F}$ = 47.6 Hz); MS (EI), *m/z* (rel intensity) 79 (100.0), 107 (32.5), 133 (23.3), 163 (42.5) (M − *tert*-butyl).

Anal. Calcd for $C_{10}H_{21}FO_2Si$: C, 54.51; H, 9.60. Found: C, 54.48; H, 9.66.

1-[(tert-Butyldimethylsilyl)oxy]-3-(fluoromethyl)-3-butene (**12**). In a flame-dried flask were placed 27.81 g (78 mmol) of triphenylphosphonium bromide and 150 mL of dry tetrahydrofuran. The flask was cooled to −78 °C, and 50.2 mL of a 1.55 M *n*-butyllithium solution in hexanes was added by syringe over a period of 20 min. The yellow suspension was allowed to warm to room temperature, and stirring was continued for 2 h, during which time the phosphonium salt dissolved. The red-brown solution was cooled again to −78 °C, and a solution of 15.6 g (71 mmol) of 4-[(*tert*-butyldimethylsilyl)oxy]-1-fluoro-2-butanone (**11**) in 100 mL of dry tetrahydrofuran was added. The solution was allowed to warm to room temperature, 400 mL of pentanes was added, and the resulting sticky precipitate was removed by filtration through a plug of glass wool. The filtrate was concentrated in vacuo, and the residue was purified by flash chromatography [9:1 (v/v) hexanes:ethyl acetate] to yield 12.1 g (79%) of a clear liquid: R_f = 0.65 [4:1 (v/v) hexanes:ethyl acetate]; IR (CCl_4) 3075, 2950, 2935, 2885, 2855, 1467, 1457, 1373, 1356, 1250, 1095, 1000, 905, 832 cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$) δ 0.07 (6 H, s, H at $SiCH_3$'s), 0.90 (9 H, s, H at *tert*-butyl CH_3's), 2.31 (2 H, t, $J_{H,H}$ = 6.0 Hz, H at C2), 3.75 (2 H, t, $J_{H,H}$ = 6.0 Hz, H at C4), 4.83 (2 H, d, $J_{H,F}$ = 46.5 Hz, H at C3′), 5.09 (2 H, m, H at C4); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ −5.43, 18.22, 25.84, 35.85, 62.08, 85.44 (d, $J_{C,F}$ = 167.1 Hz), 113.77 (d, $J_{C,F}$ = 10.9 Hz), 142.35 (d, $J_{C,F}$ = 14.5 Hz); ^{19}F NMR (282 MHz, $CDCl_3$ δ 215.8 (1 F, ddd, $J_{H,F}$ = 2.8, 1.7, 1.8 Hz, $J_{H,F}$ = 47.1 Hz); MS (EI), *m/z* (rel intensity) 67 (100.0), 73 (35.7), 77 (86.4), 89 (24.1), 107 (37.7), 115 (3.5), 119 (3.8), 133 (2.6), 145 (3.6), 147 (3.2), 161 (33.9) (M − *tert*-butyl).

Anal. Calcd for $C_{11}H_{23}FOSi$: C, 60.50; H, 10.61. Found: C, 60.63; H, 10.91.

3-(Fluoromethyl)-3-buten-1-ol (**13**). To a stirred solution of 35.8 g (114 mmol) of tetra-*n*-butylammonium fluoride trihydrate in 100 mL of tetrahydrofuran was added 12.4 g (57 mmol) of 1-[(*tert*-butyldimethylsilyl)oxy]-3-(fluoromethyl)-3-butene (**12**). Stirring was continued for 24 h. The resulting yellow solution was transferred to a liquid–liquid extractor and extracted with diethyl ether for 3 days. The ether phase (300 mL) was washed with three 20-mL portions of half-saturated brine and dried over magnesium sulfate. Solvent was removed in vacuo, and the residue was purified by flash chromatography [1:1 (v/v) hexanes:ethyl acetate] to afford 5.1 g (86%) of a clear liquid: bp 70 °C (1.0 mmHg); R_f = 0.23 [2:1 (v/v) hexanes:ethyl acetate]; IR (CCl_4) 3700–3000, 3615, 3085, 2945, 2883, 1650, 1435, 1385, 1363, 1043, 1000, 915, 863 cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$) δ 2.27 (1 H, s, OH), 2.37 (2 H, t, $J_{H,H}$ = 6.6 Hz, H at C2), 3.77 (2 H, t, $J_{H,H}$ = 6.6 Hz, H at C1), 4.84 (2 H, d, $J_{H,F}$ = 47.1 Hz, H at C3′), 5.17 (2 H, s, H at benzyl C); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 35.8, 60.9, 85.75 (d, $J_{C,F}$ = 166.8 Hz), 115.3 (d, $J_{C,F}$ = 10.9 Hz), 141.9 (d, $J_{C,F}$ = 14.6 Hz); MS (CI/isobutane), *m/z* (rel intensity) 41 (101.6), 54 (25.8), 55 (30.5), 57 (20.6), 59 (18.5), 67 (100.0), 74 (29.2), 85 (36.3), 86 (12.7), 87 (15.9) (M + 1 − H_2O).

Anal. Calcd for C_5H_9FO: C, 57.68; H, 8.71. Found: C, 57.38; H, 8.96.

2-Methyl-4-(1′-oxacyclohex-2′-yloxy)-1-butene (**14**). To a solution of 10.0 g (116 mmol) of 3-methyl-3-buten-1-ol and 2.9 g (12 mmol) of pyridinium *p*-toluenesulfonate in 300 mL of dry methylene chloride at 0 °C was added 14.6 g (174 mmol) of dihydropyran. The solution was stirred at room temperature for 4 h, diluted with 700 mL of diethyl ether, extracted with three 100-mL portions of half-saturated sodium chloride, and dried over magnesium sulfate. Solvent was removed in vacuo. The residual material was distilled at reduced pressure to afford 18.0 g (91%) of a clear liquid (Janistyn & Hansel, 1975): bp 62 °C (1.5 mmHg); ^1H NMR (300 MHz, $CDCl_3$) δ 1.53 (3 H, s, H at C2′), 1.30–1.48 (6 H, m, H at THP ring), 1.57–1.63 (2 H, m, H at C3), 3.23–3.31 (2 H, m, H at C4), 3.58–3.66 (m, H at C6′), 4.35–4.37 (1 H, m, H at C2′), 4.52 (2 H, d, $J_{H,H}$ = 9.7 Hz, H at C1); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 21.13, 24.39, 26.99, 32.23, 39.28, 63.75, 67.50, 100.15, 144.25.

2-Methyl-4-(1′-oxacyclohex-2′-yloxy)-1-butanol (**15**). To a solution of 15.0 g (88.0 mmol) of 2-methyl-4-(1′-oxacyclohex-2′-yloxy)-1-butene (**14**) in 80 mL of dry tetrahydrofuran was added 32 mL of 1.0 M BH_3/THF solution (Aldrich) over a period of 30 min. The resulting clear solution was stirred at room temperature for 4 h, after which time 9 mL of 3 M sodium hydroxide and 15 mL of 30% (w/w) aqueous hydrogen peroxide were added. The resulting mixture was stirred for 90 min, diluted with 450 mL of diethyl ether, and extracted twice with 50 mL of water. The solution was dried over magnesium sulfate and solvent removed in vacuo to afford 16.0 g (96%) of a viscous clear oil (Ladlow & Pattenden, 1985): R_f = 0.31 [1:1 (v/v) hexanes:ethyl acetate]; IR (CCl_4) 3620, 3450 (broad), 2940, 2870, 1460, 1448, 1436, 1410, 1347, 1196, 1113, 1070, 985, 903, 866 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.64 (3 H, d, $J_{H,H}$ = 6.7 Hz, H at C2′), 1.15–1.35 (6 H, m, H at THP ring), 1.35–1.58 (3 H, m, H at C2 and C3), 3.06–3.28 (3 H, m, H at C4 and OH), 3.46–3.62 (2 H, m, H at C6′), 4.28–4.34 (1 H, m, H at C2′); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 18.43, 18.62, 21.01, 21.13, 26.81, 32.06, 34.98, 35.09, 63.68, 63.81, 67.15, 67.42, 69.17, 69.28, 100.13, 100.39; MS (chemical ionization/methane), *m/z* (rel intensity) 85 (75.4), 105 (38.0) (M + 1 − THP), 189 (100.0) (M + 1).

2-Methyl-4-(1′-oxacyclohex-2′-yloxy)-1-butyl p-Toluenesulfonate (**16**). 2-Methyl-4-(1′-oxacyclohex-2′-yloxy)-1-butanol (**15**) (15.0 g, 80 mmol) was treated with *p*-toluenesulfonyl chloride (18.2 g, 96 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (12.8 g, 105 mmol) in 200 mL of methylene chloride for 20 h according to the procedure of Davisson

(Davisson et al., 1986). Following workup and flash chromatography (hexanes:ethyl acetate, 2:1), 22.9 g (84%) of a slightly yellow oil was obtained (Wolff et al., 1982): R_f = 0.36; IR (CCl_4) 2870, 2838, 1737, 1594, 1365, 1184, 1174, 1032, 967, 662 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.93 (3 H, d, $J_{H,H}$ = 6.6 Hz, H at C2′), 1.32–1.68 (8 H, m, H at C3 and at CH_2's of THP ring), 1.68–2.12 (1 H, m, H at C2), 2.43 (3 H, s, H at CH_3 of phenyl), 3.32–3.65 (2 H, m, H at CH_2O of THP ring), 3.65–3.99 (2 H, m, H at C4), 3.99 (2 H, d, $J_{H,H}$ = 6.6 Hz, H at C1), 4.48 (1 H, m, H at CH of THP ring), 7.31 (2 H, d, $J_{H,H}$ = 8.1 Hz, H at phenyl C's), 7.76 (2 H, d, $J_{H,H}$ = 8.1 Hz, H at phenyl C's); MS (chemical ionization/methane), *m/z* (rel intensity) 174 (32.0), 241 (9.9), 259 (9.1), 343 (4.6) (M + 1).

1-Fluoro-2-methyl-4-(1′-oxacyclohex-2′-yloxy)butane (**17**). To 22.9 g (67 mmol) of 2-methyl-4-(1′-oxacyclohex-2′-yloxy)-1-butyl *p*-toluenesulfonate (**16**) in 70 mL of dry acetonitrile was added 36.0 g (134 mmol) of tetra-*n*-butylammonium fluoride. The solution was stirred at 50 °C for 16 h, solvent was removed in vacuo, and the residual material was dissolved in 300 mL of diethyl ether. The resulting mixture was extracted with three 70-mL portions of water. The organic phase was dried over magnesium sulfate and concentrated in vacuo. The residue was purified by flash chromatography on silica gel [6:1 (v/v) hexanes:ethyl acetate] to afford 9.35 g (74%) of a clear liquid; no attempt was made to separate the diastereomers: R_f = 0.32; IR (CCl_4) 2940, 2870, 1550, 1197, 1120, 1113, 1070, 1018, 903 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.99 (3 H, d, $J_{H,H}$ = 6.8 Hz, H at C2′), 1.46–1.59 (6 H, m, H at CH_2's of THP ring), 1.59–1.81 (2 H, m, H at C3), 2.00–2.15 (1 H, m, H at C2), 3.42–3.54 (2 H, m, H at C4), 3.82–3.86 (2 H, m, H at CH_2O of THP ring), 4.15–4.21 (1 H, m, H at C1), 4.32–4.37 (1 H, M, H at C1), 4.56–4.59 (1 H, m, H at CHO of THP ring); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 15.82 (d, $J_{C,F}$ = 5.6 Hz), 15.96 (d, $J_{C,F}$ = 5.7 Hz), 19.57, 19.62, 25.44, 30.69, 31.37 (d, $J_{C,F}$ = 19.0 Hz), 31.43 (d, $J_{C,F}$ = 17.6 Hz), 32.31 (d, $J_{C,F}$ = 3.6 Hz), 32.40 (d, $J_{C,F}$ = 3.6 Hz), 88.06 (d, $J_{C,F}$ = 167.6 Hz), 88.19 (d, $J_{C,F}$ = 167.4 Hz), 98.59, 98.83; ^{19}F NMR (282 MHz, $CDCl_3$) δ −222.63, −222.71, −222.81, −222.88, −222.96, −223.07, −223.12, −223.22, −223.31; MS (EI), *m/z* (rel intensity) 85 (100.0), 105 (6.1) (M − THP), 135 (2.0), 189 (29) (M − 1), 190 (1.1) (M).

Anal. Calcd for $C_{10}H_{19}FO_2$: C, 63.13; H, 10.07. Found: C, 62.70; H, 10.18.

4-Fluoro-3-methylbutan-1-ol (**18**). To 1.22 g (4.9 mmol) of pyridinium *p*-toluenesulfonate in 50 mL of dry methanol was added 9.25 g (49 mmol) of 1-fluoro-2-methyl-4-(1′-oxacyclohex-2′-yloxy)butane (**17**). The resulting solution was stirred at room temperature for 16 h. Methanol was removed in vacuo. The residual material was dissolved in 200 mL of diethyl ether and extracted with 20 mL of saturated potassium carbonate. The ether phase was dried over magnesium sulfate, filtered through a short scrubber column of silica gel, and concentrated in vacuo. Flash chromatography [1:1 (v/v) hexanes:ethyl acetate] afforded 4.5 g (87%) of a clear liquid: bp 50.2 °C (2 mmHg); R_f = 0.34; IR (CCl_4) 3610, 3340, 2953, 2885, 1454, 1386, 1052, 1010, 938 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.99 (3 H, d, $J_{H,H}$ = 6.9 Hz, H at C3′), 1.19–1.72 (2 H, m, H at C2), 1.72–2.25 (1 H, m, H at C3), 2.00 [1 H, s (br), OH], 3.71 (2 H, t, $J_{H,H}$ = 6.6 Hz, H at C1), 4.28 (2 H, dd, $J_{H,F}$ = 47.1 Hz, $J_{H,H}$ = 5.7 Hz, H at C4); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 15.62 (d, $J_{C,F}$ = 5.2 Hz), 30.83 (d, $J_{C,F}$ = 19.2 Hz), 35.24 (d, $J_{C,F}$ = 4.9 Hz), 59.95, 88.12 (d, $J_{C,F}$ = 167.6 Hz); ^{19}F NMR (85 MHz, $CDCl_3$) δ −222.26 (1 F, dt, $J_{H,F}$ = 22.3 Hz, $J_{H,F}$ = 47.0 Hz).

Anal. Calcd for $C_5H_{11}FO$: C, 56.58; H, 10.45. Found: C, 56.23; H, 10.74.

3,4-Epoxybutan-1-ol (**19**). 3-Buten-1-ol (2.0 g, 28 mmol) and *m*-(chloroperoxy)benzoic acid (6.6 g, 38 mmol) were dissolved in 90 mL of dry chloroform and stirred at room temperature for 16 h. The mixture was extracted three times with 30 mL of 3 N sodium hydroxide, washed with 30 mL of water, and dried over magnesium sulfate. Solvent was removed in vacuo to afford 0.9 g (37%) of a clear liquid (Henbest & Nichols, 1957; Bats et al., 1982): R_f = 0.22 [1:2 (v/v) hexanes:ethyl acetate]; ^1H NMR (90 MHz, $CDCl_3$) δ 1.50–2.20 (2 H, m, H at C1′), 2.60 (1 H, s, OH), 2.60 (1 H, dd, $J_{H,H}$ = 4.8 Hz, $J_{H,H}$ = 3.0 Hz, H at C2), 2.80 (1 H, t, $J_{H,H}$ = 4.5 Hz, H at C2), 2.90–3.20 (1 H, m, H at C1), 3.40 (2 H, t, $J_{H,H}$ = 6.6 Hz, H at C2′).

3,4-Epoxy-3-methylbutan-1-ol (**20**). Following the procedure for synthesis of **19**, 3-methyl-3-buten-1-ol (7.0 g, 81 mmol) and *m*-(chloroperoxy)benzoic acid (16.5 g 96 mmol) were dissolved in 190 mL of dry chloroform and stirred at room temperature for 16 h. After workup, the residue was distilled at reduced pressure to afford 3.2 g (39%) of a clear liquid (Bats et al., 1982): bp 89–90 °C (10 mmHg); R_f = 0.21 [1:2 (v/v) hexanes:ethyl acetate]; ^1H NMR (90 MHz, $CDCl_3$) δ 1.37 (3 H, s, H at methyl), 1.88 (2 H, t, $J_{H,H}$ = 6.0 Hz, H at C1′), 2.52 [1 H, s (br), OH], 2.61 (1 H, d, $J_{H,H}$ = 4.2 Hz, H at C2), 2.78 (1 H, d, $J_{H,H}$ = 4.2 Hz, H at C2), 3.69 (2 H, t, $J_{H,H}$ = 6.0 Hz, H at C2′).

2,3-Epoxy-3-methylbutan-1-ol (**21**). Following the procedure for synthesis of **19**, 3-methyl-2-buten-1-ol (3.00 g, 35 mmol) and *m*-(chloroperoxy)benzoic acid (95%, 6.96 g, 38 mmol) were dissolved in 100 mL of dry chloroform and stirred at room temperature for 16 h. Upon workup, 2.50 g (70%) of a clear liquid was obtained (Pierre et al., 1969): R_f = 0.19 [1:2 (v/v) hexanes:ethyl acetate]; ^1H NMR (90 MHz, $CDCl_3$) δ 1.31 (3 H, s, H at methyl C), 1.35 (3H, s, H at methyl C), 2.97 (1 H, t, $J_{H,H}$ = 6.0 Hz, H at C1), 3.2 [1 H, s (br), OH], 3.69 (2 H, d, $J_{H,H}$ = 6.0 Hz, H at C1′).

General Procedure for Preparation of Tosylates. In a flame-dried flask under a nitrogen atmosphere were combined 1.0 equiv of *p*-toluenesulfonyl chloride and 1.2 equiv of 4-(*N*,*N*-dimethylamino)pyridine with magnetic stirring in dichloromethane (0.2 M in *p*-toluenesulfonyl chloride). To this solution was added 1.0 equiv of the appropriate alcohol. After 2–2.5 h, the mixture was diluted with a 10-volume excess of hexanes, and the resulting precipitate was removed by filtration. The filtrate was concentrated by rotary evaporation, diluted with diethyl ether, and filtered. After removal of solvent at reduced pressure, the remaining oil was used directly in the phosphorylation step. When samples were to be stored for extended periods, residual 4-(*N*,*N*-dimethylamino)pyridine was removed by passing the material through a short column of silica gel 60 using hexanes/diethyl ether as the eluent.

3,4-Epoxy-3-methyl-1-butyl p-Toluenesulfonate (**22**). 3,4-Epoxy-3-methylbutan-1-ol (**20**) (0.20 g, 2.0 mol) was treated with *p*-toluenesulfonyl chloride (0.41 g, 2.2 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (0.29 g, 2.4 mmol) in 5 mL of methylene chloride for 4 h. Following workup, 0.23 g (59%) of a colorless oil was obtained: R_f = 0.43 [1:1 (v/v) hexanes:ethyl acetate]; ^1H NMR (300 MHz, $CDCl_3$) δ 1.27 (3 H, s, H at C3), 1.91 (2 H, t, $J_{H,H}$ = 6.3 Hz, H at C2), 2.43 (3 H, s, H at CH_3 at phenyl), 2.57 [2 H, s (br), H at C4], 4.08 (2 H, t, $J_{H,H}$ = 6.3 Hz, H at C1), 7.32 (2 H, d, $J_{H,H}$ = 8.2 Hz, H at phenyl C's), 7.75 (2 H, d, $J_{H,H}$ = 8.2 Hz, H at phenyl C's); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 21.16, 21.62,

35.62, 53.54, 66.77, 127.69, 129.66, 144.79.

2,3-Epoxy-3-methyl-1-butyl p-Toluenesulfonate (**23**). 2,3-Epoxy-3-methylbutan-1-ol (**21**) (0.10 g, 1.0 mmol) was treated with *p*-toluenesulfonyl chloride (0.21 g, 1.1 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (0.14 g, 1.2 mmol) in 5 mL of methylene chloride for 4 h. Following workup, 0.16 g (64%) of a colorless oil was obtained: R_f = 0.28 [2:1 (v/v) hexanes:ethyl acetate]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.95 (3 H, s, H at C3′), 1.02 (3 H, s, H at C4), 2.18 (3 H, s, H at methyl C at phenyl), 2.70 (1 H, t, $J_{H,H}$ = 6.7 Hz), 3.80 (1 H, dd, $J_{H,H}$ = 11.2 Hz, $J_{H,H}$ = 6.4 Hz, H at C1), 3.90 (1 H, dd, $J_{H,H}$ = 11.2 Hz, $J_{H,H}$ = 7.1 Hz, H at C1), 7.10 (2 H, d, $J_{H,H}$ = 8.4 Hz, H at phenyl), 7.53 (2 H, d, $J_{H,H}$ = 7.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 20.22, 23.16, 25.82, 59.96, 61.00, 129.28, 131.34, 146.52.

3,4-Epoxy-1-butyl p-Toluenesulfonate (**24**). 3,4-Epoxybutan-1-ol (**19**) (0.20 g, 2.27 mmol) was treated with *p*-toluenesulfonyl chloride (0.48 g, 2.5 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (0.33 g, 2.7 mmol) in 5 mL of methylene chloride for 15 h. Following workup and flash chromatography [1:1 (v/v) hexanes:ethyl acetate], 0.37 g (67%) of a colorless oil was obtained: R_f = 0.44 [1:1 (v/v) hexanes:ethyl acetate]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43–1.57 (1 H, m, H at C2), 1.65–1.77 (1 H, m, H at C2), 2.18–2.23 (1 H, m, H at C3), 2.19 (3 H, s, H at methyl at phenyl), 2.47 (1 H, m, H at C4), 2.66–2.73 (1 H, m, H at C4), 3.86–3.97 (2 H, M, H at C1), 7.12 (2 H, d, $J_{H,H}$ = 7.7 Hz, H at phenyl C's), 7.53 (2 H, d, $J_{H,H}$ = 7.7 Hz, H at phenyl C's); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 23.15, 33.52, 48.36, 50.23, 68.82, 129.21, 131.33, 131.44, 146.39.

3,4-Epoxy-3-methyl-1-butyl Diphosphate (**6**). Following the procedure of Davisson (Davisson et al., 1986a), a solution of 3,4-epoxy-3-methyl-1-butyl tosylate (**22**) (100 mg, 0.39 mmol) and 0.70 g (0.78 mmol) of tris(tetra-*n*-butylammonium) hydrogen diphosphate in 2 mL of acetonitrile was stirred for 3 h. The resulting material was converted to the ammonium form with 23.4 mequiv of Dowex AG 50W-X8 (ammonium form). Flash chromatography on a 2 cm × 14 cm cellulose column [3.25:3.25:3.5 (v/v/v) 2-propanol:acetonitrile:0.1 M ammonium bicarbonate] yielded 89 mg (73%) of a white solid: R_f = 0.46 [3:3:4 (v/v/v) 2-propanol:acetonitrile:100 mM ammonium bicarbonate]; ^{1}H NMR (300 MHz, D_2O/ND_4OD] δ 1.40 (3 H, s, H at C3′), 1.80–2.12 (2 H, m, H at C2), 2.81 (1 H, d, $J_{H,H}$ = 3.9 Hz, H at C4), 2.92 (1 H, d, $J_{H,H}$ = 3.7 Hz, H at C4), 4.05 (2 H, q, $J_{H,H}$ = 6.5 Hz, H at C1); ^{13}C NMR (75 MHz, D_2O/ND_4OD)[2] δ 22.67, 39.23 (d, $J_{C,P}$ = 6.9 Hz), 57.64, 60.69, 64.86 (d, $J_{C,P}$ = 5.0 Hz); ^{31}P NMR (32 MHz, D_2O/ND_4OD] δ −5.65 (1 P, d, $J_{P,P}$ = 22.2 Hz, P2), −9.74 (1 P, d, $J_{P,P}$ = 22.1 Hz, P1).

3,4-Epoxy-1-butyl Diphosphate (**7**). Following the procedure described for synthesis of **6**, a solution of 100 mg (0.41 mmol) of 3,4-epoxy-1-butyl tosylate (**24**) and 0.74 g (0.82 mmol) of tris(tetra-*n*-butylammonium) hydrogen diphosphate in 1 mL of acetonitrile was stirred for 2 h. The resulting material was converted to the ammonium form with 27.4 mequiv of Dowex AG 50W-X8 (ammonium form). Flash chromatography on cellulose [3:3:4 (v/v/v) 2-propanol:acetonitrile:0.1 M ammonium bicarbonate] yielded 86 mg (71%) of a white solid; R_f = 0.46 [3:3:4 (v/v) 2-propanol:acetonitrile:100 mM ammonium bicarbonate]; ^{1}H NMR (300 MHz, D_2O/ND_4OD] δ (3 H, s, H at C3′), 1.80–2.12 (2 H, m, H at C2), 2.81 (1 H, d, $J_{H,H}$ = 3.9 Hz, H at C4), 2.92 (1 H, d, $J_{H,H}$ = 3.7 Hz, H at C4), 4.05 (2 H, q, $J_{H,H}$ = 6.5 Hz, H at C1); ^{13}C NMR (75 MHz, D_2O/ND_4OD)[2] δ 39.59 (d, $J_{C,P}$ = 7.1 Hz), 50.62, 54.27, 65.62 (d, $J_{C,P}$ = 5.0 Hz); ^{31}P NMR (32 MHz, D_2O/ND_4OD] δ −5.65 (1 P, d, $J_{P,P}$ = 22.2 Hz, P2), −9.74 (1 P, d, $J_{P,P}$ = 22.1 Hz, P1).

2,3-Epoxy-3-methyl-1-butyl Diphosphate (**8**). Following the procedure described for synthesis of **6**, a solution of 2,3-epoxy-3-methyl-1-butyl tosylate (**23**) (100 mg, 0.39 mmol) and 0.70 g (0.78 mmol) of tris(tetra-*n*-butylammonium) hydrogen diphosphate in 1 mL of acetonitrile was stirred for 3 h. The resulting material was converted to the ammonium form with 23.4 mequiv of Dowex AG 50W-X8 (ammonium form). Flash chromatography on cellulose [3.25:3.25:3.5 (v/v/v) 2-propanol:acetonitrile:0.1 M ammonium bicarbonate] yielded 91 mg (74%) of a white solid: R_f = 0.46 [3:3:4 (v/v) 2-propanol:acetonitrile:100 mM ammonium bicarbonate]; ^{1}H NMR (300 MHz, D_2O/ND_4OD] δ 1.40 (3 H, s, H at C3′), 1.80–2.12 (2 H, m, H at C2), 2.81 (1 H, d, $J_{H,H}$ = 3.9 Hz, H at C4), 2.92 (1 H, d, $J_{H,H}$ = 3.7 Hz, H at C4), 4.05 (2 H, q, $J_{H,H}$ = 6.5 Hz, H at C1); ^{13}C NMR (75 MHz, D_2O/ND_4OD)[2] δ 22.67, 39.23 (d, $J_{C,P}$ = 6.9 Hz), 57.64, 60.69, 64.86 (d, $J_{C,P}$ = 5.0 Hz); ^{31}P NMR (32 MHz, D_2O/ND_4OD) δ −5.65 (1 P, d, $J_{P,P}$ = 22.2 Hz, P2), −9.74 (1 P, d, $J_{P,P}$ = 22.1 Hz, P1).

[*4*-^{3}*H*]-*3*-(*Fluoromethyl*)-*3*-*buten*-*1*-*ol* ([*4*-^{3}*H*]-**13**). A break-seal ampule, containing 37 mg (258 μmol) of [^{3}H]-methyl iodide (New England Nuclear; 23.3 mCi, 90.5 mCi/mmol) was cooled in liquid nitrogen, the top of the ampule was purged with a mild stream of nitrogen, and the seal was broken. A solution of 68 mg (258 μmol) of triphenylphosphine in 0.2 mL of dry diethyl ether was added quickly by syringe, and the ampule was stoppered with a 10-mm NMR tube cap. The mixture was allowed to warm to 0 °C, maintained at that temperature for 5 h, and allowed to stand at room temperature for 6 days. To the white crystalline product (the ether had evaporated) were added 0.6 mL of dry THF and a small magnetic stir bar. The ampule was cooled again to −78 °C, and 0.16 mL of *n*-butyllithium (1.62 M in hexanes) was added by syringe. The yellow-orange mixture was warmed to room temperature and stirred for 1 h until the solid dissolved. To the deep yellow solution was added by syringe a solution containing 57 mg (258 μmol) of 4-[(*tert*-butyldimethylsilyl)oxy]-1-fluoro-2-butanone (**11**) in 0.1 mL of dry THF. The reaction mixture turned greenish brown, and a milky precipitate formed. After 15 min, the mixture was diluted with 1 mL of pentane, and the resulting slurry was filtered through a Pasteur pipet filled with 2 cm of silica gel. The pipet was rinsed with 1 mL of 2:1 THF:pentanes, and the combined eluents were concentrated in vacuo. To the residue was added a solution of 325 mg (1.03 mmol) of tetra-*n*-butylammonium fluoride trihydrate in 1 mL of THF. The mixture was stirred at room temperature for 20 min (analysis by TLC indicated the reaction was complete). The mixture was transferred to a conical glass tube. To this was added 3 mL of pentanes, and the tube was vortexed for 30 s. The pentane (top layer) was removed with a pipet and transferred to a round-bottom flask. The aqueous layer was then extracted 15 times with 1-mL portions of diethyl ether by the vortex procedure. The combined organic extracts were dried over magnesium sulfate, and the solution was concentrated with a gentle stream of nitrogen to a final volume of 2 mL (solution A). A sample of 45 μL of solution A was used to determine the specific activity of **20** as described previously (Davisson et al., 1986b).

[*4*-^{3}*H*]-*3*-(*Fluoromethyl*)-*3*-*buten*-*1*-*yl Toluenesulfonate* ([*4*-^{3}*H*]-**25**). The remainder of solution A from the previous reaction, containing 47 μmol of [4-^{3}H]-**13**, was concentrated to a final volume of 50 μL with a gentle stream of dry nitrogen.

To the residue were added 200 μL of dry methylene chloride, 13.4 mg (0.11 mmol) of 4-(*N*,*N*-dimethylamino)pyridine, and 19 mg (0.10 mmol) of *p*-toluenesulfonyl chloride. The mixture was stirred at room temperature for 16 h. The volume of the reaction mixture was reduced to 100 μL, and 100 μL of methylene chloride was added. After 3 h of additional stirring, the volume of the solution was reduced to 50 μL, and stirring was continued for 1 h. Five milligrams (41 μmol) of 4-(*N*,*N*-dimethylamino)pyridine and 100 μL of dry methylene chloride were added, and stirring was continued for 2 h. Solvent was removed with a gentle flow of nitrogen, and the residue was purified by preparative TLC on silica gel [6:1 (v/v) hexanes:ethyl acetate]. The tosylate was redissolved in 100 μL of dry acetonitrile (solution B).

[4-^{3}H]-3-(Fluoromethyl)-3-buten-1-yl Diphosphate [(*4-^{3}H*]-**3**). To a solution of 87 mg (99 μmol) of tris(tetra-*n*-butylammonium) hydrogen diphosphate in 100 μL of dry acetonitrile was added solution B from the previous reaction. The mixture was stirred at room temperature for 4 h and concentrated in vacuo. The residue was dissolved in 2 mL of water, and the resulting clear solution was passed through an ion-exchange column (20-mL plastic syringe) containing 15 mL of Dowex AG 50-X8 (ammonium form). The column was rinsed with 3 column volumes of water. The combined eluents were freeze-dried, and the remaining white solid was dissolved in 0.2 mL of 3.5:3.5:3 (v/v/v) acetonitrile:2-propanol:100 mM ammonium bicarbonate and loaded onto a 2.5 cm × 15 cm cellulose flash column which had previously been equilibrated with 50:50 (v/v) acetonitrile:2-propanol. The column was eluted with 3.5:3.5:3 (v/v/v) acetonitrile:2-propanol:100 mM ammonium bicarbonate at a flow rate of 4 mL/min. Fractions containing the product were combined, concentrated in vacuo, and freeze-dried. The residue was dissolved in 2.5 mL of water and stored at −20 °C. The radiochemical and chemical yields were 5% and 7%, respectively, based on [^{3}H]methyl iodide.

3-(Fluoromethyl)-3-buten-1-yl [^{32}*P*]*Diphosphate* ([^{32}*P*]-**3**). An ion-exchange column of 1.5 mL of Dowex AG 50W-X4 (hydrogen form) was equilibrated with water. A solution of ^{32}P-labeled disodium dihydrogen pyrophosphate (Amersham; 4.9 mg, 1.4 mCi, 63 mCi/mmol) in 0.2 mL of water was loaded onto the column and eluted with 10 mL of water. The eluent was collected in a cooled (0 °C) plastic scintillation vial containing a small stir bar. To the stirred solution was added at once 0.5 mL of 0.1 M tetra-*n*-butylammonium hydroxide, followed by dropwise addition until the pH of the solution rose to 7.2 as indicated by pH paper (1-μL aliquots were pipetted onto wet pH paper). The resulting solution was lyophilized, and the residue was rinsed with several 0.2-mL portions of acetonitrile into a 5-mL screw-cap glass vial. Acetonitrile was added to a final volume of 3.0 mL, and the solution was labeled as D.

A 1.0-mL portion of solution D was reduced in volume to 0.1 mL with a gentle stream of dry nitrogen. To the residue was added by syringe 35 μL of a solution of 27 mg (104 μmol) of 3-(fluoromethyl)-3-buten-1-yl toluenesulfonate (**25**) in 1.0 mL of acetonitrile. The solution was stirred for 2 h, solvent was removed with a gentle stream of dry nitrogen, and the residue was dissolved in 0.5 mL of water. The solution was loaded onto an ion-exchange column of 1.5 mL of Dowex AG 50W-X4 (ammonium form) and eluted with 10 mL of water. The eluent was lyophilized. The residue was rinsed from the vial with 10 0.1-mL portions of 2.5:2.5:5 (v/v/v) 2-propanol:acetonitrile:100 mM ammonium bicarbonate and loaded onto a 2 cm × 15 cm cellulose flash column that had been previously equilibrated with 1:1 (v/v) acetonitrile:2-propanol. The column was eluted with 3.5:3.5:3 (v/v/v) 2-propanol:acetonitrile:100 mM ammonium bicarbonate at a flow rate of 4 mL/min. Fractions containing the product were combined, concentrated in vacuo, and freeze-dried. The residue was dissolved in 1.0 mL of 5 mM ammonium bicarbonate and stored at −20 °C. The radiochemical and chemical yields, based on the tosylate, were 48% and 97%, respectively.

3,4-Epoxy-3-methyl-1-butyl [^{32}*P*]*Diphosphate* ([^{32}*P*]-**6**). A 1.0-mL portion of solution D (see procedure for [^{32}P]-**3**) was reduced in volume to 0.1 mL with a gentle stream of dry nitrogen. To the residue was added by syringe 40 μL of a solution of 24 mg (93 μmol) of 3,4-epoxy-3-methylbutyl toluenesulfonate (**23**) in 1.0 mL of acetonitrile. The solution was stirred for 2 h, and solvent was removed with a gentle stream of dry nitrogen. The residue was converted into the ammonium form and further purified by flash chromatography on cellulose as described for [^{32}P]-**3**. The product was dissolved in 1.0 mL of 5 mM ammonium bicarbonate and stored at −20 °C. The radiochemical and chemical yields, based on tosylate **19**, were 32% and 65%, respectively.

2,3-Epoxy-3-methyl-1-butyl [^{32}*P*]*Diphosphate* ([^{32}*P*]-**8**). A 1.0-mL portion of solution D (see procedure for [^{32}P]-**3**) was reduced in volume to 0.1 mL with a gentle stream of dry nitrogen. To the residue was added by syringe 35 μL of a solution of 27 mg (105 μmol) of 2,3-epoxy-3-methyl-1-butyl toluenesulfonate (**24**) in 1.0 mL of acetonitrile. The solution was stirred for 2 h, and solvent was removed with a gentle stream of dry nitrogen. The residue was converted into the ammonium form and further purified by flash chromatography on cellulose as described for [^{32}P]**3**. The product was dissolved in 1.0 mL of 5 mM ammonium bicarbonate and stored at −20 °C. The radiochemical and chemical yields, based on tosylate **24**, were 34% and 67%, respectively.

2-(Dimethylamino)-1-ethyl [^{32}*P*]*Diphosphate* ([^{32}*P*]-**9**). ^{32}P-labeled disodium dihydrogen pyrophosphate (Amersham; 4.0 mg, 1.2 mCi, 69.3 mCi/mmol) was converted to the tris(tetra-*n*-butylammonium) form as described previously (see procedure for [^{32}P]-**3**). The resulting material was dissolved in 100 μL of acetonitrile, and 41 μL (385 μmol) of a 0.18 M solution of 2-(dimethylamino)ethyl chloride (Burtner, 1949) in benzene was added by syringe. The solution was stirred for 24 h, and solvent was removed with a gentle stream of dry nitrogen. The remaining material was converted to the ammonium form with 3 mL of Dowex AG 50W-X4 (ammonium form) and further purified by flash chromatography on cellulose [57:43 (v/v) 2-propanol:100 mM ammonium bicarbonate] as described for [^{32}P]-**3**. The product was dissolved in 3 mL of 5 mM ammonium bicarbonate and stored at −20 °C. The radiochemical yields, based on the alcohol, were 37% and 71%, respectively.

[1,2-$^{14}C_2$]-2-(Dimethylamino)-1-ethyl Diphosphate ([*1,2-$^{14}C_2$*]-**9**). To 6.25 μL of an aqueous solution of [1,2-$^{14}C_2$]-2-(dimethylamino)ethanol (Pathfinder Laboratories, Inc.; 1.25 mg, 15 μmol; 0.1 mCi, 7.1 mCi/mmol) was added 12 μL of 4.7 M hydrochloric acid, and the resulting solution was lyophilized. To the residue was added 0.2 mL of dry tetrahydrofuran and 20 μL (31 μmol) of 1.55 M *n*-butyllithium in hexanes. The mixture was stirred at room temperature for 30 min. A solution of 5.9 mg (31 μmol) *p*-toluensulfonyl chloride in 50 μL of dry tetrahydrofuran was added, and stirring was continued for 10 min. The contents of the ampule were transferred to a 3-mL flask containing a solution of 25 mg (28 μmol) of tris(tetra-*n*-butylammonium) hydrogen diphosphate in 0.2 mL of dry acetonitrile. The mixture was

stirred at room temperature for 15 min, concentrated in vacuo, and allowed to stand for an additional 15 min. Water (0.3 mL) was added. The clear solution was loaded onto an ion-exchange column (15 mL of Dowex AG 50W-X8, ammonium form) and eluted with 30 mL of water. The eluent was lyophilized, and the residue was purified by flash chromatography on cellulose [57:43 (v/v) 2-propanol:100 mM ammonium bicarbonate] as described for [^{32}P]-**3**. The product was dissolved in 1 mL of 5 mM ammonium bicarbonate and stored at −20 °C. The total radiochemical and chemical yields, based on the alcohol, were both 6%.

2-(Methylamino)-1-ethyl Sulfate (**26**). To a stirred solution of 2-(methylamino)-1-ethanol (10.0 g, 133 mmol) in 23.3 g of 1,3-dichlorobenzene was added dropwise concentrated (96%) sulfuric acid at 60 °C. The resulting mixture was stirred at room temperature for 3 h, followed by heating at 100 °C. 1,3-Dichlorobenzene and water were distilled from the mixture at reduced pressure (12 mmHg). The oily residue was placed under high vacuum (1 mmHg) at 80 °C overnight. Upon cooling, 18.4 g (98%) of a crystalline solid was obtained (Dewey & Bafford, 1965).

N-Methylaziridine (**27**). 2-(Methylamino)-1-ethyl sulfate (**26**) (18.4 g, 130 mmol) in 30 mL of water was treated with a boiling solution of 20% (w/w) sodium hydroxide for 2 h. Distillation afforded 2.2 g (30%) of a clear liquid that was stored at −78 °C over a few pellets of sodium hydroxide: bp 30–60 °C (760 mmHg) [lit. bp 23.5 °C (739 mmHg) (Marckwald & Frobenius, 1901)]; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (2 H, m, H at C1 and C2), 1.65 (2 H, m, H at C1 and C2), 2.22 (3 H, s, H at CH_3); ^{13}C NMR (75 MHz, $CDCl_3$)[2] δ 28.4, 48.4.

[2′-^{3}H]-2-(Dimethylamino)-1-ethyl Diphosphate ([2′-^{3}H]-**9**). To 11.8 mg (83 μmol) of [^{3}H]methyl iodide (ICN Radiochemicals; 25 mCi, 300 mCi/mmol) were added 79 μL (7.14 mg, 125 μmol) of a 1.58 M solution of *N*-methylaziridine (**27**) in acetonitrile and 173.4 μL of a 0.72 M solution of silver(I) perchlorate semihydrate (Sigma, 27.0 mg, 125 μmol) in acetonitrile at −30 °C. The resulting suspension was stirred at −20 to −30 °C for 24 h. Solvent was removed at reduced pressure (1 mmHg) while the mixture was cooled at −20 °C. The yellow residue was suspended in 300 μL of dry acetonitrile, and solvent was removed again. The residue was resuspended in 200 μL of dry acetonitrile, and a solution of 226 mg (250 μmol) tris(tetra-*n*-butylammonium) hydrogen diphosphate in 0.3 mL of acetonitrile was added. The mixture was allowed to warm to room temperature, and solvent was removed in vacuo. The resulting material was converted to the ammonium form with 5 mL of Dowex AG 50W-X4 (ammonium form) and purified by flash chromatography on cellulose [57:43 (v/v) 2-propanol:100 mM ammonium bicarbonate] as described for [^{32}P]-**3**. The product was dissolved in 1.0 mL of 5 mM ammonium bicarbonate and frozen at −20 °C. Radiochemical and chemical yields, based on [^{3}H]methyl iodide, were 29% and 25%, respectively.

Isolation of Isopentenyl-diphosphate Isomerase

Initial Extraction. Freeze-dried mycelia (50 g) of *Claviceps* were ground in a large ball mill with 1 kg of ceramic stones for 2 h. Buffer (400 mL; 10 mM HEPES, 10 mM sodium metabisulfite, 10 mM sodium ascorbate, 5 mg of leupeptin, 1 mM phenylmethanesulfonyl chloride, 10 mM 2-mercaptoethanol, pH 7.0) was added, and milling was continued for 30 min. The suspension was decanted into a 1-L plastic beaker. The ball mill was rinsed with several portions of buffer (total of 300 mL). The pH of the combined extracts was adjusted to 5.5 with 3.5 M acetic acid. The suspension was transferred into six 250-mL centrifuge tubes and spun at 18900*g* for 60 min. The resulting supernatant was adjusted to pH 7.0 with 3 M ammonium hydroxide.

Ammonium Sulfate Fractionation. To the stirred supernatant (600 mL) was slowly added 155 g of ammonium sulfate over a period of 15 min followed by stirring for 15 min. The mixture was spun at 18900*g* for 60 min and filtered through cheesecloth. To the filtrate (670 mL) was slowly added 127.3 g of ammonium sulfate over a period of 15 min followed by stirring for 20 min. The mixture was spun at 16000*g* for 60 min. The precipitate was dissolved in ca. 20 mL of standard buffer (10 mM HEPES, 10 mM 2-mercaptoethanol, pH 7.0) and dialyzed overnight against two 2-L portions of standard buffer (10 mM HEPES, 10 mM 2-mercaptoethanol, pH 7.0).

n-Butyl-Sepharose Chromatography. The solution (48 mL) was loaded onto a 2.5 × 30 cm column of *n*-butyl-Sepharose equilibrated with standard buffer. The column was rinsed with 150 mL of standard buffer and developed with an 800-mL linear gradient of KCl (0–200 mM KCl in 10 mM HEPES, 10 mM 2-mercaptoethanol, pH 7.0). Six-milliliter fractions were collected, and those containing activity (49–58) were combined, saturated with ammonium sulfate, and spun at 25000*g* for 60 min. The precipitate was dissolved in standard buffer and dialyzed against two 1-L changes of standard buffer.

Fractogel TSK DEAE 650S HPLC Chromatography. A 2.5 cm × 30 cm steel column packed with Fractogel TSK DEAE 650S was equilibrated with starting buffer (25 mM HEPES, 10 mM 2-mercaptoethanol, pH 7.0). The protein was loaded with a 20-mL injector loop and eluted with a 0–100 mM linear gradient of ammonium sulfate [flow rate, 2 mL/min; 0–40 min, starting buffer; 40–180 min, 0–100 mM (linear)]. Four-milliliter fractions were collected, and those containing activity (44–53) were combined and concentrated in an Amicon stirred ultrafiltration cell to a final volume of ca. 5 mL. The solution was diluted to 50 mL with standard buffer and reconcentrated in the Amicon cell. The process was repeated and the resulting solution (ca. 5 mL) was used directly for HPLC chromatography on a Protein Pak DEAE 5PW column. Samples not used immediately were stored as a 20% ethylene glycol solution at −20 °C.

Protein Pak DEAE 5PW Chromatography. A 0.75 cm × 7.5 cm Protein Pak DEAE 5 PW column was equilibrated with 25 mM HEPES and 2 mM DTT, pH 7.0. Samples were loaded and immediately eluted with the following ammonium sulfate gradient at a flow rate of 1 mL/min: 0–4 min, starting buffer; 4–10 min, 0–30 mM (linear); 10–25 min, 30 mM; 25–35 min, 30–50 mM (linear); 35–41 min, 50–100 mM (linear). Fractions (0.7 mL) were collected, and those containing highest activity were combined, concentrated, and dialyzed in a Micro-ProDicon concentrator against 1 L of standard buffer. Solutions of the pure enzyme (0.1–1.0 mg/mL) were stored in 25% ethylene glycol containing 10 mM HEPES and 2 mM DTT at pH 7.0 at −20 °C for over 6 months without significant loss of activity.

Kinetic Measurements

Enzyme activity was measured by the acid lability method (Satterwhite, 1985). Assay mixtures contained 10 mM HEPES, 2 mM magnesium chloride, 1 mM DTT, 0.01% BSA, and 40 μM [1-^{14}C]isopentenyl diphosphate (sp act. 10 μCi/μmol), pH 7.0, in a total volume of 50 μL. The reaction was initiated by addition of enzyme, and incubation was for 10 min at 37 °C. The reaction was quenched by addition of 0.2 mL of 4:1 (v/v) methanol:concentrated hydrochloric acid, followed by a second incubation for 10 min at 37 °C. The mixture was

extracted with 1 mL of ligroin (bp 90–120 °C) by vortexing, and a 0.5-mL portion of the ligroin layer was removed for counting.

Initial rates were measured from single time points determined in triplicate under conditions where the rate of product formation was linear (typically less than 7% conversion). Kinetic constants were determined by using the programs developed by Cleland (Cleland, 1976) for a simple unimolecular reaction. Michaelis constants for IPP and DMAPP were also calculated from product inhibition studies (Segel, 1975).

Rates of inactivation were measured by preincubating the enzyme with the inhibitors followed by a 5-fold dilution into buffer containing 40 μM [1-^{14}C]-**1** and measuring isomerase activity by the acid lability assay. Residual activity was determined by comparing inhibited samples to noninhibited controls. Since small amounts of inactivation occurred during the assay for residual activity, control samples were diluted 5-fold into buffers containing the same amounts of inactivators as the diluted samples.

In a typical experiment, enzyme was added to two sets of tubes, one containing buffer and inhibitor and the other containing only buffer. The tubes were preincubated in pairs (two samples and two controls per preincubation) at 37 °C. The inactivation reaction was stopped by the addition of buffer containing saturating levels of [1-^{14}C]-**1** (40 μM) to the samples and 40 μM [1-^{14}C]-**1** plus inhibitor to the controls. The samples and controls were incubated at 37 °C for 10 min and analyzed by the acid lability procedure.

Stoichiometry of Isomerase–Inhibitor Complexes

Isomerase (14–68 μg, 0.4–2.0 nmol) was incubated at 37 °C with the radiolabeled inhibitors (1.0–80 μmol) in 100 μL of assay buffer for 30–60 min. Incubation times and inhibitor concentrations were chosen to ensure complete labeling (>98% loss of catalytic activity). The solutions were diluted to a final volume of 1 mL with 5 mM HEPES at pH 7 and transferred to microconcentrators (Centricon 10 Amicon Corp., M_r cutoff 10000). The microconcentrators were spun at 5400*g* until the original sample volume was reduced to 50–100 μL. The radioactivity of the filtrate (ca. 0.9 mL) was measured. The concentrate was repeatedly diluted to 1 mL and reconcentrated until the radioactivity in the filtrate dropped to background levels. The radioactivity of the concentrate was then measured.

Fluoride Release Assay

Reagent A. Freshly recrystallized (Dixon, 1970) eriochrome cyanine R (0.900 g; ICN Biomedicals) was dissolved in doubly deionized water to a final volume of 500 mL and stored in a Nalgene bottle at room temperature until used.

Reagent B. Zirconyl chloride octahydrate (133 mg, Aldrich) was dissolved in 25 mL of doubly deionized water. Hydrochloric acid (350 mL, sp gr 1.19) was added, and the final volume adjusted to 500 mL with doubly deionized water. The solution was stored in a Nalgene bottle at room temperature until used.

Reference Solution. To 100 mL of doubly deionized water were added 10.0 mL of reagent A and 10 mL of a hydrochloric acid solution prepared by diluting 7.0 mL of the concentrated acid to 10 mL with doubly deionized water.

Standard Sodium Fluoride Solution. Sodium fluoride (221 mg; Aldrich) was dissolved to a final volume of 1 L with doubly deionized water and stored in a Nalgene bottle at room temperature until used.

Preparation of Samples and Standards. A solution of enzyme (sp act. 100 nmol min^{-1} mg^{-1}; ca. 8.3 units) was concentrated in an Amicon (PM 10 filter) stirred ultrafiltration cell to a final volume of 5 mL. The concentrate was diluted with 50 mL of buffer (10 mM HEPES, pH 7.0, 5 mM DTT) and concentrated again to a final volume of 5 mL. This process was repeated two more times to ensure complete removal of residual salts or contaminants from the protein. The final concentrate was assayed for activity: 5 mL, 7.4 units (55 nmol of isomerase). Magnesium chloride solution (50 μL, 100 mM) was added. The following sample and standards were prepared from this solution: sample, 980 μL of protein (10.6 nmol of enzyme), 10 μL of 1.5 mM **3**, and 10 μL of 10 mM HEPES; standard 1, 980 μL of protein, 10 μL of 0.2 mM **3**, and 10 μL of 10 mM HEPES; standard 2, 980 μL of protein, 10 μL of 0.2 mM **3**, 8 μL of 10 mM HEPES, and 2 μL of 5.3 mM NaF; standard 3, 980 μL of protein, 10 μL of 0.2 mM **3**, 6 μL of 10 mM HEPES, and 4 μL of 5.3 mM NaF; and standard 4, 980 μL of protein, 10 μL of 0.2 mM **3**, 2 μL of 10 mM HEPES, and 8 μL of 5.3 mM NaF.

The components were mixed in polypropylene tubes, which were cooled on ice. The protein in the standards was denatured by heating at 100 °C for 5 min before the addition of **3**, HEPES, and fluoride. The tubes were incubated at 37 °C for 60 min and then cooled on ice. The contents were transferred with a plastic pipet into microconcentrators (Centricon 10 Amicon Corp., M_r cutoff 10000, that had been rinsed twice with 2 mL of doubly deionized water by spinning at 5400*g*). The microconcentrators were spun at 5400*g* at 4 °C for 6 h. To the filtrates were added 100 μL of reagent A and 100 μL of reagent B. The contents were mixed and transferred with a plastic pipet into UV cuvettes. Absorbance was measured at 533 nm against a reference solution without fluoride.

Table I: Isolation of Isomerase from *C. purpurea*

purification step	total units[a]	SA[b]	overall yield (%)	purification (*x*-fold)
pH 5.5 supernatant	75	0.01		
$(NH_4)_2SO_4$ precipitation	61	0.03	81	3
n-butyl-Sepharose	46	0.16	61	16
Fractogel TSK 650S	16	0.57	21	57
Protein Pak DEAE 5PW	3	3.6	4	330

[a] Units in μmol min^{-1}. [b] Specific activity in μmol mg^{-1} min^{-1}.

Results

Purification and Properties of Isopentenyl-diphosphate Isomerase from C. purpurea. Isopentenyl-diphosphate isomerase from several different sources has been studied, and extensive purifications of the enzyme are reported from pig liver (Banthorpe et al., 1977), *Claviceps* (Bruenger et al., 1986), and *Saccharomyces cerevisiae* (Reardon & Abeles, 1986). The procedure we report for isomerase from *C. purpurea* is summarized in Table I. Activity for the enzyme increased rapidly from 50 μmol min^{-1} g^{-1} of dry mycelia at day 4 of a stationary culture to 275 μmol min^{-1} g^{-1} at day 6. After day 6, the specific activity in the initial extract decreased, although the total units of activity increased until day 12. We typically elected to maximize specific activity and were able to obtain 0.01 μmol min^{-1} mg^{-1} in initial extracts from 6-day-old cultures. The specific activity of purified isomerase was 3.6 μmol min^{-1} mg^{-1}, a value similar to those previously reported for the enzyme from *Claviceps* sp (3.0 μmol min^{-1} mg^{-1}) (Bruenger et al., 1986) and *Saccharomyces cerevisiae* (2.7 μmol min^{-1} mg^{-1}) (Reardon & Abeles, 1986). The material from our purification was >90% pure, as judged from the intensity of bands on SDS gels stained with Coomassie Blue.

The Michaelis constant for isopentenyl diphosphate (**1**) was determined in the standard manner from double-reciprocal

Table II: Kinetic Constants and Substrate Protection for Irreversible Inhibition of Isomerase

inhibitor (I)	k_2 (min^{-1})	K_I (μM)	substrate protection [I] (μM)	substrate protection [1] (μM)	substrate protection $t_{1/2}/t_{1/2}^1$
3	0.22 ± 0.07	0.085 ± 0.04	0.10	1.0	0.42
4	0.40 ± 0.04	0.59 ± 0.09	0.22	1.0	0.37
5	0.97 ± 0.52	0.97 ± 0.55	0.15	1.0	0.48
6	0.13 ± 0.01	0.011 ± 0.004	0.02	1.0	0.38
7	0.17 ± 0.02	0.017 ± 0.001	0.02	1.0	0.45
8	0.24 ± 0.06	8.74 ± 1.83	5.0	2.0	0.55
9	1.17 ± 0.33	15.2 ± 4.75	5.0	2.0	0.75
iodoacetamide	*a*	*a*	2×10^3	50	1.0

[a] Not determined. A double-reciprocal plot of k_{inact} versus [iodoacetamide] was not linear; $k_{inact} = 1 \times 10^{-3}$ s^{-1} at 2 mM iodoacetamide.

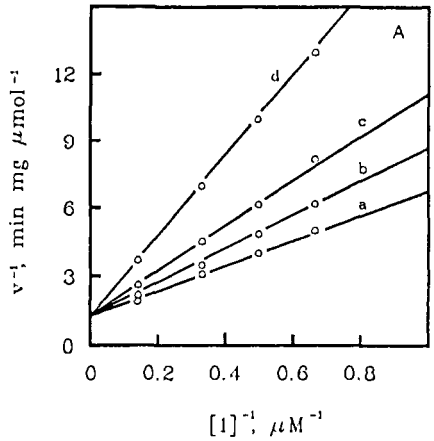


FIGURE 1: Double-reciprocal plot of initial velocity versus [1] at different fixed [2]: (a) 4.0 μM; (b) 5.3 μM; (c) 7.8 μM; (d) 15 μM.

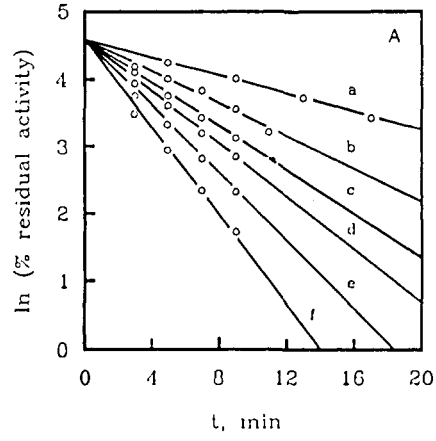


FIGURE 2: Plots of ln (% residual activity) for isomerase versus time at different fixed concentrations of **4**: (a) 0.063 μM; (b) 0.13 μM; (c) 0.17 μM; (d) 0.21 μM; (e) 0.27 μM; (f) 0.34 μM.

plots of initial velocities versus substrate concentrations. The value of K_M^1 = 2.4 μm for our enzyme was similar to K_M^1 = 5.4 μM reported from *Claviceps* sp (Bruenger et al., 1986) and substantially below K_M^1 = 35 μM reported for the yeast enzyme (Reardon & Abeles, 1986). The Michaelis constant for dimethylallyl diphosphate (**2**) cannot be determined in a straightforward manner because there is no convenient assay for measuring the initial velocity of the isomerization reaction in the reverse direction. The constant can, however, be calculated from product inhibition studies of initial velocities in the forward direction using the relationship for a uni-uni reaction given in eq 1 (Segal, 1975). Initial rates for the ap-

$$V = \frac{V_{max}[1]}{K_M^1\left(1 + \frac{[2]}{K_M^2}\right) + [1]} \quad (1)$$

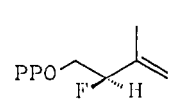
pearance of radioactivity from [1-^{14}C]-**1** into unlabeled **2** were measured at varying concentrations of **1** and **2**. Plots of v^{-1} versus $[1]^{-1}$ at different concentrations of **2** gave a family of straight lines (see Figure 1) with a common intercept on the v^{-1} axis (V_{max}) and a series of apparent K_M^1's on the $[1]^{-1}$ axis. A replot of K_{Mapp}^1 versus [**2**] also gave a straight line with a y intercept of K_M^1 and an x intercept of $-K_M^2$. The values of K_M^1 and K_M^2 calculated from the product inhibition experiments were 1.1 and 0.9 μM, respectively. The Michaelis constants determined for isopentenyl diphosphate by the two procedures were in reasonably close agreement. $V_{max}^{reverse} \cong$ 1.5–3.2 μmol min^{-1} mg^{-1} for the *C. purpurea* enzyme was obtained from the Haldane relationship (eq 2) by using the Michaelis constants for **1** and **2**, V_{max} for **1** ($V_{max}^{forward}$), and K_{eq} = 3.

$$\frac{K_M^1 V_{max}^{forward}}{K_M^2 V_{max}^{reverse}} = K_{eq} \quad (2)$$

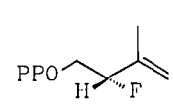
Kinetic Constants for Inactivation. Treatment of isomerase with diphosphate analogues **3**–**9** resulted in a first-order time-dependent loss of activity. The rate constants for inactivation (k_{inact}) were determined from plots of the natural logarithm of residual activity versus time as illustrated in Figure 2 for **4**. A plot of $(k_{inact})^{-1}$ versus $[I]^{-1}$ afforded the rate constants k_2 for inactivation at saturating levels of inhibitor and the inhibition constants K_I listed in Table II (Walsh, 1982).

As expected for an active-site-directed process, incubations conducted in the presence of **1** showed substrate protection of the enzyme. The degree of protection afforded by **1** expressed as the ratio of the half-life of the enzyme in the absence ($t_{1/2}$) and presence ($t_{1/2}^1$) of **1** and inhibitors **4**–**9** is also given in the table. In addition, iodoacetamide irreversibly inhibited isomerase. However, **1** did not afford substrate protection against iodoacetamide, even when present at saturating (50 μM) concentrations. This result agrees with the previous report by Bruenger (Bruenger et al., 1986) for the enzyme from *Claviceps* sp but contrasts with the observations of Reardon and Abeles (Reardon & Abeles, 1986) with isomerase from *Saccharomyces*.

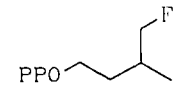
4-Fluoro-3-methyl-1-butyl diphosphate and (*R*)- and (*S*)-2-fluoroisopentenyl diphosphate were not irreversible inhibitors. Exposure of isomerase to 100 μM concentrations



(R)-28



(S)-28



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of the compounds for 90 min at 37 °C did not irreversibly inactivate the enzyme. In contrast, the half-life for inactivation

Table III: Stoichiometry of Irreversible Inhibitors in Isomerase Complexes

inhibitor	stoichiometry[a]	inhibitor	stoichiometry[a]
[4-^{3}H]-**3**	1.09, 0.43[b]	[^{32}P]-**8**	0.58
[^{32}P]-**3**	0.97	[2′-^{3}H]-**9**	1.00, 0.99[b]
[1-^{3}H]-**5**	0.81	[1,2-$^{14}C_2$]-**9**	1.13
[^{32}P]-**6**	0.81	[^{32}P]-**9**	0.98

[a] Stoichiometry is expressed as the ratio of moles of inhibitor to moles of active sites of isomerase. [b] After complete inactivation with 10 mM iodoacetamide at 37 °C.

of isomerase in the presence of 1 μM **9**, the least potent inhibitor listed in Table II, was 6.5 min at 37 °C.

Stoichiometry of Isomerase–Inhibitor Complexes. The stoichiometry of irreversible inhibition was studied by using radiolabeled **3**, **5**, **6**, **8**, and **9**. The results are presented in Table III. Except for **8**, 1 mol of inhibitor was bound per mole of enzyme. Two of the inhibitors, allylic fluoride **3** and ammonium analogue **9**, also contained radioactivity in the diphosphate and hydrocarbon moieties. Again the stoichiometry was 1:1, demonstrating that the diphosphate and the hydrocarbon residues both are sequestered in the enzyme–inhibitor complex.

Treatment of isomerase with 10 mM iodoacetamide at 37 °C for 30 min inactivated the enzyme. When the inactivated enzyme was then treated with **3**, the molar ratio of bound fluorinated analogue to enzyme dropped to 0.42. No further reductions in the stoichiometry of binding for **3** were seen when the pretreatment with iodoacetamide was extended to 1 h. In a related experiment, pretreatment of isomerase with iodoacetamide for 30 min at 37 °C did not alter the binding stoichiometry of **9**.

An additional set of experiments was conducted to demonstrate that the binding of **3** and **9** by iodoacetamide-inactivated isomerase was an active-site phenomenon. The incorporation of radioactivity from **3** and **9** into iodoacetamide-inactivated enzyme at 37 °C was measured in the presence of 50 μM IPP and compared to incorporations in the absence of IPP. Isomerase was inactivated by treatment with 10 μM iodoacetamide for 30 min. Excess iodoacetamide was removed by five cycles of microconcentration–dilution. Separate samples of the enzyme were then treated with radiolabeled **3** (1 μM), **9** (90 μM), or a mixture of each inhibitor and **1** (50 μM). Samples containing **3** were incubated for 10 min and those containing **9** for 30 min, conditions known to produce maximal incorporation. Excess inhibitor was again removed by microconcentration–dilution and radioactivity in the enzyme determined. The presence of **1** in the buffer reduced the levels of incorporation of **3** and **9** by 60% and 54%, respectively.

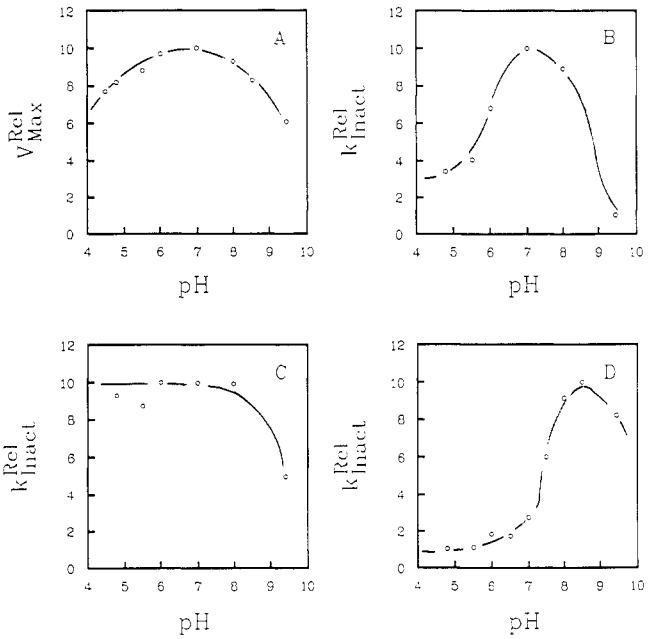


FIGURE 3: (A) Plot of relative V_{max} versus pH for isomerization of **1** to **2**; plots of relative first-order rate constants for inactivation of isomerase (k_{inact}) versus pH for 60 nM **3** (B), 20 nM **6** (C), and 20 μM **9** (D). Buffers used were sodium malate (pH 4.8–6.8) and HEPES (pH 6.8–9.5).

pH Dependence of Inactivation Rates. pH–rate profiles for the normal isomerization reaction and for inactivation of isomerase by **3**, **6**, and **9** were determined, and the results are summarized in Figure 3. The normal reaction had a broad maximum between pH 5 and 9. The pH dependence for inactivation by fluoro analogue **3** had an optimum at pH 7.0. In contrast, inactivation by epoxide **6** was maximal from pH 4.8 to 8.0, and the rate dropped sharply above pH 8, while the rate for amine **9** was minimal from pH 4.8 to 7.0 and then rose sharply. Above pH 9.0, k_{inact}[9] appeared to decrease slightly.

Stability of Isomerase–Inhibitor Complexes. The stabilities of isomerase–inhibitor complexes were studied for compounds **3**, **4**, **6**, and **9** under a variety of conditions. The results are summarized in Table IV. In each experiment, isomerase was incubated with an excess of radiolabeled inhibitor to generate the complex. Excess inhibitor was removed, 2 mL of buffer was added, and the sample was concentrated to a final volume of 45 μL repeatedly until radioactivity in the filtrate dropped to background levels. The material was removed from the microconcentrator, radioactivity was measured (retained dpm), and the sample was treated as described in Table IV. Two milliliters of buffer was added, the sample was concentrated

Table IV: Stability of Isomerase–Inhibitor Complexes

	% dpm released upon treatment[a]											
inhibitor	A	B	C	B and C	D	E	F	G	H	I	J	K
[4-^{3}H]-**3**	0.3	2.1	4.1[b]	30.0[b]	0.3	1.0	14.7[b]	4.8				
[^{32}P]-**3**		0.2	15.2[b]	48.0[b]								
[1-^{3}H]-**5**	0.9				0.5							
[^{32}P]-**6**		0.8	15.1[b]	34.2[b]	0.2							
[1,2-$^{14}C_2$]-**9**		27.7[b]	34.2[b]	45.2[b]	0.3						>99.9	2.8
[^{32}P]-**9**		29.5[b]	48.8[b]	69.4[b]	0.2							
[2′-^{3}H]-**9**	0.3				0.9			9.9	83.0[b]	>99.9	>99.9	

[a] To 50 μL of complex in 5 mM HEPES, pH 7.0, was added 5 μL of reagent to produce conditions as described in A–K: A, 6 M urea, 37 °C, 60 min; B, 5% BME, 37 °C, 60 min; C, 0.015 M SDS, 37 °C, 60 min; D, 5 mM HEPES, pH 7.0, 37 °C, 60 min; E, 6 M urea, 0.05 M NaOH, 37 °C, 60 min; F, 6 M urea, 0.20 M, NaOH, 37 °C, 60 min; G, 6 M urea, 400 mM KP_i, 100 mM NaCl, pH 6.8, 37 °C, 180 min; H, 6 M urea, 0.01 M NaOH, 37 °C, 30 min; I, 6 M urea, 0.20 M NaOH, 37 °C, 15 min; J, 0.20 M NaOH, 37 °C, 15 min; K, 5 mM HEPES, 10.0 mM **9**, pH 7.0, 20 °C, 72 h. After treatment, the complex was diluted with 5 mM HEPES or 50 mM $(NH_4)_2HCO_3$ to a final volume of 2 mL and concentrated through a microconcentrator to a final volume of 45 μL. [b] Increased with incubation time.

to 45 μL, and radioactivity in the filtrate (released dpm) was measured.

In all instances the complexes were stable to prolonged standing in buffer (treatments D, K). The fluorinated and epoxy inhibitors formed complexes that were resistant to 6 M urea (treatment A) or 0.015 M SDS (treatment C). However, substantial releases of radioactivity were observed upon incubation with SDS and 5% 2-mercaptoethanol (treatment B + C). The complex with ammonium analogue **9** was stable in the presence of 6 M urea (treatment A) and only lost 10% of its radioactivity when the urea treatment was combined with high salt (treatment G). In contrast, high levels of 2-mercaptoethanol (treatment B), SDS (treatment C), and a combination of the two resulted in significant releases of radioactivity.

The isomerase complex with **3** was also resistant to treatment with 6 M urea and 0.2 M sodium hydroxide (treatment G). The behavior of the isomerase–**9** complex was, however, markedly different. In the presence of 6 M urea and 0.01 M sodium hydroxide, 83% of the radioactivity was lost after incubation for 30 min at 37 °C (treatment H), and all of the radioactivity was lost within 15 min when the concentration of hydroxide was increased to 0.2 M (treatment I). We also discovered that quantitative release of radioactivity occurred within 15 min when urea was not included in the buffer (treatment J).

The chromatographic properties of complexes between isomerase and [4-^{3}H]-**3**, [1-^{3}H]-**4**, and [^{32}P]-**6** were compared to those of the native enzyme during ion-exchange chromatography (Waters Protein Pak 5PW) upon elution with a linear ammonium sulfate gradient. Approximately 70–80% of the total radioactivity of the complexes was located in fractions that had retention volumes identical with those for catalytically active isomerase chromatographed under identical conditions. The remaining radioactivity eluted from the column with a high-salt wash. SDS gel electrophoresis of [^{32}P]-**3** and [^{32}P]-**6** complexes confirmed this result.

Cell-free extracts containing isomerase activity were also treated with radiolabeled **3** and **6**. After excess of inhibitor was removed by microconcentration, the complexes were electrophoresed on a polyacrylamide gel under denaturing conditions (SDS) at low concentrations of 2-mercaptoethanol (high levels of 2-mercaptoethanol cause loss of label). The gel was fixed under standard acidic conditions and dried onto paper. Autoradiography showed a single major band with a molecular weight of 35 000 and several very faint bands of slightly lower molecular weights. Other lanes on the same gel loaded with equivalent amounts of [^{32}P]-**3** and [^{32}P]-**6** did not give an image. On the basis of the above results, there is no doubt that **3** and **6** form covalent adducts with isomerase.

Experiments designed to demonstrate that the complex with **9** was stable to electrophoresis failed. Therefore, it became necessary to establish whether the radioactive material released from the isomerase–inhibitor complex was still **9**. Two double-labeling experiments were conducted. In the first, isomerase was labeled with a mixture of [^{32}P]- and [1,2-$^{14}C_2$]-**9** (^{14}C/^{32}P = 0.79). As expected, identical incorporations of ^{32}P and ^{14}C were found in the complex (^{14}C/^{32}P of complex = 0.79). The complex was treated with 0.015 M SDS and 5% 2-mercaptoethanol at 37 °C for 60 min, after which time 54% of ^{14}C activity and 87% of ^{32}P activity were released from the complex (^{14}C/^{32}P = 0.48). The released material was then chromatographed on a cellulose TLC plate. Only 27% of the ^{32}P radioactivity and 43% of the ^{14}C radioactivity comigrated with **9**, and a control sample containing [^{14}C, ^{32}P]-**9** without isomerase migrated as a single spot (>90% of radioactivity, no change in ^{14}C/^{32}P) after a similar treatment. In the second experiment, isomerase was inactivated with a mixture of [2′-^{3}H]- and [1,2-$^{14}C_2$]-**9**. Again, incorporations of ^{3}H and ^{14}C were identical. The complex was then treated with 0.20 M NaOH at 37 °C for 15 min, during which time greater than 99% of the ^{3}H and ^{14}C radioactivity was released. The ^{3}H/^{14}C ratios for radioactivity in the enzyme–inhibitor complex and in the released material were identical within experimental error (3.4 and 3.3, respectively). The released material was chromatographed on a cellulose TLC plate, and radioactivity comigrated with an authentic sample of **9**. A control sample of a [^{3}H, ^{14}C]-**9** mixture treated under identical conditions as the isomerase–NIPP complex migrated as a single spot on cellulose TLC that contained more than 90% of the total radioactivity.

Fluoride Release during Reaction of Isomerase with 3-(Fluoromethyl)-3-buten-1-yl Diphosphate. When it became evident that inactivation of isomerase by **3** must involve covalent linkage to the enzyme, nucleophilic displacement of fluoride by an active-site residue that covalently linked the inhibitor to the enzyme with concomitant release of fluoride ion was an obvious mechanism for inactivation. We therefore decided to examine the incubation mixture for the presence of fluoride ion. A spectrophotometric procedure based on fluoride's ability to destroy the orange-colored zirconium eriochrome cyanine R complex, λ_{max} 533 μM, sensitive in the 0.5–1.0 μM range, was selected (Megregian, 1978).

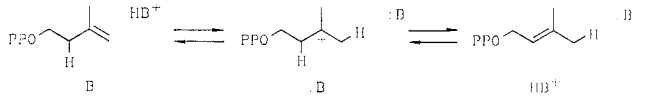
Isomerase (11 nmol) was incubated with 15 μM **3** at 37 °C for 60 min, protein was removed by microconcentration, and the amount of fluoride ion in the filtrate was determined from a standard curve of micrograms of fluoride versus A_{533} of the dye complex. The amount of fluoride released upon inactivation of the enzyme (12 nmol) was identical, within experimental error, with the amount of isomerase used in the experiment (11 nmol).

Determination of K_{dis} for the 2-(Dimethylamino)-1-ethyl Diphosphate–Isomerase Complex. Inhibition and stability experiments indicated that ammonium analogue **9** inactivated isomerase by a tight electrostatic interaction rather than by a covalent attachment. In theory this is a reversible process, and we were interested in measuring the dissociation constant, K_{dis}, of ammonium analogue **9** in the inactive isomerase–**9** complex.

K_{dis} was estimated from the ratio of the rate constants of complex formation (k_{on}) and complex dissociation (k_{off}) (Williams & Morrison, 1979). Apparent k_{on}'s were determined from inactivation constants at various concentrations of **9**. The true value for the bimolecular rate constant k_{on} was determined from the slope of a plot of apparent rate constant versus their corresponding inhibitor concentrations.. The value $k_{on} = 5.4 \times 10^4$ min^{-1} M^{-1} was obtained. The unimolecular rate constant k_{off} for the dissociation of the complex was estimated from the rate of release of radioactivity from the isomerase–**9** complex. The labeled complex was incubated with a large excess of unlabeled **9**, and the release of radioactivity into the pool of **9** was determined as described in Table IV (treatment K). From these data, k_{off} was estimated to be $\leq 6.6 \times 10^{-6}$ min^{-1}, and a value $K_{dis} \leq 1.2 \times 10^{-10}$ M was calculated.

Discussion

Several lines of evidence suggest that the isomerization of isopentenyl diphosphate to dimethylallyl diphosphate proceeds by a carbocationic mechanism.



In analogy with linear free energy studies of farnesyl-diphosphate synthetase, which catalyzes an electrophilic alkylation via carbocationic intermediates (Poulter et al., 1981), substitution of hydrogens at C2 and C3′ of **1** and C2, C3′, and C4 of **2** with fluorine greatly retards the rate of isomerization. In our product and kinetic studies we saw no evidence for isomerization of fluoro analogues **3**, **4**, or **5** prior to inactivation of isomerase. Likewise, both (*R*)- and (*S*)-2-fluoroisopentenyl diphosphate (**28**) were not alternate substrates. These results agree with the recent reports by Reardon and Abeles (Reardon & Abeles, 1986) that (*Z*)-3-(trifluoromethyl)-2-butenyl diphosphate is at least 10^6 times less reactive than the normal substrate. In nonenzymatic model reactions we found that a single fluorine at C3′ retards the solvolysis of allylic derivatives by 10^3, and a trifluoromethyl moiety, by 10^7 (Poulter et al., 1981). A tertiary carbocation should be destabilized to a similar extent.

Additional support for the mechanism is provided by the inhibitory properties of 2-aminoethyl diphosphate derivatives. We and Reardon and Abeles (Reardon & Abeles, 1986) discovered that 2-(dimethylamino)ethyl diphosphate (**9**) inactivates isomerase in a time-dependent manner. The binding of **9** to the *Claviceps* enzyme is extremely tight ($K_i < 10^{-10}$ M). Reardon and Abeles reported an even lower value, $K_i < 10^{-11}$ M, for **9** and yeast isomerase. However, under stringent denaturing conditions the ammonium analogue was released, while the fluoro and epoxy inhibitors were not. There are also significant differences in the behavior of *Claviceps* isomerase and yeast isomerase toward **9**. Reardon and Abeles (Reardon & Abeles, 1986) reported that **9** was released intact when the yeast enzyme–inhibitor complex was treated with 6 M urea and 400 mM potassium phosphate at 37 °C. Under conditions where they found greater than 90% release of inhibitor, we saw about 10% release with the *Claviceps* enzyme. Harsher treatments with SDS gave 30–70% release of radioactivity, but analysis of the products from ^{32}P, ^{14}C (ethylene carbons), and ^{3}H (methyls) labeled material revealed that between 60 and 70% of the inhibitor had degraded. Thus, it was possible that isomerase catalyzed the decomposition of **9** and that fragments were tightly bound noncovalently or covalently attached through a labile linkage. This question was resolved by brief treatment of the complex with 6 M urea at high pH, where the ammonium analogue was rapidly released intact. The reluctance of isomerase to release the ammonium analogue suggests that **9** is a tight-binding, slowly released analogue of the proposed tertiary cationic species. At the present time we cannot distinguish between a stepwise mechanism for isomerization with a tertiary cation as an intermediate or a concerted protonation–deprotonation sequence through a highly charged transition state.

Six other analogues of isopentenyl diphosphate inhibited the *Claviceps* enzyme irreversibly. Allylic fluorides **3–5** and epoxides **6–8** behaved as expected for active-site-directed irreversible inhibitors that become covalently attached to the protein. Inactivation of isomerase exhibited pseudo-first-order kinetics and saturation at high levels of inhibitor, a 1:1 inhibitor to active-site stoichiometry, and substrate protection. Inhibitor–isomerase complexes were stable during extended dialysis, treatment with urea and SDS, and SDS–polyacrylamide gel electrophoresis. Inhibition constants for **3–9** were in the low to submicromolar range. The most potent analogue, epoxide **6**, had an inhibition constant of 11 nM and a saturation inactivation rate constant of 3.7×10^{-3} s^{-1}.

Inhibitors **3–5** each contained an allylic fluoride moiety, and an obvious mechanism for inactivation is an S_N2 or S_N2' displacement of fluoride by a nucleophile in the active site. In our study, formation of the enzyme–inhibitor adduct with **3** was accompanied by the stoichiometric release of fluoride. Three other fluorinated analogues, both enantiomers of 2-fluoro-3-buten-1-yl diphosphate [(*R*)-**28** and (*S*)-**28**] and saturated analogue **29** were not irreversible inhibitors. Lack of inhibition by (*R*)- and (*S*)-**28** may reflect differences in reactivity between primary and secondary allylic centers toward S_N2 displacement or perhaps binding of the inhibitors in orientations incompatible with direct attack by an active-site nucleophile. Diphosphate **29**, which lacks the C3–C4 double bond found in **3**, would be expected to be considerably less reactive toward S_N2 displacement, perhaps by as much as a factor of 100 (DeWolfe & Young, 1956). In addition, the presence of a double bond may be important in binding. A logical mechanism for inactivation by epoxides **7** and **8** involves suicidal protonation of the oxirane oxygen followed by nucleophilic attack at carbon.

Because isomerization involves an antarafacial transposition of a proton, it has been suggested that a conjugate acid–base pair works in tandem to catalyze the reaction. pH–rate profiles for isomerase support this proposal. Isomerase from *Claviceps* and yeast has a broad maximum between pH 6 and 8. Reardon and Abeles (Reardon & Abeles, 1986) identified two kinetically important groups with pK_a's at 5.6 and 9.4, respectively. However, on the basis of V/K profiles they concluded that the ionization at 5.6 was due to the diphosphate moiety in the substrate. Plots of k_{inact} versus pH for fluoro analogue **3**, epoxide **6**, and ammonium analogue **9** presented in Figure 3 show somewhat different behavior. Analogue **3** gave a bell-shaped plot with a well-defined maximum at pH 7, while the plot for epoxide **6** had a broad maximal plateau between pH 5 and 8 with an abrupt decrease at higher pH. This behavior supports our contention that the oxirane ring is activated by protonation prior to nucleophilic attack. Analogue **9** was minimally active at low pH. However, k_{inact} increased rapidly above pH 7 and then dropped above pH 9.

These results are consistent with at least three different forms of the enzyme between pH 5 and 9. The transition near pH 6 for inactivation by **3** (Figure 3B) is consistent with the unmasking of an active-site nucleophile, which displaces the allylic fluoride. The profile seen for epoxide **6** in Figure 3C suggests the inhibitor reacts with singly or doubly protonated forms of isomerase and that activation of the oxirane ring by a proton transfer is required for subsequent covalent attack by an active-site nucleophile. In contrast, ammonium analogue **9** (see Figure 3D) appears to bind selectively to the doubly deprotonated form of isomerase.

Similar behavior for **9** was reported by Reardon and Abeles (Reardon & Abeles, 1986). The loss of inhibitory power by ammonium analogue **9** above pH 9 can be attributed to deprotonation of the ammonium moiety. Reardon and Abeles found that the related trimethylammonium derivative, which cannot deprotonate, retained activity up to pH 9.5. In addition, they discovered that the trimethyl analogue is a much less effective inhibitor ($K_D = 7 \times 10^{-6}$ M) than **9** ($K_D = 1 \times 10^{-11}$ M). This difference could result from different steric properties in the vicinity of the nitrogen or perhaps by selective formation of a hydrogen bond between the ammonium moiety in **9** and an acceptor in the catalytic site. Bartlett and Marlowe (Bartlett & Marlowe, 1987) recently proposed that a strong hydrogen bond between tight-binding phosphonoamidate

peptide analogues and thermolysin was responsible for approximately 4 kcal/mol of stabilization.

It should also be pointed out that isomerase, like proline racemase (Belasco et al., 1986), should have two monoprotonated forms. In one form the base adjacent to C4 of the isoprene unit is protonated and the enzyme catalyzes the isomerization of **1** to **2**, while in the other form the proton is on the base adjacent to C2 and the enzyme catalyzes isomerization in the reverse direction. There is no a priori reason to expect symmetry in the catalytic site, and the different pK_a's observed near pH 7 for k_{inact} versus pH profiles of **3**, **6**, and **9** may well reflect selective binding to the different monoprotonated isomers.

Isomerase is sensitive to iodoacetamide, and it has been suggested that a sulfhydryl moiety is part of the catalytic machinery of the enzyme. Reardon and Abeles (Reardon & Abeles, 1986) confirmed the earlier studies and reported that methyl methanethiosulfonate also inactivates the enzyme. They found that substrate protected against sulfhydryl inactivation and that inactivated enzyme did not bind ammonium analogue **9**. Bruenger and co-workers (Bruenger et al., 1986) found that *Claviceps* isomerase was inactivated by iodoacetamide, but in contrast to previous studies, the enzyme was not protected by substrate.

We repeated the experiments reported for the *Claviceps* enzyme and found that iodoacetamide inactivated isomerase with pseudo-first-order kinetics and that the enzyme was not protected by saturating levels of substrate. Furthermore, we found that catalytically inactive enzyme obtained by pretreatment with iodoacetamide bound **9**, while a heat-treated control did not. We also discovered that iodoacetamide-inactivated isomerase bound fluoro analogue **3**, although under the conditions of the experiment only 0.5 mol of inhibitor was bound per catalytic site. In view of these results we believe that it is premature to conclude that a sulfhydryl moiety is involved in proton transfer. Although we do not know the type of covalent bond formed between the irreversible inhibitors and isomerase, it is interesting to note that a small amount of catalytic activity can be restored to enzyme inactivated by **3** and **4** upon treatment with DTT.[3] This was accomplished by removing excess inhibitor by microconcentration. The inactive complex was then treated with 100 mM DTT for 20 min at 37 °C in standard buffer. Control samples treated in a similar manner in buffer without DTT had no detectable activity, while those incubated with DTT regained 10% of their original activity. This suggests that the enzyme–inhibitor linkage is reactive and can be ruptured by a strong nucleophile. Although we have no direct information about which amino acid furnishes the nucleophile, incubation of inhibited isomerase in the presence of strong base (0.05 and 0.2 M NaOH) for 1 h at 37 °C released less than 15% of the bound radioactivity. These results suggest that the inhibitor is not attached through an ester linkage.

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Mechanism-Based Inactivation of Rabbit Muscle Phosphoglucomutase by Nojirimycin 6-Phosphate†

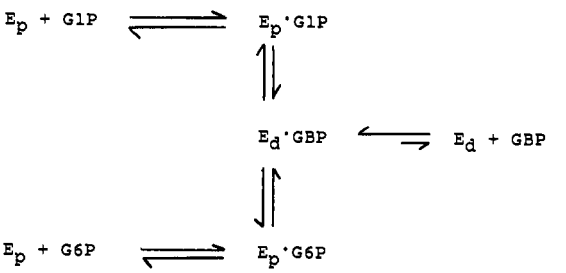
Sung Chun Kim and Frank M. Raushel*
Departments of Chemistry and Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843
Received April 8, 1988; Revised Manuscript Received June 1, 1988

ABSTRACT: Nojirimycin 6-phosphate (N6P) was tested as a substrate and inhibitor for phosphoglucomutase (PGM). In the absence of glucose 1,6-bisphosphate (GBP), the incubation of PGM and N6P resulted in the complete inactivation of all enzyme activity. When equimolar amounts of N6P and GBP were incubated together with PGM, the GBP was quantitatively converted to glucose 6-phosphate (G6P) and phosphate. At higher ratios of GBP and N6P (>100) the final concentration of G6P produced was found to be 19 times the initial N6P concentration. These results have been interpreted to suggest that the phosphorylated form of PGM catalyzes the phosphorylation of N6P at C-1. This intermediate rapidly eliminates phosphate to form an imine and the dephosphorylated enzyme. The dephosphorylated enzyme is rapidly rephosphorylated by GBP and forms G6P. The imine is nonenzymatically hydrated back to N6P. Occasionally (5%) the imine isomerizes to a compound that is not processed by PGM.

Phosphoglucomutase (PGM)[1] catalyzes the interconversion of glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P). The reaction catalyzed by phosphoglucomutase is activated by glucose 1,6-bisphosphate (GBP). Ray and Roscelli (1964) have proposed the mechanism shown in Scheme I. PGM has two forms; one is the phosphorylated enzyme (E_p), and the other is the dephosphorylated enzyme (E_d). Only the phosphorylated enzyme is active since it can phosphorylate either G1P or G6P to GBP in the enzyme active site. The newly formed GBP rephosphorylates the enzyme again, and the product, either G1P or G6P, is released from the phosphorylated enzyme. The thermodynamic equilibrium constant is 17.2 in favor of G6P in the pH range 6.2–7.5 at 30 °C (Najjar, 1962; Colowick & Sutherland, 1942). GBP can phosphorylate the dephosphorylated enzyme to the fully active phosphorylated form (Ray & Peck, 1972).

Nojirimycin is an antibiotic isolated from fermentation broths of several strains of *Streptomyces* such as *S. roseochromogenes* R-468, *S. lavendulae* SF-425, and *S. nojiriensis* n.sp. SF-426 (Inouye, 1968). Nojirimycin has been shown to be a potent inhibitor for α- and β-glucosidase (Legler, 1984; Niwa et al., 1970). Nojirimycin 6-phosphate (N6P) is the product from the reaction of nojirimycin with hexokinase and ATP (Drueckhammer & Wong, 1985). In an attempt to use PGM to synthesize nojirimycin 1-phosphate we found that in the absence of GBP the incubation of PGM with N6P resulted in the inactivation of enzyme activity. When equimolar amounts of N6P and GBP were incubated together with PGM, the GBP was quantitatively converted to G6P. In this paper, the inhibitory mechanism of PGM by N6P and the role of GBP in this mechanism are elucidated.

Scheme I



Materials and Methods

Hexokinase, glucose-6-phosphate dehydrogenase (G6PDH), GBP, G6P, PGM (300 units/mg), and G1P were purchased from Sigma Chemical Co. Nojirimycin was obtained from Dr. C.-H. Wong. Rabbit muscle PGM was the generous gift of Professor William J. Ray. [γ-^{32}P]ATP was purchased from ICN Radiochemicals. All other chemicals were purchased from either Sigma or Aldrich.

Preparation of Nojirimycin 6-Phosphate. The free form of nojirimycin was prepared from the nojirimycin bisulfite

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* Address correspondence to this author at the Department of Chemistry.

[1] Abbreviations: PGM, phosphoglucomutase; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GBP, glucose 1,6-bisphosphate; N6P, nojirimycin 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase.

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