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3 **Steric enforcement of cis-epoxide formation in the radical C–O-coupling reaction by which**
4 **(S)-2-hydroxypropylphosphonate epoxidase (HppE) produces Fosfomicin**
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21 **ABSTRACT:** (S)-2-hydroxypropylphosphonate [(S)-2-HPP, **1**] epoxidase (HppE) reduces H₂O₂
22 at its non-heme-iron cofactor to install the oxirane “warhead” of the antibiotic fosfomicin. The net
23 replacement of the C1 *pro-R* hydrogen of **1** by its C2 oxygen, with inversion of configuration at
24 C1, yields the cis epoxide of the drug [(1*R*,2*S*)-epoxypropylphosphonic acid (*cis*-Fos, **2**)]. Here we
25 show that HppE achieves ~ 95% selectivity for C1 inversion and cis-epoxide formation via steric
26 guidance of a radical-coupling mechanism. Published structures of the HppE•Fe^{II}•**1** and
27 HppE•Zn^{II}•**2** complexes reveal distinct pockets for C3 of the substrate and product and identify
28 four hydrophobic residues – Leu120, Leu144, Phe182, and Leu193 – close to C3 in one of the
29 complexes. Replacement of Leu193 in the substrate C3 pocket with the bulkier Phe enhances
30 stereoselectivity (*cis*:*trans* ~ 99:1), whereas the Leu120Phe substitution in the product C3 pocket
31 diminishes it (~ 82:18). *Retention* of C1 configuration and *trans*-epoxide formation become
32 predominant with the bulk-reducing Phe182Ala substitution in the substrate C3 pocket (~ 13:87),
33 trifluorination of C3 (~ 23:77), or both (~ 1:99). The effect of C3 trifluorination is counteracted by
34 the more constrained substrate C3 pockets in the Leu193Phe (~ 56:44) and Leu144Phe/Leu193Phe
35 (~ 90:10) variants. The ability of HppE to epoxidize substrate analogues bearing halogens at C3,
36 C1, or both is inconsistent with a published hypothesis of polar cyclization via a C1 carbocation.
37 Rather, specific enzyme-substrate contacts drive inversion of the C1 radical – as proposed in a
38 recent computational study – to direct formation of the more potently antibacterial *cis* epoxide by
39 radicaloid C–O coupling.
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54 **KEY WORDS:** iron, peroxidase, stereochemistry, oxirane, antibacterial
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INTRODUCTION

The strained oxirane (epoxide) moiety readily undergoes ring-opening by nucleophilic attack and can thus serve as the basis for covalent modification of drug targets.¹ The naturally occurring antibiotic, fosfomycin [(*1R,2S*)-epoxypropylphosphonic acid; *cis*-Fos, **2**], provides a classic example.² Widely used to treat urinary tract infections, fosfomycin targets UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), a key enzyme in bacterial peptidoglycan biosynthesis.³ Attack of a cysteine residue in the active site of MurA opens the epoxide, resulting in covalent coupling and enzyme inactivation.^{3,4}

Enzymatic installation of an epoxide proceeds by one of two main routes: cyclic coupling of an oxygen-atom equivalent to both carbons of an olefin or cyclization of an alcohol.⁵ The epoxide warhead of fosfomycin is installed by the latter route.⁶ The enzyme *S*-2-hydroxypropylphosphonate [(*S*)-2-HPP, **1**] epoxidase (HppE) removes a hydrogen atom (H•) from C1 of **1** and couples this carbon to the alcohol oxygen on C2 (Scheme 1A).⁷ For many years, HppE was thought to activate dioxygen at its non-heme Fe(II) cofactor to generate the H•-abstracting intermediate.⁶ However, more recent work showed that the oxidizing co-substrate of HppE is actually hydrogen peroxide (Scheme 1A).⁷ This same study speculated that O–O-bond heterolysis following addition of the peroxide to the Fe(II) cofactor would produce an Fe(IV)-oxo (ferryl) intermediate (Scheme S1, *route A*),⁷ known in other systems to abstract H• from unactivated aliphatic carbon centers.⁸ However, a more recent computational study questioned the feasibility of such a pathway and suggested, instead, cofactor-promoted *homolysis* of the peroxide bond to form a hydroxyl radical, oriented for abstraction of H• from C1 by hydrogen bonding to the Fe(III)-coordinated hydroxide derived from the other peroxide oxygen (Scheme S1, *route B*).⁹ To date, no experimental data to distinguish between these two possibilities have been reported.

Multiple mechanisms have also been considered for the ensuing C1 ↔ O–C2 coupling step (Scheme 1B),^{6, 9-10} and no consensus has yet been reached. In the reactions of several other non-heme-iron enzymes that cleave strong C–H bonds to install heteroatoms (e.g., isopenicillin *N* synthase, the aliphatic halogenases, and several radical-SAM enzymes), carbon-centered radicals attack the Fe(III)-coordinated heteroatoms (X) to form the new C–X bonds, in the process reducing

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4 the Fe(III) to Fe(II).¹¹⁻¹⁹ A recent study of an inorganic model complex demonstrated ether C–O-
5 bond formation by this mechanism.²⁰ Given that the iron cofactor of HppE also coordinates the
6 (C2) oxygen of **1** that couples to C1 (Figure 1A), an analogous radical-coupling mechanism would
7 seem likely for its cyclization step (Scheme 1B, *blue arrows*). However, two experimental
8 observations – the unexpected stereochemical course of the native epoxidation reaction²¹ and the
9 nature of a distinct oxidation of a substrate analog²²⁻²³ – have led to consideration of more complex
10 C–O-coupling mechanisms. HppE removes the *pro-R* hydrogen from C1 of **1**,²⁴ consistent with
11 the orientation of the substrate in published x-ray crystal structures of the complex (Figure 1A).²⁵⁻²⁶
12 Coupling of the C2 oxygen to C1 in the site vacated by the abstracted H• would yield the *1S*
13 configuration and *trans* epoxide (*trans*-Fos, **3**), rather than the *1R* configuration and *cis* epoxide of
14 the actual antibiotic, **2**. In other words, C1 ↔ O–C2 coupling proceeds with inversion of
15 configuration at C1.⁷ By contrast, in the simplest radical-coupling mechanism, retention of
16 configuration at C1 would be anticipated (Scheme 1B, *blue arrows*). Consequently, a mechanism
17 involving an initial radical coupling of the coordinated phosphonate oxygen (with retention at C1)
18 and subsequent nucleophilic attack (S_N2) of the C2 oxygen upon the carbon of the unstable C-P-
19 O heterocycle (with inversion), was proposed (*purple arrows*).⁷

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35 HppE is capable of a number of alternative outcomes when challenged with structural isomers
36 of its substrate (Scheme S2). In addition to its native epoxidation of **1** (Scheme S2A), it can
37 dehydrogenate the alcohol groups of (*R*)-2-HPP and (*S*)-1-HPP to carbonyls (Schemes S2B-C).²²⁻
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23 The former outcome implies that the enzyme can also remove H• from C2, when differences in
substrate structure position this carbon appropriately. More interestingly, (*R*)-1-HPP undergoes an
oxidative 1,2-phosphonate migration, yielding 2-phosphonopropanal (Scheme S2D).²² This
outcome was rationalized in terms of H• abstraction from C2, electron transfer from this radical to
an oxidized form of the iron cofactor, and migration of the phosphonate to quench the resulting
C2 carbocation.^{10, 22} An analogous pathway – H• abstraction from C1 of **1**, electron transfer from
the C1 radical to the cofactor, and polar C1⁺ ↔ ⁻O–C2 coupling – was suggested for the native
epoxidation reaction.²² At that time, O₂ was still thought to be the oxidizing co-substrate, leading

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4 to the proposal of H• abstraction by a superoxo-Fe(III) complex and electron transfer to a ferryl
5 form of the cofactor, but an analogous mechanism via C1 carbocation can be formulated with H₂O₂
6 as the oxidant (Scheme 1B, *orange arrows*).⁷ The hypothesis of a planar C1 carbocation
7 intermediate would, in principle, explain why the expectation of strict retention in the ring-closure
8 step is not met, but it would not, *a priori*, lead one to expect high selectivity for inversion. The
9 aforementioned computational study put forth a different explanation – facile inversion of the C1
10 radical⁹ – for the stereochemical course of the ring-closure step (Scheme 1B, *red arrows*), thus
11 potentially rationalizing this crucial aspect of the reaction within the framework of the chemical
12 mechanism that would, in light of precedent, seem most probable.

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21 In this study, we sought to clarify the mechanism of the C–O-coupling step in the HppE
22 reaction by assessing (i) whether substrate analogs harboring electron withdrawing groups, which
23 would be expected to disfavor C1-carbocation formation, can be epoxidized and (ii) whether
24 sterically consequential modifications to the substrate or enzyme can change the stereochemical
25 course of the reaction. The ability of the enzyme to epoxidize analogs bearing halogens at C3 (1-
26 3 fluorine atoms), C1 (fluorine or chlorine), or both weighs heavily against the carbocation
27 mechanism (Scheme 1B, *orange arrows*), and the demonstration that modifications to the substrate
28 or enzyme, chosen rationally on the basis of published x-ray crystal structures, can completely
29 reverse the ring-closure stereochemistry provides argument against a chemically enforced C1
30 inversion, as in the mechanism involving intermediary radical coupling and S_N2 heterocycle
31 interconversion (Scheme 1B, *purple arrows*). The data thus drastically limit the mechanistic
32 possibilities, decisively favoring a sterically enforced inversion of the C1 radical and subsequent
33 radicaloid C1 ↔ O–C2 coupling (Scheme 1B, *red arrows*). The observation of diminished
34 antibiotic activities of the trans-epoxide compounds generated either from the 3-F₃-substituted
35 analog of (*S*)-2-HPP by the wild-type enzyme or from the native substrate by site-directed variants
36 implies that the ability of HppE to direct C1 inversion and cis-epoxide formation in the ring-closure
37 step is essential to its biological role, which is, presumably, to produce a competition-suppressing,
38 antimicrobial secondary metabolite.

RESULTS AND DISCUSSION

Mixed stereochemistry in the C–O-coupling (cyclization) step of the HppE reaction. Although published work has established that the predominant outcome in the oxidation of **1** by HppE is, effectively, replacement of the C1 *pro-R* hydrogen by the C2 oxygen with *inversion of configuration at C1* (producing *cis*-Fos, **2**; Scheme 1A and Figure 2A),⁶⁻⁷ one study examining production of Fos *in vivo* reported observation of a small quantity of the trans epoxide, [(1*S*,2*S*)-epoxypropylphosphonic acid; *trans*-Fos, **3**],²⁷ potentially arising from *competing retention at C1* in the HppE-catalyzed cyclization (Scheme 1B, *blue arrows*, and Figure 2A). Because even a small degree of stereo-ambiguity in this step would rule out the mechanism enforcing inversion (Scheme 1B, *purple arrows*), we deemed it important to test directly for *in vitro* production of **3** by HppE. Accordingly, we synthesized the racemic trans isomer (*rac*-**3**, composed of **3** and its 1*R*,2*R* enantiomer; see Supplemental Methods in the *Supporting Information* for details of the synthetic procedure) in order to compare its ¹H- and ³¹P-NMR spectra to those of the commercially available *cis* compound, **2**). *Rac*-**3** is readily distinguished from **2** by (i) the diminished downfield shifts of its C1-¹H and C2-¹H resonances and ~ 2-fold weaker vicinal coupling²¹ (³*J*_{H-H}; Figure 2B-C, *top and middle spectra*) and (ii) the increased downfield shift of its ³¹P resonance (Figure 2D, *top and middle spectra*) and stronger ³¹P-¹H geminal coupling (²*J*_{P-H}; Figure 2C, *top and middle spectra*). These distinctions afforded the tools to determine relative yields of the *cis* and *trans* products in the reactions of all combinations of modified substrates and variant enzymes examined in this work.

¹H-NMR (Figure 2B-C, *bottom spectra*) and ³¹P-NMR (Figure 2D, *bottom spectrum*) spectra of the products from the HppE reaction with **1** (see *SI, Sec 2.2* for detailed procedures) confirm that, indeed, the enzyme does produce a small quantity (3-5 % of the total epoxide) of *trans*-Fos, **3**, suggesting that ring closure with retention competes to a minor extent with the predominant C1-inverting pathway. To verify that formation of **3** still involves removal of the *pro-R* hydrogen,^{6-7,24} we synthesized both 1-[²H₁]-(*S*)-2-HPP diastereomers (*SI Sec 2.3 and 6.1*; Figure S2) and subjected them to oxidation by HppE. Analysis by ³¹P-NMR spectroscopy (Figure 3; Figure S3)

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4 showed that, as expected, the (1*S*,2*S*) diastereomer, **1d**, yields the monodeuterated cis- and trans-
5 epoxide products, **2d** and **3d**, in the characteristic ~ 95:5 ratio (panel B, *top*), whereas the (1*R*,2*S*)
6 diastereomer, **1e**, yields the same ratio of cis and trans epoxides (**2** and **3**) with protium at C1 (panel
7 B, *bottom*). These results confirm that the cis and trans products form by initial removal of the
8 same (*pro-R*) hydrogen and subsequent ring-closure with opposite stereochemistry, weighing
9 against the proposed mechanism involving an intermediary coupling step via the C–P–O
10 heterocycle (Scheme 1B, *purple arrows*).
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17 *Structure-guided mutagenesis to alter cyclization stereochemistry.* The above evidence
18 against the sole mechanism that could enforce net inversion in the ring-closure step implies that
19 the enzyme controls the reaction stereochemistry sterically, by favoring either (i) radicaloid ring
20 closure from the inverted configuration of the C1 radical (Scheme 1B, *red arrows*) or (ii) the
21 “back-side” trajectory for attack of the C2 oxygen on the planar C1 carbocation (Scheme 1B,
22 *orange arrows*). We examined the published HppE structures^{25, 28} (Figure 1) for active-site
23 residues and enzyme-substrate/product interactions that might contribute to this steric control.
24 From the best available structures of the HppE•**1** (A) and HppE•**2** (B) complexes, we identified
25 four hydrophobic residues in the active site that are within 5 Å of the C3 methyl group in one of
26 the two complexes and ≥ 0.5 Å closer in one complex than in the other. Leu193 (L193) and Phe182
27 (F182) are markedly (~ 1.5 Å) closer to C3 in the model of the substrate complex than in that of
28 the product complex, and L144 appears to be slightly (~ 0.5 Å) closer. These three residues make
29 up the substrate-C3 binding pocket. Conversely, L120 is nearly 3 Å closer in the model of the
30 product complex and largely defines the product-C3 pocket. It appears – although there is no
31 available structure to confirm – that the trans-epoxide product, **3**, generated by ring closure with
32 retention of configuration at C1, would project its C3 group toward the substrate-C3 pocket, and
33 so we reasoned that amino acid substitutions contracting this pocket (by increasing the volume of
34 the side chains lining it) might enhance selectivity for C1 inversion, whereas expansion of the
35 pocket (by bulk-reducing substitutions to the residues defining it) might diminish the
36 stereoselectivity. Conversely, we expected that bulk-enhancing substitutions of L120 would
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4 constrict the product-C3 pocket and diminish selectivity for C1 inversion and cis-epoxide (**2**)
5 formation. We prepared the L193F and L144F/L193F variant proteins to evaluate the effect of
6 constriction, and the F182A variant to evaluate the effect of expansion, of the substrate-C3 pocket.
7 Similarly, we prepared the L120F variant to assess the impact of a constricted product-C3 pocket.
8 All four proteins were sufficiently soluble and stable to be characterized after production in and
9 purification from *Escherichia coli*, although the F182A protein was seen to lose activity upon
10 freeze-thaw cycles. All four proteins proved capable of multiple turnovers of (*S*)-2-HPP with H₂O₂
11 as oxidizing co-substrate, although the total turnover numbers (TTNs) achieved with the variant
12 proteins were diminished from that obtained with the wild-type enzyme (~ 40) to a minimum of ~
13 3 for the L144F/L193F double variant (Figure S4). For the two variant proteins that exhibited the
14 most striking effects on the reaction stereochemistry (F182A and L193F; *vide infra*), we
15 determined the H₂O₂:product stoichiometries to compare to that of wild-type HppE, which we
16 previously reported⁷ and verified here (Figure S5A-C) to be indistinguishable from the theoretical
17 value of unity. Both variant proteins retain the ability to fully couple H₂O₂ reduction to (*S*)-2-HPP
18 oxidation (Figure S5D-I).

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33 Characterization of the reaction products by ³¹P-NMR spectroscopy (Figures 4B and S6)
34 revealed that, as predicted from analysis of the published structures, expansion of the substrate-C3
35 pocket by replacement of the phenyl group of F182 with hydrogen (i.e., the F182A substitution)
36 diminishes selectivity for C1 inversion in the ring-closure step. The F182A variant produces
37 predominantly the *trans*-Fos isomer, **3** (Figure 4B, *bottom*, Figure S6). A cis:trans (**2**:**3**) ratio of
38 12:88 was obtained by averaging 3 replicates of the ³¹P-NMR analysis; standard deviations are
39 given in parentheses. By use of the stereospecifically 1-[²H₁]-labeled substrates, **1d** and **1e**, we
40 confirmed that the variant protein still abstracts the *pro-R* hydrogen (Figure S7A): the former
41 substrate yields the 12:88 mixture of monodeuterated epoxide products (**2d**:**3d**), whereas the latter
42 substrate yields the equivalent mixture of epoxides lacking deuterium (**2**:**3**; Figure S7A, *middle*).
43 Increased bulk in the product-C3 pocket, imparted by the L120F substitution, was also seen to
44 diminish selectivity (Figure 4B, *second from bottom*, Figure S6), albeit to a more modest extent
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4 (2:3 ~ 82:18) than the F182A substitution. We saw the opposite effect on ring-closure
5 stereoselectivity upon introducing the L193F substitution (Figure 4B, *top*; Figure S6), predicted
6 by the structural analysis to constrict the substrate-C3 pocket. This variant protein is, remarkably,
7 *more stereoselective than the wild-type enzyme*, producing a fraction of the trans epoxide, **3**, so
8 small (~ 1%) as to be challenging to detect. We again verified that this variant removes the *pro-R*
9 hydrogen in the cyclization reaction (Figure S7B). The accuracy of these several predictions
10 regarding ring-closure stereochemistry confirms the hypothesis on which they were founded – that
11 HppE controls the outcome by a steric mechanism rather than by leveraging a multi-step pathway
12 (Scheme 1B, *purple arrows*) that enforces inversion.
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21 *Use of halogen substitution to distinguish between polar and radicaloid C–O-coupling*
22 *mechanisms.* We next sought to distinguish whether the enzyme directs C1 inversion (i) by tuning
23 the relative stabilities within the active site of the two configurations of the C1 radical (Scheme
24 1B, *red arrows*) or (ii) by impacting the relative barrier heights of the two possible trajectories for
25 attack of the C2 oxygen on the C1 carbocation (Scheme 1B, *orange arrows and green arrows*).
26 We reasoned that carbocation formation would be disfavored by the presence of strongly electron-
27 withdrawing groups on the substrate, whereas the radicaloid mechanism would be more robust to
28 such modifications.¹⁰ We therefore synthetically incorporated halogen atoms at C3, C1, or both
29 and evaluated the ability of HppE to cyclize these analogues. Comparison of the ¹H-NMR spectra
30 of the products generated by the wild-type enzyme from the 3-F₃-substituted (*S*)-2-HPP analog
31 (**1c**) to the spectra of the corresponding synthetic cis- and trans-epoxide standards (**2c** and **3c**,
32 respectively) showed that replacement of even all three C3 hydrogens by fluorine does not prevent
33 HppE-catalyzed epoxidation (Figure S8). Intriguingly, trifluorination of C3 *does* markedly impact
34 the stereochemistry: ¹H-NMR (Figure S8), ³¹P-NMR (Figure 4C, *middle spectrum*; Figure S10),
35 and ¹⁹F-NMR (Figure S11) spectra were all consistent with a cis:trans (**2c**:**3c**) product ratio of ~
36 23:77, a selectivity reversed from that seen with the native substrate. All three fluorine atoms are
37 required for this effect on the stereoschemistry: we found the 3-F- (**1a**) and 3-F₂- (**1b**) substrates
38 to yield cis:trans ratios (**2a,b**:**3a,b**) similar to those obtained with the native substrate, **1** (Figure
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4 4D-E; Figures S12-16). With the C3-trifluorinated analogue (**1c**), the more cis-stereoselective
5 L193F variant produces more of the cis-epoxide product (**2c:3c** ~ 56:44; Figure 4C, *second from*
6 *top*; Figures S10-11) than does the wild-type enzyme, and the L144F/L193F double variant is even
7 more selective for this product (Figure 4C, *top*; Figures S10-11), giving a cis:trans (**2c:3c**) ratio (~
8 90:10) that approaches that for the wild-type enzyme with the native substrate. Conversely, the
9 less cis-selective F182A variant produces almost exclusively (~ 1:99) trans epoxide **3c** (Figure 4C,
10 *bottom*; Figures S10-11). Thus, the trans-favoring effect of 3-F₃ substitution is both counteracted
11 by constriction of the substrate-C3 pocket and potentiated by expansion of the pocket. The parallel
12 responses of the 3-F₃-substituted and native substrates to these active-site amino acid substitutions
13 implies that cyclization of the two compounds proceeds by the same mechanism. Most likely, the
14 energetic cost of eclipsing the trifluoromethyl and phosphonate groups in the trajectory²⁹ for C1
15 inversion/cis-epoxide formation is greater than that incurred with the unsubstituted C3 methyl
16 group (Figure S9), resulting in more C1 retention and trans-epoxide production with the analog.
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29 Fluorine substitution markedly destabilizes organic cations,^{10, 30-31} even when the carbon atom
30 bearing the halogen is separated from the cationic site by an “insulating” *sp*³-hybridized carbon,
31 as would be the case here for a polar cyclization mechanism. For example, the *pK*_a of the
32 ethylammonium cation is 10.8, whereas that of the 2,2,2-trifluoroethylammonium cation is 5.6.³²⁻
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37 ³³ In this case, trifluorination diminishes the *pK*_a of the β nitrogen substituent by ~ 5 units,
38 corresponding to a relative destabilization of the cation by almost 30 kJ/mol (at 20 °C).³⁴
39 Extrapolated to the proposed C1 carbocation intermediate in the HppE reaction, this effect would
40 correspond to an increase in its reduction potential of ~ 300 mV. It seems unlikely that carbocation
41 formation would remain robust to such a severe perturbation, implying that a radicaloid
42 mechanism for C2–O ↔ C1 coupling is more likely. To provide additional evidence for this
43 conclusion, we tested the (*S*)-2-HPP analogs containing either (i) halogens at both C3 (F₃) and C1
44 (chlorine) or (ii) fluorine at C1 for the ability to serve as HppE substrates. In the case of the 1-C1-
45 3-F₃ analogue, we were unable to obtain the single 1*S*,2*S* diastereomer that would be expected to
46 project its C1-H toward the iron cofactor appropriately for H• abstraction, but we did verify by
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¹⁹F-NMR analysis that the pure 1*R*,2*S* compound (**1f**) – expected to direct the chlorine toward the cofactor – is not a substrate for wild-type HppE (Figure 5, *top*; Figure S17B-C). Moreover, we found (also by ¹⁹F-NMR) that the racemic anti compound (*rac*-**1g**, consisting of a mixture of 1*S*,2*S*-**1g** and 1*R*,2*R*-**1g**) could be converted by HppE and its F182A variant to a mixture of the 2-ketone (**4g**), cis-epoxide (**2g**) and trans-epoxide (**3g**) products (Figure 5, *bottom*). Presumably, 1*R*,2*R*-**1g** is, by analogy to the reaction of (*R*)-2-HPP,²³ dehydrogenated to the 2-ketone (**4g**), and 1*S*,2*S*-**1g** is successfully cyclized to the mixture of epoxides (Figure 5B, *second from bottom*). As for the native and 3-F_n-substituted substrates (**1**, **1a-c**), we found that the F182A variant makes less of the cis epoxide (**2g**) from the *rac*-**1g** than does the wild-type enzyme (Figure 5B, *bottom*; Figure S17E-F).

We did succeed in synthesizing the desired single diastereomer of the 1-fluoro analogue, (1*R*,2*S*)-1-F-HPP (**1h**; note that the substituent priority order changes in the 1-F analog relative to the 1-Cl compound, Figure 6A). High-resolution mass spectra (HRMS; Figure 6B) of **1h** (*top*), its reactions with wild-type HppE (*second from top*) or the trans-epoxide-favoring F182A variant (*third from top*), and the synthetic 2-ketone derivative, **4h** (*bottom*), revealed that both HppE proteins effect a net dehydrogenation (removal of two hydrogens) of the 1-F-substituted analog. The corresponding ³¹P-NMR spectra (Figure 6C; Figure S18B-E) reveal a pair of major oxidation products with ³¹P resonances at $\delta \sim 2.5$ ppm and ~ 3.5 ppm. These resonances are 6-7 ppm upfield from that of **1h** (Figure 6C), similar to the 8-11 ppm shifts seen upon epoxidation of the 3-F_n-substituted compounds (**1**, **1a-c**). The ³¹P spectra of both products reveal large geminal hyperfine couplings to ¹⁹F (²*J*_{P-F} ~ 98 and 102 Hz) – as also seen for **1h** and **4h** – but neither product-associated resonance exhibits an obvious geminal coupling to ¹H, unlike in **1h** and **4h** (Figure 6C; Figure S18C-E). This latter observation implies that one of the two hydrogens removed in the transformation of **1h** to the pair of major products is from C1, as occurs in epoxidation of the other substrates. The NMR data thus show that HppE can cyclize **1h**, despite the expectation that a hypothetical C1-carbocation intermediate would be severely destabilized by the fluorine substituent.

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4 For each of the four pairs of 3-F_n-substituted (n = 0-3) cis and trans epoxides produced by
5 HppE from the corresponding 3-F_n-(S)-2-HPP analog, the cis epoxide (**2**, **2a-c**) has the lesser ³¹P
6 δ, and the trans epoxide has the greater ³¹P δ. For the cases of **2/3** and **2c/3c**, this trend was verified
7 directly, by characterization of synthetic or commercially available standards, whereas for **2a/3a**
8 and **2b/3b**, the rank order of the vicinal ¹H-¹H couplings (³J_{H-H}) in each pair confirmed that the
9 trend holds throughout the 3-F_n (n = 0-3) series. This trend would imply that the product from **1h**
10 with δ ~ 2.5 ppm is the cis epoxide, **2h**, and the product with δ ~ 3.5 ppm is the trans epoxide, **3h**.
11 The absence of a ¹H nucleus on C1 in these products precludes use of the rank order of ³J_{H-H} values
12 for confirmation of the assignment, but the ¹⁹F spectra of the products (Figure 6D-E) provide
13 further verification. The two major products, tentatively assigned above as **2h** and **3h**, exhibit ¹⁹F
14 resonances at δ ~ -137 and ~ -159 ppm, respectively. Blow-ups of their ¹⁹F spectra (Figure 6E)
15 reveal, in addition to the major doublet splitting from the geminal ³¹P nucleus (²J_{F-P}), smaller
16 hyperfine couplings on each line of the doublet arising from ¹H nuclei on C2 (³J_{F-H}, doublet) and
17 C3 (⁴J_{F-H}, quartet). Deeper (derivative) analysis of the spectra revealed that the resonance with ¹⁹F
18 δ ~ -137 ppm has the larger of the two vicinal F-H couplings (³J_{F-H} 4.2 Hz compared to 1.5 Hz),
19 suggesting that the associated product is the cis epoxide, **2h**. With this assignment confirmed, it
20 can be seen that, as in epoxidation of **1**, **1a-c**, and **1g**, wild-type HppE markedly favors the cis
21 product (**2h:3h** ~ 93:7), whereas the F182 variant markedly favors the trans product (**2h:3h** ~ 10:90)
22 (Figure 6C-D; Figure S18). The consistent, pronounced shift to C1 retention and cis-epoxide
23 formation caused by the F182A substitution implies that all of the analogs undergo epoxidation by
24 a common mechanism. The combined results thus decisively favor a radicaloid ring-closure
25 mechanism (Scheme 1B, *red*) – as supported by the computational analysis of Shaik and co-
26 workers⁹ – over the previously proposed polar coupling via the C1 carbocation (Scheme 1B,
27 *orange and green*).

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51 *Antimicrobial potencies of the (halogenated) cis and trans epoxide products.* The
52 accumulated sets of substrate analogues and HppE variants afforded a panel of cis- and trans-
53 epoxide compounds, which we evaluated for antimicrobial potency in comparison to the clinical
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4 antibiotic, Fos. We measured the areas of the zones of growth inhibition created by spotting
5 reaction solutions on a lawn of *Escherichia coli* K12.^{2, 28} We first established a linear correlation
6 between the area of inhibition and the concentration of commercial Fos within the range of 0.15
7 mM < [Fos] < 2 mM (Figure S19). We then validated use of raw reaction solutions for testing
8 potency by confirming that the solution with wild-type HppE, 1.5 mM of the native substrate, and
9 excess H₂O₂ (producing ~ 1.42 mM **2** and 0.08 mM **3**) gave a zone of inhibition indistinguishable
10 from that of 1.5 mM of pure commercial Fos. We next measured inhibition areas created by
11 reactions that were, by choice of substrate (each at 1.5 mM with excess H₂O₂) and HppE variant,
12 enriched in either **3** or the cis- or trans-epoxide product of 3-F- (**2a** or **3a**), 3-F₂- (**2b** or **3b**), or 3-
13 F₃-(*S*)-2-HPP (**2c** or **3c**) (Figure S20, 21). The reaction solution enriched in trans epoxide, **3**
14 [F182A variant with native (*S*)-2-HPP], was ~ 50% less potent than that enriched in **2**, and
15 reactions enriched in any of the C3-fluorinated epoxides (either cis or trans) were at least 50% less
16 potent than those enriched in **3**. The uniformly deleterious impact of C3 fluorination suggests that
17 increased electrophilicity of the epoxide warhead does not, at least in this bioassay, potentiate its
18 activity. The fluorinated compounds may have diminished affinity for MurA or may suffer from
19 increased decomposition (e.g., by hydrolysis) prior to reaching their target. More importantly, the
20 implication of the diminished potency of the trans epoxide, **3**, is that the steric enforcement of C1
21 inversion and cis-epoxide formation by HppE is important for its biological function. For the case
22 of this enzyme, it appears that necessity was indeed the mother of invention.

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41 *Possible implications for mechanism of H•-abstraction step.* The mechanism deduced here for
42 the HppE reaction – H• abstraction, radical inversion, and radicaloid ring closure – shares aspects
43 of that previously elucidated for the first of two reactions catalyzed by the iron(II)- and 2-
44 oxoglutarate-dependent (Fe/2OG) oxygenase, carbapenem synthase (CarC). CarC promotes the
45 stereoinversion (epimerization) of C5 of (3*S*,5*S*)-carbapenam-3-carboxylate, yielding the 3*S*,5*R*
46 compound, before desaturating between C2 and C3 to generate its (5*R*)-carbapenem final product.
47 In the stereoinversion, H• is removed from C5 by a ferryl intermediate, and Tyr165 then donates
48 H• to the opposite side of the bicyclic system.³⁵ Calculations have suggested that the structure of
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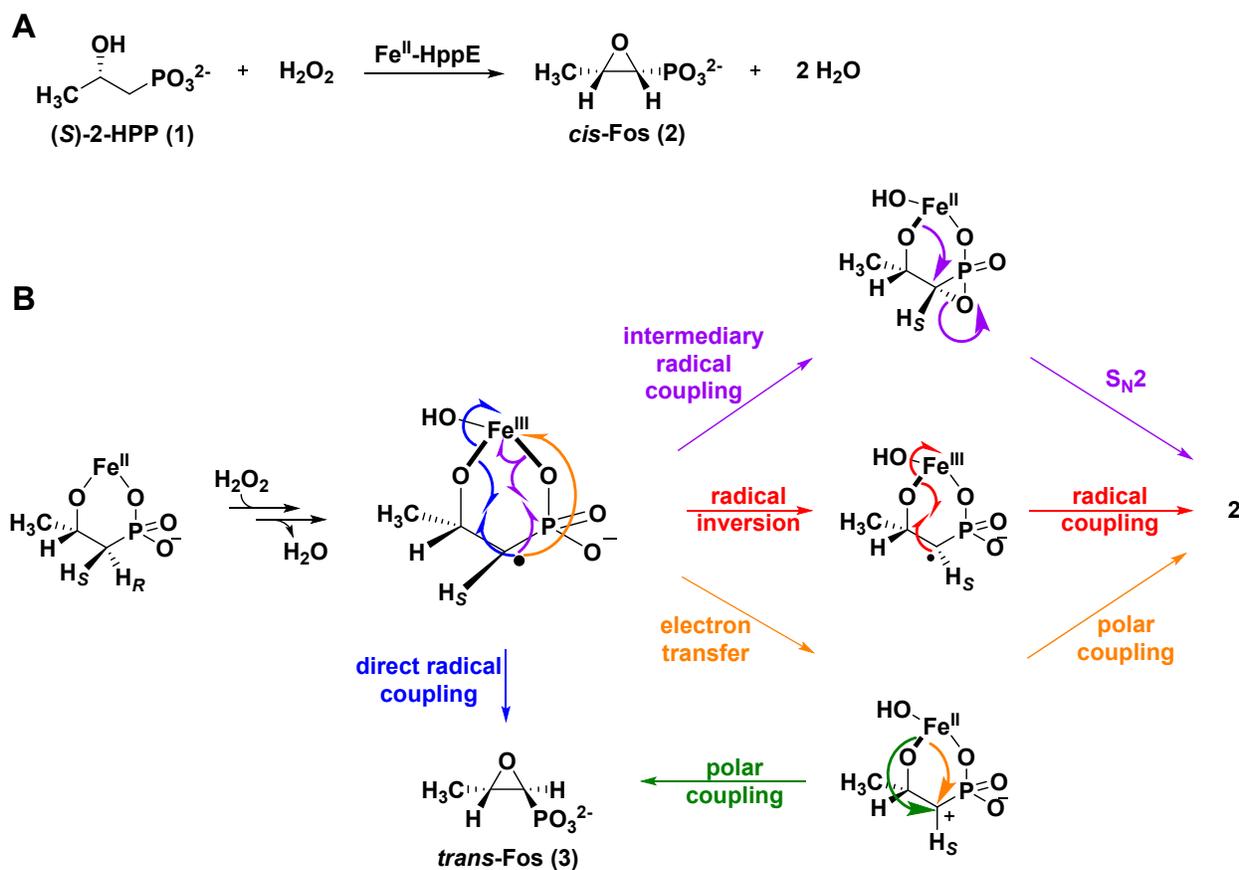
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4 the intermediate itself drives the facile inversion of the initial 5*S* radical to the 5*R* radical in a
5 butterfly-like motion of the bicyclic system that is predicted to have a relatively low activation
6 barrier (~ 24 kJ/mol) and a negative free-energy change (~ - 7 kJ/mol). It was proposed that this
7 motion, which would be expected to move C5 away from the iron cofactor, is crucial to the
8 avoidance of C5 hydroxylation via the generally facile coupling of the initial 5*S* radical to the
9 Fe(III)-coordinated hydroxide ligand, a step known generally as “oxygen rebound.” One could
10 envisage also for the possibility of H• abstraction by a ferryl complex in HppE (Scheme S1A) that
11 the demonstrated inversion of the C1 radical – driven in this case by steric contacts with the
12 enzyme – might be essential for avoidance of oxygen rebound. However, for the cases of the HppE
13 variant and (*S*)-2-HPP analogs that yield ring closure with predominant retention of C1, one might
14 then expect oxygen rebound to compete with cyclization, resulting in formation of some of the C1-
15 hydroxylated product. It is tempting to interpret the fact that we did not, for any combination of
16 variant enzyme and substrate analog, observe such competition from C1 hydroxylation as evidence
17 against H• abstraction by a ferryl complex (Scheme S1A) and in favor of the proposal of Shaik
18 and co-workers that H• abstraction is effected a by a hydrogen-bonded hydroxyl radical (Scheme
19 S1B), a mechanism that could, by virtue of greater separation of the cofactor and C1 radical,
20 potentially preclude oxygen rebound. Definitive resolution of this key mechanistic issue awaits
21 experimental determination of the oxidation state of the cofactor (+IV or +III) immediately before
22 H• abstraction.

41 CONCLUSIONS

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43 A remarkable robustness of HppE to both sterically unconservative amino-acid substitutions
44 in its active site and stereo-electronically perturbing modifications to its substrate has enabled
45 elucidation of the mechanism of its C–O-coupling step. The results experimentally validate two of
46 the principal conclusions from the recent computational study of Shaik and co-workers, who
47 posited that facile inversion of the C1 radical and radicaloid C–O-coupling produce the cis epoxide
48 of the antibiotic.⁹ Moreover, the experimental results both identify specific enzyme-
49 substrate/product contacts that drive C1-radical inversion and show that perturbation of these
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4 contacts allows the outcome to be tuned from vastly predominant C1-inversion and cis-epoxide
5 formation to vastly predominant C1-retention and trans-epoxide formation. The conclusion of a
6 radicaloid coupling step is likely to apply also to reactions of other non-heme-iron enzymes that
7 form C–O and other carbon-heteroatom bonds. For example, the Fe/2OG enzyme, hyoscyamine
8 6 β -hydroxylase (H6H), installs the epoxide of the plant-derived anesthetic drug, scopolamine, by
9 sequential hydroxylation and cyclization steps.³⁶ Because the radicaloid nature of the epoxidation
10 step would almost certainly require iron coordination of the C6 oxygen that undergoes coupling to
11 C7 in the second step, the analysis predicts that coordination of the heteroatom to the ferryl
12 complex, its Fe(III)–OH successor, or both, might be observable in H6H and, more generally, in
13 other, analogous enzymatic carbon-heteroatom-coupling reactions.
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Scheme 1. (A) Predominant transformation of (*S*)-2-HPP by HppE and (B) possible mechanisms for this outcome.



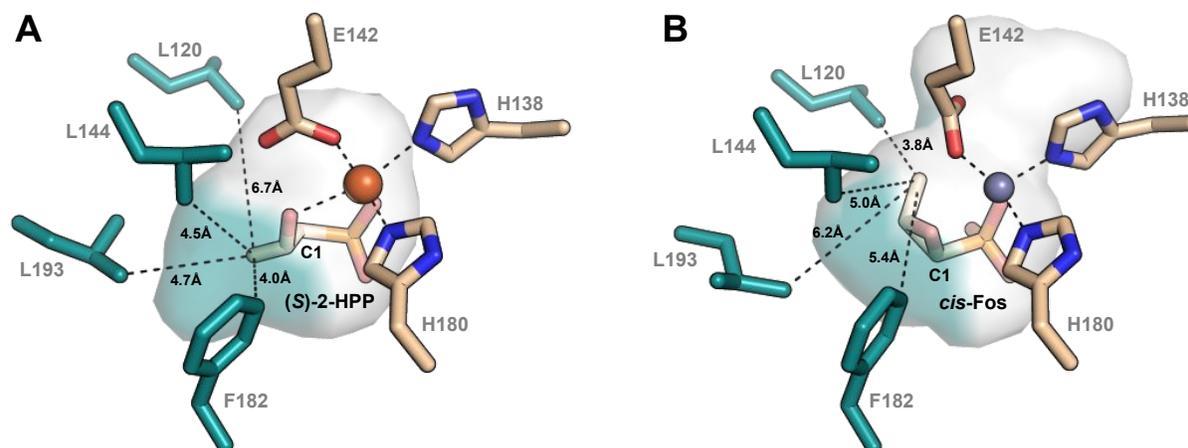


Figure 1. Structural models of the HppE substrate and product complexes from x-ray crystallographic studies. **(A)** Model of the HppE•Fe^{II}•(S)-2-HPP complex (PDB ID: 1ZZ8). **(B)** Model of the HppE•Zn^{II}•fosfomycin complex (PDB ID: 2BNN).

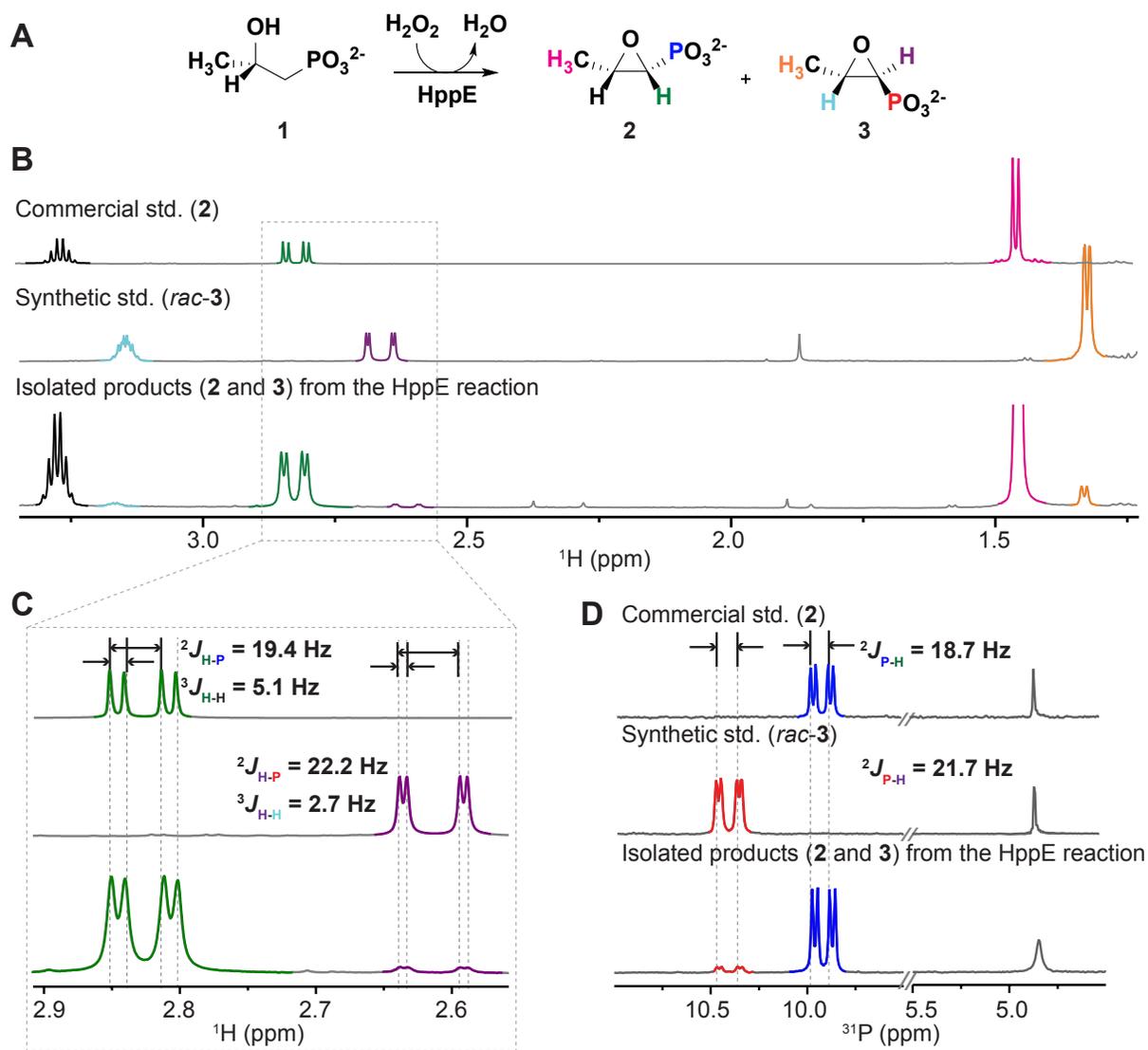


Figure 2. Re-examination of the stereochemical selectivity in Fos production by HppE. (A) *Cis*-Fos (**2**) and *trans*-Fos (**3**) products of ring-closure with inversion and retention, respectively, of C1 configuration. The atoms are color-coded to facilitate interpretation of the ^1H -NMR spectra in the lower panels. (B) ^1H NMR spectra of commercial *cis*-Fos (*top*), synthetic standard *trans*-Fos (*middle*), and the isolated epoxide products from the HppE reaction (*bottom*). (C) Blow-up of the peaks arising from the C1- ^1H showing that the vicinal coupling constant, $^3J_{\text{H-H}}$, to the C2- ^1H in *cis*-Fos ($^3J_{\text{H-H}} = \sim 5.1$ Hz) is almost twice that in *trans*-Fos ($^3J_{\text{H-H}} = \sim 2.7$ Hz). For ease of comparison, minor adjustments in peak positions (≤ 0.08 ppm) have been made to account for vari-

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4 ations in δ with differences in pH, counter ion composition, and concentration. (**D**) Corresponding
5 ^{31}P -NMR spectra measured in 100 mM NaOD in D_2O . The dd feature ($^2J_{\text{P-H}} = \sim 21.7$, $^3J_{\text{P-H}} = \sim 5.1$
6 Hz) at ~ 10.4 ppm is from *trans*-Fos, and the dd feature ($^2J_{\text{P-H}} = \sim 18.7$, $^3J_{\text{P-H}} = \sim 5.3$ Hz) at ~ 9.9
7 ppm is from *cis*-Fos. The singlet at ~ 4.8 ppm is from the internal standard (PO_4^{3-}).
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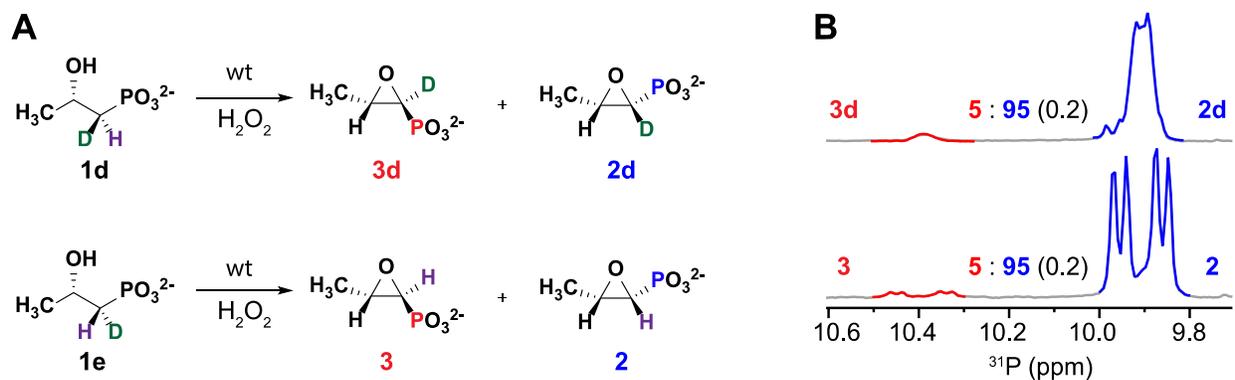


Figure 3. Stereochemical courses of the transformations of stereospecifically 1- d_1 labeled 2-HPP diastereomers by wild-type (wt) HppE. (**A**) Summary of reaction outcomes and (**B**) ^{31}P NMR analysis of the products in reactions of wt HppE with the (1*S*,2*S*)-1- d_1 -2-HPP (**1d**) and (1*R*,2*S*)-1- d_1 -2-HPP (**1e**). Each **trans**:**cis** ratio is the mean value from three ^{31}P -NMR assays, and the standard deviations given in parentheses are errors on each percentage value [i.e., **5:95** (0.2) implies a **trans**:**cis** ratio in the range of **4.8:95.2** – **5.2:94.8**].

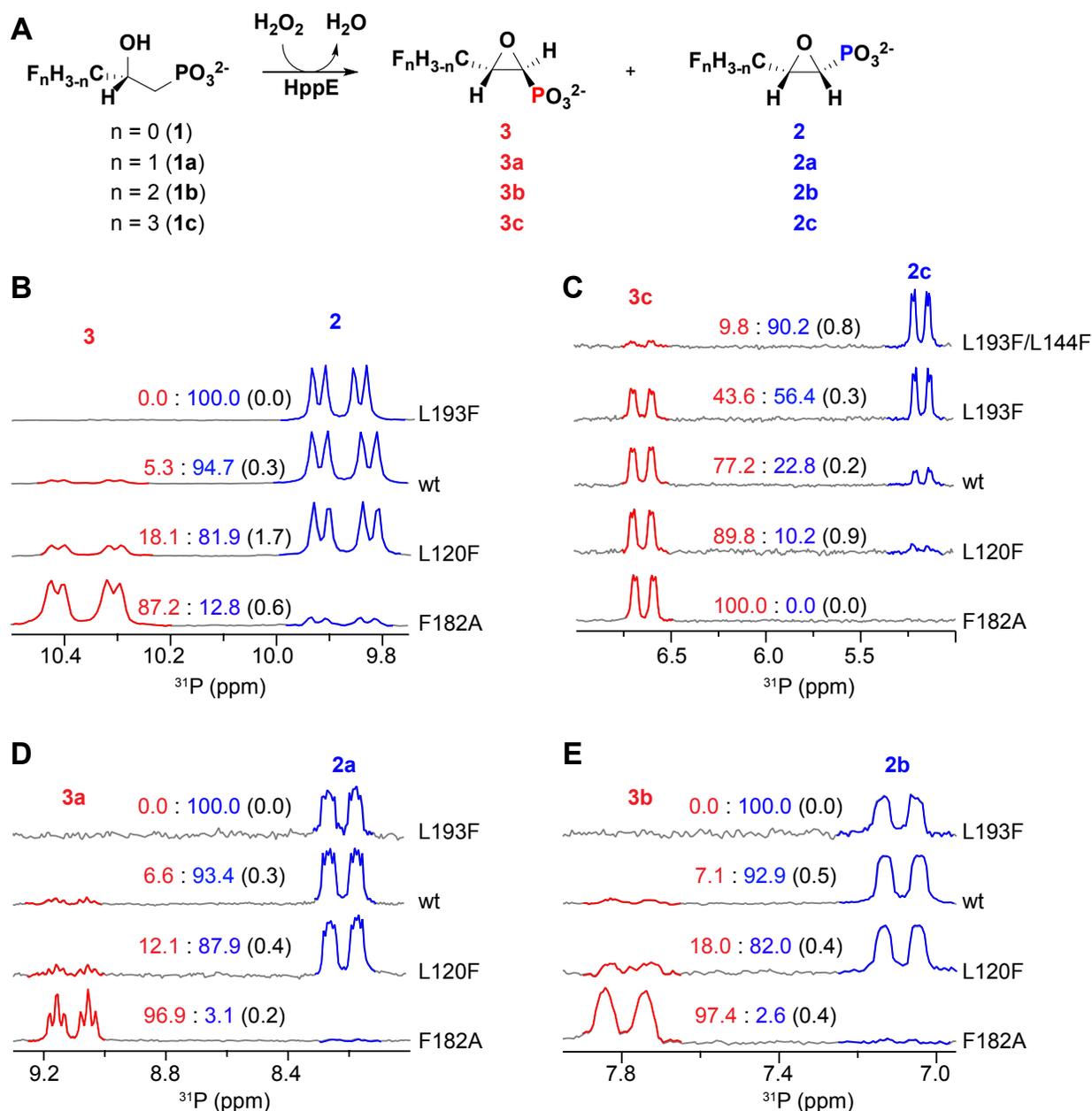


Figure 4. Analysis by ^{31}P -NMR spectroscopy of the product profiles in reactions of HppE and its variants with the 3- F_n -substituted ($n = 0$ -3) (*S*)-2-HPP substrates (**1**, **1a-c**). (**A**) Reaction scheme and explanation of product nomenclature. (**B-E**) ^{31}P -NMR spectra from the reactions with the $n = 0$ -3 compounds and the HppE variants indicated to the right of each spectrum. The **trans**:**cis** ratios shown at the center of the spectra were determined by comparison of the integrated intensities of the more down-field dd features of the **trans**-epoxide products and the more upfield dd features of

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4 the **cis**-epoxide compounds. Each value is the mean of three trials, with standard deviations shown
5 in parentheses. Details of the analytical procedures are provided in the *Supporting Information*.
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7 For compounds with at least one fluorine (**C-E**), **trans:cis** ratios were also calculated by comparison
8 of the intensities of the appropriate features in the ^{19}F -NMR spectra and were found to be
9 quantitatively consistent.
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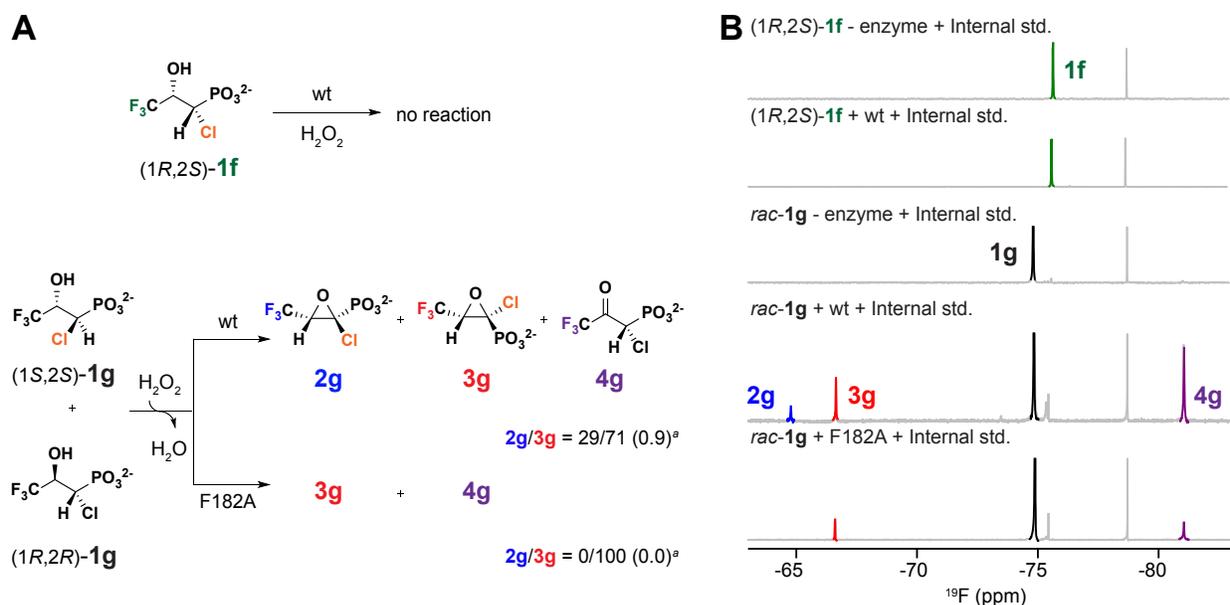


Figure 5. Summary of outcomes and ¹⁹F NMR analysis in the reactions of the wild-type (wt) and F182A HppE proteins with (1*R*,2*S*)-1-Cl-3-F₃-HPP [(1*R*,2*S*)-**1f**] and *rac*-1-Cl-3-F₃-2-HPP [(1*S*,2*S*)- and (1*R*,2*R*)-**1g**] compounds. (A) Reaction schemes depicting C1 *pro-R* hydrogen abstraction and formation of the *cis* and *trans* epoxides (**2g** and **3g**, respectively) by the two proteins. ^aThe **2g:3g** ratio given is the mean value from three ¹⁹F -NMR assays with the standard deviations given in parentheses. (B) ¹⁹F-NMR spectra of the reactions of (1*R*,2*S*)-**1f** with HppE (*second from top*), *rac*-**1g** with HppE and the F182A variant (*second from bottom* and *bottom*). Assignment of structures of products **2g**, **3g**, and **4g** refers ¹⁹F NMR analyses of *cis*-, *trans*-F₃-Fos and 3-F₃-2-keto compounds (**2c**, **3c** and **4c**). Single peaks of **4g** (*second from bottom*) and **4c** (table S11) at ~-81~-87 ppm correspond to C2 dehydrogenation. The doublets at -64.7 and -66.6 ppm correspond to *cis*- and *trans*-1-Cl-3-F₃-Fos (**2g** and **3g**), respectively, in analogy with the doublets at -64.2, -74.1 ppm of *cis* and *trans*-3-F₃-Fos (**2c** and **3c**). The single peak at -78.7 is from the internal standard sodium triflate.

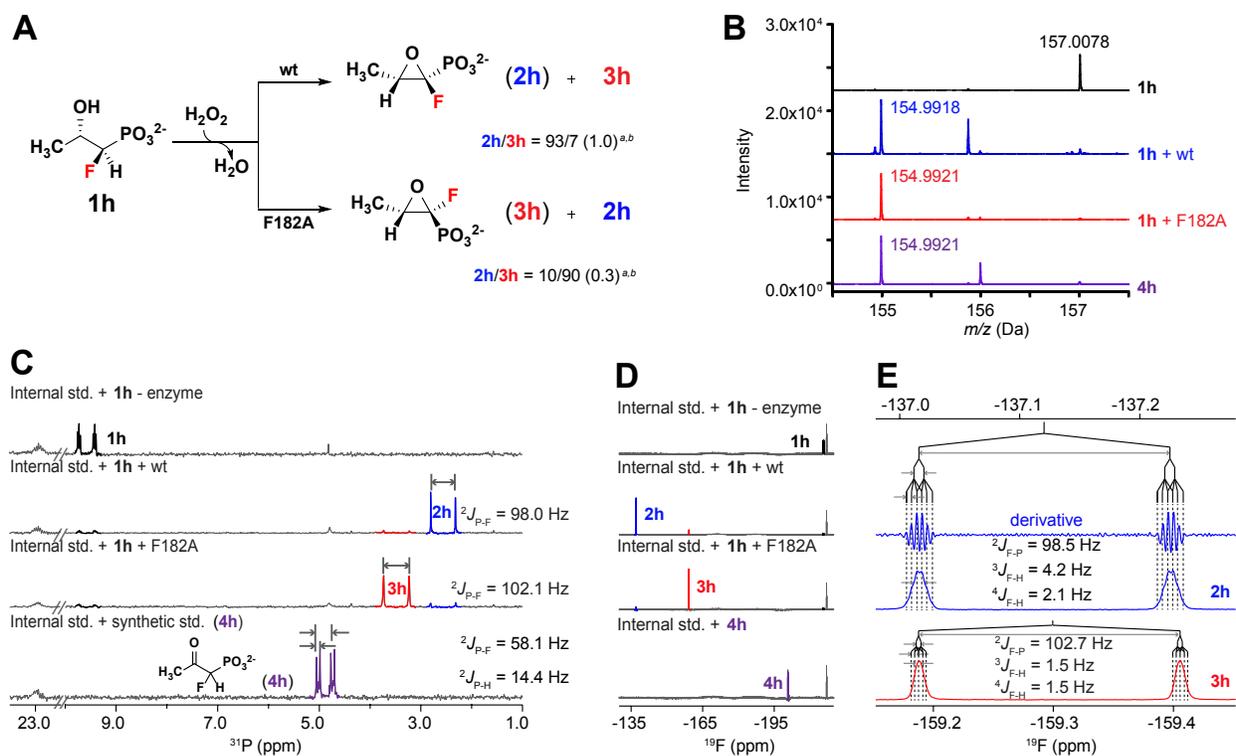


Figure 6. Summary of stereochemistry, mass-spectrometric and NMR-spectroscopic characterization of the products generated by wild-type HppE and its F182A variant from (1*R*,2*S*)-1-F-HPP (**1h**). (**A**) Reaction schemes depicting C1 *pro-R* hydrogen abstraction and formation of the cis and trans epoxides (**2h** and **3h**, respectively) by the two proteins. ^aEach cis:trans ratio given is the mean value from three ^{31}P -NMR assays; ^bValues given were determined by ^{31}F -NMR analysis, and the standard deviations given in parentheses are errors associated with each percentage value. (**B**) HRMS of synthetic **1h** (top), the reactions of **1h** with wild-type HppE (second from top) and its F182A variant (third from top), and synthetic 1-fluoro-2-oxo-propylphosphonate, **4h** (bottom), another possible product of HppE-mediated oxidation (from C2 dehydrogenation) of **1h**. (**C**) ^{31}P NMR spectra showing production of primarily cis epoxide, **2h**, in the reaction of the wild-type enzyme (second from top) and trans epoxide, **3h**, in the reaction of the F182A variant (third from top). The flanking spectra are – as in panel **B** – of the **1h** (top) and **4h** (bottom) standards. Note that both **1h** and **4h** give rise to doublet-of-doublets (dd) spectra as a result of large geminal couplings of the ^{31}P nucleus to the ^{19}F (larger coupling) and ^1H (smaller

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4 coupling) nuclei that are also bonded to C1, whereas both **2h** and **3h** give rise to doublet (d)
5 features lacking the smaller geminal coupling to the C1 ¹H, which is removed in their formation
6 from **1h**. The multiplet at 22.9 ppm is from the internal standard, sodium propylphosphonate. (**D**)
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8 ¹⁹F-NMR spectra of the same samples as in panel **B**. The triplet at -217 ppm is from the internal
9 standard, sodium fluoroacetate. (**E**) Blow-up of the ¹⁹F-NMR spectra of **2h** and **3h**, showing
10 geminal couplings (²J_{F-P}) consistent with those seen in the ³¹P-NMR spectra.
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ASSOCIATED CONTENT

Supporting Information. Synthetic methods and spectroscopic data on compounds; procedures for preparation of wild-type and variant HppE enzymes; procedures and data for quantifying enzyme reaction outcomes; Tables assigning NMR resonances of synthetic and enzyme products; methods and data to determine antimicrobial potencies of Fos analogs; NMR spectra of all compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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