

Figure 2. Mims ENDOR spectra of $[4Fe-4S]^+$ cluster of aconitase (A) without substrate and (B) with substrate (citrate). Spectra are plotted as $\delta\nu = \nu - \nu_D$. Experimental conditions: T = 2 K; $\nu_e = 9.14$ (A), 9.09 (B) GHz; $H_0 = 3525$ (A), 3645 (B) G; microwave pulse width, 16 ns; rf pulse width, 50 μ s; $\tau_{12} = 380$ (A), 440 (B) ns; (A) 168, (B) 880 scans.

ENDOR spectra from which hyperfine and quadrupole splittings can be determined directly. Figure 1B is a single-crystal-like^{5a,b} ²H Mims ENDOR spectrum taken^{7b} at the high-field (g_3) edge of the Anabaena Fd EPR absorption envelope, using the same small sample used at the Q-band.^{5d} It shows a pair of peaks centered at ν_D and separated by 0.60 MHz. This splitting, which is far too large to be associated with the quadrupolar interaction, represents a deuteron hyperfine coupling, $A^D \approx 0.60$ MHz.¹⁰ The peaks further show a partially resolved quadrupole splitting of $2P^D \approx 0.1$ MHz. As no exogenous ligands are associated with the cluster, this local deuteron can be assigned to a hydrogen N-H...S bond, presumably one of the two putative strong H bonds seen in the crystal structure of the oxidized protein.^{6a} arginine 42 H-bonded to a cysteinyl mercaptide sulfur bound to iron or arginine 258 H-bonded to a bridging clu: et S²⁻.

The ²H splitting varies little in the ENDOR spectra taken at magnetic field values across the EPR envelope (data not shown), which indicates that the coupling is nearly isotropic.^{5a,b} This is equivalent to an isotropic proton interaction, $A^{\rm H} = (g_{\rm H}/g_{\rm D})A^{\rm D} = 6.51A^{\rm D} \approx 3.9$ MHz, whose size indicates that this H bond must have significant covalency. Variations in the quadrupole term indicate that $K^{\rm D} \equiv 3e^2qQ/2h \lesssim 0.2$ MHz, in agreement with the expectation from model compounds.¹⁰

The explanation for the difference in resolution of the CW and pulsed deuterium signals is likely as follows. In the former, distant ENDOR signals from noninteracting deuterons^{9,5d} overwhelm the local ENDOR of H-bonded deuterons. In pulsed ENDOR, an individual pulse sequence ($\leq 50 \ \mu s$) is shorter than the time for the spin diffusion processes that are the basis of the distant CW ENDOR response; thus the distant ENDOR response is "quenched", and local ENDOR signals become visible.

The $[4Fe-4S]^+$ cluster of aconitase has one iron ion (Fe_a) that has been shown by ¹⁷O CW ENDOR to be coordinated to a solvent species, $H_xO^{2a,5c}$ The enzyme shows a rhombic EPR signal, with $g_{1,2,3} = 2.06$, 1.93, and 1.86 in the absence of substrate, and $g_{1,2,3}$ = 2.04, 1.85, and 1.78 in the presence of substrate (citrate). Figure 2A is a single-crystal-like ²H Mims ENDOR spectrum of the enzyme in D₂O without substrate, taken at the high-field edge

 $(g_3 = 1.86)$ of the EPR absorption envelope. It shows one pair of peaks from D_xO at $\delta v_{\pm} = v_{\pm} - v_D \approx \pm 0.25$ MHz, with each showing a further small splitting of ~ 0.12 MHz. We assign the spectrum to a deuteron with hyperfine interactions, $A^{\rm D} \approx 0.50$ $(A^{\rm H} \approx 3.25)$ MHz, and quadrupole splitting, $2P^{\rm D} \approx 0.12$ MHz. The pulsed ENDOR spectrum taken at $g_3 = 1.78$ for the enzyme with substrate (nondeuterated citrate) in D₂O (Figure 2B) shows a more complex pattern, with four deuterium peaks at $\delta v_{\pm} \approx \pm 0.61$ and ± 0.23 MHz. An assignment to a single deuterium with $A^{\rm D}$ = 0.84 MHz and quadrupole splitting $2P^{D} = 0.38$ MHz can be discounted because the latter is improbably large;¹⁰ instead, the peaks are assigned to two inequivalent nuclei that have $A^{D} = 1.22$ and 0.46 (A^{H} = 7.9, 3.0) MHz and no observable quadrupole splitting at this field. Comparison with the ¹H resonances lost upon H/D exchange^{5c} confirms the latter assignment. The ²H Mims ENDOR spectrum of enzyme without substrate has significantly better resolution than the corresponding O-band CW ENDOR spectrum; that of enzyme with substrate also is improved.5c These high-resolution spectra support the earlier suggestion that the H_vO species coordinated to the [4Fe-4S]⁺ cluster of the enzyme with substrate is a water molecule, whereas it is a hydroxyl ion in the enzyme without substrate.^{2a,5c}

The data presented here clearly show that ²H Mims pulsed ENDOR examination of H bonds and H_xO coordinated to metal clusters provides an important complement to multifrequency CW ENDOR⁵ and its pulsed analogue, Davies ENDOR,^{4d,7b} which typically do best with larger couplings, and to ESEEM, which is well-suited for measuring distances to and numbers of dipolecoupled exchangeable protons in the active-site vicinity.^{3c}

Acknowledgment. This work could not have been performed without the expert assistance of Mr. Clark E. Davoust and supportive discussions with Dr. Peter E. Doan. The Anabaena 7120 Fd was kindly provided by Professor John L. Markley. This work was supported by the NIH [HL 13531 (B.M.H.), GM 34812 (H.B.)], NSF [DBM-8907559 (B.M.H.)], and USDA [90-37120-5604 (B.M.H.)]. Equipment was purchased with a grant from the NIH (DRR 04936) and received support from the Materials Research Center of Northwestern University (DMR-88 21571).

Stereochemical Analysis of Totally Stereoselective, Competing [1,2]- and [2,3]-Wittig Rearrangements. Inversion at the Lithium-Bearing Carbon Atom

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We present the first stereochemical analysis of $[1,2]^{-1}$ and $[2,3]^{-2}$ Wittig rearrangements which includes the stereochemical change that occurs at the lithium-bearing carbon atom. Counterintuitively, the [2,3]-rearrangement requires a trans disposition of the two substituents in a 2-lithio-6-vinyltetrahydropyran, and both rearrangements occur with *inversion of configuration at the lithium-bearing carbon atom.*³ This inversion in the case of the

^{(10) (}a) Recall (refs 5a,b) that ENDOR frequencies for ${}^{2}H(I=1)$ have the form $\nu_{\pm}(m) \approx |\pm A^{D}/2 + \nu_{D} + P^{D}(2m-1)|$, m = 0, 1, for a given field orientation. (b) Studies on model compounds (ref 10c) show that the maximum deuterium quadrupole splitting falls in the range $2|P_{1:1}| \equiv K^{D} \approx 0.26-0.30$ MHz for $R_{1}R_{2}ND$ that is H-bonded, $2|P_{1:1}| \approx 0.20-0.36$ MHz for OD⁻, and $2|P_{1:1}| \approx 0.30-0.38$ MHz for D₂O. (c) Edmonds, D. T. *Phys. Rep.* **1974**, 29(4), 233.

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Scheme I



Table I. Wittig Rearrangements of 2-Lithio-6-(1-propenyl)tetrahydropyrans 2

| $\begin{array}{c} R^{2} \\ R^{1} \\ R^{1} \\ R^{2} \\ L_{i} \text{ or b78 °C, 12 h} \\ R^{3} \\ R^{2} \\ R^{2} \\ R^{3} \\ R^{3} \\ R^{3} \\ R^{3} \\ R^{3} \\ R^{1} \\ R^{1} \\ R^{3} \\ H \\ R^{1} \\ R^{3} \\ H \\ R^{3} \\ $ | | | | | | |
|--|-----------------|-----------------|-----------------------|------|--------|--------|
| compd | R ¹ | R ² | R ³ | 3, % | 4, % | method |
| 2A | CH ₃ | Н | Н | 21 | 45 | a or b |
| 2B | H | CH ₃ | H | 28 | 3 (4A) | a |
| 2C | CH ₃ | Н | CH_3 | 79 | 0 | a |

[2,3]-rearrangement has recently been anticipated by Wu, Houk, and Marshall⁴ on the basis of theoretical calculations; we have arrived at essentially the same transition state on the basis of experimental results.

Earlier work⁵ indicated that a wide variety of α -lithio ethers could be generated by reductive lithiation of α -(phenylthio) ethers, and we⁶ and others⁷ have used the resulting anions in Wittig rearrangements. The finding⁸ that the proximate product of reductive lithiation of readily produced 2-(phenylthio)tetrahydropyrans possesses an axial carbon-lithium bond has now allowed the determination of the stereochemistry of these rearrangements. The method of preparation of the 2-lithio-6-vinyltetrahydropyrans used in the present study is outlined in Scheme I (LDBB is lithium 4,4'-di-tert-butylbiphenylide⁹).¹⁰ The expected^{8,11} trans arrangement of the Li and vinyl substituents was shown to be virtually complete by ¹H NMR analysis of the products obtained by quenching 2D with CH₃OD and 2C with CH₃OH, processes that occur with configurational retention.^{8,12,13}

Upon being warmed, 2A-C yield as the only identified compounds [1,2]- and [2,3]-rearrangement products (3 and 4, respectively) (eq 1, Table I).¹⁴ In the ring contraction product 3. one of the two chiral centers of the substrate has been inverted and one retained. Since the literature contains many examples of partial retention of the migrating group in [1,2]-Wittig rearrangements,¹ it is likely that this group retains its configuration and that the lithium-bearing carbon atom becomes inverted, as

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indicated in eq 1. This was shown to be the case by conducting the rearrangement of nonracemic 2E prepared by the use of nonracemic 1E, generated by Sharpless resolution.15,16

The usual assumption² that the [2,3]-rearrangement is concerted seems particularly likely in the case of 2A since it is the major reaction path despite the undoubtedly more crowded conformation of the precursor (see below). Since the propenyl group must undergo a suprafacial change, an inversion must have occurred at the lithium-bearing carbon atom. These stereochemical results are readily rationalized in Scheme II.

The equatorial C-Li bond is aligned antiperiplanar to the C-O bond that cleaves in both processes, and in fact, the σ, σ^* overlap is a substantial contributor to the stabilization of these conformers.¹⁷ The widely accepted Lansbury¹⁸ mechanism nicely explains the stereochemistry of the [1,2]-process. Unlike other cases whose stereochemistry has been studied, the two radical centers of 5, generated by homolysis of the C-O bond, are held together by a tether, accounting for the fact that recombination, very rapid in any case, 1c,19 occurs before bond rotations.19 Thus retention at the migrating chiral center and inversion at the terminus occur. The undoubtedly far less stable endo conformer of **2A** is the only one capable of undergoing a concerted [2,3]-Wittig rearrangement, assuming the necessity of an equatorial C-Li bond. Such a process yields a cis olefin as can be seen by noting the juxtaposition of the circled hydrogen atoms. This six-electron process is consistent with the rules of orbital symmetry²⁰ in that it involves a suprafacial process and two inversions, one at the carbon atom bearing the lithium and the other at the oxygen atom $(\pi 2_s + \sigma 2_a + \sigma 2_a)$. The importance of this transfer of Li⁺ from C to O during the [1,2]-process has been highlighted by the depression in rate that is observed when Li⁺ is replaced by K⁺.1c

This picture of the [2,3]-process leads to several testable predictions. The endo conformation should be greatly destabilized by steric congestion if a *cis*-propenyl group were used as in 2B or if the hydrogen atom on the lithium-bearing carbon atom were replaced by a methyl group as in 2C. In fact, the concerted [2,3]-process is inhibited in both cases (Table I); only 2B produces a trace of cycloheptenol, and it is not 4B but 4A, presumably formed by a nonconcerted process from the exo conformer. Finally, we have found²¹ that an analogue in which the 2-lithio and 6-propenyl substituents are cis to each other and trans to a 4tert-butyl group undergoes no [2,3]-rearrangement even though it is undoubtedly born in the 2,6-diaxial conformation. This result rules out the possibility that such a conformer, generated by a thermal inversion^{8a,11} of the lithium-bearing carbon atom, is an intermediate in the rearrangements of 2A-C.

The full paper will explore the synthetic aspects of these rearrangements, including the preparation of the hydrazulene ring system by this type of ring expansion.

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Acknowledgment. We thank the National Institutes of Health for financial support and Steven Norton and Professors Peter Wipf and Russell Petter for helpful suggestions.

Supplementary Material Available: Experimental procedures and spectral data (5 pages). Ordering information is given on any current masthead page.

Synthesis of the Unusual Metabolite Carboxyphosphonoenolpyruvate. Cloning and Expression of Carboxyphosphonoenolpyruvate Mutase

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The carbon-phosphorus bond of most naturally occurring phosphonates derives from a 1,3-phospho group transfer reaction between phosphoenolpyruvate (1) and phosphonopyruvate (2).¹ The equilibrium for this rearrangement, which is catalyzed by phosphoenolpyruvate mutase (EC 6.4.2.9), lies predominantly toward 1.¹ This enzyme is not, however, responsible for the



formation of either of the two carbon-phosphorus bonds in bialaphos, a powerful herbicide isolated from *Streptomyces hy*groscopicus. Thus Seto and his collaborators^{2,3} have shown that the biosynthetic pathway to bialaphos involves carboxyphosphonoenolpyruvate mutase (CPEP mutase), which catalyzes the formation of (hydroxyphosphinyl)pyruvate (5) from carboxyphosphonoenolpyruvate (3, CPEP). By analogy with phosphoenolpyruvate mutase, the first step of the CPEP mutase reaction presumably generates the new carbon-phosphorus bond by carboxyphospho group migration to give 4. This reaction seems likely



to be energetically unfavorable (as is the conversion of 1 to 2). In the second step, the intermediate 4 would decarboxylate, thus driving the reaction toward 5. Mechanistic studies on this interesting enzyme will be possible only with a supply of the substrate 3, a continuous product assay for 5, and ready access to the enzyme. We report here the chemical synthesis of the unusual phosphonate 3, the cloning of the mutase gene from S. hygroscopicus and its expression at high levels in Escherichia coli, and a convenient assay for the product, 5.

Despite earlier suggestions that 3 is "extremely unstable",³ the chemical synthesis of 3 proceeded smoothly, the key step⁴ involving

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a Perkov reaction between bis(trimethylsiloxy)(methoxycarbonyl)phosphine⁵ (6) and ethyl bromopyruvate to give thetriester 7. Although recent studies on the hydrolysis of trialkyl



esters of phosphonoformate have shown that the carbon-phosphorus bond is readily cleaved by attack at carbonyl,⁶ treatment of 7 with aqueous base first removes the reactive $SiMe_3$ group to give the diester, in which the carbon-phosphorus bond is much less vulnerable. The triester 7 was readily converted to 3 by the careful addition of 3 equiv of aqueous NaOH.⁷ The spectroscopic data⁷ were consistent with those reported for the natural product.² The trisodium salt of 3 is stable in water over several days at room temperature. Carboxyphosphonoenolpyruvate (3) will be useful for studies of both CPEP mutase and the enzyme that catalyzes CPEP formation from phosphoenolpyruvate and phosphonoformate.⁸

To develop an assay for CPEP mutase, a sample of $5^{9,10}$ was prepared by the transamination of (hydroxyphosphinyl)alanine¹¹ with glyoxylic acid-Cu(OAc)₂.¹² We had earlier shown that **2** is a relatively poor substrate for malate dehydrogenase (MDH), having a high K_m of 11 mM. In terms of both size and charge, 5 should be a better mimic for oxaloacetate than **2**. As predicted, 5 is a good substrate for MDH, having a K_m of 0.68 mM and a k_{cat} of 164 s⁻¹.¹³ Interestingly, the product of this reaction, (hydroxyphosphinyl)lactate, has been found in extracts of *S*. *hygroscopicus*.¹⁴ Using MDH/NADH, therefore, a continuous coupled enzyme assay can be established for CPEP mutase.

Using the partial gene sequence reported by Hidaka et al.,² we have cloned the gene for CPEP mutase from S. hygroscopicus into E. coli. The open reading frame encodes a protein of 295 amino acids, the calculated molecular weight of which (32 700) agrees with that determined for the purified enzyme (32000 \pm 1000).² The gene was expressed in E. coli using the T7 pET11 vector, which produced CPEP mutase at 20% of the total cell

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Freeman, S. J. Chem. Soc., Perkin Trans. 2 1991, 1297. (7) Addition of methanol to the aqueous solution caused precipitation of trisodium carboxyphosphonoenolpyruvate (3) in 53% yield: mp 275-280 °C dec; ¹H NMR (250.1 MHz, D₂O) δ 5.54 (t, J_{PH} = J_{gem} = 1.7 Hz, 1 H), 5.20 (t, J_{PH} = J_{gem} = 1.8 Hz, 1 H); ¹³C NMR (62.9 MHz, D₂O) δ 179.6 (d, J_{PC} = 235.4 Hz, PCOO⁻), 173.4 (d, J_{PC} = 6.1 Hz, CCOO⁻), 151.8 (d, J_{PC} = 9.2 Hz, C=CH₂), 106.0 (d, J_{PC} = 4.8 Hz, C=CH₂); ³¹P NMR (101.3 MHz, D₂O) δ -1.77 (s); IR (KBr) 1603 cm⁻¹. Calcd for C₄H₂O₇PNa₃: C, 18.34; H, 0.77; P, 11.82. Found: C, 18.00; H, 1.01; P, 11.48.

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