Isopentenyldiphosphate Isomerase. Mechanism of Active-Site-Directed Irreversible Inhibition by 3-(Fluoromethyl)-3-butenyl Diphosphate

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Abstract: Isopentenyldiphosphate:dimethylallyldiphosphate isomerase catalyzes an antarafacial [1.3] proton migration by an electrophilic addition–elimination mechanism. Incubation of yeast isomerase with 3-(fluoromethyl)-3-butenyl diphosphate (**3**) led to rapid inactivation by an active-site-directed irreversible inhibition accompanied by covalent modification of the enzyme. Examination of ^{1}H, ^{13}C, and ^{19}F NMR spectra during irreversible inhibition with [4-^{13}C]-3-(fluoromethyl)-3-butenyl diphosphate indicated that displacement of fluorine incorporated the label into a protein-bound, unsymmetrically disubstituted olefinic methylene unit as expected for inactivation by an S_N2 mechanism.

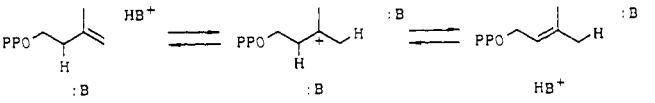
The isomerization of isopentenyl diphosphate (**1**) to dimethylallyl diphosphate (**2**) is a major activation step in the early stages of isoprenoid metabolism which transforms the unreactive homoallylic substrate into a potent electrophile.[1] Dimethylallyl diphosphate, in turn, serves as the primer for all subsequent prenyl transfer reactions used to construct carbon skeletons for a variety of important classes of compounds, including sterols, carotenoids, dolichols, and ubiquinones. Isopentenyldiphosphate:dimethylallyldiphosphate isomerase (EC 5.3.3.2) catalyzes the antarafacial [1.3] transposition of allylic protons in **1** and **2** by a protonation–elimination mechanism[1-3] (see Scheme I). The stereochemistry of the reaction suggests that two bases, one of which is in the conjugate acid form, are required to introduce and remove the protons.[2,3]

In the course of mechanistic studies with isomerase, we attempted to use fluorinated derivatives of **1** and **2** as alternate substrates for linear free energy correlations of enzyme-catalyzed rates with appropriate nonenzymatic models. Although none of the analogues were alternate substrates, several were active-site-directed irreversible inhibitors of isomerase.[2] 3-(Fluoromethyl)-3-butenyl diphosphate (**3**) was the most potent of the fluorinated analogues with K_I = 85 nM. Formation of an enzyme–inhibitor adduct between isomerase and **3** was accompanied by stoichiometric release of fluoride. Inhibition with [^{3}H]- or [^{32}P]-**3**, followed by denaturation of the inactivated enzyme, demonstrated that the remainder of the inhibitor remained covalently attached to isomerase. We also found that the double bond in **3** was essential for inactivation. These observations suggested that inhibition occurred by covalent attachment of the inhibitor to the enzyme as the result of an S_N2 or S_N2' displacement of fluoride from **3** by a nucleophile in the catalytic site of isomerase. We now report NMR studies with ^{13}C-labeled **3** which support an S_N2 mechanism.

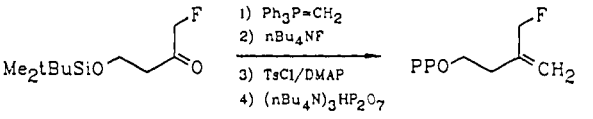
Results and Discussion

We recently demonstrated that incubation of isomerase with **3** resulted in a time-dependent inactivation of the enzyme by an S_N2 or S_N2', displacement of fluorine from the inhibitor.[2] To distinguish between the two possibilities, isomerase was inactivated with [4-^{13}C]-**3** using sufficient quantities of enzyme and inhibitor for analysis by NMR spectroscopy. ^{13}C was introduced by a Wittig condensation as shown in Scheme II according to the route developed by Muehlbacher and Poulter[2] for unlabeled **3**. Although our previous inhibition studies utilized isomerase from *Claviceps*, we decided to switch to the yeast enzyme because of the large quantity of material required for NMR experiments and the availability of yeast from commercial sources. The sequence of steps used to purify the enzyme described under Experimental Section combined features of procedures recently reported by Muehlbacher and Poulter[2] for *Claviceps* and by Reardon and Abeles[3] for yeast. In this manner we were able to obtain 7.5 mg of isomerase (sp ac. 4.9 μmol min^{-1} mg^{-1}).

Scheme I. Two-Base Mechanism for Isomerization of **1** to **2**



Scheme II. Synthesis of [4-^{13}C]-**3**



A ^{1}H-decoupled ^{13}C NMR spectrum of a 0.5 mM solution of [4-^{13}C]-**3** in 5 mM potassium phosphate, 1 mM DTT,[4] and 10% D_2O, pH 7.0, gave a closely spaced doublet ($J_{^{13}C^{19}F}$ = 11 Hz) which appeared as a single sharp peak at 117.7 ppm at wide sweep widths. The ^{13}C spectrum of isomerase shown in Figure 1A (7.5 mg, M_r 39000, 0.9 mM) in 200 μL of the same buffer at 4 °C showed broad regions of absorption from 10 to 65 ppm for the aliphatic carbons, from 125 to 135 ppm for aromatic carbons, and from 170 to 180 ppm for carbonyl resonances. Addition of [4-^{13}C]-**3** to the sample at a final concentration of 1 mM gave a spectrum (Figure 1B) that was identical with superimposed spectra of enzyme and inhibitor. However, when the sample was warmed to 37 °C and allowed to incubate for 3.5 h (Figure 1C), the intensity of the signal at 117.7 ppm decreased, and a new, broad resonance appeared slightly downfield at 119.6 ppm. Addition of more [4-^{13}C]-**3** (2 mM on the basis of total added inhibitor) followed by incubation at 37 °C for 8.5 h produced no further substantial changes in the spectrum. At this point the sample was removed from the NMR tube, placed in a microconcentrator, and spun as previously described in our earlier inhibition study.[2] A ^{13}C NMR spectrum of the filtrate had a single peak at 117.8 ppm characteristic of unreacted [4-^{13}C]-**3**. The sample remaining in the microconcentrator was diluted with D_2O and spun again. This procedure was repeated four times to remove unbound inhibitor. D_2O was then added to a final volume of 200 μL. A ^{13}C spectrum of the sample (Figure 1D) had a broad peak at 119.6 ppm, but the sharp resonance at 117.8 ppm had disappeared.

The ^{13}C spectra presented in Figure 1, in conjunction with our previous studies with radiolabeled **3**, indicated that the ^{13}C label resided in an olefinic methylene unit now covalently attached to

(1) Poulter, C. D.; Rilling, H. C. *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; pp 209-219.
(2) Muehlbacher, M.; Poulter, C. D. *Biochemistry* **1988**, *27*, 7315-7328.
(3) Reardon, J. E.; Abeles, R. H. *Biochemistry* **1986**, *25*, 5609-5616.

(4) Abbreviations: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; DTT, dithiothrietol; SDS, sodium dodecyl sulfate.

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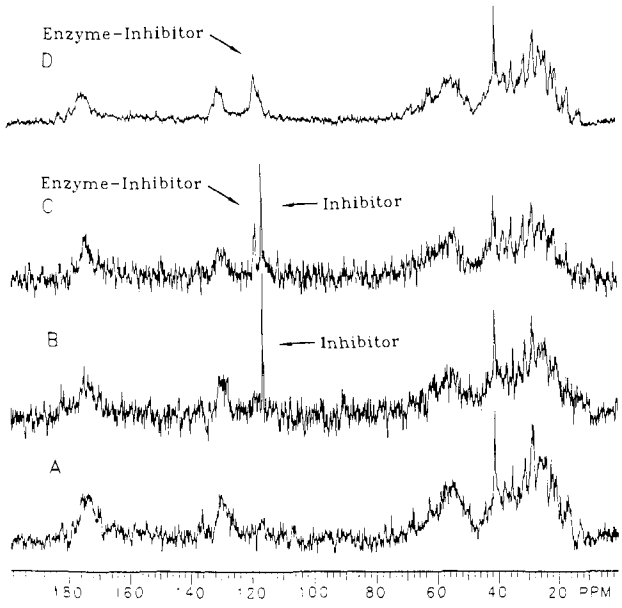


Figure 1. ^{1}H-decoupled ^{13}C NMR spectra of isomerase and isomerase treated with [4-^{13}C]-**3**: (A) 0.9 mM isomerase in 5 mM potassium phosphate, 1 μM DTT, and 10% D_2O, pH 7.0 at 4 °C; (B) after addition of [4-^{13}C]-**3** (1 mM total concentration) at 4 °C; (C) after incubation at 37 °C for 3.5 h; (D) inhibited enzyme after washing with D_2O to remove free [4-^{13}C]-**3**.

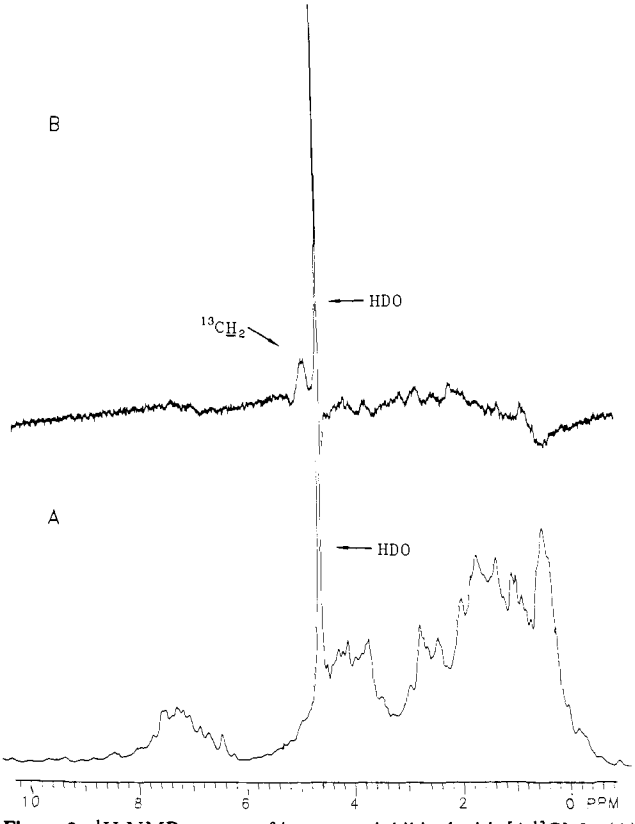


Figure 2. ^{1}H NMR spectra of isomerase inhibited with [4-^{13}C]-**3**: (A) ^{1}H NMR spectrum of 0.9 mM isomerase treated with 2 mM [4-^{13}C]-**3** and washed with D_2O to remove free inhibitor; (B) ^{1}H–^{13}C double resonance difference spectrum with ^{13}C decoupling at 119.6 ppm.

isomerase. This conclusion was confirmed by a ^{1}H–^{13}C chemical shift correlation study. A ^{1}H NMR spectrum of inhibited isomerase after the D_2O washes (the sample that gave the spectrum shown in Figure 1D) is presented in Figure 2A. Aliphatic protons appear in a series of envelopes from 0.5 to 4.3 ppm, and aromatic signals are clustered from 6.0 to 7.5 ppm. In addition, a sharp resonance from residual HDO was seen at 4.65 ppm. A ^{1}H–^{13}C

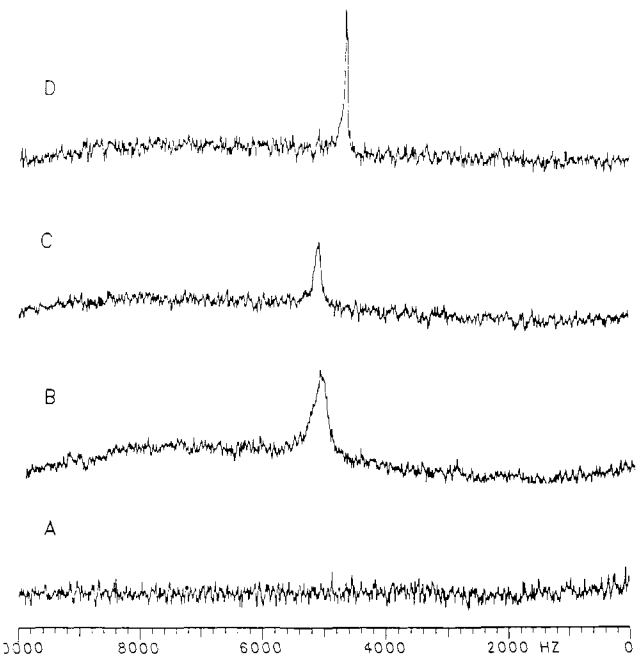


Figure 3. ^{19}F NMR spectra of isomerase treated with [4-^{13}C]-**3**: (A) 0.9 mM isomerase and 1 mM [4-^{13}C]-**3** in 5 mM potassium phosphate, 10 μM DTT, and 20% D_2O, pH 7.0 at 4 °C; (B) after addition of more [4-^{13}C]-**3** (2 mM total concentration) and incubation for 10 h at 37 °C; (C) filtrate from microconcentrator after incubation; (D) control sample of 0.5 mM potassium fluoride in 5 mM potassium phosphate, 10 μM DTT, and 10% D_2O, pH 7.0 at 37 °C.

difference spectrum employing single-frequency ^{13}C decoupling at 119.6 ppm is shown in Figure 2B.

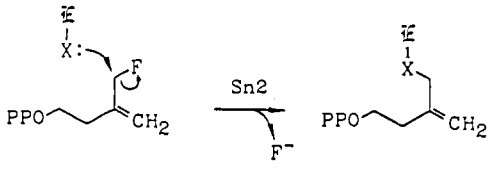
For samples enriched with a magnetically active isotope normally found in low natural abundance such as ^{13}C, the difference spectrum results in cancellation of resonances for protons not attached to the magnetically active heteronucleus, leaving only those signals in ^{1}H–^{13}C units.[5,6] In our study cancellation was not perfect as indicated by residual signals for the HDO peak and protons in the aliphatic and aromatic regions. However, a major new resonance appeared at 5.02 ppm in the difference spectrum in a region where no significant intensity was seen in the normal ^{1}H spectrum. The observation of a ^{1}H/^{13}C correlated peak at 5.02/119.6 ppm confirms the olefinic methylene nature of the labeled carbon in the inhibited enzyme.

^{19}F spectra were also obtained for some of the samples. [4-^{13}C]-**3** gave a 12-line pattern consisting of a doublet of triplets ($J_{^1H^{19}F}$ = 3.7 Hz, $J_{^{13}C^{19}F}$ = 10.6 Hz, and $J_{^1H^{19}F}$ = 47.2 Hz) at 213.5 ppm. As expected, a ^{19}F spectrum of isomerase was devoid of signals in this region. Furthermore, the fluoride region in the vicinity of 116 ppm for samples of [4-^{13}C]-**3**, isomerase, and 0.9 mM isomerase–1 mM [4-^{13}C]-**3** held at 4 °C (Figure 3A) was silent. However, a sample containing isomerase and 2 mM inhibitor that had been allowed to incubate for a total of 12 h at 37 °C had a broad peak centered at 116 ppm. The peak was also present in the first filtrate obtained by microcentrifugation (Figure 3C), thereby demonstrating that fluoride was not bound to the enzyme. When EDTA was added to a final concentration of 20 mM, the ^{19}F resonance sharpened noticeably and moved upfield by approximately 600 Hz (Figure 3D). The ^{19}F chemical shift of the EDTA-treated filtrate was identical with that of a control containing 0.5 mM potassium fluoride that had been subjected to similar treatment.

Our ^{1}H, ^{13}C, and ^{19}F studies establish that **3** becomes attached to isomerase by direct displacement of fluorine. Given the stability of the inhibitor in aqueous buffers upon prolonged incubation at 37 °C in the absence of enzyme,[7] it is unlikely that the dis-

(5) Griffey, R. H.; Davis, D.; Yamaizumi, Z.; Nishimura, S.; Bax, A.; Hawkins, B.; Poulter, C. D. *J. Biol. Chem.* **1985**, *260*, 9734–9741.
(6) Griffey, R. H.; Redfield, A. G. *Q. Rev. Biophys.* **1987**, *19*, 51–82.

Scheme III. Mechanism for Inactivation of Isomerase by [4-^{13}C]-**3**



placement is an S_N1 process. Alternatively, we propose an S_N2 reaction (see Scheme III) involving a nucleophile in the active site of isomerase. Although the nucleophile has not yet been identified, a carboxylate oxygen or a thioether sulfur are unlikely candidates. We have shown that the isomerase–**3** adduct is stable to treatment with base under conditions where an ester linkage should be hydrolyzed.[2] Furthermore, we do not favor the sulfur in methoinine because the resulting allylic sulfonium salt should be unstable toward solvolysis when the inhibited protein is denatured.[8] At the present time our NMR experiments do not permit us to distinguish among hydroxyl, sulfhydryl, and amine nucleophiles, and studies to address this question are now in progress.

Experimental Section

General. [1-^{14}C]Isopentenyl diphosphate for assays was purchased from Amersham and used directly or diluted to a specific activity to 10 μCi/μmol with synthetic material. All other materials were obtained from Sigma or Aldrich Chemical Co. and were of reagent grade or higher. Instafluor was purchased from Packard Instrument Co., and liquid scintillation spectrometry was on a Packard Tricarb Model 4530 spectrometer.

All steps in the purification of isomerase were at 4 °C except for HPLC which was at room temperature. Plasticware 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 membranes (M_r cutoff 10000). SDS–polyacrylamide gel electrophoresis of proteins employed the Laemli discontinuous buffer system. Gels were stained with Coomassie Brilliant Blue R or silver nitrate. Enzyme activity was determined according to the acid-lability assay.[2]

Isopentenyldiphosphate:dimethylallyldiphosphate isomerase was purified from fresh baker's yeast (*Saccharomyces cerevisiae*, Westco Distributing Co., Salt Lake City, UT) by combination of previously reported procedures. Four 1.5-kg batches were carried through ammonium sulfate precipitation and DE-52 chromatography, combined, and then carried through subsequent steps (chromatography on butyl-Sepharose, chromatofocusing, and chromatography on Sephacryl-200 columns) to yield 7.5 mg (sp act. 4.9 μmol mg^{-1} min^{-1}) of enzyme for NMR experiments.[2,3]

NMR Spectroscopy. NMR spectra were recorded on a Varian VXR 500-MHz spectrometer. A 5-mm broad-band probe was used for all experiments with ^{1}H and ^{19}F observation through the ^{1}H-decoupling coil. This permitted ^{1}H, ^{13}C, and ^{19}F spectra to be acquired during the course of the enzymatic reaction without removing the sample from the insert. ^{1}H and ^{13}C spectra were referenced downfield to internal DSS, ^{19}F upfield to external $CFCl_3$, and ^{31}P downfield to external 85% phosphoric acid.

^{13}C spectra were collected with a spectral width of 25 kHz into 32K complex points which were zero-filled to 64K prior to transformation. Approximately 2 W of radio-frequency power was used with WALTZ-16 modulation to decouple protons. The ^{13}C 90° pulse width was 11 μs.

^{19}F spectra were collected with a spectral width of 10 kHz into 8K points which were zero-filled to 16K points prior to transformation. A relatively narrow sweep width was used to observe fluorine bound to carbon or free fluoride since it was not possible to cover the entire range in a single experiment. The ^{19}F 90° pulse width was 30 μs.

^{1}H and ^{13}C difference decoupled spectra were obtained by collection of 14000 transients with single-frequency ^{13}C decoupling at 119.6 ppm (on-resonance) and 14000 transients with the ^{13}C-decoupling field 75 kHz off-resonance. Spectra were collected in blocks of 64 transients in an interleaved fashion and then subtracted to produce the difference spectrum. The residual HDO peak and the most intense protein peaks did not cleanly cancel. The ^{1}H 90° pulse width was 20 μs.

Synthesis of [4-^{13}C]-3-(Fluoromethyl)-3-butenyl Diphosphate ([4-^{13}C]-3). [4-^{13}C]-3-(Fluoromethyl)-3-butenyl diphosphate ([4-^{13}C]-**3**) was synthesized from [^{13}C]triphenylmethylphosphonium iodide according to the procedure developed by Muehlbacher and Poulter[2] for unlabeled material. The phosphonium salt was prepared from [^{13}C]methyl iodide (Sigma) by the following procedure. Triphenylphosphine (1.83 g, 7 mmol) was dissolved in 5 mL of ether in a heavy-walled ampule. The vial containing methyl iodide (1.00 g, 7 mmol) was opened, 1 mL of chilled ether was added, and the contents were transferred to the ampule. The procedure was repeated twice with 1-mL portions of ether. The resulting solution was cooled to −78 °C, and the ampule was sealed. The reaction mixture was allowed to stand for 3 days at room temperature before the ampule was opened, and the resulting white suspension was filtered. The residue was washed with 4:1 hexane/ethyl acetate and dried under vacuum for 6 h to yield 2.33 g (82%) of [^{13}C]triphenylmethylphosphonium iodide, which was used in the subsequent reactions to give 200 mg (21% based on [^{13}C]methyl iodide) of a white powder; ^{1}H NMR (300 MHz, D_2O) 2.24 (2 H, q, J = 6.1 Hz, H at C2), 3.85 (2 H, q, J = 6.1 Hz, H at C1), 4.71 (2 H, dd, $J_{^1H^{13}C}$ = 5.7 Hz, $J_{^1H^{19}F}$ = 46.1 Hz, fluoromethyl H), 4.96 (1 H, d, $J_{^1H^{13}C}$ = 157.9 Hz, H at C4), and 5.01 ppm (1 H, d, $J_{^1H^{13}C}$ = 157.2 Hz, at C4); ^{13}C NMR (75 MHz, D_2O, ^{1}H decoupled) 117.72 ppm ($J_{^{13}C^{19}F}$ = 10.8 Hz); ^{19}F NMR (470 MHz, D_2O) 213.52 ppm (dt, $J_{^1H^{19}F}$ = 46.1 Hz, $J_{^1H^{19}F}$ = 9.3 Hz); ^{31}P NMR (121 MHz, D_2O, ^{1}H decoupled) −7.02 (1 P, d, J = 21.2 Hz) and −9.96 ppm (1 P, d, J = 21.2 Hz).

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(7) No decomposition of **3** was detected after 24 h at 37 °C in aqueous buffer.

(8) Trost, B. M.; Conway, P.; Stanton, J. *Chem. Commun.* **1971**, 1639–1640.

Communications to the Editor

Solid-State Chemistry in Antimony Pentafluoride Matrices. Infrared Spectra of Reactive Intermediates†

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On the basis of Olah's fundamental work[1] which demonstrated the advantage of superacidic media for the study of carbocations we developed a method for matrix deposition of suitable precursors in SbF_5 and subsequent IR analysis of some carbocations and carbocation-like species in the solid state. Our technique is a combination of Saunders' "molecular beam" method[2] and the cold window codeposition technique of Craig et al.[3] Using a liquid

† Dedicated to the memory of Dr. Zdenko Majerski (1937–1988).

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