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## $\beta$ -Lactone Synthetase Found in Olefin Biosynthesis Path-way

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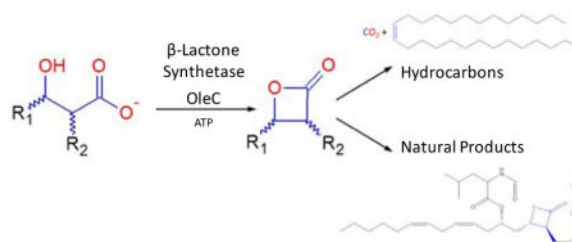
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### Abstract

The first  $\beta$ -lactone synthetase enzyme is reported, creating an unexpected link between the biosynthesis of olefinic hydrocarbons and highly functionalized natural products. The enzyme OleC, involved in the microbial biosynthesis of long-chain olefinic hydrocarbons, reacts with *syn*- and *anti*- $\beta$ -hydroxy acid substrates to yield *cis*- and *trans*- $\beta$ -lactones, respectively. Protein sequence comparisons reveal that enzymes homologous to OleC are encoded in natural product gene clusters that generate  $\beta$ -lactone rings, suggesting a common mechanism of biosynthesis.

### Table of Contents Artwork



The  $\beta$ -lactone (2-oxetanone) substructure is well known in organic synthesis and microbial natural products, some of which are presently being investigated for anti-obesity, anti-cancer, and antibiotic properties<sup>1–5</sup>. Although multiple organic synthesis routes exist for  $\beta$ -lactones<sup>6</sup>, no specific enzyme that catalyzes the formation of this functional group had been identified. While defining the chemistry of a well-known olefinic hydrocarbon biosynthesis pathway, we identified a  $\beta$ -lactone synthetase whose presence extends into natural product biosynthesis.

The olefin biosynthesis pathway is encoded by a four-gene cluster, *oleABCD*, and is found in over 250 divergent bacteria<sup>7</sup>. Ole enzymes produce long-chain hydrocarbon *cis*-alkenes

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#### Notes

The authors declare no competing financial interest.

from activated fatty acids through the reported chemistry shown in Figure 1<sup>8,9</sup>. OleA, the first enzyme of the pathway, has been studied in *Xanthomonas campestris* (*Xc*) and found to catalyze the “head-to-head” coupling of CoA-activated fatty acids (**1**) to unstable  $\beta$ -keto acids (**2**)<sup>10</sup>. The second enzyme, OleD, couples the reduction of **2** with NADPH oxidation to yield stable  $\beta$ -hydroxy acids (**3**) as defined in *Stenotrophomonas maltophilia* (*Sm*)<sup>11</sup>. Finally, using GC detection methods, we have observed and others have reported that *Sm* OleC catalyzes an apparent decarboxylative dehydration reaction to generate the final *cis*-olefin product<sup>12</sup>. Together, these findings indicate that there is no defined purpose for the ever-present fourth gene in the cluster, *oleB*.

We now report that the observation of *cis*-alkenes as products of OleC is an artifact, a misleading observation caused by GC-analytical methods used to identify the high-boiling point products of OleC. Rather, using <sup>1</sup>H NMR, we demonstrate that OleC from four different bacteria produce thermally-labile  $\beta$ -lactones from  $\beta$ -hydroxy acids in an ATP-dependent reaction; no alkenes are observed. Further analyses of gene clusters for  $\beta$ -lactone-containing natural products reveal *oleC* homologues that likely perform this previously unknown biological  $\beta$ -lactone ring closure reaction.

The first suggestions of  $\beta$ -lactone synthetase activity arose when monitoring reactions of *Xc* OleC with ATP, MgCl<sub>2</sub>, and a synthetic, diastereomeric mixture of **3** by GC. Full <sup>1</sup>H NMR spectra of all synthesized compounds from this study are shown in Supplemental Figures S1–S6. Two peaks were observed by GC, coupled to both a mass spectrometer (MS) and flame ionization detector (FID), with mass spectra and retention times identical to synthetic *cis*- and *trans*-olefin standards (**5a** and **5b**, respectively). However, the GC/FID peak area of the enzymatically-generated olefin varied significantly with GC inlet temperature and inlet liner purity, while synthetic standards were unaffected, suggesting that the observed olefin may be a thermal decomposition product of the actual OleC product.

To test this hypothesis, *Xc* OleC reactions with **3** were scaled to generate sufficient quantities for <sup>1</sup>H NMR. No resonances consistent with the prepared olefin standards (Figures S1 and S2) (**5a**, **5b**) were observed; rather, four distinct multiplets, appearing as doublet of doublets of doublets, arose (Figures S7 and S8). These resonances were consistent with the two hydrogens of *cis*- and *trans*- $\beta$ -lactone rings and perfectly matched our authentic standards of *cis*- and *trans*-3-octyl-4-nonyloxetan-2-one (**4a**, **4b**) (Figures S3 and S4). Furthermore, when **4a** and **4b** were analyzed by GC, retention times and mass spectra matched the olefin standards **5a** and **5b**, with sensitivity to inlet conditions being observed. The thermal decarboxylation of *cis*- and *trans*- $\beta$ -lactone to *cis*- and *trans*-olefin, respectively, is well known<sup>13,14</sup>. We believe that thermal decomposition during GC/MS analysis caused the product of OleC catalysis to be misidentified. Additionally, when reviewing supplemental NMR data from the literature report of *Sm* OleC characterization, resonances of the *cis*- and *trans*- $\beta$ -lactones, consistent with those synthesized here, are visible<sup>12</sup>.

The stereochemical origins of **4a** and **4b** were then investigated by reacting *Xc* OleC with *syn*- and *anti*- $\beta$ -hydroxy acids (Figures S9 and S10). HPLC was used to separate **3** into its *syn*- and *anti*-diastereomeric pairs (**3a** and **3b**) (Figure 2). Examining **3a** and **3b** by <sup>1</sup>H NMR

and GC/MS, post-methylation, demonstrated each contained <10% of the opposite racemic diastereomer (Figures S5 and S6). When reacting with *Xc* OleC, **3a** produced **4a**, while **3b** generated **4b** (Figure 3). GC/MS analysis supported this conclusion, as OleC reactions with **3a** and **3b** yielded the  $\beta$ -lactone breakdown products, **5a** and **5b**, respectively. OleC consumed greater than 90% of substrates **3a** and **3b** by GC/MS, supporting the conclusions of Kancharla, et al. that all four  $\beta$ -hydroxy acid isomers are utilized by OleC<sup>12</sup>. Taken together, *Xc* OleC represents the first reported  $\beta$ -lactone synthetase, converting  $\beta$ -hydroxy acid substrates to  $\beta$ -lactones in the presence of ATP and MgCl<sub>2</sub>. MgATP is likely required to activate the hydroxyl or carboxyl group and promote  $\beta$ -lactone ring formation.

To determine if  $\beta$ -lactone synthetase activity is a common enzymatic step in long-chain olefin biosynthesis, we obtained four *oleC* genes from *oleABCD* gene clusters in divergent microorganisms (Table 1). Purified OleC enzyme from the four organisms was reacted overnight with ATP and **3**, then analyzed by <sup>1</sup>H NMR and GC/MS. The products of OleC proteins from the bacteria *Stenotrophomonas maltophilia*, *Arenimonas malthae*, and *Lysobacter dokdonensis* were both **4a** and **4b**  $\beta$ -lactones (Figures S11–S13), with no **5a** or **5b** olefins being observed, indicating that OleC enzymes from diverse sources are  $\beta$ -lactone synthetases. The Gram-positive bacterium *Micrococcus luteus* (*MI*) was specifically chosen because its sequence is very divergent from *Xc* OleC, and it contains a natural fusion of the *oleB* and *oleC* genes. This natural *oleBC* fusion is found in all actinobacteria, which comprise ~30% of the microorganisms that contain identifiable *oleABCD* genes. Reaction of purified *MI* OleBC fusion with MgCl<sub>2</sub>, ATP, and **3** produced  $\beta$ -lactones **4a** and **4b** as well as small amounts of *cis*-olefin, **5a** (Figure S14). No trace of *trans*-olefin, **5b**, was detected. Further characterization is ongoing, but we believe that OleB performs a *syn*-elimination of *cis*- $\beta$ -lactone to form the final *cis*-olefin product. This is consistent with previous studies of microorganisms expressing *ole* genes that contain olefins with a *cis*-relative configuration exclusively<sup>7–9</sup>. These data also demonstrate that an enzyme domain with only 35% amino acid identity to the *Xc* OleC generates  $\beta$ -lactones, indicating that this activity is likely common among all olefinic hydrocarbon biosynthesis OleC homologues.

Establishing the widespread nature of lactone synthetase activity within olefinic hydrocarbon biosynthesis led us to search sequence databases for OleC homologues in other biosynthetic pathways. OleC is a member of the ubiquitous AMP-dependent ligase/synthetase enzyme superfamily; as such, homologues are found in all organisms<sup>15</sup>. As of November 2016, a BLAST search of NCBI's non-redundant protein sequence database identified over 900 sequences with greater than 35% sequence identity and over 16,000 with greater than 25% sequence identity to *Xc* OleC.

Of the sequences examined, two *Xc* OleC homologues were clearly encoded in gene clusters known to produce  $\beta$ -lactone natural products (Figure 4). The first, LstC, is an uncharacterized enzyme found in the lipstatin biosynthesis pathway from *Streptomyces toxytricini*. Lipstatin is the precursor to Orlistat, the only over-the-counter, FDA-approved anti-obesity drug. LstC is a member of the AMP-dependent ligase/synthetase superfamily, and its protein sequence is 38% similar to *Xc* OleC, more similar than the sequence of the  $\beta$ -lactone synthetase domain of *MI* OleBC (35%). Surprisingly, further investigation revealed homologues to OleA and OleD encoded by the lipstatin gene cluster, suggesting that the two

gene clusters have a common ancestry. The synthesis of both lipstatin and olefinic hydrocarbons are initiated by the condensation of two fatty acyl-CoAs to form a  $\beta$ -keto acid. In the case of lipstatin, the two fatty acids are 3-hydroxy-linoleic and octanoic acid<sup>1</sup>. The hydroxyl group of linoleic acid is later functionalized by LstE and LstF with a modified valine<sup>1</sup>. LstD and OleD likely perform the same NADPH-dependent reduction of the  $\beta$ -keto group to a hydroxyl group. Formation of the *trans*- $\beta$ -lactone is likely accomplished by the OleC homologue LstC, to generate the final product in lipstatin biosynthesis. Olefin biosynthesis is completed by the putative OleB-dependent elimination of CO<sub>2</sub> to generate the final olefin product. The lipstatin gene cluster lacks any gene product that shows homology to OleB, consistent with the accumulation of the  $\beta$ -lactone natural product and further supporting our hypothesis that OleB performs the final step in the biosynthetic pathway to olefins.

The gene cluster responsible for the biosynthesis of ebelactone A, a commercially available esterase inhibitor, in *Streptomyces aburaviensis* shows a gene, *orf1*, with 46% amino acid sequence identity to *Xc* OleC and is directly adjacent to *ebeA-G* (Figure 4). Unlike lipstatin, ebelactone A is formed partly by a polyketide synthase multi-domain protein rather than fatty acid condensation; as such, OleA and OleD homologues are not encoded in the surrounding gene cluster. Literature reports suggest that the  $\beta$ -lactone ring of ebelactone A is formed spontaneously from the final, enzyme-linked,  $\beta$ -hydroxy-thioester intermediate<sup>5</sup>. While a spontaneous  $\beta$ -hydroxy-thioester cyclization is mechanistically plausible,  $\beta$ -lactone formation from  $\beta$ -hydroxy-thioesters in ubiquitous pathways such as fatty acid oxidation or synthesis has not been reported to our knowledge<sup>16</sup>. Additionally,  $\beta$ -hydroxy-thioester intermediates are extremely common in polyketide synthesis pathways, while  $\beta$ -lactone formation is comparatively rare. An Orf1-independent cyclization would require a unique property of ebelactone A precursors or a novel polyketide domain architecture to promote  $\beta$ -lactone ring cyclization. However, in favor of an Orf1-independent mechanism is that no thioesterase domain exists in the final polyketide synthesis domain, suggesting that no free  $\beta$ -hydroxy acid is released for the putative ATP-dependent Orf1 to act on. Other polyketide-type  $\beta$ -lactone gene clusters, such as those for salinosporamide A, cinnabaramide, and oxazolomycin, do not encode an OleC homologue with high sequence similarity (>35%). Polyketide-derived  $\beta$ -lactones are thought to form by the cyclization of the final thioester, enzyme-linked intermediate, but this has never been characterized<sup>2,17-19</sup>. It is reasonable to hypothesize that specialized polyketide synthase domains represent a second mechanism of  $\beta$ -lactone formation. Regardless, the discovery of a stand-alone  $\beta$ -lactone synthetase here creates new opportunities for the natural product field. Preliminary screening of *Streptomyces* and *Nocardia* genomes suggests that  $\beta$ -lactone natural products may be more widespread than currently realized.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

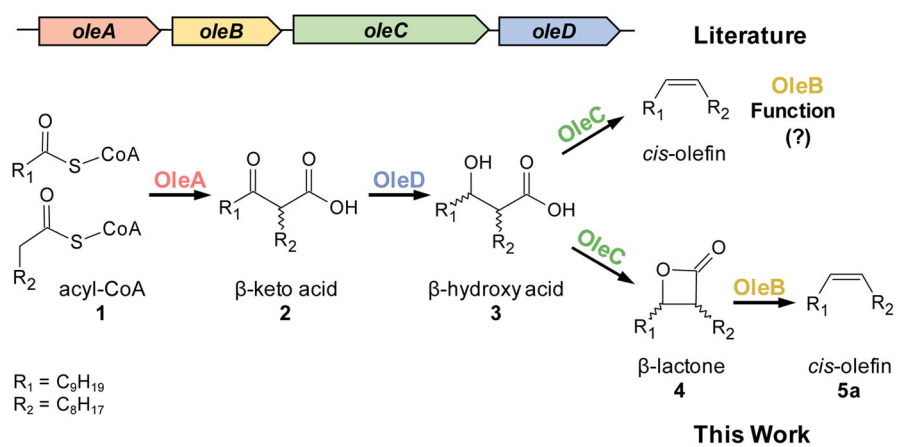
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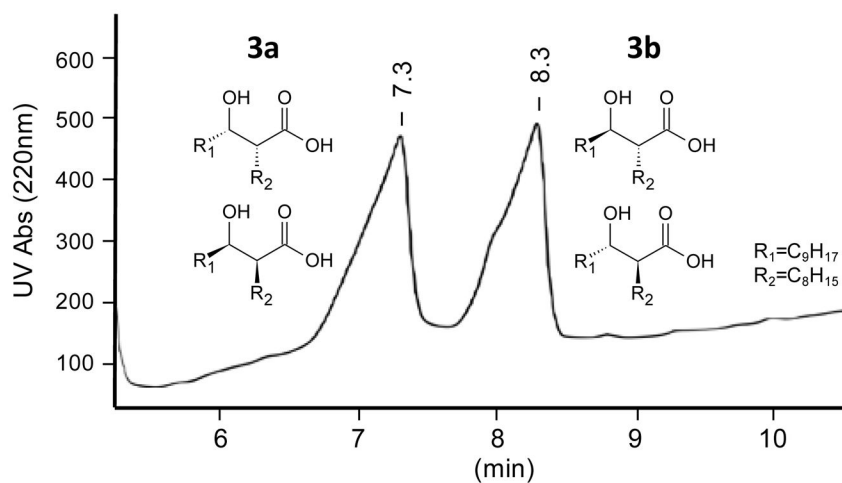
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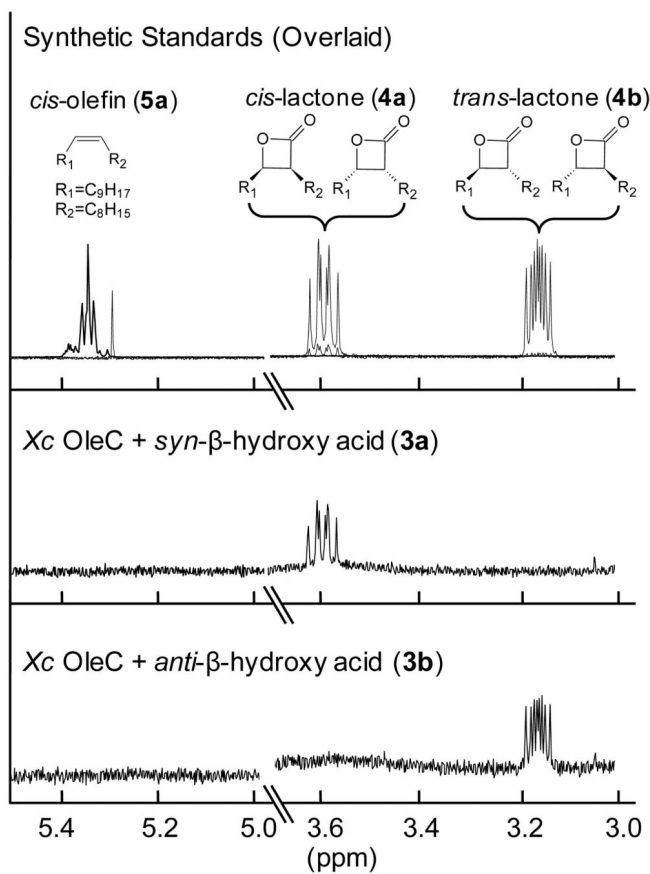
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**Figure 1.**  
*ole* gene cluster and enzymatic pathway.

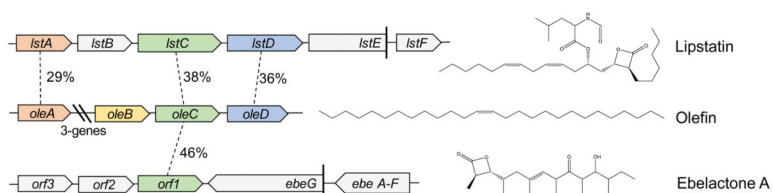


**Figure 2.** HPLC separation of *syn*- and *anti*-β-hydroxy acids (**3a** and **3b**, respectively) for use as *Xc* OleC substrates. The rise in baseline is caused by impurities from the MTBE gradient that absorb at 220 nm.



**Figure 3.**  $^1\text{H}$  NMR (400 MHz;  $\text{CDCl}_3$ ) analyses of *Xc* OleC products with  $\beta$ -hydroxy acid substrates.





**Figure 4.** Homologous OleC enzymes encoded in  $\beta$ -lactone biosynthesis gene clusters. Percent identity is based on amino acid sequences. The E values for OleC to LstC and Orf1 are  $2e-72$  and  $1e-143$ , respectively. Lipstatin is the precursor to the anti-obesity drug Orlistat. Ebelactone A is a commercially available general esterase inhibitor.

**Table 1**Other OleC enzymes make  $\beta$ -lactones

Organism	Ascension #	% ID <sup>a</sup>
<i>Xanthomonas campestris</i>	WP_011035474.1	100
<i>Stenotrophomonas maltophilia</i>	AFC01244.1	77
<i>Arenimonas malthae</i>	WP_043804215.1	73
<i>Lysobacter dokdonensis</i>	WP_036166093.1	70
<i>Micrococcus luteus</i> <sup>b</sup>	WP_010078536.1	35

<sup>a</sup>% identity based on amino acid sequence<sup>b</sup>OleC and OleB are a natural fusion in *MI*.