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On the Stereochemistry of 2-Hydroxyethylphosphonate Dioxygenase

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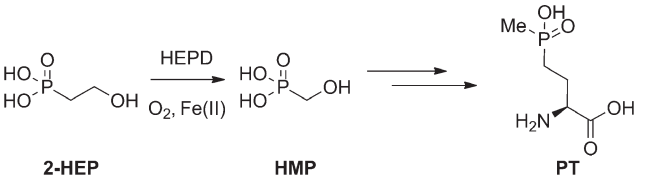
Supporting Information

ABSTRACT: Stereochemical investigations have shown that the conversion of 2-hydroxyethylphosphonate to hydroxymethylphosphonate by the enzyme HEPD involves removal of the pro-*S* hydrogen at C2 and, surprisingly, the loss of stereochemical information at C1. As a result, the mechanisms previously proposed for HEPD must be re-evaluated.

Natural-product phosphonates and phosphinates have found wide use in medicine and agriculture.[1] One such compound, phosphinothricin (PT),[2,3] is the active component in commercial herbicides (Liberty, Basta, and Ignite) that are used in conjunction with transgenic crops such as corn, cotton, soy, and canola.[4−6] Recent genetic and biochemical studies on PT biosynthesis have shown that 2-hydroxyethylphosphonate (2-HEP) is converted to hydroxymethylphosphonate (HMP) by hydroxyethylphosphonate dioxygenase (HEPD), the gene product of *phpD* (Scheme 1).[7,8]

Investigations of this unique carbon−carbon bond cleavage reaction have shown that HEPD is a non-heme mononuclear iron-dependent dioxygenase that requires only ferrous iron and molecular oxygen for activity.[8] Studies using 2-HEP isotopologues, $^{18}O_2$, and $H_2{}^{18}O$ identified formate as an additional product and led to two proposed mechanisms (Scheme 2).[8] In each case, O_2 activation is postulated to generate a ferric superoxo species.[9] After H-atom abstraction from C2 of 2-HEP to generate substrate radical **I**, the mechanisms differ in that the substrate is either hydroxylated with formation of an Fe(IV)═O intermediate or hydroperoxylated to form Criegee intermediate **III**. Experiments in which substrate analogues were incubated with HEPD resulted in the direct observation of Criegee rearrangement products.[10] These results, in combination with the expected high pK_a of the anion in intermediate **II**, suggesting a high-energy intermediate,[11,12] previously led us to favor the hydroperoxylation mechanism.[10] However, this proposal requires a very unusual hydrolysis of the intermediate formyl ester to account for the partial incorporation of oxygen from the solvent into the hydroxyl group of HMP.[8] To further investigate the mechanism of the reaction, in this study we examined the stereochemical course of catalysis at C1 and C2 of 2-HEP. If the reaction proceeds by a hydroperoxylation mechanism, net inversion of configuration should occur at C1 with retention during the Criegee rearrangement[13] and inversion in the hydrolysis step.

Scheme 1. 2-Hydroxyethylphosphonate (2-HEP) Is Converted to Hydroxymethylphosphonate (HMP) by HEPD during Phosphinothricin (PT) Biosynthesis



A previous investigation showed that both hydrogen atoms at C1 were retained in HMP when 2-[1-$^2H_2$]-HEP was used as a substrate.[8] To complement this experiment, 2-[2-$^2H_2$]-HEP was synthesized (Scheme S1 in the Supporting Information) and incubated with HEPD. The HMP and formate produced were characterized as previously described,[8,10] and it was found that the formate contained deuterium but the HMP did not (Figure S1 in the Supporting Information), thus confirming that the putative hydrogen atom abstraction is regioselective. To test the stereospecificity of this step, (*R*)- and (*S*)-2-[2-$^2H_1$]-HEP were synthesized[16] and incubated with HEPD. The formate produced from (*R*)-2-[2-$^2H_1$]-HEP contained deuterium, whereas that produced with the *S* enantiomer did not (Figure S2). These observations show that the pro-*S* hydrogen is abstracted, which agrees well with the active-site geometry and bidentate substrate binding observed in the cocrystal structure of Cd(II)−HEPD with 2-HEP.[8]

We next prepared (*R*)- and (*S*)-2-[1-$^2H_1$]-HEP (84 and 86% ee, respectively; 99% isotopic purity for both compounds)[17] as substrates for HEPD. Moreover, authentic (*R*)- and (*S*)-[$^2H_1$]-HMP with 99% ee were prepared from (*R*)- and (*S*)-diisopropyl hydroxy-[$^2H_1$]-methylphosphonate[18] (Scheme S2). The compounds were deprotected using TMSBr/allylsilane[19] followed by hydrolysis of the formed silyl esters. For storage and later use, the free phosphonic acids were converted to the ammonium salts. To prepare the (*R*)-Mosher esters [i.e., the esters of (*R*)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA)], the free acids were esterified at the phosphonate group with a distilled ethereal solution of diazomethane to produce dimethyl hydroxy-[$^2H_1$]-methylphosphonates, and the hydroxyl groups were subsequently esterified with (*S*)-MTPACl/pyridine.

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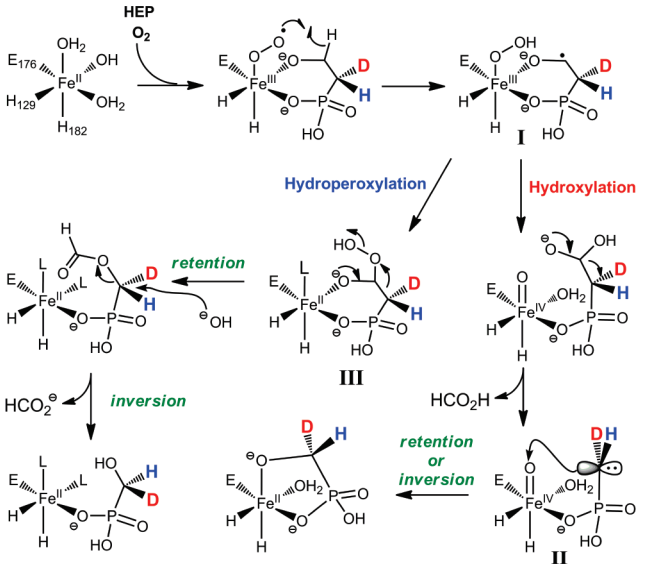
Scheme 2. Previously Proposed Mechanisms for the HEPD-Catalyzed Conversion of 2-HEP to HMP Showing the Predicted Stereochemistry with Stereoselectively Labeled 2-[1-$^2H_1$]-HEP[a]



[a] If the reaction proceeds through hydroxylation and involves two-electron chemistry, retention or inversion of configuration may be expected depending whether attack of intermediate **II** on the Fe(IV)=O proceeds from the same (as shown) or opposite face as the loss of formate. This step was expected to be stereospecific on the basis of precedents with enzymatic reactions involving resonance-stabilized methyl anions (enolates).[14,15]

Synthetic (*R*)- and (*S*)-2-[1-$^2H_1$]-HEP were incubated with HEPD, and the produced [$^2H_1$]-HMP was derivatized to the dimethyl ester, which was purified to afford milligram quantities of dimethyl [$^2H_1$]-HMP. The stereochemistry of this product was assessed by conversion to the corresponding Mosher's ester as described above and subsequent ^{1}H NMR analysis. Unexpectedly, comparison with the Mosher's esters prepared from authentic (*R*)- and (*S*)-[$^2H_1$]-HMP showed that HEPD converted both (*R*)- and (*S*)-2-[1-$^2H_1$]-HEP into near-racemic [$^2H_1$]-HMP (Figure 1 and Scheme 3a). Because of this unanticipated outcome, further experiments were conducted to ensure that the stereochemical information was not lost during conversion of HMP to its phosphonate dimethyl ester or its Mosher's ester.

Previous experiments demonstrated that HMP is also a substrate for HEPD that is slowly converted to phosphate and formate.[10] We first determined the stereochemistry of this reaction. When (*R*)-[$^2H_1$]-HMP was incubated with HEPD, the generated formate contained no deuterium, whereas when (*S*)-[$^2H_1$]-HMP was used, the formate produced contained 1 equiv of deuterium. After it had been established that oxidation of HMP is stereospecific, (*R*)- and (*S*)-2-[1-$^2H_1$]-HEP were incubated with HEPD until full conversion of both 2-HEP and the initial HMP product to phosphate and 2 equiv of formate was achieved (Scheme 3b). If the conversion of 2-HEP to HMP were stereospecific at C1, the total formate produced from (*R*)- and (*S*)-2-[1-$^2H_1$]-HEP should contain either 50% deuterium or no deuterium. However, we observed experimentally that both enantiomers of 2-[1-$^2H_1$]-HEP resulted in 25% deuterium incorporation in the formate. This finding is consistent with

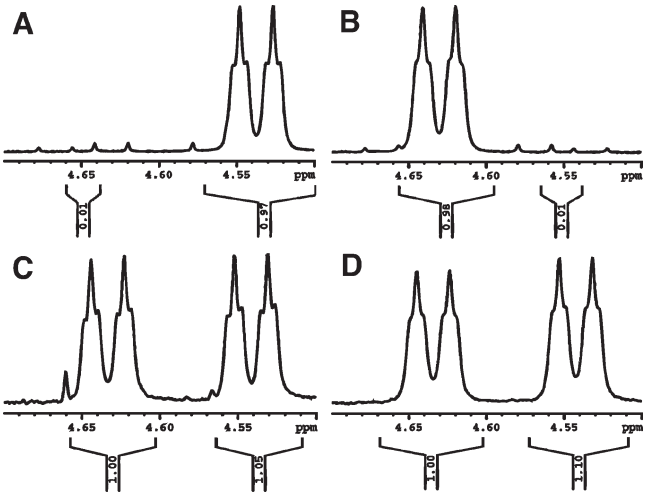
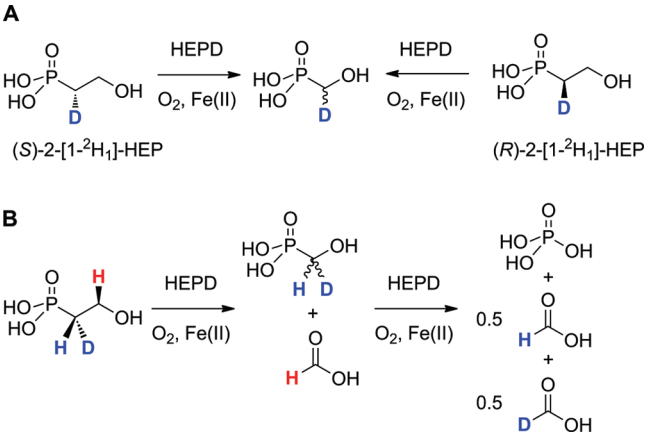


Figure 1. ^{1}H NMR spectra of the PCHD groups of the (*R*)-Mosher esters prepared from (*R*)- and (*S*)-hydroxy-[$^2H_1$]-methylphosphonic acid (panels A and B, respectively) and from the products of incubation of (*R*)- and (*S*)-2-hydroxy-[1-$^2H_1$]-ethylphosphonic acid with HEPD (panels C and D, respectively). The spectra were taken at (A, B) 400.13 and (C, D) 400.27 MHz in $CDCl_3$.

Scheme 3. (A) Both (*S*)-2-[1-$^2H_1$]-HEP and (*R*)-2-[1-$^2H_1$]-HEP Result in Racemic [$^2H_1$]-HMP; (B) Conversion of (*S*)-2-[1-$^2H_1$]-HEP to Phosphate and 2 equiv of Formate Results in 75% Unlabeled Formate and 25% Deuterium-Labeled Formate



racemization at C1 during the conversion of 2-HEP to HMP (Scheme 3b).

A large kinetic isotope effect (KIE) on H/D abstraction from HMP could potentially be a complicating factor in these experiments. Therefore, we measured the kinetics of oxidation of (*R*)-[$^2H_1$]-HMP in comparison with unlabeled HMP using an oxygen electrode. As shown in Figure 2, this reaction exhibited a substantial primary KIE of 7.6 ± 0.4 at saturating HMP concentrations. This observed isotope effect provided the opportunity to confirm racemization at C1 with one more experiment. (*R*)- and (*S*)-2-[1-$^2H_1$]-HEP were separately incubated with HEPD until all of the 2-HEP was consumed, as determined by ^{31}P NMR spectroscopy. The resulting deuterium-labeled HMP was then used as substrate for a kinetics experiment, and

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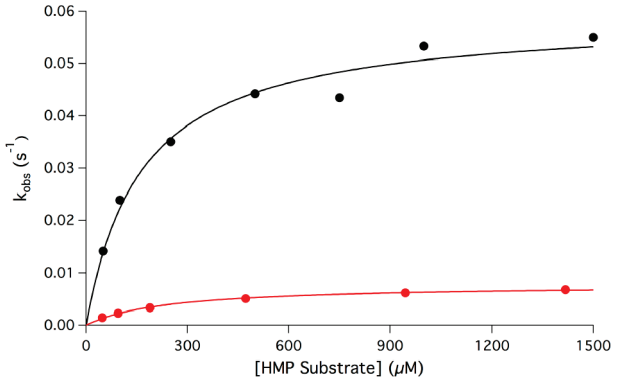
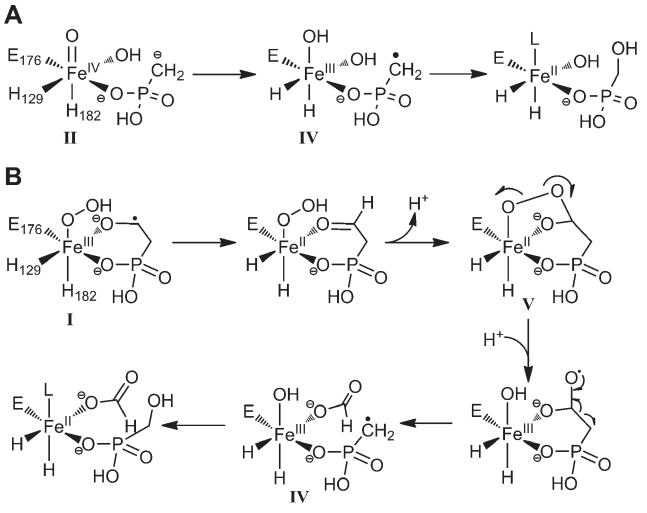


Figure 2. Michaelis−Menten curves for the oxidations of HMP (black) and (*R*)-[$^2H_1$]-HMP (red).

Scheme 4. Alternate Mechanisms That Could Account for the Observed Racemization of Substrate Enantioselectively Labeled at C1



the rate of oxidation was measured at saturating HMP concentrations. Comparison with the observed rate of oxidation of unlabeled HMP under identical conditions showed that the HMP produced from (*S*)-2-[1-$^2H_1$]-HEP resulted in an observed KIE of 2.4 ± 0.2 and the HMP produced from (*R*)-2-[1-$^2H_1$]-HEP displayed an observed KIE of 2.5 ± 0.3. Importantly, a 1:1 mixture of authentic (*R*)- and (*S*)-[$^2H_1$]-HMP displayed an observed KIE of 2.2 ± 0.2 under the same conditions, agreeing well with the expected value of 2.4 for a racemic mixture (see the Supporting Information). Thus, all of the experiments point to the same conclusion: the stereochemical integrity at C1 of 2-HEP is lost during the transformation to HMP.

The loss of stereochemistry can be rationalized in several ways. First, the hydroxylation mechanism in Scheme 2 may still be operational, but the carbanion formed upon the retro-Claisen-like step may have a lower barrier for rotation than an enolate, resulting in loss of stereochemical information. Alternatively, the electron-rich carbanion in intermediate **II** may be oxidized by proton-coupled electron transfer to provide ferric hydroxide and product radical **IV**, which could recombine in much the same way as a rebound-type mechanism often invoked for other iron-dependent enzymes (Scheme 4A).[20,21] The radical would be expected to have a lower barrier for rotation than the carbanion, potentially explaining the observed racemization.

An alternative explanation for racemization is shown in Scheme 4B. Here the initial radical at C2 in intermediate **I** could undergo electron transfer to the iron center to generate an aldehyde. Subsequent nucleophilic attack by the peroxide at the carbonyl would then produce bridged alkylperoxide **V**. Similar mechanisms have been proposed for isopenicillin N synthase-catalyzed oxidation of a substrate analogue,[22] for the reaction catalyzed by CloR,[23,24] for *myo*-inositol oxygenase (MIOX),[25] and very recently for HEPD in a computational study.[26] In the last of these reports, the bridged peroxide was proposed to cleave homolytically,[26] resulting in a geminal diolate radical that could generate the same intermediate **IV** depicted in Scheme 4A, in which the stereochemical information at C1 may be lost.

In summary, this investigation has provided new insights into the novel chemistry catalyzed by HEPD. Abstraction of a hydrogen atom from C2 has been shown to proceed stereospecifically. Interestingly, however, all of the stereochemical information at C1 of 2-HEP is lost in HMP. Thus, the hydroperoxylation mechanism, previously proposed on the basis of the direct observation of the product of a Criegee rearrangement with the substrate analogue 1-HEP,[10] cannot be operational in the conversion of 2-HEP to HMP.

■ ASSOCIATED CONTENT

Supporting Information. Synthetic procedures, spectral characterization of compounds, and procedures for enzymatic assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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