ChemCatChem
Chemistry Europe
European Chemical Societies Publishing

RESEARCH ARTICLE OPEN ACCESS

Expanding the Alkene-Forming Capability of the *Pseudomonas Savastanoi* Ethylene-Forming Enzyme

Siddhant Dhingra | Yihong Sun | Zhihong Zhang | Christopher J. Schofield | Lennart Brewitz

Chemistry Research Laboratory, Department of Chemistry and the Ineos Oxford Institute for Antimicrobial Research, University of Oxford, Oxford, UK

Correspondence: Christopher J. Schofield (christopher.schofield@chem.ox.ac.uk) | Lennart Brewitz (lennart.brewitz@chem.ox.ac.uk)

Received: 20 August 2025 | **Revised:** 4 December 2025 | **Accepted:** 9 December 2025

Keywords: 1-aminocyclopropyl-1-carboxylic acid oxidase | ACCO | 2-oxoglutarate/α-ketoglutarate dependent dioxygenases | alkene | biocatalysis | ethylene | ethylene-forming enzyme

ABSTRACT

Ethylene is an established signaling molecule in plants and other organisms; however, the biosynthesis and biological roles of gaseous alkenes other than ethylene are less well defined. The *Pseudomonas savastanoi* ethylene/succinate-forming enzyme (PsEFE) catalyzes ethylene production from 2-oxoglutarate (2OG), though does not catalyze formation of alkenes from C4 alkyl- or hydroxyl-substituted 2OG derivatives. Here we report studies on the reactivity of L206 PsEFE variants with C4-substituted 2OG derivatives. Spectroscopic evidence reveals that L206V and L206A PsEFE react with C4-substituted 2OG derivatives to give diacid and alcohol products, similarly to wildtype (wt) PsEFE. Importantly, L206V PsEFE, but not L206A and L206G PsEFE, catalyzed production of low levels of acetaldehyde and propylene from the natural metabolites 4-hydroxy-2OG and 4-methyl-2OG, respectively. By contrast, L206A PsEFE, but not L206V and L206G PsEFE, catalyzed formation of low levels of 1-butylene from 4-ethyl-2OG. Together with studies from others, the combined results indicate the potential of bump-and-hole studies to modify the substrate and product selectivities of PsEFE reactions, provided that the PsEFE variant:2OG derivative pairs are matched. The results suggest that wildtype 2OG oxygenases other than PsEFE may catalyze production of gaseous alkenes other than ethylene.

1 | Introduction

2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenases catalyze two-electron oxidations of their substrates, a process typically coupled to 2OG oxidation, producing succinic acid and CO_2 [1]. 2OG oxygenases share a characteristic distorted double-stranded β-helix (DSBH) core fold which, together with other conserved secondary structure elements, they use to form Fe(II) and 2OG binding sites; there is more variation in the modes of prime substrate binding [2, 3]. Oxygenases with the distorted DSBH fold have important functions inter alia in plant, fungal, and bacterial ethylene production [4, 5].

Ethylene has validated signaling roles in plant development [6–9], whereas its functions in bacteria and fungi are less well defined [10–15]. In seeding plants, 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACCO) catalyzes oxidation of ACC giving ethylene, along with hydrogen cyanide and CO_2 [5, 16, 17]. Although ACCO has a DSBH fold and belongs to the 2OG oxygenase superfamily, it does not use 2OG as a cosubstrate for productive ACC turnover giving ethylene [4, 5, 18].

Bacteria and fungi employ enzymes other than ACCOs to catalyze ethylene production [4, 19–27], including ethylene/succinate-forming enzymes (EFEs) which catalyze oxidative fragmentation

Siddhant Dhingra and Yihong Sun contributed equally to this work.

of 2OG to give ethylene and CO_2 [28–35]. The EFE of *Pseudomonas savastanoi* (previously: *P. syringae*) pv. *phaseolicola* PK2 (i.e., PsEFE) [33, 34] not only catalyzes fragmentation of 2OG to give ethylene [33, 36], but also catalyzes low levels of 2OG fragmentation to give 3-hydroxypropionic acid and CO_2 [29, 32, 37]. Both of these PsEFE reactions require *L*-arginine to proceed efficiently [37–39]. PsEFE additionally employs 2OG as a cosubstrate in a more typical 2OG oxygenase reaction involving C5 hydroxylation of *L*-arginine to give succinic acid, CO_2, H_2O, guanidine, and *L*-Δ^1-pyrroline-5-carboxylic acid (P5C), the latter likely produced from *L*-glutamate-5-semialdehyde, formed via dehydration of nascent 5-hydroxyarginine (Figure 1a) [36, 38]. PsEFE also catalyzes oxidation of 2OG to give succinic acid and CO_2 uncoupled from *L*-arginine substrate oxidation, though not efficiently [29, 32].

Recent work on the (co)substrate scope of wildtype (wt) PsEFE has shown that it can employ certain C3- and/or C4-substituted 2OG derivatives to catalyze *L*-arginine oxidation in the absence of 2OG to give diacid products (Figure 1b) [40, 41]. This property is reminiscent of the cosubstrate flexibility manifested by, at least, some other 2OG oxygenases [43–48]. wt PsEFE has also been observed to catalyze formation of an alcohol product with some, but not all, of the tested 2OG derivatives, with the 2OG substitution pattern regulating the observed reaction outcomes (Figure 1b) [40, 41]. By contrast, wt PsEFE did not catalyze formation of alkene products from any of the tested C3- and/or C4-substituted 2OG diacid derivatives with the exception of fluoroethylene from 4-fluoro-2OG [40].

Analysis of PsEFE:2OG complex crystal structures reveals that side chains of hydrophobic amino acid residues (e.g., L173, F175, L206, V270, A279) help shape the 2OG binding site, likely stabilizing 2OG binding via hydrophobic interactions with the 2OG C3 and C4 methylene groups (Figures 1 and S1) [42, 49–51], with the L206 side chain methyl groups being proximate with the 2OG C4 carbon (distances: 4.1 and 4.8 Å) (Figure 1). Altering enzyme active site geometries using site-directed mutagenesis to reduce/increase steric bulk proximate to the substrate is an established approach to modify reaction outcomes of wt enzymes with (co)substrate derivatives (the bump-and-hole strategy) [52], including for 2OG oxygenases [53]. Recent studies with L206V PsEFE have revealed its potential to catalyze propylene formation from 4-methyl-2OG [37]. However, the reactivity of L206V PsEFE with C4-substituted 2OG derivatives other than 4-methyl-2OG was not investigated in this work [37].

Here, we report on the reactivity of L206V, L206A, and L206G PsEFE with five C4-substituted 2OG derivatives [45]. The results reveal that, in addition to the reported propylene formation from 4-methyl-2OG [37], L206V PsEFE catalyzes formation of acetaldehyde from the natural metabolite [54] 4-hydroxy-2OG. Notably, L206A PsEFE, but not L206V or L206G PsEFE, catalyze 1-butylene formation from 4-ethyl-2OG. The combined results indicate that active site mutation studies with PsEFE have potential to enable formation of products not produced by wildtype PsEFE, including 1-butylene, from non-native substrates, provided that the 2OG derivative:PsEFE variant pairs are matched.

2 | Results

Reactivity of L206 PsEFE variants with 2OG. The L206V, L206A, and L206G PsEFE variants (UNIPROT ID: P32021; EC:1.13.12.19) [33, 34] were produced in >90% purity, as judged by SDS-PAGE and MS analyses (Figure S2), to investigate whether altering the steric bulk at residue-206 can affect reaction outcomes with C4-substituted 2OG derivatives. Initially, the reactivities of the three isolated recombinant L206 PsEFE variants were investigated using 2OG as a (co)substrate together with *L*-arginine using a reported ^{1}H NMR-based assay [41]. This assay enables quantification of 2OG/2OG derivative consumption and 2OG/2OG-derived (co)product formation by comparing signals with an internal standard (i.e., 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid [55], TMSP-d_4); the assay also enables qualitative product identification by comparison with authentic standards.

^{1}H NMR analysis (700 MHz) of reactions of L206V, L206A, and L206G PsEFE with 2OG and *L*-arginine reveals that all of these variants were less active than wt PsEFE: the efficiency of PsEFE-variants, as measured by depletion of the characteristic 2OG methylene triplet peaks, decreased with decreasing steric bulk at residue-206 in the order: L206 (wt) >V206>A206>G206, with L206G PsEFE being inactive under the tested conditions (Figure 2). Analysis of wt, L206V, L206A, and L206G PsEFE by circular dichroism (CD) spectroscopy indicates that substituting L206 did not affect the overall PsEFE fold (Figure S3). There was no evidence accrued for formation of substantial amounts of any 2OG-derived products other than ethylene and succinic acid (Figure S4), including a lack of 3-hydroxypropionic acid, that is, the low level alcohol product of the wt PsEFE reaction with 2OG [29, 40]. This observation contrasts with the reported detection of 3-hydroxypropionic acid from reactions of L206V PsEFE with 2OG by infrared spectroscopy and LCMS assays, possibly reflecting differences in assay method sensitivity and/or assay conditions [37].

The estimated succinic acid:ethylene ratios following reaction of L206V and L206A PsEFE with 2OG were similar to that reported for wt PsEFE (~1:1) (Figure 2) [41]. P5C formation was not quantified, because the abundance of its characteristic singlet ($\delta \sim 7.70$ ppm) apparently varied over time [41]. Experiments in the absence of *L*-arginine indicated that L206V and L206A PsEFE, like wt PsEFE, require *L*-arginine for, at least, efficient 2OG oxidation (Figure S5).

L206 PsEFE variants catalyze oxidation of C4-substituted 2OG derivatives. Having validated that L206V and L206A PsEFE catalyze 2OG oxidation, we next investigated the ability of five representative C4-substituted 2OG derivatives (all as racemates) to sustain catalysis by L206V, L206A, and L206G PsEFE. ^{1}H NMR analysis (700 MHz) reveals that L206V and L206A PsEFE react with 4-methyl-2OG (**1**), 4,4-dimethyl-2OG (**2**), 4-hydroxy-2OG (**3**), 4-ethyl-2OG (**4**), and 4-propyl-2OG (**5**) to give both the corresponding diacid and alcohol products (Figures 3 and S6–S10), as reported for wt PsEFE [40, 41]. Note that the stereochemistry of the products was not assigned. By contrast, L206G PsEFE did not catalyze conversion of any of the 2OG derivatives **1-5** within 2 h (Figures 3 and S6–S10). Experiments in the absence of *L*-

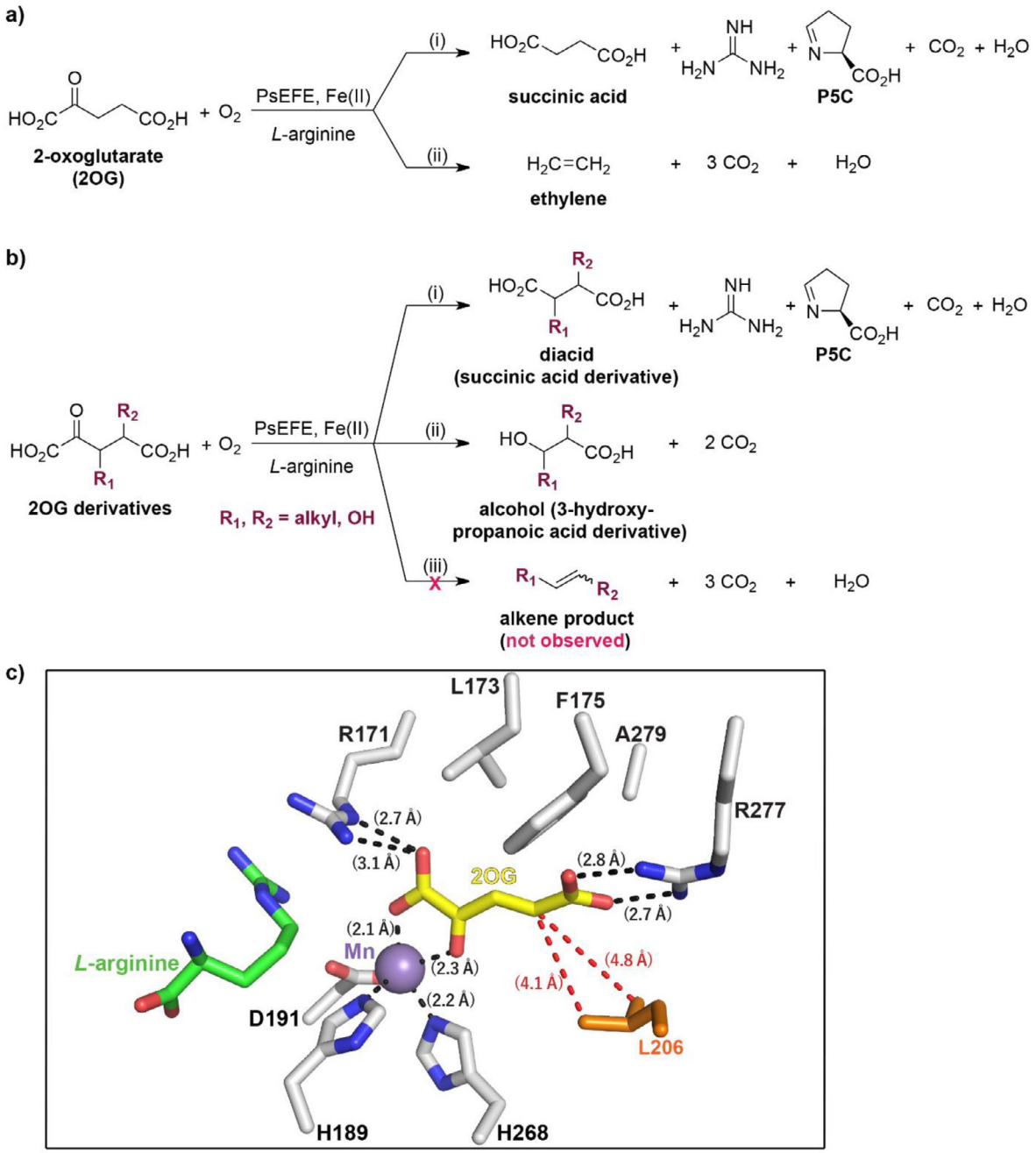

FIGURE 1 | The wildtype PsEFE substrate scope. (a) Wildtype (wt) PsEFE reacts with 2OG to give ethylene and succinic acid as major products, with formation of succinic acid being coupled to C5 oxidation of *L*-arginine to give guanidine and P5C [33, 36]; note that low levels of an alcohol product (3-hydroxypropionic acid) have also been observed [29, 32, 37]. (b) wt PsEFE reacts with C3/C4-alkyl/hydroxy-substituted 2OG derivatives to give diacid (coupled to P5C formation) and alcohol, but not alkene, products [40, 41]. (c) View from a wt PsEFE:Mn:2OG:arginine crystal structure (with Mn substituting for catalytically active Fe(II); PDB ID: 5V2Y [42]) showing that the side-chain methyl groups of L206 are close to the 2OG C4 methylene group (dotted red lines), suggesting that reducing the steric bulk at residue-206 may affect the conformation of C4-substituted 2OG derivatives at the active site. Dotted black lines indicate polar interactions of PsEFE residues with 2OG and Mn.

arginine indicated that the three L206 PsEFE variants require *L*-arginine for, at least, efficient oxidation of the C4-substituted 2OG derivatives (Figures S13–S15).

The results indicate that, in general, L206V and L206A PsEFE reacted less efficiently with the five tested C4-substituted 2OG derivatives **1**-**5** than wt PsEFE (Figure 3) [41]. Notable exceptions were the reactions of L206V PsEFE with racemic 4-methyl-2OG (**1**) and 4-propyl-2OG (**5**), which were equally or more efficient than those reported with wt PsEFE [41], giving 2-methylsuccinic acid (**1a**)/*β*-hydroxyisobutyric acid (**1b**) and 2-propylsuccinic acid (**5a**)/2-(hydroxymethyl)pentanoic acid (**5b**), respectively.

Analysis of the diacid:alcohol ratios for the reactions of L206V and L206A PsEFE with 2OG derivatives **1**-**5** reveals that the nature of the L206 variant did not substantially affect the diacid:alcohol

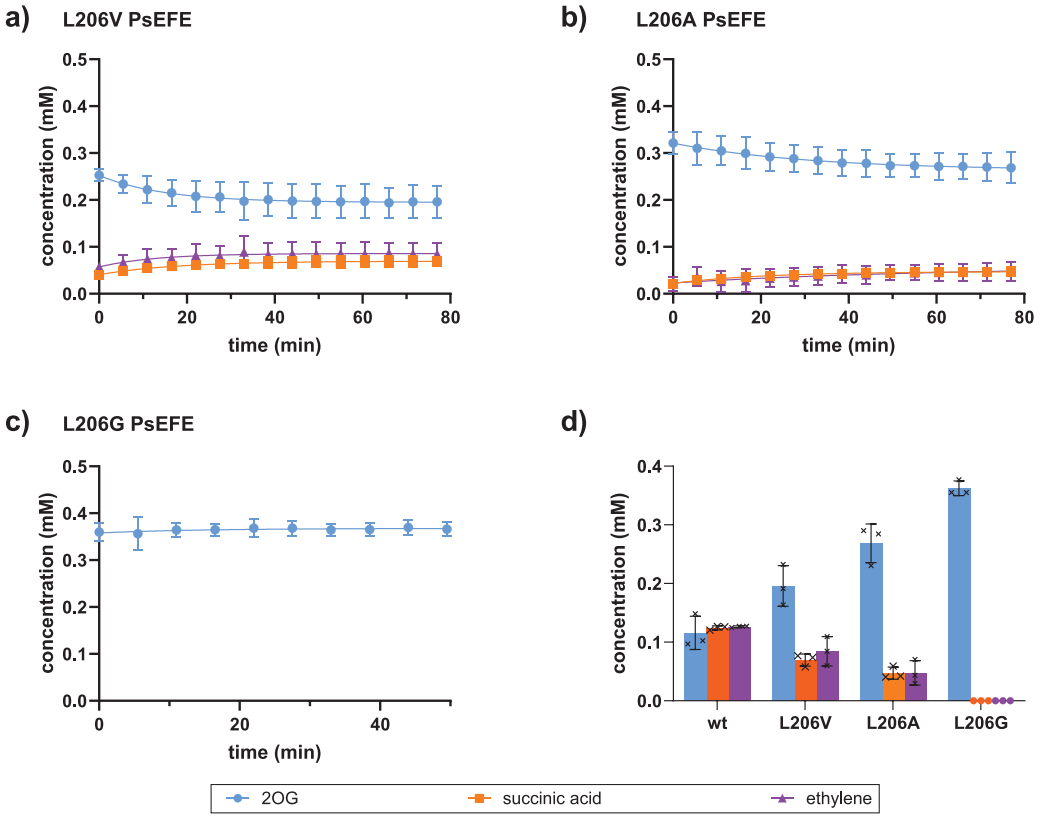

FIGURE 2 | Substitutions at PsEFE residue-206 influence reactivity with 2OG. Reactions of (a) L206V PsEFE, (b) L206A PsEFE, and (c) L206G PsEFE with 2OG. (d) Outcomes of L206 PsEFE variant reactions with 2OG compared to that reported for wt PsEFE [41]. Ethylene formation was estimated by subtracting the integral of the succinic acid (normalized to TMSP-d_4) from the starting concentration of 2OG. The time-scales were calibrated to the end of the acquisition of the first ^{1}H NMR experiment following PsEFE addition to the reaction mixture (t = 0 min), by which time low levels of 2OG conversion were manifest. Results are means of independent triplicates (n = 3; mean ± standard deviation, SD). Conditions: 400 μM 2OG, 500 μM *L*-arginine, 500 μM *L*-ascorbic acid, 50 μM Fe(II), 800 μM TMSP-d_4, and L206 PsEFE variant (10 μM) or wt PsEFE (2 μM) [41] in buffer (50 mM sodium phosphate, pH 7.4, 10% v/v D_2O).

product ratios. The diacid:alcohol ratios for the reactions of L206V and L206A PsEFE with **1**-**5** matched those reported for wt PsEFE (i.e., ~1:1 for **2**; ~2:1 for **3**; ~2:1 for **4**; ~3:2 for **5**); the exception was the product ratios obtained with **1**, which were ~1:1 for L206V and L206A PsEFE, but ~2:1 for wt PsEFE (Figure 3) [41].

L206 PsEFE variants catalyze alkene formation from C4-substituted 2OG derivatives. Importantly, ^{1}H NMR analyses of the L206V and L206A PsEFE reactions with 2OG derivatives **1**-**5** not only revealed diacid and alcohol formation, but also the formation of relatively low levels of alkene products from 4-methyl-2OG (**1**), 4-hydroxy-2OG (**3**) (to give acetaldehyde), and 4-ethyl-2OG (**4**) (Figures S16–S18). In accord with reported work [37], we observed L206V PsEFE catalyzed formation of propylene from **1**, as supported by direct comparison with an authentic sample of propylene (Figure 4). Note that alkene formation was not directly quantified by ^{1}H NMR, because alkenes may evaporate from the aqueous reaction mixture [56]; in some cases, overlapping peaks (e.g., the peaks of the propylene methyl group and the C4 methylene group of *L*-arginine; Figure 4) further complicated quantification of alkene formation. The alkene levels were thus approximated by subtracting the normalized integral of the diacid and alcohol products (obtained via comparison with TMSP-d_4) from that of **1** because no substantial amounts of additional products derived from **1** were detected and because diacid formation appeared to be mostly coupled to *L*-arginine oxidation [40, 41]. By contrast with L206V PsEFE, L206A and L206G PsEFE (like wt PsEFE [41]) did not catalyze formation of propylene from **1**, possibly reflecting their substantially reduced reactivity with **1** compared to that of L206V PsEFE.

Like wt PsEFE [41], L206V, L206A, and L206G PsEFE did not catalyze formation of propylene from 2-oxoadipate (2OA) and 3-methyl-2OG (**6**), the regioisomer of **1**, within 2 h (Figures 3, S11 and S12). None of the L206 PsEFE variants appeared to catalyze isobutene production from 4,4-dimethyl-2OG (**2**) within 2 h (Figure S6), in accord with the reported reactivity of wt PsEFE with **2** [41]; note the possibility of formation of low amounts of isobutylene cannot be excluded due to potential overlap with peaks from *L*-arginine (Figure S6).

L206V PsEFE not only catalyzed propylene formation from 4-methyl-2OG (**1**) but also catalyzed production of relatively low levels of acetaldehyde from 4-hydroxy-2OG (**3**); note that we observed no evidence for formation of pyruvate via the potential retroaldol reaction of **3** (Figure S8). Neither L206A, L206G, nor wt PsEFE [41] catalyzed acetaldehyde formation from **3** (Figure S7). By contrast, analysis of the reaction products of L206A PsEFE, but not of L206V, L206G, or of wt PsEFE [41], showed

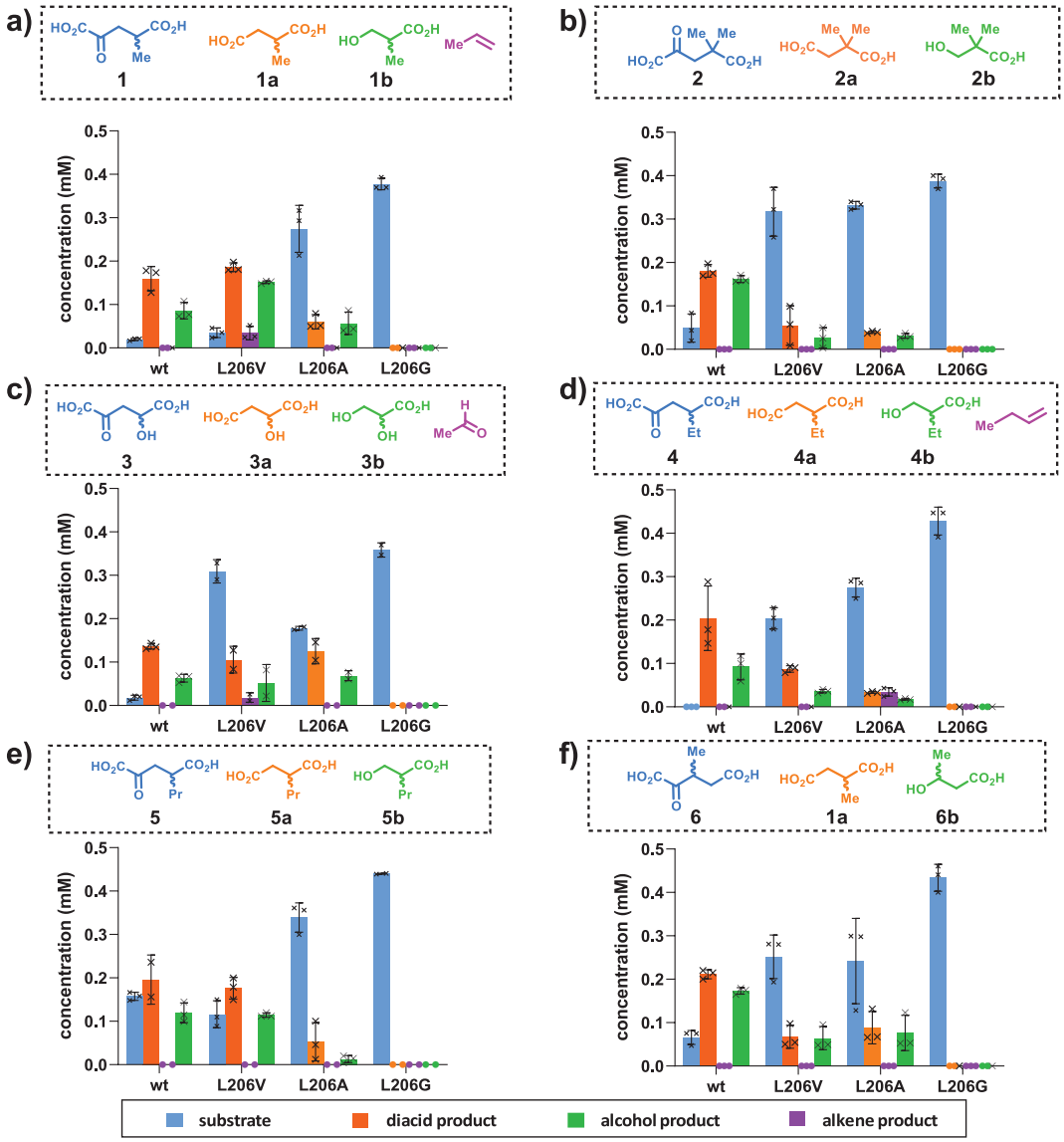

FIGURE 3 | L206 PsEFE variants can employ C4-substituted 2OG derivatives as (co)substrates. Outcomes of the reaction of L206 PsEFE variants with: (a) 4-methyl-2OG (**1**), (b) 4,4-dimethyl-2OG (**2**), (c) 4-hydroxy-2OG (**3**), (d) 4-ethyl-2OG (**4**), (e) 4-propyl-2OG (**5**), and (f) 3-methyl-2OG (**6**) compared to that reported for wt PsEFE [41]. Results are means of independent triplicates ($n = 3$; mean ± SD); ^{1}H NMR spectra are shown in Figures S6–S11. Conditions: 400 μM 2OG derivative, 500 μM *L*-arginine, 500 μM *L*-ascorbate, 50 μM Fe(II), 800 μM TMSP-d_4, and 10 μM L206 PsEFE variant in buffer (50 mM sodium phosphate, pH 7.4, 10%$_{v/v}$ D_2O). Note that the stereochemistry of the diacid and alcohol products was not assigned.

evidence for formation of relatively low levels of 1-butylene from 4-ethyl-2OG (**4**), as manifest by peaks for the methyl group of 1-butylene (Figure S9). The results suggest that the C4 ethyl group of **4** requires more space in proximity to the PsEFE residue-206 relative to the methyl group of **1** in order to adopt a conformation enabling alkene formation. Consistent with this proposal, none of the L206 PsEFE variants catalyzed formation of 1-pentene from 4-propyl-2OG (**5**) (Figure S10).

3 | Discussion

Plants and/or microorganisms are reported to emit low mass gaseous alkenes other than ethylene; however, it is unclear whether all of these alkenes are produced in an enzyme-catalyzed manner [57–59]. The effects of these alkenes on plant signaling can differ from those of ethylene [60–63]. Pioneering work with fruit extracts on the substrate scope of ACCOs revealed the possibility of ACCO-catalyzed propylene and 1-butylene production from naturally occurring C-alkylated ACC derivatives [64–67]. By contrast, reported studies on the substrate scope of wt PsEFE indicate that it does not catalyze propylene production from 2-oxoadipate, 4-methyl-2OG (**1**), or 3-methyl-2OG (**6**) [39–42], although wt PsEFE catalyzed production of fluoroethylene from 4-fluoro-2OG [40], a reaction likely lacking biological significance as 4-fluoro-2OG is not a naturally occurring cellular metabolite.

The results presented here show that decreasing the steric bulk at PsEFE residue-206 can enable the capacity to catalyse production of alkenes, that is, propylene from the natural product [68–70] **1** and 1-butylene from 4-ethyl-2OG (**4**), as well as acetaldehyde from the metabolite [54] 4-hydroxy-2OG (**3**) (Figure 3), which is

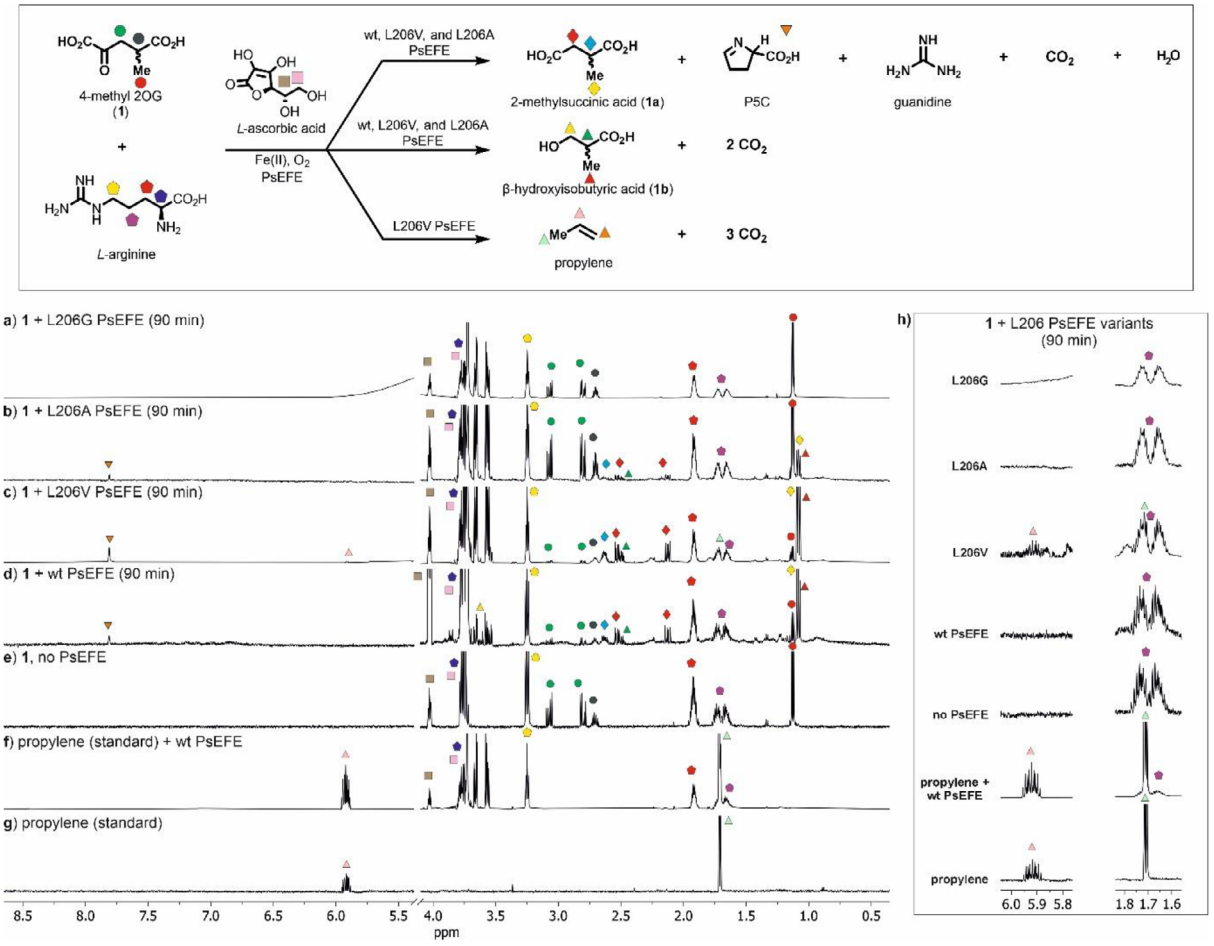

FIGURE 4 | L206V PsEFE catalyzes production of propylene from 4-methyl-2OG (1). (a–d) ^{1}H NMR analysis (~0.5 to ~4.0 ppm and ~5.5 to ~8.5 ppm) for ~90 min incubations of 4-methyl-2OG (**1**) with: (a) L206G PsEFE, (b) L206A PsEFE, or (c) L206V PsEFE. (d) Reported ^{1}H NMR analysis of (~90 min incubation) for reaction of **1** with wt PsEFE [41]. (e) Reported ^{1}H NMR spectrum of **1** under standard conditions without PsEFE [41]. (f) ^{1}H NMR spectrum of an authentic sample of propylene under assay conditions. Note that the C1 alkene protons of propylene were not observed, likely because they coincide with the HOD peak. (g) ^{1}H NMR spectrum of an authentic propylene in buffer. (h) ^{1}H NMR spectra (~5.8 to ~6.0 ppm and ~1.6 to ~1.8 ppm) showing that L206V PsEFE catalyzed propylene production from **1**. Conditions: 400 μM **1**, 500 μM *L*-arginine, 500 μM *L*-ascorbic acid, 50 μM Fe(II), 800 μM TMSP-d_4, and 10 μM PsEFE variant in buffer (50 mM sodium phosphate, pH 7.4, 10%$_{v/v}$ D_2O). Peaks at ~3.6 and ~3.7 ppm are from glycerol; the singlet at ~3.7 ppm corresponds to Tris buffer. Note that the stereochemistry of products was not assigned. Representative spectra of independent triplicates are shown.

formed in cells as an intermediate of 4-hydroxyproline catabolism [71, 72]. They confirm the recently reported ability of L206V PsEFE to catalyze propylene production from **1** [37]. Thus, the biocatalytic potential of PsEFE variants extends beyond catalyzing production of ethylene, diacids, and alcohols [40, 41, 73, 74] to production of aldehydes and alkenes other than ethylene, which are commodities [74–77]. L206V and L206A PsEFE likely catalyze alkene formation from C4-substituted 2OG derivatives via a radical Grob-type mechanism similar to that proposed for the reaction of wt PsEFE with 2OG [37, 40, 78, 79]. The reaction of L206V PsEFE with **3** might involve transient production of vinyl alcohol giving acetaldehyde following tautomerization.

The observations: (i) that L206V PsEFE, but not L206A, L206G, and wt PsEFE [41], catalyzed propylene formation from **1**, (ii) that the tested L206 PsEFE variants did not catalyze formation of propylene and isobutylene from **6** and 4,4-dimethyl-2OG (**2**), respectively, and (iii) that L206A PsEFE, but not L206V, L206G, and wt PsEFE [41], catalyze formation of 1-butene from **4,** suggest matching of specific 2OG derivative:PsEFE variant pairs is required to enable substantial levels of novel PsEFE reactivities (Figure 3). Thus, there is potential for design of PsEFE variants which catalyze alkene and/or acetaldehyde formation more efficiently than the L206 PsEFE variants tested in this work and for bump-and-hole studies with PsEFE to enable formation of (novel) products from 2OG derivatives by varying PsEFE residues other than residue-206.

Aside from the work on wt ACCO, the ability of substitutions in the PsEFE active site to induce capacity for production of alkenes from C4-substituted 2OG derivatives is precedented by work on nitrogenases which catalyze ethylene production from acetylene in their wildtype form [23, 24, 80], as substitutions in the active site of nitrogenases enabled production of propylene from propyne [81, 82]. The combined evidence highlights the importance of second coordination sphere residues in influencing

reactivity of mechanistically unrelated ethylene-producing nitrogenases [83] and PsEFE [84, 85], as is also the case for more typical 2OG oxygenases [86–89]. Thus, mutations beyond the simple bump-and-hole type studies reported here and elsewhere will likely be required to optimize formation of specific PsEFE products including alkenes. Given that the alkene products can be measured by headspace analysis, a forced-evolution approach [90, 91] (taking into account substrate stereochemistry) may be particularly productive.

The combined results on the reactivity of the L206 PsEFE variants and the observation that disease-associated variations in the active sites of certain human 2OG oxygenases can alter their reactivity with 2-oxoacids [92, 93] raises the question as to whether 2OG oxygenase(s) can catalyze alkene formation from naturally abundant C3- and/or C4-substituted 2OG derivatives in their wildtype form. To our knowledge, L206V/A (equivalent) PsEFE variants have not yet been identified in microbes.

4 | Conclusion

The results reveal L206V and L206A PsEFE have capacity to catalyze production of alkenes other than ethylene, including propylene and 1-butylene, from 2OG-derived substrates, including from natural products. L206V PsEFE also catalyzed low levels of acetaldehyde formation from the abundant metabolite 4-hydroxy-2OG (**3**). The combined results further highlight the remarkable versatility of the PsEFE reaction with respect to 2-oxoacid oxidation. They suggest that plants and/or microorganisms may have potential to produce gaseous alkenes other than ethylene in an enzyme-catalyzed manner, possibly to exert signaling effects distinct from those of ethylene.

5 | Experimental Section

Protein production. Plasmid DNA encoding for the L206V, L206A, and L206G PsEFE variants was obtained from our reported wt PsEFE construct [49] by a standard site-directed mutagenesis procedure (Table S1). The L206V, L206A, and L206G PsEFE variants were produced and purified as reported for wt PsEFE [41, 49]. The L206 PsEFE variants were >90% pure by SDS-PAGE analysis and had the anticipated masses (Figure S2). They were stored at −70°C, at ~20–25 mg protein/mL.

2OG derivatives. 2-Oxoglutarate (2OG), 2-oxoadipate (2OA), and racemic 4-hydroxy-2OG (**3**) were from Sigma-Aldrich. 4-Methyl-2OG (**1**), 4,4-dimethyl-2OG (**2**), 4-ethyl-2OG (**4**), 4-propyl-2OG (**5**), and 3-methyl-2OG (**6**) were synthesized as reported [45]; chiral 2OG derivatives were prepared as racemic mixtures.

^{1}H NMR assays. ^{1}H NMR assays were performed as reported [41], typically using: 2OG derivative (400 μM), *L*-arginine (500 μM), *L*-ascorbic acid (500 μM), Fe(II) (50 μM), TMSP-d_4 (800 μM), and L206 PsEFE variant (10 μM) in buffer (50 mM sodium phosphate, pH 7.4, 10%$_{v/v}$ D_2O), if not specified otherwise in the figure legends. Freshly thawed aliquots of L206 PsEFE variants were used for all experiments. A Bruker AVIII 700 MHz NMR spectrometer with a 5-mm inverse triple-resonance-inverse cryoprobe was used for analysis. The water peak in ^{1}H NMR experiments was suppressed using the perfect echo-modified WATERGATE solvent suppression method [94].

Author Contributions

Siddhant Dhingra and Yihong Sun produced enzymes; they performed assays; all authors analyzed data; Lennart Brewitz conceived the project together with Siddhant Dhingra; Lennart Brewitz, Zhihong Zhang, and Christopher J. Schofield supervised the research; Lennart Brewitz, Siddhant Dhingra, and Christopher J. Schofield wrote the manuscript with help from all authors.

Acknowledgments

This research was funded in part by the Wellcome Trust (106244/Z/14/Z). For the purpose of open access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission. The authors thank the Biotechnology and Biological Sciences Research Council (BB/J003018/1 and BB/R000344/1) for funding and Prof. Dr. C. Redfield, Dr. C. Mycroft, and L. Harrington for helpful discussions.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

References

1. *2-Oxoglutarate-Dependent Oxygenases* (Ed.: R. P. Hausinger, C. J. Schofield), (The Royal Society of Chemistry, 2015).

2. S. Kal and L. Que, "Dioxygen Activation by Nonheme Iron Enzymes With the 2-His-1-Carboxylate Facial Triad That Generate High-Valent Oxoiron Oxidants," *JBIC Journal of Biological Inorganic Chemistry* 22 (2017): 339–365, https://doi.org/10.1007/s00775-016-1431-2.

3. W. Aik, M. A. McDonough, A. Thalhammer, R. Chowdhury, and C. J. Schofield, "Role of the Jelly-Roll Fold In Substrate Binding by 2-Oxoglutarate Oxygenases," *Current Opinion in Structural Biology* 22 (2012): 691–700, https://doi.org/10.1016/j.sbi.2012.10.001.

4. R. P. Hausinger, S. B. J. S. Rifayee, M. G. Thomas, S. Chatterjee, J. Hu, and C. Z. Christov, "Biological Formation of Ethylene," *RSC Chemical Biology* 4 (2023): 635–646, https://doi.org/10.1039/D3CB00066D.

5. M. Houben and B. Van de Poel, "1-Aminocyclopropane-1-Carboxylic Acid Oxidase (ACO): The Enzyme That Makes the Plant Hormone Ethylene," *Frontiers in Plant Science* 10 (2019): 695, https://doi.org/10.3389/fpls.2019.00695.

6. B. M. Binder, "Ethylene Signaling in Plants," *Journal of Biological Chemistry* 295 (2020): 7710–7725, https://doi.org/10.1074/jbc.REV120.010854.

7. X. Wang, H. Wen, A. Suprun, and H. Zhu, "Ethylene Signaling in Regulating Plant Growth, Development, and Stress Responses," *Plants* 14 (2025): 309, https://doi.org/10.3390/plants14030309.

8. B. Van de Poel, E. D. Cooper, C. F. Delwiche, and C. Chang, "An Evolutionary Perspective on the Plant Hormone Ethylene," *Ethylene in Plants* In (Eds: CK. Wen), Springer, Dordrecht (2015): 109–134, https://doi.org/10.1007/978-94-017-9484-8.

9. B. Van de Poel and J. de Vries, "Evolution of Ethylene as an Abiotic Stress Hormone in Streptophytes," *Environmental and Experimental Botany* 214 (2023): 105456, https://doi.org/10.1016/j.envexpbot.2023.105456.

10. M. Ravanbakhsh, R. Sasidharan, L. A. C. J. Voesenek, G. A. Kowalchuk, and A. Jousset, "Microbial Modulation of Plant Ethylene Signaling: Ecological and Evolutionary Consequences," *Microbiome* 6 (2018): 52, https://doi.org/10.1186/s40168-018-0436-1.

11. N. Papon and B. M. Binder, "An Evolutionary Perspective on Ethylene Sensing in Microorganisms," *Trends in Microbiology* 27 (2019): 193–196, https://doi.org/10.1016/j.tim.2018.12.002.

12. B. Bidon, S. Kabbara, V. Courdavault, et al., "Cytokinin and Ethylene Cell Signaling Pathways From Prokaryotes to Eukaryotes," *Cells* 9 (2020): 2526.

13. I. Camehl, I. Sherameti, Y. Venus, et al., "Ethylene Signalling and Ethylene-Targeted Transcription Factors are Required to Balance Beneficial and Nonbeneficial Traits in the Symbiosis Between the Endophytic Fungus *Piriformospora Indica* and *Arabidopsis Thaliana*," *New Phytologist* 185 (2010): 1062–1073, https://doi.org/10.1111/j.1469-8137.2009.03149.x.

14. V. Chagué, *Handbook of Hydrocarbon and Lipid Microbiology* (Berlin Heidelberg, 2010): 3011–3020.

15. S. Gu, L. Xie, Q. Guan, X. Sheng, Y. Fang, and X. Wang, "Effect of Ethylene Production by Four Pathogenic Fungi on the Postharvest Diseases of Green Pepper (*Capsicum Annuum* L.)," *International Journal of Food Microbiology* 418 (2024): 110729, https://doi.org/10.1016/j.ijfoodmicro.2024.110729.

16. A. J. Hamilton, G. W. Lycett, and D. Grierson, "Antisense Gene That Inhibits Synthesis of the Hormone Ethylene in Transgenic Plants," *Nature* 346 (1990): 284–287, https://doi.org/10.1038/346284a0.

17. Z. Zhang, J.-S. Ren, I. J. Clifton, and C. J. Schofield, "Crystal Structure and Mechanistic Implications of 1-Aminocyclopropane-1-Carboxylic Acid Oxidase—The Ethylene-Forming Enzyme," *Chemistry & Biology* 11 (2004): 1383–1394, https://doi.org/10.1016/j.chembiol.2004.08.012.

18. J. G. Dong, J. C. Fernández-Maculet, and S. F. Yang, "Purification and Characterization of 1-Aminocyclopropane-1-Carboxylate Oxidase From Apple Fruit," *Proceedings of the National Academy of Sciences* 89 (1992): 9789–9793, https://doi.org/10.1073/pnas.89.20.9789.

19. C. C. Lee, Y. Hu, and M. W. Ribbe, "Vanadium Nitrogenase Reduces CO," *Science* 329 (2010): 642, https://doi.org/10.1126/science.1191455.

20. Y. Hu, C. C. Lee, and M. W. Ribbe, "Extending the Carbon Chain: Hydrocarbon Formation Catalyzed by Vanadium/Molybdenum Nitrogenases," *Science* 333 (2011): 753–755, https://doi.org/10.1126/science.1206883.

21. C. C. Lee, Y. Hu, and M. W. Ribbe, "Tracing the Hydrogen Source of Hydrocarbons Formed by Vanadium Nitrogenase," *Angewandte Chemie International Edition* 50 (2011): 5545–5547, https://doi.org/10.1002/anie.201100869.

22. Y. Hu, C. C. Lee, M. Grosch, J. B. Solomon, W. Weigand, and M. W. Ribbe, "Enzymatic Fischer–Tropsch-Type Reactions," *Chemical Reviews* 123 (2023): 5755–5797, https://doi.org/10.1021/acs.chemrev.2c00612.

23. L. C. Seefeldt, Z.-Y. Yang, D. A. Lukoyanov, et al., "Reduction of Substrates by Nitrogenases," *Chemical Reviews* 120 (2020): 5082–5106, https://doi.org/10.1021/acs.chemrev.9b00556.

24. B. M. Barney, R. Y. Igarashi, P. C. Dos Santos, D. R. Dean, and L. C. Seefeldt, "Substrate Interaction at an Iron-Sulfur Face of the FeMo-Cofactor During Nitrogenase Catalysis," *Journal of Biological Chemistry* 279 (2004): 53621–53624, https://doi.org/10.1074/jbc.M410247200.

25. J. A. North, A. R. Miller, J. A. Wildenthal, S. J. Young, and F. R. Tabita, "Microbial Pathway for Anaerobic 5′-Methylthioadenosine Metabolism Coupled to Ethylene Formation," *Proceedings of the National Academy of Sciences* 114 (2017): E10455–E10464, https://doi.org/10.1073/pnas.1711625114.

26. J. A. North, A. B. Narrowe, W. Xiong, et al., "A Nitrogenase-Like Enzyme System Catalyzes Methionine, Ethylene, and Methane Biogenesis," *Science* 369 (2020): 1094–1098, https://doi.org/10.1126/science.abb6310.

27. A. Lago-Maciel, J. C. Soares, J. Zarzycki, et al., "Methylthio-Alkane Reductases use Nitrogenase Metalloclusters for Carbon–Sulfur Bond Cleavage," *Nature Catalysis* 8 (2025): 1086–1099, https://doi.org/10.1038/s41929-025-01426-2.

28. Y. Cui, Y. Jiang, M. Xiao, et al., "Discovery of Five New Ethylene-Forming Enzymes for Clean Production of Ethylene in *E. Coli*," *International Journal of Molecular Sciences* 23 (2022): 4500, https://doi.org/10.3390/ijms23094500.

29. S. Chatterjee, J. A. Rankin, M. A. Farrugia, et al., "Biochemical, Structural, and Conformational Characterization of a Fungal Ethylene-Forming Enzyme," *Biochemistry* 64 (2025): 2054–2067, https://doi.org/10.1021/acs.biochem.5c00038.

30. H. Fukuda, T. Fujii, and T. Ogawa, "Preparation of a Cell-free Ethylene-Forming System From Penicillium Digitatum," *Agricultural and Biological Chemistry* 50 (1986): 977–981, https://doi.org/10.1080/00021369.1986.10867507.

31. T. Hottiger and T. Boller, "Ethylene Biosynthesis in *Fusarium Oxysporum* f. sp. Tulipae Proceeds From Glutamate/2-Oxoglutarate and Requires Oxygen and Ferrous Ions In Vivo," *Archives of Microbiology* 157 (1991): 18–22, https://doi.org/10.1007/BF00245329.

32. S. Chatterjee, J. A. Rankin, M. A. Farrugia, et al., "Ancestral Sequence Reconstruction of the Ethylene-Forming Enzyme," *Biochemistry* 64 (2025): 3432–3445, https://doi.org/10.1021/acs.biochem.5c00334.

33. K. Nagahama, T. Ogawa, T. Fujii, et al., "Purification and Properties of an Ethylene-Forming Enzyme From *Pseudomonas Syringae* pv. Phaseolicola PK2," *Journal of General Microbiology* 137 (1991): 2281–2286, https://doi.org/10.1099/00221287-137-10-2281.

34. H. Fukuda, T. Ogawa, K. Ishihara, et al., "Molecular Cloning in *Escherichia Coli*, Expression, and Nucleotide Sequence of the Gene for the Ethylene-Forming Enzyme of *Pseudomonas Syringae* pv. Phaseolicola PK2," *Biochemical and Biophysical Research Communications* 188 (1992): 826–832, https://doi.org/10.1016/0006-291X(92)91131-9.

35. N. Johansson, K. O. Persson, C. Larsson, and J. Norbeck, "Comparative Sequence Analysis and Mutagenesis of Ethylene Forming Enzyme (EFE) 2-Oxoglutarate/Fe(II)-Dependent Dioxygenase Homologs," *BMC Biochemistry* 15 (2014): 22, https://doi.org/10.1186/1471-2091-15-22.

36. H. Fukuda, T. Ogawa, M. Tazaki, et al., "Two Reactions are Simultaneously Catalyzed by a Single Enzyme: The Arginine-Dependent Simultaneous Formation of Two Products, Ethylene and Succinate, From 2-Oxoglutarate by an Enzyme From *Pseudomonas Syringae*," *Biochemical and Biophysical Research Communications* 188 (1992): 483–489, https://doi.org/10.1016/0006-291X(92)91081-Z.

37. E. J. Burke, R. A. Copeland, Y. Dixit, C. Krebs, and J. M. Bollinger Jr., "Steric Perturbation of the Grob-Like Final Step of Ethylene-Forming Enzyme Enables 3-Hydroxypropionate and Propylene Production," *Journal of the American Chemical Society* 146 (2024): 1977–1983, https://doi.org/10.1021/jacs.3c09733.

38. K. Nagahama, T. Ogawa, T. Fujii, M. Tazaki, M. Goto, and H. Fukuda, "l-Arginine is Essential for the Formation In Vitro of Ethylene by an Extract of *Pseudomonas Syringae*," *Journal of General Microbiology* 137 (1991): 1641–1646, https://doi.org/10.1099/00221287-137-7-1641.

39. S. Martinez and R. P. Hausinger, "Biochemical and Spectroscopic Characterization of the Non-Heme Fe(II)- and 2-Oxoglutarate-Dependent Ethylene-Forming Enzyme From *Pseudomonas Syringae* pv. Phaseolicola PK2," *Biochemistry* 55 (2016): 5989–5999, https://doi.org/10.1021/acs.biochem.6b00890.

40. R. A. Copeland, S. Zhou, I. Schaperdoth, T. K. C. Shoda, J. M. Bollinger, and C. Krebs, "Hybrid Radical-Polar Pathway for Excision of Ethylene From 2-Oxoglutarate by an Iron Oxygenase," *Science* 373 (2021): 1489–1493, https://doi.org/10.1126/science.abj4290.

41. S. Dhingra, Z. Zhang, C. T. Lohans, L. Brewitz, and C. J. Schofield, "Substitution of 2-Oxoglutarate Alters Reaction Outcomes of the *Pseudomonas Savastanoi* Ethylene-Forming Enzyme," *Journal of Biological Chemistry* 300 (2024): 107546, https://doi.org/10.1016/j.jbc.2024.107546.

42. S. Martinez, M. Fellner, C. Q. Herr, A. Ritchie, J. Hu, and R. P. Hausinger, “Structures and Mechanisms of the Non-Heme Fe(II)- and 2-Oxoglutarate-Dependent Ethylene-Forming Enzyme: Substrate Binding Creates a Twist,” *Journal of the American Chemical Society* 139 (2017): 11980–11988, https://doi.org/10.1021/jacs.7b06186.

43. K. Majamaa, H. M. Hanauske-Abel, V. Günzler, and K. I. Kivirikko, “The 2-Oxoglutarate Binding Site of Prolyl 4-Hydroxylase,” *European Journal of Biochemistry* 138 (1984): 239–245, https://doi.org/10.1111/j.1432-1033.1984.tb07907.x.

44. C. J. Cunliffe, T. J. Franklin, N. J. Hales, and G. B. Hill, “Novel Inhibitors of Prolyl 4-Hydroxylase. 3. Inhibition by the Substrate Analog N-Oxaloglycine and its Derivatives,” *Journal of Medicinal Chemistry* 35 (1992): 2652–2658, https://doi.org/10.1021/jm00092a016.

45. L. Brewitz, Y. Nakashima, and C. J. Schofield, “Synthesis of 2-Oxoglutarate Derivatives and Their Evaluation as Cosubstrates and Inhibitors of Human Aspartate/Asparagine-β-Hydroxylase,” *Chemical Science* 12 (2021): 1327–1342, https://doi.org/10.1039/D0SC04301J.

46. Y. Nakashima, L. Brewitz, A. Tumber, E. Salah, and C. J. Schofield, “2-Oxoglutarate Derivatives can Selectively Enhance or Inhibit the Activity of Human Oxygenases,” *Nature Communications* 12 (2021): 6478, https://doi.org/10.1038/s41467-021-26673-2.

47. A. Tumber, E. Salah, L. Brewitz, T. Corner, and C. J. Schofield, “Kinetic and Inhibition Studies on Human Jumonji-C (JmjC) Domain-Containing Protein 5,” *RSC Chemical Biology* 4 (2023): 399–413, https://doi.org/10.1039/D2CB00249C.

48. G. Fiorini, S. A. Marshall, W. D. Figg, W. K. Myers, L. Brewitz, and C. J. Schofield, “Human Prolyl Hydroxylase Domain 2 Reacts With O2 and 2-Oxoglutarate to Enable Formation of Inactive Fe(III).2OG.Hypoxia-Inducible-Factor α Complexes,” *Scientific Reports* 14 (2024): 26162, https://doi.org/10.1038/s41598-024-75761-y.

49. Z. Zhang, T. J. Smart, and H. Choi, et al., “Structural and Stereoelectronic Insights Into Oxygenase-Catalyzed Formation of Ethylene From 2-Oxoglutarate,” *Proceedings of the National Academy of Sciences* 114 (2017): 4667–4672, https://doi.org/10.1073/pnas.1617760114.

50. R. A. Copeland, K. M. Davis, T. K. C. Shoda, et al., “An Iron(IV)–Oxo Intermediate Initiating l -Arginine Oxidation but Not Ethylene Production by the 2-Oxoglutarate-Dependent Oxygenase, Ethylene-Forming Enzyme,” *Journal of the American Chemical Society* 143 (2021): 2293–2303, https://doi.org/10.1021/jacs.0c10923.

51. S. Chatterjee, M. Fellner, J. Rankin, et al., “Structural, Spectroscopic, and Computational Insights From Canavanine-Bound and Two Catalytically Compromised Variants of the Ethylene-Forming Enzyme,” *Biochemistry* 63 (2024): 1038–1050, https://doi.org/10.1021/acs.biochem.4c00031.

52. K. Islam, “The Bump-and-Hole Tactic: Expanding the Scope of Chemical Genetics,” *Cell Chemical Biology* 25 (2018): 1171–1184, https://doi.org/10.1016/j.chembiol.2018.07.001.

53. M. Breski, D. Dey, S. Obringer, B. Sudhamalla, and K. Islam, “Engineering Biological C–H Functionalization Leads to Allele-Specific Regulation of Histone Demethylases,” *Journal of the American Chemical Society* 138 (2016): 13505–13508, https://doi.org/10.1021/jacs.6b08653.

54. D. Siegel, A. C. Meinema, H. Permentier, G. Hopfgartner, and R. Bischoff, “Integrated Quantification and Identification of Aldehydes and Ketones in Biological Samples,” *Analytical Chemistry* 86 (2014): 5089–5100, https://doi.org/10.1021/ac500810r.

55. L. Pohl and M. Eckle, “Sodium 3-Trimethylsilyltetradeuteriopropionate, a New Water-Soluble Standard for 1 H-NMR,” *Angewandte Chemie International Edition in English* 8 (1969): 381, https://doi.org/10.1002/anie.196903811.

56. C. McAuliffe, “Solubility in Water of Paraffin, Cycloparaffin, Olefin, Acetylene, Cycloolefin, and Aromatic Hydrocarbons 1,” *Journal of Physical Chemistry* 70 (1966): 1267–1275, https://doi.org/10.1021/j100876a049.

57. H. Fukuda, T. Fujii, and T. Ogawa, "Microbial Production of C_3- and C_4-Hydrocarbons Under Aerobic Conditions," *Agricultural and Biological Chemistry* 48 (1984): 1679–1682, https://doi.org/10.1080/00021369.1984.1086638.

58. M. A. K. Khalil and R. A. Rasmussen, “Forest Hydrocarbon Emissions: Relationships Between Fluxes and Ambient Concentrations,” *Journal of the Air & Waste Management Association* 42 (1992): 810–813, https://doi.org/10.1080/10473289.1992.10467033.

59. T. D. Sharkey and S. Yeh, “ISoprene Emission from Plants,” *Annual Review of Plant Physiology and Plant Molecular Biology* 52 (2001): 407–436, https://doi.org/10.1146/annurev.arplant.52.1.407.

60. A. E. H. W. Crocker and P. W. Zimmerman, "Similarities in the Effects of Ethylene and the Plant Auxins,"*Contributions from Boyce Thompson Institute* 7 (1935): 231–248.

61. E. J. McMurchie, W. B. McGlasson, and I. L. Eaks, “Treatment of Fruit With Propylene Gives Information About the Biogenesis of Ethylene,” *Nature* 237 (1972): 235–236, https://doi.org/10.1038/237235a0.

62. G. Stavroulakis and E. Sfakiotakis, “Regulation of Propylene-Induced Ripening and Ethylene Biosynthesis by Oxygen in ‘Hayward’ Kiwifruit,” *Postharvest Biology and Technology* 10 (1997): 189–194, https://doi.org/10.1016/S0925-5214(97)00006-9.

63. T. Suzuki, T. Yazawa, N. Morishita, A. Maruyama, and H. Fuse, “Genetic and Physiological Characteristics of a Novel Marine Propylene-Assimilating *Halieaceae* Bacterium Isolated From Seawater and the Diversity of Its Alkene and Epoxide Metabolism Genes,” *Microbes and Environments* 34 (2019): 33–42, https://doi.org/10.1264/jsme2.ME18053.

64. N. E. Hoffman, S. F. Yang, A. Ichihara, and S. Sakamura, “Stereospecific Conversion of 1-Aminocyclopropanecarboxylic Acid to Ethylene by Plant Tissues,” *Plant Physiology* 70 (1982): 195–199, https://doi.org/10.1104/pp.70.1.195.

65. M. C. Pirrung and G. M. McGeehan, “Ethylene Biosynthesis. 6. Synthesis and Evaluation of Methylaminocyclopropanecarboxylic Acid,” *Journal of Organic Chemistry* 51 (1986): 2103–2106, https://doi.org/10.1021/jo00361a032.

66. M. C. Pirrung, “Ethylene Biosynthesis From 1-Aminocyclopropanecarboxylic Acid,” *Accounts of Chemical Research* 32 (1999): 711–718, https://doi.org/10.1021/ar960003.

67. J. E. Baldwin, R. M. Adlington, G. A. Lajoie, and B. J. Rawlings, “On the Biosynthesis of Ethylene. Determination of the Stereochemical Course Using Modified Substrates,” *Journal of the Chemical Society, Chemical Communications* (1985): 1496–1498, https://doi.org/10.1039/c39850001496.

68. R. Díaz, H. Gallart-Ayala, J. V. Sancho, et al., "Told Through the Wine: A Liquid Chromatography–Mass Spectrometry Interplatform Comparison Reveals the Influence of the Global Approach on the Final Annotated Metabolites in non-Targeted Metabolomics," *Journal of Chromatography A* 1433 (2016): 90–97, https://doi.org/10.1016/j.chroma.2016.01.010.

69. D. Mukherjee and M. M. Laloraya, “Metabolism of γ-Methyl-α-Ketoglutaric Acid, γ-Methylene-α-Ketoglutaric Acid and Other Keto Acids During the Seedling Growth in Tamarindus Indica,” *Biochemie Und Physiologie Der Pflanzen* 166 (1974): 429–436, https://doi.org/10.1016/S0015-3796(17)30079-3.

70. A. I. Virtanen and A.-M. Berg, “New Aminodicarboxylic Acids and Corresponding Alpha-Keto Acids in Phyllitis Scolopendrium,” *Acta Chemica Scandinavica* 9 (1955): 553–554, https://doi.org/10.3891/acta.chem.scand.09-0553.

71. T. J. Riedel, L. C. Johnson, J. Knight, R. R. Hantgan, R. P. Holmes, and W. T. Lowther, “Structural and Biochemical Studies of Human 4-hydroxy-2-oxoglutarate Aldolase: Implications for Hydroxyproline Metabolism in Primary Hyperoxaluria,” *PLoS One* 6 (2011): e26021, https://doi.org/10.1371/journal.pone.0026021.

72. T. J. Riedel, J. Knight, M. S. Murray, D. S. Milliner, R. P. Holmes, and W. T. Lowther, “4-Hydroxy-2-Oxoglutarate Aldolase Inactivity in Primary Hyperoxaluria Type 3 and Glyoxylate Reductase Inhibition,” *Biochimica*

Et Biophysica Acta (BBA)—Molecular Basis of Disease 1822 (2012): 1544–1552, https://doi.org/10.1016/j.bbadis.2012.06.014.

73. C. Eckert, W. Xu, W. Xiong, et al., "Ethylene-Forming Enzyme and Bioethylene Production," *Biotechnology for Biofuels* 7 (2014): 33, https://doi.org/10.1186/1754-6834-7-33.

74. J. Wilson, S. Gering, J. Pinard, R. Lucas, and B. R. Briggs, "Bio-Production of Gaseous Alkenes: Ethylene, Isoprene, Isobutene," *Biotechnology for Biofuels* 11 (2018): 234, https://doi.org/10.1186/s13068-018-1230-9.

75. R. Jira, "Acetaldehyde From Ethylene—A Retrospective on the Discovery of the Wacker Process," *Angewandte Chemie International Edition* 48 (2009): 9034–9037, https://doi.org/10.1002/anie.200903992.

76. J. A. Keith and P. M. Henry, "The Mechanism of the Wacker Reaction: A Tale of Two Hydroxypalladations," *Angewandte Chemie International Edition* 48 (2009): 9038–9049, https://doi.org/10.1002/anie.200902194.

77. V. Kazimírová and M. Rebroš, "Production of Aldehydes by Biocatalysis," *International Journal of Molecular Sciences* 22 (2021): 4949.

78. S. B. Jaber Sathik Rifayee, M. G. Thomas, and C. Christov, "Revealing the Nature of the Second Branch Point in the Catalytic Mechanism of the Fe(ii)/2OG-Dependent Ethylene Forming Enzyme," *Chemical Science* 16 (2025): 7667–7684, https://doi.org/10.1039/D4SC08378D.

79. C. Wang and E. R. Sayfutyarova, "Diverging Reaction Pathways and Key Intermediates in Ethylene Forming Enzyme," *Journal of Physical Chemistry B* 129 (2025): 4335–4349, https://doi.org/10.1021/acs.jpcb.5c02007.

80. C. C. Lee, Y. Hu, and M. W. Ribbe, "Unique Features of the Nitrogenase VFe Protein From *Azotobacter Vinelandii*," *Proceedings of the National Academy of Sciences* 106 (2009): 9209–9214, https://doi.org/10.1073/pnas.0904408106.

81. S. M. Mayer, W. G. Niehaus, and D. R. Dean, "Reduction of Short Chain Alkynes by a Nitrogenase α-70Ala-Substituted MoFe ProteinBased on the Presentation Given at Dalton Discussion No. 4, 10–13th January 2002, Kloster Banz, Germany. Research Supported by National Institutes of Health Grant R01-GM59087," *Journal of the Chemical Society, Dalton Transactions* (2002): 802–807, https://doi.org/10.1039/b107336b.

82. P. C. Dos Santos, S. M. Mayer, B. M. Barney, L. C. Seefeldt, and D. R. Dean, "Alkyne Substrate Interaction Within the Nitrogenase MoFe Protein," *Journal of Inorganic Biochemistry* 101 (2007): 1642–1648, https://doi.org/10.1016/j.jinorgbio.2007.05.007.

83. S. T. Stripp, B. R. Duffus, V. Fourmond, et al., "Second and Outer Coordination Sphere Effects in Nitrogenase, Hydrogenase, Formate Dehydrogenase, and CO Dehydrogenase," *Chemical Reviews* 122 (2022): 11900–11973, https://doi.org/10.1021/acs.chemrev.1c00914.

84. S. S. Chaturvedi, R. Ramanan, J. Hu, R. P. Hausinger, and C. Z. Christov, "Atomic and Electronic Structure Determinants Distinguish Between Ethylene Formation and l -Arginine Hydroxylation Reaction Mechanisms in the Ethylene-Forming Enzyme," *ACS Catalysis* 11 (2021): 1578–1592, https://doi.org/10.1021/acscatal.0c03349.

85. M. G. Thomas, S. B. Jaber Sathik Rifayee, and C. Z. Christov, "How Do Variants of Residues in the First Coordination Sphere, Second Coordination Sphere, and Remote Areas Influence the Catalytic Mechanism of Non-Heme Fe(II)/2-Oxoglutarate Dependent Ethylene-Forming Enzyme?," *ACS Catalysis* 14 (2024): 18550–18569, https://doi.org/10.1021/acscatal.4c04010.

86. M. G. Thomas, S. B. Jaber Sathik Rifayee, S. S. Chaturvedi, et al., "The Unique Role of the Second Coordination Sphere to Unlock and Control Catalysis in Nonheme Fe(II)/2-Oxoglutarate Histone Demethylase KDM2A," *Inorganic Chemistry* 63 (2024): 10737–10755, https://doi.org/10.1021/acs.inorgchem.4c01365.

87. M. E. Neugebauer, E. N. Kissman, J. A. Marchand, et al., "Reaction Pathway Engineering Converts a Radical Hydroxylase Into a Halogenase," *Nature Chemical Biology* 18 (2022): 171–179, https://doi.org/10.1038/s41589-021-00944-x.

88. A. Papadopoulou, J. Meierhofer, F. Meyer, et al., "Re-Programming and Optimization of a L -Proline Cis -4-Hydroxylase for the Cis -3-Halogenation of its Native Substrate," *ChemCatChem* 13 (2021): 3914–3919, https://doi.org/10.1002/cctc.202100591.

89. S. S. Chaturvedi, S. B. Jaber Sathik Rifayee, S. O. Waheed, et al., *JACS Au* 2 (2022): 2169–2186.

90. C. Zeymer and D. Hilvert, "Directed Evolution of Protein Catalysts," *Annual Review of Biochemistry* 87 (2018): 131–157, https://doi.org/10.1146/annurev-biochem-062917-012034.

91. Y. Wang, P. Xue, M. Cao, T. Yu, S. T. Lane, and H. Zhao, "Directed Evolution: Methodologies and Applications," *Chemical Reviews* 121 (2021): 12384–12444, https://doi.org/10.1021/acs.chemrev.1c00260.

92. M. Mukherji, N. J. Kershaw, C. H. MacKinnon, et al., "Chemical co-Substrate Rescue′ of Phytanoyl-CoA 2-Hydroxylase Mutants Causing Refsum's Disease," *Chemical Communications* (2001): 972–973, https://doi.org/10.1039/b101039p.

93. C. X. Hou, A. Brasnett, P. Rabe, C. J. Schofield, and L. Brewitz, "Structural and Functional Consequences of Aspartate/asparagine-β-hydroxylase Variants Causing Traboulsi Syndrome," *Journal of Biological Chemistry*: 111008, https://doi.org/10.1016/j.jbc.2025.111008.

94. R. W. Adams, C. M. Holroyd, J. A. Aguilar, M. Nilsson, and G. A. Morris, "Perfecting″ WATERGATE: Clean Proton NMR Spectra From Aqueous Solution," *Chemical Communications* 49 (2013): 358–360, https://doi.org/10.1039/C2CC37579F.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information File 1: cctc70501-sup-0001-SuppMat.pdf.