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p-Nitrophenyl esters provide new insights and applications for the thiolase enzyme OleA

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ABSTRACT

The OleA enzyme is distinct amongst thiolase enzymes in binding two long ($\geq C_8$) acyl chains into structurally-opposed hydrophobic channels, denoted A and B, to carry out a non-decarboxylative Claisen condensation reaction and initiate the biosynthesis of membrane hydrocarbons and β-lactone natural products. OleA has now been identified in hundreds of diverse bacteria via bioinformatics and high-throughput screening using *p*-nitrophenyl alkanoate esters as surrogate substrates. In the present study, *p*-nitrophenyl esters were used to probe the reaction mechanism of OleA and shown to be incorporated into Claisen condensation products for the first time. *p*-Nitrophenyl alkanoate substrates alone were shown not to undergo Claisen condensation, but co-incubation of *p*-nitrophenyl esters and CoA thioesters produced mixed Claisen products. Mixed product reactions were shown to initiate via acyl group transfer from a *p*-nitrophenyl carrier to the enzyme active site cysteine, C143. Acyl chains esterified to *p*-nitrophenol were synthesized and shown to undergo Claisen condensation with an acyl-CoA substrate, showing potential to greatly expand the range of possible Claisen products. Using *p*-nitrophenyl 1-^{13}C-decanoate, the Channel A bound thioester chain was shown to act as the Claisen nucleophile, representing the first direct evidence for the directionality of the Claisen reaction in any OleA enzyme. These results both provide new insights into OleA catalysis and open a path for making unnatural hydrocarbon and β-lactone natural products for biotechnological applications using cheap and easily synthesized *p*-nitrophenyl esters.

1. Introduction

Biohydrocarbons and β-lactone natural products molecules are of current interest in biotechnology. Hydrocarbons have been studied as petroleum product replacements and β-lactone natural products have demonstrated anti-obesity, anti-tumor, and antibiotic properties [1–7]. β-Lactones are analogs of β-lactams, both of which are chemical antagonists produced by living things, typically bacteria and fungi, to inhibit other organisms [8,9]. As a class, β-lactams are the major clinical antibiotics that have saved millions of lives but their effectiveness has been diminished in recent years by widespread pathogen resistance due to β-lactamases [10,11]. β-Lactones may fill in the some of the gaps for new antibiotics and anticancer treatments. For example, salinosporamide is in Phase three clinical trials for the treatment of multiple myeloma [12,13]. β-Lactones are end-products in some bacteria and metabolic intermediates in others. In the latter, they typically undergo decarboxylation to make olefinic hydrocarbons [14,15]. The intertwined pathways use homologous enzymes that are denoted as Ole, designating olefinic hydrocarbons, or they are named for their respective natural product; for example, LstAB in the case of lipstatin [16]. OleA-type thiolase enzymes initiate biosynthesis of both the membrane hydrocarbons and many β-lactone natural products [5,17].

OleA is a homodimer in the thiolase superfamily of enzymes and catalyzes a non-decarboxylative Claisen condensation of two acyl-CoA substrates consisting of C8-C16 carbon chains to produce hydrocarbons and natural products [17] (Fig. 1A). Thiolase

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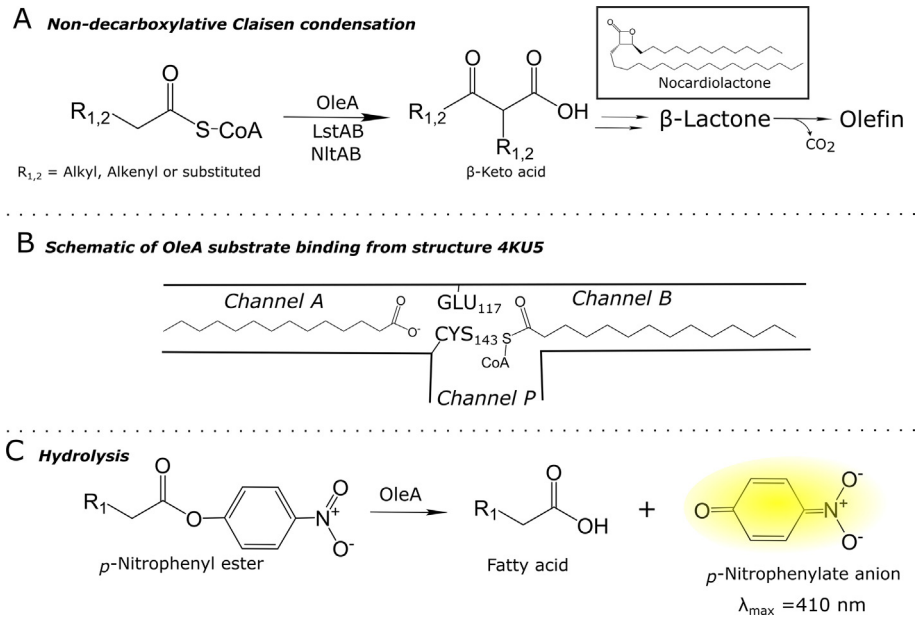

Fig. 1. *Reactions catalyzed by OleA and homologous enzymes.* (A) Physiological reactions catalyzed by OleA and homologous enzymes LstAB and NltAB. (B) Schematic of OleA reaction showing three channel architecture of enzyme. (C) OleA screening reaction with *p*-nitrophenyl hexanoate making *p*-nitrophenol.

enzymes are involved in many different pathways in the metabolism of fatty acids, polyhydroxybutyrate storage, and natural product biosynthesis [18–22]. OleA homologs that produce natural products include NltAB, a heterodimer biosynthesized by a *Nocardia* species that produces the β-lactone nocardiolactone [23] (Fig. 1, *inset*). Another homologous protein pair, LstAB, is a heterodimer made by *Streptomyces toxytricini* that produces lipstatin, which is hydrogenated to make the antiobesity drug tetrahydrolipstatin, known commercially as Orlistat or Xenical [16,24]. The best studied enzyme catalyzing these condensation reactions with long chain substrates is the OleA from the plant pathogen *Xanthomonas campestris* [17,25–28].

X-ray structures of the *X. campestris* OleA enzyme have been solved to 1.8 Å resolution, helping to reveal features of the Claisen condensation reaction [25–28]. Initial studies with native enzyme and inhibitors revealed the reactivity of Cys143 and the occupation of a hydrophobic tunnel denoted as Channel A (Fig. 1B). Structures were solved with irreversible inhibitors iodoacetamide and cerulenin, shown to be be covalently bonded to Cys143 with the hydrocarbon moiety extended toward and into Channel A, respectively. Additional structures were determined with an inactive Cys143Ser mutant in which a fatty acid was bound in Channel A and an acyl chain covalently bonded to Coenzyme A were bound in Channel B and Channel P, respectively (Fig. 1B). This was not a reaction complex since the fatty acid is not an activated intermediate but it showed the general binding mode of the two acyl chains and the Coenzyme A carrier in their respective channels. Further mutagenesis studies revealed that the Claisen reaction required E117 or, with lower activity, D117 [27]. The carboxylate side chain is proposed to act as a general base to deprotonate a carbon adjacent to the acyl carbonyl carbon that acts as the Claisen reaction nucleophile.

Progress on OleA had initially been slowed by a cumbersome and time-demanding assay and was recently accelerated by the development of a high throughput readout of OleA activity, both *in vitro* and *in vivo* [29] (Fig. 1C). The new assay was based on the observation that OleA catalyzes hydrolysis of *p*-nitrophenyl hexanoate at rates of hundreds of nmol per min, generating intensely yellow *p*-nitrophenol, that can be observed in seconds and determined quantitatively in a microtiter well-plate format. This assay has paved the way to conduct a broader palette of in vitro experiments with OleA. The method can also be used *in vivo* since *p*-nitrophenyl hexanoate diffuses into recombinant *E. coli* cells expressing OleA enzymes, while other cellular enzymes have relatively low activity with this substrate [29].

The new assay was leveraged, along with bioinformatics identifying thousands of putative OleA orthologs, to broaden OleA enzyme studies [29,30]. The method allowed identification of 72 additional OleA enzymes based on both bioinformatics criteria and *p*-nitrophenyl hexanoate hydrolysis activity (Fig. 1C). Hexanoyl chains are shorter than those found in substrates preferred for Claisen condensation by the well-studied OleA from *X. campestris*. So it has remained an open question as to whether *p*-nitrophenyl esters could act as Claisen condensation donors or acceptors, or both.

The production of Claisen products starting with *p*-nitrophenyl esters rather than CoA esters would have numerous advantages in cost, ease of substrate synthesis, and expanding the ability to generate chemical libraries of hydrocarbons and natural products. *p*-Nitrophenyl esters are typically <1% of the cost of corresponding CoA thioesters if purchased and they can be generated from thousands of available carboxylic acids via a high-yield synthetic procedure using *p*-nitrophenyl chloroformate [30,31] (Table S1). With the availability of many cheap and easy-to-synthesize substrates for OleA, we could potentially make large product libraries to expand upon the range of products made by OleA enzymes. In a previous study, we found OleA enzymes that would react with more than one dozen *p*-nitrophenyl ester substrates containing aromatic, heterocyclic, and other functionalities that could provide useful pharmacophores if OleA would accept *p*-nitrophenyl-donated acyl groups and make Claisen condensation products.

The present study was conducted to explore the reactivity of *p*-nitrophenyl esters with OleA beyond the hydrolysis of *p*-nitrophenyl hexanoate (Fig. 1C) to further probe the reaction mechanism and potentially produce new natural products (Fig. 1A). The OleA from *X. campestris* was chosen for this study because the enzyme is significantly promiscuous in utilizing different substrates and X-ray structures are available. The results with

this enzyme indicate that the *p*-nitrophenyl group can deliver an acyl chain into Channel A. A second *p*-nitrophenyl alkanoate is not competent for Claisen condensation but an acyl-CoA molecule can react to generate a mixed Claisen product. The ability to discriminate chains from discrete donors with a fixed channel occupancy allowed us to determine the directionality of the Claisen reaction for the first time. Previously undescribed chemical compounds were produced by co-reacting *p*-nitrophenyl and CoA esters. This study provided key insights into the OleA mechanism and opens the door for introducing unique functionality into the OleA condensation reaction to make new products of biotechnological importance.

2. Methods

2.1. Chemicals and Reagents.

p-Nitrophenyl octanoate, decanoate, laurate, myristate, and palmitate, octanoyl-CoA, decanoyl-CoA, lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA, 10-nonadecanone, 12-tricosanone, and 14-heptacosanone were all obtained from Sigma-Aldrich (St Louis, MO). *p*-Nitrophenyl hexanoate was obtained from Tokyo Chemical Industry. *p*-Nitrophenyl heptanoate, nonanoate, 6-azidohexanoate, 3-(4-chlorophenoxy)propanoate and 1-^{13}C-decanoate were synthesized by the method of Engström *et al* [31]. Briefly, in a 10 mL flask, the respective carboxylic acid (0.5 mmol) and 4-nitrophenol chloroformate (0.5 mmol) were stirred under nitrogen 10 min. in cold (0 °C, 1 mL) anhydrous dichloromethane (DCM). A solution of 4-dimethylaminopyridine (0.05 mmol DMAP decarboxylation catalyst) and triethylamine (0.55 mmol) in 1.5 mL dry DCM was slowly added and stirred for 2 h. The reaction flask was nearly filled then with DCM and stirred rapidly with a drop of 6 N aq. HCl. The pH was adjusted to 6 (1 N NaOH), then the DCM phase and DCM washes were filtered through 2 g of silica gel collecting UV active eluate. The *p*-nitrophenol esters, analyzed by ^{1}H NMR and including *p*-nitrophenyl 1-^{13}C decanoate, were generally obtained in ≥98% purity and yield. The purity of *p*-nitrophenyl 1-^{13}C-decanoate used in this study was 90–95%.

2.2. Bacterial strains, vectors and genes encoding OleA wild-type and mutant proteins

All genes used in this study were for *Xanthomonas campestris* Ole A wild-type (NP_635607.1) and mutant derivatives C143S, C143A, and T292M. All OleA genes were expressed in *Escherichia coli* T7 Express cells from vector pET28b+ with a T7 promoter. The T292M mutant OleA gene was obtained from the Department of Energy's Joint Genome Institute (JGI) and expressed in the same vector. All contained an N-terminal 6x His-tag. *E. coli* cells were grown to an OD of 0.4 and induced at 16 °C overnight prior to protein purification.

2.3. Protein purification and handling

Xanthomonas campestris Ole A wild-type (NP_635607.1) and mutant derivatives C143S, C143A, and T292M were lysed by French Press and proteins purified by the general method described by Frias *et al* [17] using Ni-column chromatography. The column was washed with a buffer of 20 mM Tris·HCl at pH 7.5 containing 500 mM NaCl. The column was subsequently washed with two column volumes each of 30 mM, 100 mM and 400 mM imidazole in the same buffer. OleA eluted half-way through the last 400 mM column wash. Protein was then eluted through a Sephadex G-10 column to remove imidazole that can catalyze hydrolysis of *p*-nitrophenyl esters. Protein was then flash frozen and stored at −80 °C until use. Yields of protein were typically 15–20 mg per liter. SDS-PAGE showed protein homogeneity as previously reported [17]. Mutant proteins were purified via the same methods and showed similar behavior upon handling.

2.4. General assay for p-nitrophenyl ester hydrolysis

50 mM Tris·HCl pH 8.0 was added to a 96-well Flat Bottom Suspension Culture Plate (Cat#:25–104 Genesee Scientific) containing 30 μg of OleA C143S or C143A, 5% ethanol, and 200 μM *p*-nitrophenyl hexanoate in a 200 μL total volume. A SpectraMax Plus 384 Microplate Reader (Molecular Devices, San Jose, CA) was used, and *p*-nitrophenol absorbance was read at 410 nm. Absorbance was read at 410 nm every 5 min for at least 2 h at 37 °C. A standard curve was developed in a buffer containing 50 mM Tris·HCl at pH 8.0. The extinction coefficient for *p*-nitrophenol was determined to be 15,548 $M^{-1}cm^{-1}$, and the path length through the 200 μL liquids in microtiter wells was experimentally determined to be 0.58 cm. All reactions were run in parallel on the same microtiter plates with 3–5 replicates for each data point. Controls of each *p*-nitrophenyl alkanoate chain length containing no protein were used to correct for any non-enzymatic hydrolysis in buffer. For wells containing wild type OleA, only 4 μg of protein was added to the wells, and measurements were taken every minute for 30 min. Other mutant and inhibited assays were varied as described below.

2.5. p-Nitrophenyl ester hydrolysis with inhibited or mutant enzymes

The assays were run as generally described for wild-type OleA with the following changes. For assays containing inhibited wild type enzyme or C143A/S mutants, 30 μg of OleA was used, and absorbance was determined over a 2 hr time course. For assays with wild-type enzyme inhibited with cerulenin and iodoacetamide, 1.25 mM of the inhibitor was added to 30 μg OleA enzyme and buffer and allowed to react at room temperature for 1 hr prior to addition of *p*-nitrophenyl hexanoate.

For reactions comparing OleA wild type and T292M mutants, reactions were run in parallel. Both sets of reactions tested C_6, C_7, C_8, C_9, C_{10}, C_{12}, and C_{14} alkanoate esters of *p*-nitrophenol. The buffer, pH and other parameters were as described for the general assays. Reactions contained 6 μg of enzyme and were initiated by the addition of the respective *p*-nitrophenyl ester.

2.6. GC–MS conditions for screening Claisen condensation activity with p-nitrophenyl esters

Gas chromatography-mass spectrometry (GC–MS) was carried out on an Agilent 7890a gas chromatograph and an Agilent 5975c mass spectrometer (Santa Clara, CA). 500 μL reactions were run using 8 μg OleA, 5% ethanol, and 200 μM *p*-nitrophenyl esters in Tris·HCl 50 mM pH 8.0. The reaction was allowed to proceed overnight at room temperature. The product was extracted into 500 μL of methoxy-*t*-butyl ether (MTBE). Methylation was performed using 100 μL diazomethane in diethyl ether. The resultant product was injected into the gas chromatograph using both flame ionization (FID), and electron impact mass spectrometry detection at 70 V. The inlet temperature was 250 °C. The elution program was as follows: hold 60 °C for 8 min followed by a 15 °C increase per minute until 320 °C was attained and held constant for 5 min.

The products of the Claisen condensation are β-keto acids that completely decarboxylate in the gas chromatograph to yield ketones [17]. The resultant ketones of different molecular weights are thermostable under the GC regime described above and yield very characteristic mass spectra. To determine the lowest level of activity that was detectable in the assay, standard curves were

obtained for commercially-obtained 10-nonadecanone, 12-tricosanone, and 14-heptacosanone via GC–MS (Fig. S1-3). Standards were dissolved in MTBE at 400 nM, 250 nM, 200 nM, 150 nM, 100 nM, 50 nM concentrations and 1 μL standards were run using the same protocol detailed in the GC–MS condensation assay. Ketone peaks were recorded at 17.1 min for 10-nonadecanone, 19.6 min for 12-tricosanone, and 21.9 min for 14-heptacosanone. Peaks were integrated to generate the standard curves.

2.7. p-Nitrophenyl 1-^{13}C-decanoate and myristoyl-CoA used to investigate the Claisen nucleophile

500 μL reactions were run using 8 μg OleA, 5% ethanol, 100 μM *p*-nitrophenyl 1-^{13}C-decanoate and 100 μM myristoyl-CoA in Tris·HCl 50 mM pH 8.0. The reaction was allowed to proceed for 30 min. To detect the intact β-keto acid products, the enzyme reactions were extracted with MTBE after 30 min, the product(s) methylated by diazomethane in ether via the standard extraction protocol, and subjected to GC–MS as previously described. An unlabeled *p*-nitrophenyl decanoate substrate control was run in parallel at the same time. The mass spectra are presented in the results section. In separate experiments, the β-keto acid decarboxylation products, aliphatic ketones, were analyzed omitting the methylation step and carrying out GC–MS directly. The half-life of decarboxylation of the β-keto acids to ketones was previously determined to be ~8 h and, if not methylated, they undergo complete decarboxylation in the GC injection inlet [17].

2.8. Computational methods

The OleA T292M variant was modelled using the Mutagenesis tool in PyMOL, Version 2.0 (Schrödinger) as shown in Fig. 2. The specific rotamer chosen for the methionine variant came from a backbone-dependent rotamer library and was the only rotamer that could directly add steric effects to substrate binding. Modelling of the enzyme thioester intermediate was done by placing the myristyl carbonyl carbon linked to the sulfur atom of OleA residue C143 in the existing ligand electron density from structure PDB 4KU3 using Coot, v0.8.9.2 [32]. The carbonyl moiety of the thioester intermediate was positioned manually into the oxyanion hole formed by H285 and N315 and the OleA-thioester complex was refined using default restraints using the Refmac5 tool within the software, CCP4, version 7.0 [33]. The refinement minimized the energy of the bonded and non-bonded interactions of the thioester intermediate which resulted in the final model shown in Fig. 6. Chemical substances were searched using SciFinder [34,35].

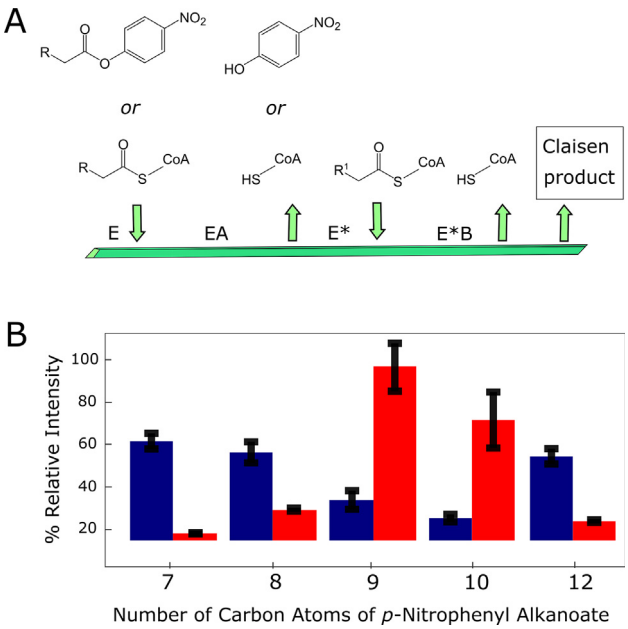

Fig. 3. *OleA catalyzes reaction between p-nitrophenyl ester and CoA ester.* (A) Cleland diagram illustrating ping-pong mechanism in which transesterification from a *p*-nitrophenyl or CoA ester leads to an identical intermediate E* enzyme state. (B) Bar graph showing relative products from condensation of two CoA esters (blue) and a CoA ester and a *p*-nitrophenyl ester (red). The CoA ester was myristoyl-CoA and the *p*-nitrophenyl ester chain length was varied as shown on the X-axis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. No Claisen condensation with p-nitrophenyl esters alone

p-Nitrophenyl esters react rapidly with OleA to release *p*-nitrophenol and this has been used to identify OleA enzymes in

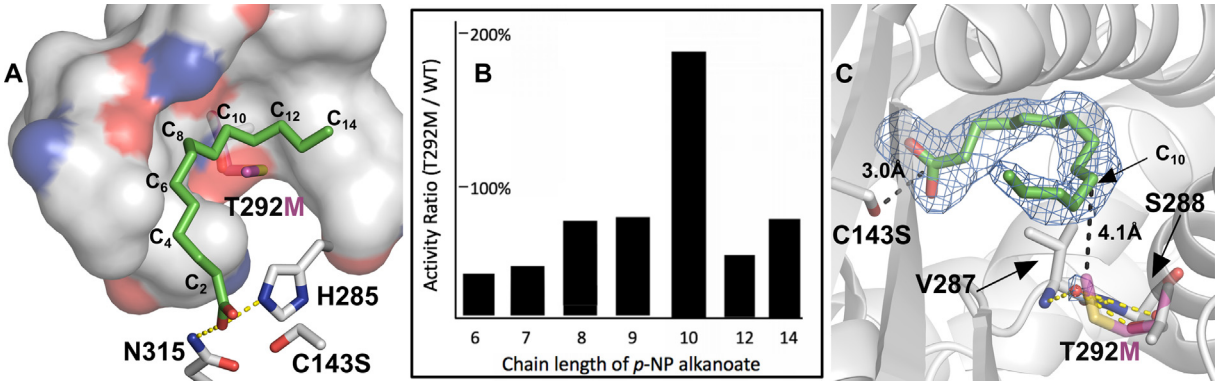

Fig. 2. *Mutation in Channel A effects length of p-nitrophenyl ester chain accepted.* (A) The "bend" of myristic acid bound in OleA channel A. Surface representation of the channel A cavity with the T292M variant modelled. The carboxylic acid moiety of myristic acid is proximate to the catalytic cysteine, C143, and H285, N315 that form the oxyanion hole. The C143S variant inactivates OleA and allows for co-crystallization with myristic acid (PDB 4KU3) (B) Activity of the T292M variant over wild-type OleA versus *p*-nitrophenyl ester chain length. Activity measured as described in the Methods section (C) Modeled binding mode of T292M variant. Channel A of OleA with a Fo-Fc map contoured at 3σ carved around myristic acid bound in the channel (PDB 4KU3). The T292M variant when modelled may occlude a highly coordinated water molecule (red sphere) and have increased Van der Waals interactions with a C_{10} *p*-nitrophenyl alkanoate. In the wild-type, the hydroxyl of T292 and the backbone atoms of V287 and S288 coordinate the water molecule and occlusion of binding this water is proposed to alter the channel A cavity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dozens of different bacteria and to probe OleA substrate specificity [29,30]. Here, we wished to produce Claisen condensation products using *p*-nitrophenyl esters. In the present study, *p*-nitrophenyl esters were reacted in sixteen different combinations and analyzed for Claisen product formation. All combinations of straight-chain C_9, C_{10}, C_{12} and C_{14} alkanoic acid esters were examined. No Claisen products were detected in any reaction. Available standards for the analyzable Claisen condensed products were run in parallel. The standards were readily detected at a level of 35 pmol (Figure S1-S3). Positive controls with C_{10}, C_{12} and C_{14}-CoA thioesters, the physiologically relevant substrates [14], gave expected levels of Claisen condensation products.

3.2. p-Nitrophenyl ester reactions require the OleA active site cysteine

With wild type *X. campestris* OleA, acyl-CoA substrates undergo acyl group transfer to Cys143 and subsequently two reactions: (i) a Claisen condensation with an acyl chain bound in another channel or (ii) a hydrolysis of the acyl enzyme to generate a fatty acid [17]. The ratio of the reactions varies with the length of the acyl chain and reactions occur on a time scale of minutes. In some cases, the hydrolysis product predominates over the Claisen product. OleA single-site mutants, C143S or C143A, do not undergo Claisen condensation [26]. Additionally, the C143 mutants catalyze a very slow hydrolysis of acyl-CoA thioesters, requiring overnight incubations to observe significant fatty acid product [26]. While rates were too slow to measure precisely, they are estimated to be less than one percent of the rates of the wild-type Claisen and hydrolysis reactions, consistent with the C143 acylation reaction preceding both Claisen condensation and hydrolysis of CoA thioesters [26]. We believe this is likely due to direct hydrolysis by a water molecule. Also, consistent with the role of C143, iodoacetamide and cerulenin were shown to be potent irreversible inhibitors of OleA and X-ray studies revealed they inhibit by covalently modifying the cysteine [25].

In the present study, the significant reaction of *p*-nitrophenyl esters with OleA was seen to similarly be dependent on C143. Reaction of the wild-type enzyme with iodoacetamide and cerulenin virtually eliminated the hydrolysis reaction, determined here at <0.1% of the rate of uninhibited enzyme (Table 1; Fig. S4). Similarly, C143S and C143A mutants were severely impaired with the rates of hydrolysis <1% of the wild-type rate (Table 1; Fig. S5). The alkyl chain of cerulenin, following cysteine alkylation, was previously shown by X-ray crystallographic studies to occupy what has been denoted as the Channel A, in which the first acyl substrate binds to OleA [25]. These studies are consistent with a transfer of alkyl chains from *p*-nitrophenyl esters to C143 with the hydrophobic alkyl moiety extending into Channel A.

3.3. Mutation in Channel A effects length of p-nitrophenyl ester chain accepted

Similarities in the OleA-catalyzed hydrolysis reactions with acyl-CoAs and acyl-*p*-nitrophenyl esters suggested that both undergo transesterification to Cys143 with the acyl group occupying Channel A. To further investigate these commonalities, we made mutations in Thr292 that forms part of the Channel A binding site. Here Thr292 was mutated to a methionine, a change expected to alter the binding and reactivity of *p*-nitrophenyl alkyl esters (Fig. 2A).

The T292M mutant was stable and could be purified in reasonable yield and showed clearly altered chain selectivity of for *p*-nitrophenyl alkyl esters, further supporting the conclusion that *p*-nitrophenyl alkanotes occupy Channel A. The wild type enzyme has the lowest activity with *p*-nitrophenyl decanoate in a C6 through C12 panel of substrates [17] whereas the T292M mutant showed comparatively high activity with *p*-nitrophenyl decanoate (Fig. 2B). Indeed, while the mutant is lower in activity against all other chain lengths, it shows nearly double the activity of the wild-type with the C10 alkanoate ester. Mutant OleA enzymes with larger, more hydrophobic side chains, T292I and T292V and T292F, were tested in lysed cells and failed to show any significant hydrolysis activity with either *p*-nitrophenyl hexanoate or *p*-nitrophenyl dodecanoate. These larger hydrophobic side-chains may result in the Channel A not opening sufficiently to accommodate a *p*-nitrophenyl ester.

X-ray structures with C_{12} and C_{14} acyl chains bound show a distinct bend in the chain between carbon atoms C_8-C_{11}, the region of T292 (Fig. 2A). Note that the chain is likely displaced further down the channel in the X-ray structure PDB 4KU3 that contains a bound carboxylic acid. In a true reaction complex, for which no experimental structure exists currently, the acyl chain would be directly bonded to C143, eliminating the second oxygen atom of the carboxylate. This would effectively position the chain such that the C_9-C_{10} atoms start the bend region depicted in Fig. 2A and 2C. In that region of Channel A, T292 participates in coordinating a tightly bound water, shown as a red sphere, along with the backbone atoms of V287 and S288. Our modeling indicates that all these interactions would be disrupted by a methionine residue in position 292. We propose that the T292M mutation displaces the bound water and the methionine can provide a favorable Van der Waals interaction with the terminal methyl group of the C_{10} *p*-nitrophenyl substrate. This configuration would be consistent with favorable binding of a C_{10}-chain with the mutant compared to a more unfavorable binding by the wild-type, and this is what was observed experimentally.

3.4. Mixed p-nitrophenyl ester and CoA-thioester reactions

The present study suggested that *p*-nitrophenyl acyl chains bind in Channel A prior to hydrolytic cleavage, and previous data support the OleA-catalyzed Claisen reaction following a ping-pong mechanism like other thiolases [18–22,25]. Via that mechanism, acyl transfer from the *p*-nitrophenyl ester to C143 is followed by leaving of the first product, *p*-nitrophenol (Fig. 3A). This intermediate state would be identical to the covalent enzyme intermediate (E* in Fig. 3A) formed from acyl transfer from acyl-CoA and leaving of CoA. This led us to the hypothesis that a subsequent acyl-CoA substrate can occupy Channel B and undergo Claisen condensation with the Channel A acyl chain, regardless of whether the first chain comes from a CoA or a *p*-nitrophenol donor.

This was tested with mixed *p*-nitrophenyl alkanoate + acyl-CoA incubations in which the chain lengths differ, allowing differentiation from condensation reactions occurring from two acyl-CoA substrates of the same chain lengths. Our choice of *p*-nitrophenyl ester chain lengths was guided by two factors: (1) much higher solubility of shorter chain length *p*-nitrophenyl esters (Table 2S), and (2) previous observations that Claisen reactions occur with intermediate chain length acyl-CoA substrates (C_8-C_{12}). In this context, we tested C_7, C_8, C_9, C_{10}, and C_{12} straight chain alkyl esters of *p*-

Table 1
Enzyme activity measured with *p*-nitrophenyl hexanoate as described in Methods for wild-type enzyme, wild-type enzyme reacted with Channel A inhibitors and mutant enzymes.

Enzyme form	Standard assay or additive	Specific Activity (nmol/min per mg)	k_{cat} (min^{-1})
Wild-type	Standard	270 ± 0.05	10
Wild-type	Iodoacetamide	0.16 ± 0.08	0.006
Wild-type	Cerulenin	0.26 ± 0.09	0.010
Mutant, C143A	Standard	0.75 ± 0.08	0.029
Mutant, C143S	Standard	0.69 ± 0.08	0.026

nitrophenol, each separately in a mixture with a preferred chain-length second substrate, myristoyl-CoA (C_{14}).

The mixtures were allowed to react until completion, extracted with methyl-*t*-butyl ether, and analyzed by GC–MS for the Claisen product. There was extensive hydrolysis but significant Claisen products were observed, including mixed Claisen products derived from one *p*-nitrophenyl ester and the CoA thioester (Fig. 3B; Fig. S6). When examining the mixed condensation products only, product derived from a *p*-nitrophenyl nonanoate had the greatest relative amount observed (100%), while the product derived from *p*-nitrophenyl decanoate also yielded a substantial amount (70%) (Fig. 3B). Other condensed products were significantly less, due largely to hydrolytic cleavage of the *p*-nitrophenyl ester. These data are consistent with *p*-nitrophenyl acyl chains binding in Channel A, with a kinetic competition between hydrolysis of the enzyme C143 thioester intermediate and acyl-CoA binding in Channel B to allow for the Claisen condensation to proceed.

3.5. Mixed incubations of p-nitrophenol [^{13}C]-ester and CoA thioester

The mixed Claisen reaction could be occurring in either of two ways. In one way, the α-carbon of the acyl chain delivered by *p*-nitrophenol to C143 and in Channel A is activated to attack the carbonyl of the chain bound in Channel B (Fig. 4A, left). In the second way, the α-carbon of the acyl chain covalently attached to coenzyme A and in Channel B is activated for the Claisen condensation (Figure A, right). To differentiate between those mechanisms, it was necessary to use different chain length *p*-nitrophenyl and CoA esters with one chain labeled. We could then analyze the unstable mixed Claisen product β-keto acid directly and, alternatively after decarboxylation, both by mass spectrometry. To that end, *p*-nitrophenyl 1-^{13}C-decanoate was synthesized and a protocol was developed to methylate the β-keto acid using diazomethane to stabilize it for GC–MS analysis. We then conducted an experiment with *p*-nitrophenyl 1-^{13}C-decanoate and

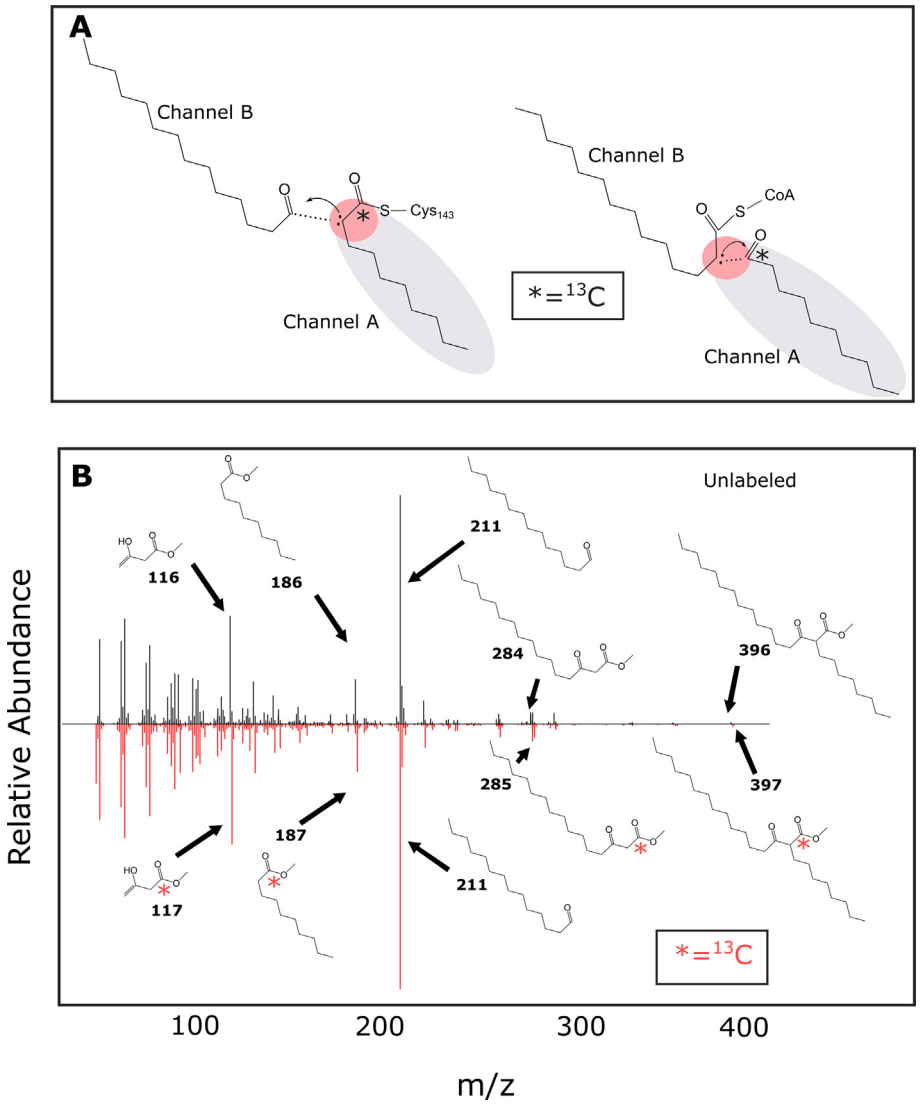

Fig. 4. *Directionality of OleA Claisen reaction demonstrated with a 1-^{13}C-p-nitrophenyl decanoate that only reacts from binding in Channel A.* (A) The two possible mechanisms of chain activation and C–C bond formation in *X. campestris* OleA. (B) Mass spectrum of the methylated β-keto acid product emanating from the condensation of *p*-nitrophenyl 1-^{13}C-decanoate and myristoyl-CoA. (red), or p-nitrophenyl decanoate and myristoyl-CoA (black). Major fragments of the β-keto acid product are depicted, along with their mass and arrows pointing to their corresponding peak. A red asterisk present indicates the ^{13}C label. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

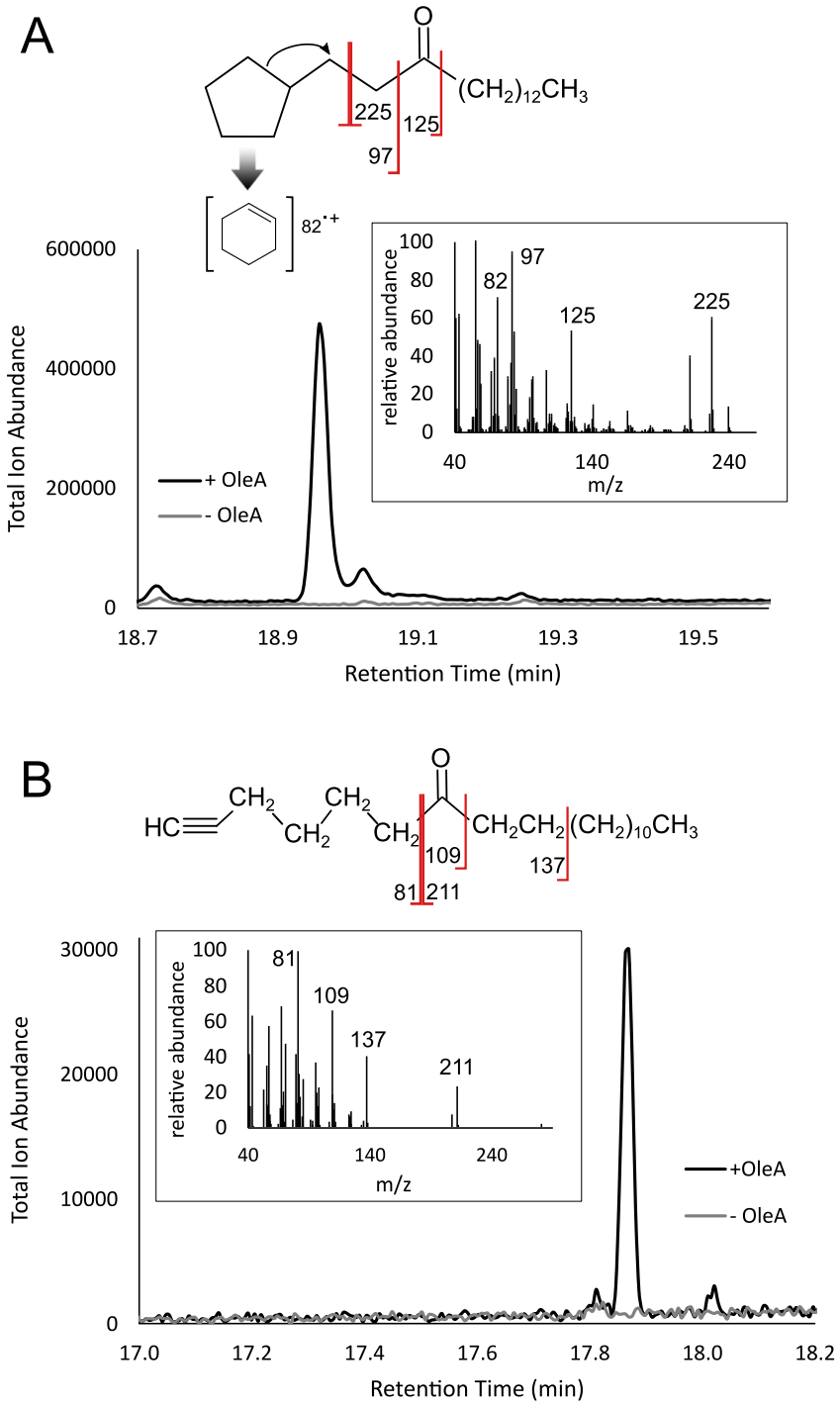

Fig. 5. *Identification of OleA-catalyzed Claisen condensation products combining acyl groups delivered by p-nitrophenol and CoA, respectively.* The CoA ester was myristoyl-CoA condensed with either *p*-nitrophenyl 3-cyclopentylpropionate (A) or *p*-nitrophenyl 6-heptynoate (B). Both (A) and (B) show gas chromatograph traces of the condensed product (+OleA) and a no enzyme control (- OleA). The inset boxes are electron impact mass spectra of the major peaks. Product structures are shown above that. Red bars and numbers indicate mass fragments (*m*/*z*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

myristoyl-CoA, analyzing the C_{24} beta-keto acid thus formed. A control without ^{13}C-labeling was conducted in parallel for direct comparison.

The mass spectrum of 2-octyl-3-ketohexadecanoate methyl ester showed a small parent ion that contained only one ^{13}C-label (Fig. 4B). The mass, *m*/*z* = 397, was consistent with a β-keto acid of 24 carbons plus the methyl group from diazomethane. This indicated that the product was formed from one C_{10}-*p*-nitrophenyl ester and one C_{14}-CoA thioester. The fragmentation pattern further revealed that the ^{13}C -label was found exclusively in the carboxyl group of the product. Typically, ketones fragment at the bond adjacent to the carbonyl carbon, as is observed here. Moreover, unlabeled and ^{13}C-labeled mass spectra are highly comparable, with exception of a mass shift of one for the identified fragments. The major fragment with *m*/*z* = 211 is consistent with a fragment of $CH_3(CH_2)_{12}C(O)$ derived from

myristoyl-CoA and that is not expected to shift when using ^{13}C-1-decyl-*p*-nitrophenol based on the mechanism shown in Fig. 4A, on the left. The corollary to this is that fragments containing the carbonyl carbon of *p*-nitrophenyl 1-^{13}C-decanoate would be expected to shift up by one mass unit and this is observed in Fig. 4B.

In a separate experiment, we reacted *p*-nitrophenyl 1-^{13}C-decanoate and myristoyl-CoA and directly chromatographed the product via GC–MS. β-Keto acid products are known to rapidly decarboxylate in the heat of the GC [17]. If the ^{13}C -label is in the carboxyl group, that will be lost as carbon dioxide and the mass spectra of the ketone products from ^{13}C-labelled and nonlabelled substrates would be identical. Indeed, we observed identical mass spectra, no hint of a mass shift of all identifiable fragments (Fig. S7). Collectively, these data demonstrated that the chain from the *p*-nitrophenyl ester that is tethered to the cysteine and bound in Channel A is activated to carry out the Claisen reaction as shown in Fig. 4A, left.

3.6. Generating novel OleA products

The OleA-catalyzed *p*-nitrophenyl ester hydrolysis reaction has, in the past, proven useful mainly for screening purposes, but it can now be extended with the new insights obtained in this work. Novel *p*-nitrophenyl acyl chains can be introduced into Claisen products because they uniquely go into Channel A and condense with a CoA ester bound in Channel B. This greatly expands the range of Claisen products that can be generated by OleA enzymes, which have been shown to have a promiscuous Channel A binding site.

Moreover, the mixed Claisen products can be generated more efficiently and inexpensively via *p*-nitrophenyl esters in place of CoA esters. Acyl-CoA thioesters are expensive to purchase, > $30,000 per gram (Table S1), and their synthesis requires a non-ideal coupling of the highly water soluble CoA moiety with an activated carboxylic acid that can hydrolyze in water, making for low to modest yields of product [36]. Alternatively, fatty acid CoA ligase enzymes and ATP can be used to biosynthesize specific CoA esters [37,38]. By contrast, *p*-nitrophenyl esters can be made in high yields using *p*-nitrophenyl chloroformate in direct reaction with thousands of cheap and commercially available carboxylic acids, opening the door to making novel substrates for OleA-catalyzed reactions. Here we synthesized 4-nitrophenyl 3-cylopentylpropionate and 4-nitrophenyl 6-heptynoate using a facile one-pot reaction as previously described [30,31]. These compounds were predicted to bind in Channel A and undergo reaction with co-incubated myristoyl-CoA substrates that would bind in Channel B.

The compounds reacted with OleA to form Claisen products that are derived from one each of the *p*-nitrophenyl donors and myristoyl-CoA as the second substrate (Fig. 5). GC–MS was used to separate and identify products via mass fragments. The β-keto acid products are unstable in the gas chromatograph and undergo decarboxylation to yield ketones, as illustrated in Fig. 5. Unique peaks were observed in each OleA reaction mixture that were absent in chromatograms of incubations lacking OleA. Each product was a distinct ketone with 20 or 21 carbon atoms, which was expected to elute in the 17–20 min range, as was observed here. Each compound was subjected to electron impact mass spectrometry. The cyclopropyl ketone and alkyne ketone mass spectra both showed highly diagnostic fragmentation patterns (Fig. 5 insets and top). Neither of the ketone compounds, nor the β-keto carboxylic acids from which they are derived, have been described in the chemical literature. Their novelty was determined by searching SciFinder, which contains more than one hundred million chemical substances and is regularly updated [34,35].

3.7. Enzyme modeling to explain mechanism.

In addition to offering a way to make novel β-keto acids, and hence β-lactones and lipids, the use of *p*-nitrophenyl esters to drive chain condensation provides new insights into the Claisen mechanism of OleA. Prior to this study, there was no direct evidence for the directionality of the Claisen condensation reaction. The use of *p*-nitrophenyl esters and ^{13}C-labeling allowed new insights to be obtained. OleA reaction-intermediate structures were modeled to better understand these new insights.

The positioning of E117 in OleA and its role in the directionality of the Claisen condensation reaction is re-examined here in light of these new findings. Thiolases demonstrated or predicted to be OleA enzymes show a [LIVYF]-E-P-X-X-[VIL] motif where the E corresponds to E117 in the *X. campestris* OleA studied here (Fig. 6). E117 interposes into the active site catalytic triad of C143, H285 and N315 from the opposite subunit of the OleA homodimer. It has been proposed to be the catalytic base that abstracts a hydrogen atom to activate one of the chains. Consistent with that, a E117D mutation results in greatly diminished Claisen condensation activity, while mutation to an alanine completely eliminates this activity [27].

Overlay of the two subunits in the OleA structure PDB 4KU3, where channel B is bound with myristoyl-CoA or is unbound in either subunit, shows that residue E117 has multiple conformations. Modelling the thioester intermediate bound in Channel A and linked to the catalytic cysteine, C143, it appears that the E117 conformer, with Channel B unoccupied, is positioned well to act as a base that could deprotonate the methylene carbon of the thioester intermediate. When the acyl-CoA is bound in Channel B, the E117 conformer found in crystal structures is placed in a way that would least obstruct substrate entry or product release from either channel.

A previous X-ray crystallographic experiment with lauryl- and myristoyl-CoA revealed structures with a fatty acid bound in Channel A and acyl-CoA in Channel B [26]. In those structures, PDB 4KU5 and 4KU3, the general base E117 [25,27] was somewhat closer to the Channel B acyl-CoA methylene carbon than the corresponding Channel A fatty acid methylene carbon. This led to an earlier proposal that the Claisen condensation might occur via the Channel B chain attack on the Channel A carbonyl carbon,

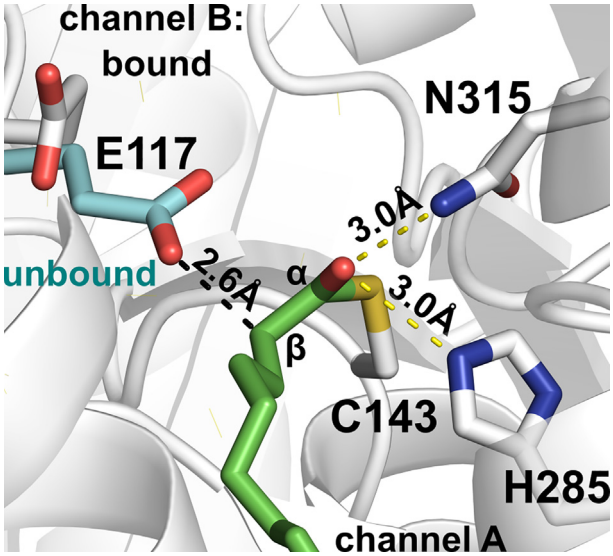

Fig. 6. E117 position in the unbound form of OleA suggests its role in deprotonation of the A-Channel chain with stabilization of deprotonated β-methylene carbon by enolization facilitated by the oxyanion hole formed by H285 and N315.

opposite to what was demonstrated here. Note that the structures 4KU5 and 4KU3 do not represent reaction intermediates. The fatty acid chain is not tethered to C143 and an intervening oxygen molecule positions the methylene carbon hydrogen atoms considerably further away from the other reactive components. Moreover, other structures show a different positioning of E117. These alternative positions are depicted in Fig. 6.

In active sites with Channel B not occupied with an acyl-coA, E117 is positioned closer to the catalytic residues and when modelling the enzyme thioester intermediate of the fatty acyl chain bound to C143 in these active sites, E117 is well positioned (2.6 Å) to abstract a proton from the methylene carbon of the fatty acyl chain (Fig. 6). When the proton is abstracted, the deprotonated methylene carbon can be further stabilized in its enol form by the oxyanion hole formed by residues H285 and N315. Then, E117 can swing back to make room for the acyl-CoA to come into Channel B, positioning both chains for the subsequent Claisen reaction.

4. Conclusions

The present study provides new insights into the mechanism of, and applications for, the thiolase enzyme OleA. The acyl chains from *p*-nitrophenyl esters are transferred onto C143 and extend into Channel A, subsequently being activated to attack an acyl chain bound to Coenzyme A (CoA) in Channel B. Since *p*-nitrophenyl esters are much less expensive to purchase and can be more readily synthesized than CoA esters, these findings also provide a means to introduce novel functionality into OleA products. OleA may then be used with additional enzymes, OleBCD, to synthesize novel lipids and β-lactone natural products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

MDS and LPW conceived the study. MDS, LJT, TB, JER, LPW participated in research design. MDS, LJT, TB, JER performed the experiments. All authors analyzed data. MDS and LPW wrote the original draft of the manuscript and all authors approved the final version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.05.031.

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