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## Trapping of Intermediates with Substrate Analog HBOCoA in the Polymerizations Catalyzed by Class III Polyhydroxybutyrate (PHB) Synthase from *Allochromatium Vinosum*

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### Abstract

Polyhydroxybutyrate (PHB) synthases (PhaCs) catalyze the formation of biodegradable PHB polymers that are considered as an ideal alternative to petroleum-based plastics. To provide strong evidence for the preferred mechanistic model involving covalent and noncovalent intermediates, a substrate analog HBOCoA was synthesized chemoenzymatically. Substitution of sulfur in the native substrate HBCoA with an oxygen in HBOCoA enabled detection of  $(\text{HB})_n\text{OCoA}$  ( $n = 2-6$ ) intermediates when the polymerization was catalyzed by wild-type (*wt*-)PhaEC<sub>AV</sub> at 5.84 hr<sup>-1</sup>. This extremely slow rate is due to thermodynamically unfavorable steps that involve formation of enzyme-bound PHB species (thioesters) from corresponding CoA oxoesters. Synthesized standards  $(\text{HB})_n\text{OCoA}$  ( $n = 2-3$ ) were found to undergo both reacylation and hydrolysis catalyzed by the synthase. Distribution of the hydrolysis products highlights the importance of the penultimate ester group as previously suggested. Importantly, the reaction between primed synthase [<sup>3</sup>H]-sT-PhaEC<sub>AV</sub> and HBOCoA yielded [<sup>3</sup>H]-sTet-O-CoA at a rate constant faster than 17.4 s<sup>-1</sup>, which represents the first example that a substrate analog undergoes PHB chain elongation at a rate close to that of the native substrate (65.0 s<sup>-1</sup>). Therefore, for the first time with a *wt*-synthase, strong evidence was obtained to support our favored PHB chain elongation model.

Polyhydroxyalkanoates (PHAs) are energy storage polymers that are synthesized by a variety of bacteria under nutrient-limited conditions except for carbon.<sup>1,2</sup> Accumulation of PHA can reach up to 90% of dry cell weight.<sup>3</sup> When the conditions become hospitable, these polymers are degraded to release energy for other biological processes.<sup>1</sup> PHAs have received

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**Supporting Information Available.** Synthesis of analogs and their characterization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

much attention because they are considered as an ideal alternative to petroleum-based plastics that are nonbiodegradable.<sup>4, 5</sup> Huge amounts of plastic waste are generated each year and a significant portion ends up in oceans, which will eventually destroy the marine ecosystem and in turn our planet if nothing is done to prevent it.

In contrast, PHAs are biodegradable and can be produced from renewable sources.<sup>1, 2</sup> Approximately 150 different monomers are found to be incorporated into PHAs that can be developed into various applications such as packaging, drug delivery, and tissue engineering.<sup>2, 6</sup> However, progress in PHA commercialization has been limited due to high cost of production.<sup>7-9</sup> Thus, study of PHA biosynthesis will facilitate us to engineer relevant enzymes so that PHAs can be produced as economically as the petroleum-based plastics.

As depicted in Scheme 1, PHAs are generated from 3-hydroxyalkanoate (HA) coenzyme A (CoA) catalyzed by PHA synthases, PhaCs.<sup>10, 11</sup> Depending on the size of the R group, the resulting polymers can have properties ranging from thermoplastics to elastomers. When R is a methyl group, the polymer is named polyhydroxybutyrates (PHBs). It is well known that PhaCs are crucial enzyme in PHA biosynthesis as they determine polymer productivity as well as the properties such as composition, length, and polydispersity.<sup>6, 12, 13</sup>

Bacterial PhaCs are divided into four classes according to their subunit composition and substrate specificity.<sup>10-12</sup> The synthase from *Allochromatium Vinosum*, PhaEC<sub>AV</sub> represents a prototypical class III PHB synthase. It consists of two subunits PhaC and PhaE, each having a molecular weight (MW) at 39 and 40K Da, respectively.<sup>14</sup> No homology has been found for PhaE in any other proteins. However, its presence is required in order for PhaC to catalyze polymerization reactions.<sup>15</sup>

Two mechanistic models have been proposed for PHB chain elongation as depicted in Scheme 2.<sup>10, 11</sup> Model A is based on the analogy to fatty acid biosynthesis,<sup>16</sup> in which there is one PHB chain per dimer of PhaC and the growing chain alternates between the two monomers as each additional 3-hydroxybutyrate (HB) unit is added. According to this model, the active site has to be close to the surface in order to facilitate chain transfer. However, sequence alignment involving all four classes of PhaCs suggests that they have strong homology with the  $\alpha/\beta$ -hydrolase superfamily and particularly lipases.<sup>17, 18</sup> Available crystal structures of lipases have indicated that the active site is deeply buried.<sup>19</sup> Therefore, model B based on the polyketide synthases is proposed.<sup>20</sup> For both models, the triad consisting of Cys, His, and Asp is catalyzing the polymerization reactions, in which Asp activates the 3-OH of a second HBCoA for ester bond formation. However, the difference between the two models is the way the second HBCoA is loaded to the synthases. While model A involves a covalent catalysis with the second HBCoA, model B requires noncovalent catalysis with the second HBCoA as well as the generation of a noncovalent chain-extension intermediate. Therefore, detection of the noncovalent intermediates (boxed in Scheme 2B) will provide an effective way to distinguish between these two models.

Differentiation of chain elongation modes has significant implications in polymer length control. Such efforts have been reported for revealing the MW control of galactans.<sup>21</sup> Processive and distributive mechanisms are commonly referred in template-independent



group in **7**. Depending on the structure of acid **7**, this step was accomplished in the presence of different coupling reagents. When the benzyl (Bn) protected monomeric HB acid **7a** was employed, *N,N*-dicyclohexylcarbodiimide (DCC) and catalytic 4-dimethylaminopyridine (DMAP) were used to promote the ester formation **8a** in a 85% yield. However, combination of DCC and DMAP resulted in low yields for other acids (**7b-e**). Thus, they were first converted to acyl chloride in the presence of oxalyl chloride and catalytic amount of *N,N*-dimethylformamide (DMF) followed by nucleophilic acyl substitution with **6** to afford esters **8b-e** in good yields. Synthesis of **7a-d** has been reported elsewhere.<sup>17</sup> The protecting groups in **8** and **6** were removed by hydrogenation in excellent yields to give enzymatic precursors **9** and **10**, respectively.

Introduction of adenosine 3',5'-triphosphate was accomplished with three enzymes involved in CoA biosynthesis: a pantothenate kinase from *Staphylococcus aureus* (*SaPanK*),<sup>26</sup> a phosphopantetheine adenylyltransferase (*EcCoaD*),<sup>27</sup> and a dephospho-CoA kinase (*EcCoaE*) from *Escherichia coli*.<sup>28</sup> Various CoA analogs have been prepared by this method and employed as inhibitors or mechanistic probes to investigate CoA-dependent enzymes.<sup>23, 25, 29-33</sup> The final compounds were isolated by reverse-phase HPLC in reasonable yields and characterized by <sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR and HRMS.

It should be noted that since the pantothenamide **10** contains two terminal hydroxyl groups, concerns of regioselectivity were raised for the enzymatic conversion. Although CoAOH **4** was previously synthesized from a pre-CoA thioester,<sup>34</sup> only a low resolution MS was reported for its characterization. To make sure phosphate installed at the correct hydroxyl group by *SaPanK*, both the amides **6** and **10** were subject to phosphorylation. It was found that no reaction occurred with **6** while **10** was converted to phosphopantothenamide according to HPLC profile (data not shown). Thus, demonstrating that the phosphorylation occurred at the desired hydroxyl group. The identity of **4** was fully characterized and is reported in Supporting Information.

#### Detection of (HB)<sub>n</sub> CoA (n=2–6) Intermediates in the Reaction between HBOCoA and *wt*-PhaEC<sub>AV</sub>

To look for (HB)<sub>n</sub>OCoA intermediates, *wt*-PhaEC<sub>AV</sub> and HBOCoA **1** were incubated at 30 °C and the reaction was monitored by HPLC at 260 nm (Figure 1A). A decrease in the amount of HBOCoA (retention time *t*<sub>R</sub> = 17.7 min) was observed. Additionally, three new peaks at 9.80, 29.0, and 37.7 min appeared. UV-Vis scan of these peaks revealed that they all had a maximal absorbance at 260 nm.

The new species were isolated, concentrated, and characterized by low-resolution MALDITOF MS in a negative reflective mode. The results are shown in Figure 1B I–III. The species eluted at 9.80, 29.0, and 37.7 min (Figure 1A) were identified as CoAOH **4**, (HB)<sub>2</sub>OCoA **2a**, and (HB)<sub>3</sub>OCoA **2b**, respectively. Their MS are identical to the synthesized standards (data not shown). The assignment of the mass peaks is summarized in Figure 1C. Further confirmation arises from their respective co-elution with the synthesized standards.

Since PHB is highly hydrophobic, it is predicted that CoA analogs attached with a large number of HB units will be eluted at high percentages of methanol. Examination of HPLC

profiles at 260 nm did not find apparent peaks between 40.0 and 60.0 min. Nevertheless, the eluents at 40.0–60.0 min were collected, concentrated, and analyzed by MALDI-TOF MS. The result is shown in Figure 1B IV with the peak assignment summarized in Figure 1C. CoA analogs containing up to hexameric HB units were identified. Additionally, peaks corresponding to H<sub>2</sub>O elimination were observed in MS, suggesting that the terminal HB unit had been converted into a crotonate. Because MALDI-TOF MS uses soft ionization, elimination was not expected. Thus, formation of crotonate was attributed to the presence of acidic matrix during sample preparation as shown in Figure 1D. The terminal hydroxyl group is protonated by matrix  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA). A subsequent E2 elimination will generate the analog with an end crotonate unit.

### Determination of Kinetic Parameters of HBOCoA and Rates of the Formation of Intermediates (HB)<sub>n</sub>OCoA (n = 2–3)

Since the analog HBOCoA was recognized as a substrate, its kinetic parameters were determined with *wt*-PhaEC<sub>AV</sub>. The analog underwent monophasic release of CoAOH with a  $K_M$  and  $k_{cat}$  at 2.38 mM and 5.84 hr<sup>-1</sup>, respectively (Figure 2A). This translates into catalytic efficiency ( $k_{cat}/K_M$ ) of 0.68 M<sup>-1</sup>s<sup>-1</sup>, which is 7.35×10<sup>5</sup>-fold slower than for the native substrate HBCoA ( $k_{cat}/K_M = 5.00 \times 10^5$  M<sup>-1</sup>s<sup>-1</sup>). Furthermore, the turnover number of HBOCoA is about 4.00×10<sup>5</sup>-fold slower than that of HBCoA. The dramatically lowered catalytic efficacy and extremely slow rate are consistent with the prediction that the reaction between the Cys and (HB)<sub>n</sub>OCoA (an oxoester) to produce an enzyme-bound PHB (a thioester) is thermodynamically unfavorable (Scheme 3A).

Kinetic competence implies that the rates of formation and breakdown of the intermediates must be equal to or faster than the overall rate of the reaction ( $k_{cat}$ ). To examine that, the rates of formation of (HB)<sub>2</sub>OCoA and (HB)<sub>3</sub>OCoA were measured under the condition that the synthase was saturated with the substrate ( $8K_M$ ). As shown in Figure 2B, (HB)<sub>2</sub>OCoA and (HB)<sub>3</sub>OCoA were formed at a rate constant of 3.47 and 0.35 hr<sup>-1</sup>, respectively. Thus, the overall rate of intermediate formation is 7.99 hr<sup>-1</sup> ( $3.47 \times 2 + 0.35 \times 3 = 7.99$  hr<sup>-1</sup>) plus the rate of formation of PHB attached to the enzyme. The latter is unknown due to lack of the radioactivity. Nevertheless, the rate of formation of intermediates (>7.99 hr<sup>-1</sup>) is indeed faster than the overall rate of the reaction ( $k_{cat} = 5.84$  hr<sup>-1</sup>), which suggests that these intermediates could be kinetically competent.

### Products from Incubation of (HB)<sub>n</sub>OCoA (n = 2–3) with *wt*-PhaEC<sub>AV</sub>

As described above, kinetic competence of an intermediate also requires that the rate of its utilization is greater than or equals to the rate of substrate consumption. Therefore, efforts were made to measure the reacylation rates of (HB)<sub>n</sub>OCoA (n=2–3) with *wt*-PhaEC<sub>AV</sub>. It has been reported that (HB)<sub>2</sub>CoA and (HB)<sub>3</sub>CoA can function as primers to PHB synthases by covalently attaching (HB)<sub>n</sub> (n=2–3) chain to the active site Cys with the concurrent release of CoA.<sup>35</sup> However, when the oxo analogs (HB)<sub>n</sub>OCoA (n=2–3) were incubated with *wt*-PhaEC<sub>AV</sub> followed by HPLC analysis, new species were found in addition to the expected release of CoAOH ( $t_R = 9.80$  min). As shown in Figure 3A, incubation with dimer (HB)<sub>2</sub>OCoA (solid line,  $t_R = 29.0$  min) resulted in an additional species that corresponded to HBOCoA ( $t_R = 17.7$  min). Similarly, when trimer (HB)<sub>3</sub>OCoA (dash line,  $t_R = 37.7$  min)

was employed as the substrate, both HBOCoA ( $t_R = 17.7$  min) and  $(\text{HB})_2\text{OCoA}$  ( $t_R = 29.0$  min) were identified along with CoAOH ( $t_R = 17.7$  min). Characterization of these species was confirmed with MALDI-TOF MS and co-elution with authentic standards. Their rates of formation are summarized in Figure 3B.

Formation of these products is postulated in Figure 3C. Acylation of the synthase by  $(\text{HB})_n\text{OCoA}$  ( $n=2-3$ ) will release the expected CoAOH (red arrows). Their rates were estimated at  $\sim 2$ -fold faster than the overall rate of the reaction ( $5.84 \text{ hr}^{-1}$ ), which suggests that  $(\text{HB})_n\text{OCoA}$  ( $n=2-3$ ) could serve as kinetically competent intermediates during polymerization reactions. Additionally, the rate of acylation of  $(\text{HB})_3\text{OCoA}$  is slightly faster than that of  $(\text{HB})_2\text{OCoA}$ , which is consistent with previous observations that rate of reacylation increases as the chain elongates.<sup>22, 36</sup>

Products other than CoAOH generated during incubation are attributed to enzyme-catalyzed hydrolysis. It is known that PHB synthases are hydrolytic.<sup>17</sup> As shown in Figure 3A, the major hydrolysis product is HBOCoA though the starting materials were different. This means the water preferentially attacks the penultimate HB unit (black solid arrows in Figure 3C) independent of primer length, which coincidentally agrees with the hydrolysis studies performed recently with  $[\text{}^3\text{H}]\text{-sT-PhaEC}_{\text{AV}}$  (structure shown in Scheme 3B).<sup>23, 37</sup> It was discovered that the penultimate HB unit of the sT chain attached to a synthase is preferentially attacked by a nucleophile.<sup>23, 37</sup> However, the rate of hydrolysis of sT chain is  $\sim 2$ - $5$ -fold slower than that of  $(\text{HB})_n\text{OCoA}$  ( $n=2-3$ ), suggesting that the synthase-bound PHB chain is oriented for elongation, the native function of the enzyme.

### Rapid Formation of Elongated Products with Radiolabeled Primed Synthase $[\text{}^3\text{H}]\text{-sT-PhaEC}_{\text{AV}}$

It is known that the rate of PHB chain elongation is much faster than that of initiation.<sup>38</sup> This poses a challenge to detect noncovalently bound intermediates due to heterogeneous formation of multiple species in undetectable amounts. In order to overcome this problem, artificial primers such as saturated trimeric-CoA (sTCoA) were developed to uniformly load the synthases so that the elongation process could be examined.<sup>15</sup>

Such a strategy has recently been employed with the analog  $\text{HBCH}_2\text{CoA}$  to investigate PHB chain elongation. However, the attempt was unsuccessful, possibly due to misalignment of the substrate binding.<sup>23</sup> In the present study, intermediates  $(\text{HB})_n\text{OCoA}$  ( $n=2-6$ ) were trapped during polymerization, suggesting that the binding mode of HBOCoA is similar to the native substrate and might overcome the misalignment associated with  $\text{HBCH}_2\text{CoA}$  in the reaction with a primed synthase. As described in Scheme 3B and since  $k_{\text{cat}}$  of the native substrate is  $65.0 \text{ s}^{-1}$ ,<sup>39</sup> it is predicted that the proposed reaction will be rapid for the first turnover as it does not involve formation of enzyme-bound PHB that is thermodynamically unfavorable.

Thus, the *wt-PhaEC*<sub>AV</sub> was acylated with radiolabeled  $[\text{}^3\text{H}]\text{-sTCoA}$  and subsequently purified by Sephadex G-50 size-exclusion chromatography (SEC) to remove excess  $[\text{}^3\text{H}]\text{-sTCoA}$ . The primed synthase was immediately reacted with HBOCoA. The reaction was terminated at selected times using RFQ. After the protein precipitate was separated from the

mixture, the supernatant was neutralized, concentrated, and analyzed by HPLC and scintillation counting. The profiles of elution at 260 nm and radioactivity are shown in Figure 4A.

Indeed, the results were consistent with our prediction. The eluents at 47.0–49.0 min contained the radioactivity (bar in Figure 4A) and had maximal absorbance at 260 nm (solid line in Figure 4A). Mass analysis by MALDI-TOF under a negative reflective mode showed a value of 1078.818 (Figure 4B), which corresponds to the  $[M-H]^-$  ions (calcd. 1078.283) of predicted elongation product  $[^3H]$ -sTet-O-CoA **3b** (Scheme 3B). This species also eluted at the same  $t_R$  (47.5 min) as the synthesized authentic compound (dash line in Figure 4A).

Moreover, it has to be pointed out that sT-O-CoA **3a** (Scheme 4) was also prepared as a standard, which has a  $t_R$  at 44.1 min (dash line in Figure 4A). Recent study with HBCH<sub>2</sub>CoA has revealed that the oxoester group that is next to thioester bond can be attacked by a nucleophile.<sup>23</sup> To determine whether compound **3a** was produced, eluents at 43.5–45.5 min were collected, concentrated, and analyzed by MALDI-TOF MS and scintillation counting. The results suggested that sT-O-CoA was not generated during the reaction.

The rate of formation of  $[^3H]$ -sTet-O-CoA was measured for the reaction between 32.0  $\mu$ M primed synthase and 5.00 mM HBOCoA. It was observed that only 0.13 equiv of the synthase ( $\sim$ 4.20  $\mu$ M) was attached with a radiolabelled  $[^3H]$ -sT chain ( $[^3H]$ -sT-PhaEC<sub>AV</sub>) after SEC purification. This agrees with previous established study that 0.15 equiv per PhaEC<sub>AV</sub> was radiolabelled.<sup>15</sup> The reaction was stopped by perchloric acid from 10 to 300 ms. The total recovery of radioactivity was more than 85% after HPLC separation. A typical radioactivity profile is shown as the bars in Figure 4A. The data were fitted to a single exponential rise to the maximum and gave a  $k_{obs}$  of 17.4 s<sup>-1</sup> (Figure 4C). Due to limited amount of  $[^3H]$ -sTCoA and HBOCoA, the experiment was only performed once. Although the obtained 17.4 s<sup>-1</sup> is less than the  $k_{cat}$  of native substrate (65.0 s<sup>-1</sup>), it could be due to the fact that the enzyme was not saturated with the substrate analog. Thus, for the first time, we have designed a mechanistic probe that can be used with a primed *wt*-synthase in a kinetically competent fashion to interrogate PHB chain elongation.

## CONCLUSION

This study demonstrates a new way to study the challenging PHB polymerization processes using the substrate analog HBOCoA. Incubation of the analog with *wt*-PhaEC<sub>AV</sub> resulted in detection of multiple intermediates (HB)<sub>n</sub>OCOA (n=2-6) that have been proven chemically and kinetically competent. This represents the first example of employing a *wt*-synthase to provide direct evidence to support the preferred model involving covalent and noncovalent intermediates. The rate of polymerization with HBOCoA is extremely slow (5.84 hr<sup>-1</sup>) due to a step that is thermodynamically unfavorable (from oxoester to thioester).

Moreover, reaction between *wt*-PhaEC<sub>AV</sub> and (HB)<sub>n</sub>OCOA (n=2-3) yielded hydrolysis products in addition to the expected release of CoAOH from reacylation. The distribution suggests that not only are the hydrolysis products independent of the primers being used, but

also the penultimate HB unit (from the side of CoA moiety) is susceptible to synthase-catalyzed hydrolysis. The latter observation coincidentally agrees with previous studies done by us and others showing that the penultimate HB of a sT chain attached to synthases is preferentially attacked by a nucleophile.<sup>23, 37</sup>

Reactions between the primed synthase [<sup>3</sup>H]-sT-PhaEC<sub>Av</sub> and HBOCoA formed the elongated product [<sup>3</sup>H]-sTet-O-CoA at a rate of at least 17.4 s<sup>-1</sup>. This represents the first example that a *wt*-synthase catalyzes the polymerization with an analog at the rate close to that of the native substrate. To the best of our knowledge, it is by far the most decisive evidence to support our favored model shown in Scheme 2.

Modes of PHB polymerizations will facilitate the determination of other factors in addition to PhaCs that are required for controlling polymer length and polydispersity. The distributive character associated with our preferred chain elongation model suggests that PHB should have a high PDI. Although no report was found that addressed the polymer properties obtained from native *A. vinosum*, studies with other PHB-producing organisms suggest that the polydispersity of PHB is relatively low.<sup>40-44</sup> Thus, our results presented here provide additional evidence to support the hypothesis that PHB polymer length and polydispersity are controlled by multiple factors in addition to PhaCs.<sup>45-47</sup> Such factors could include phasin proteins PhaP,<sup>48</sup> recently identified membrane-associated protein PhaM,<sup>49</sup> and others yet to be identified. Understanding their relationships to PhaCs is essential in order to engineer polymer properties as well as to lower the cost of production.

## METHODS

### Chemoenzymatic Synthesis of HBOCoA 1 and Related Analogs

The chemoenzymatic approaches to synthesize HBOCoA **1** and related analogs are shown in Schemes 3. The experimental details are summarized in Supporting Information.

### General HPLC Conditions

The HPLC was performed on a Waters Breeze 2 system equipped with a 1525 pump and 2998 PDA detector. Except for compound purification, all HPLC were performed on a Luna C18-2 column (5 μm, 4.6×250 mm) that was eluted with 40.0 mM NH<sub>4</sub>OAc (pH 4.70) and MeOH at 1.0 mL/min using a linear gradient (1) 5-30% MeOH in 0-30 min; and (2) 30-90% MeOH in 30-60 min.

### Reaction of HBOCoA with *wt*-PhaEC<sub>Av</sub> and Analysis of Small Molecules by HPLC and MALDI-TOF

In a final volume of 100 μL, 100 μM *wt*-PhaEC<sub>Av</sub> in 200 mM KPi (pH 7.80) was incubated with 20.0 mM HBOCoA at 30 °C. Aliquots of 13.0 μL were withdrawn at selected time intervals (1.0 to 6.0 hr) and the enzyme was deactivated by heat treatment at 95 °C for 5 min. At the end of the time course, the reaction mixture was centrifuged and the supernatant was removed and placed on ice. The precipitated protein was washed with ddH<sub>2</sub>O (100 μL ×2). The pooled washes were combined with the supernatant and then filtered using a Whatman microsyringe filter. The filtrate was subject to HPLC. Each peak was lyophilized

to dryness, re-dissolved in minimal ddH<sub>2</sub>O, and desalted by a Zip Tip. The concentrated peak was analyzed by MALDI-TOF MS in a negative mode using  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) as the matrix.

### Determination of Kinetic Parameters of HBOCoA and Rate of the Formation of Intermediate (HB)<sub>n</sub>OCOA (n=2-3)

Reactions and HPLC analysis were conducted in the same way as described above except that adenosine 5'-triphosphate (ATP) was added as an internal standard. The HBOCoA concentration varied from 0.50 to 20.0 mM and each concentration was performed in duplicate. The kinetic parameters were determined by fitting the data to Michaelis-Menten equation 1 using SigmaPlot. Rate of the formation of intermediate (HB)<sub>n</sub>OCOA (n=2-3) was determined when the enzyme was saturated with the substrate ([HBOCoA] = 20.0 mM).

$$v = \frac{v_{max} [S]}{K_M + [S]} \quad (1)$$

### Incubation of (HB)<sub>n</sub>OCOA (n=2-3) with wt-PhaEC<sub>AV</sub>

In a final volume of 150  $\mu$ L, 50.0  $\mu$ M wt-PhaEC<sub>AV</sub> in 200 mM KPi (pH 7.80) was incubated with 5.00 mM (HB)<sub>n</sub>OCOA (n=2-3) at 30 °C. Aliquots of 13.0  $\mu$ L were removed at different time points (10 min to 3.0 hrs) and the enzyme was deactivated by heat treatment at 95 °C for 5 min. Each sample was added ATP as an internal standard and then processed and analyzed by HPLC and MALDI-TOF MS as described above. The rate of formation of each peak was determined by initial slope of the reaction.

### Reaction of HBOCoA with Primed Synthase [<sup>3</sup>H]-sT-PhaEC<sub>AV</sub> and Analysis of Small Molecules by HPLC and MALDI-TOF MS

In a final volume of 50  $\mu$ L, 1.30 mM wt-PhaEC<sub>AV</sub> and 13.0 mM [<sup>3</sup>H]-sT-CoA (832 cpm/nmol) in 200 mM KPi (pH 7.80) were incubated at 25 °C for 3 min before loading the mixture onto a Sephadex G-50 column (5  $\times$  80 mm) pre-equilibrated with 200 mM KPi (pH 7.80) at 4 °C. The acylated protein [<sup>3</sup>H]-sT-PhaEC<sub>AV</sub> was isolated in 90% yield and then immediately reacted with 5.00 mM HBOCoA. The reaction was performed with KinTeK RQF-3 at room temperature. The final concentration of synthase was 32.0  $\mu$ M, of which 0.13 equiv was attached with a [<sup>3</sup>H]-sT chain (4.20  $\mu$ M). At defined time points (10-300 ms), the reaction was quenched with 2% HClO<sub>4</sub>. Each sample was then centrifuged and the supernatant was removed and placed on ice. The precipitated protein was washed with ddH<sub>2</sub>O (75  $\mu$ L  $\times$  3), redissolved in 100  $\mu$ L 20% SDS, and analyzed by scintillation counting. The pooled washes were combined with supernatant. The pH of the solution was adjusted to ~6.0 using pre-determined amount of chilled 2M NaOH. The solution was then centrifuged at 4 °C to remove any insoluble particles. Aliquots of 410  $\mu$ L were injected onto HPLC column. Fractions of 1.00 mL were collected and each fraction was analyzed by scintillation counting. The total recovery yields of radioactivity (including supernatants and protein precipitates) ranged from 85% to 90%.

Eluents at 43.5–45.5 min and 47.0–49.0 min were combined. They were processed and analyzed by MALDI-TOF MS in the same way as described above.

The observed rate of formation of [ $^3\text{H}$ ]-sTet-O-CoA was determined by fitting the data to exponential rise to maximum  $y=a(1-e^{-bx})$  using SigmaPlot.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENT

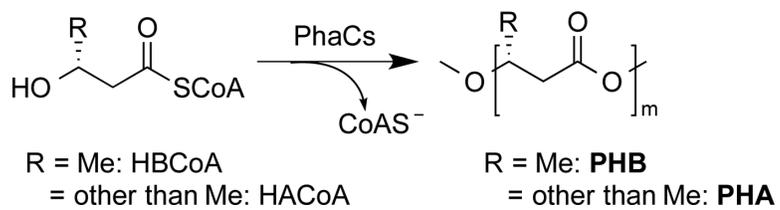
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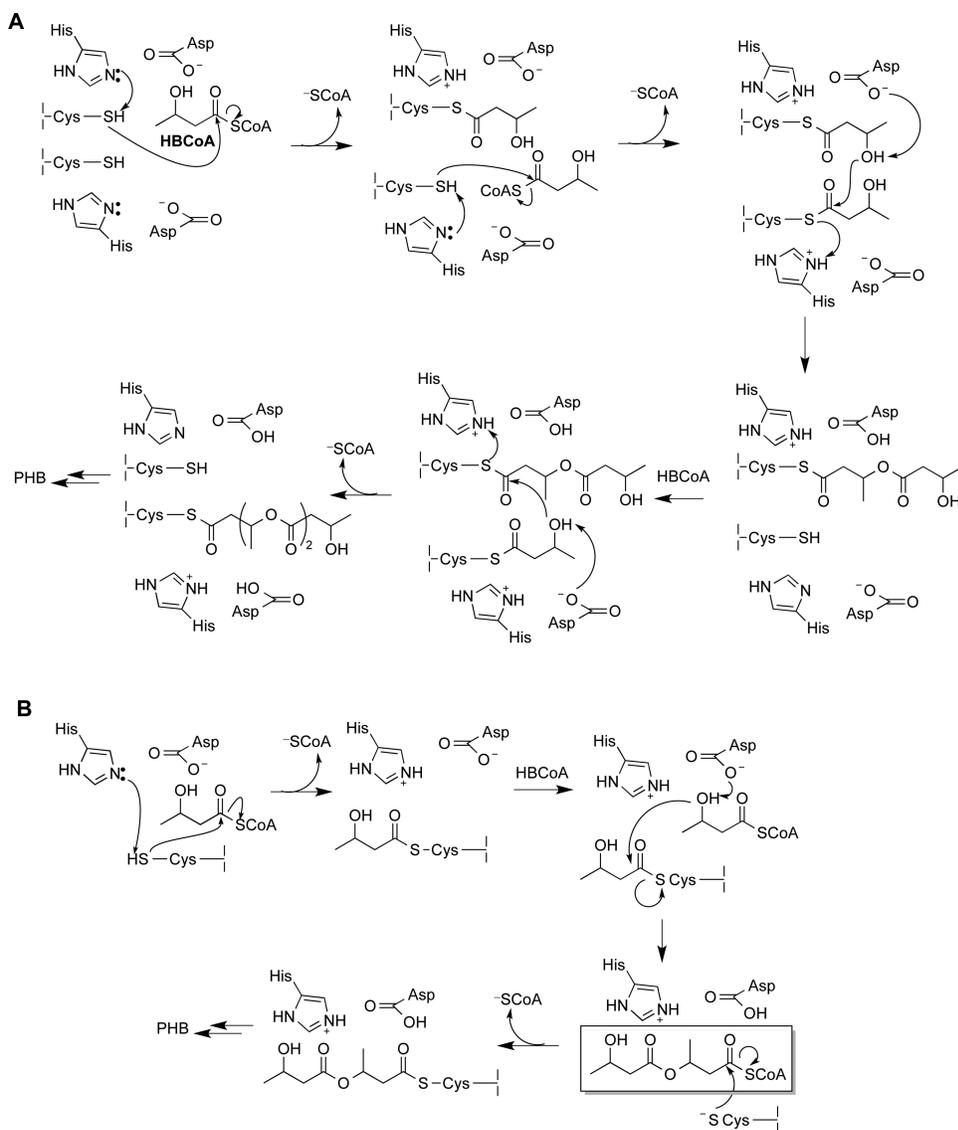
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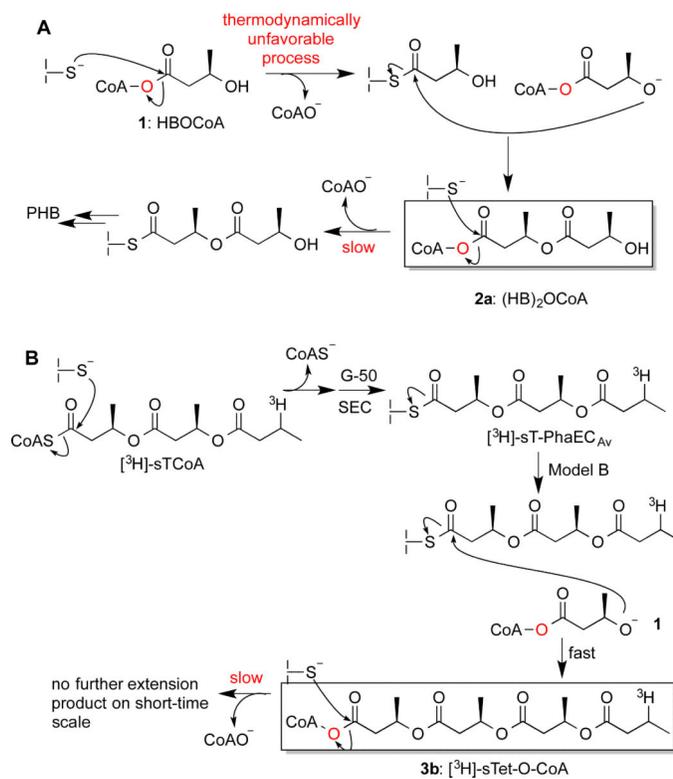
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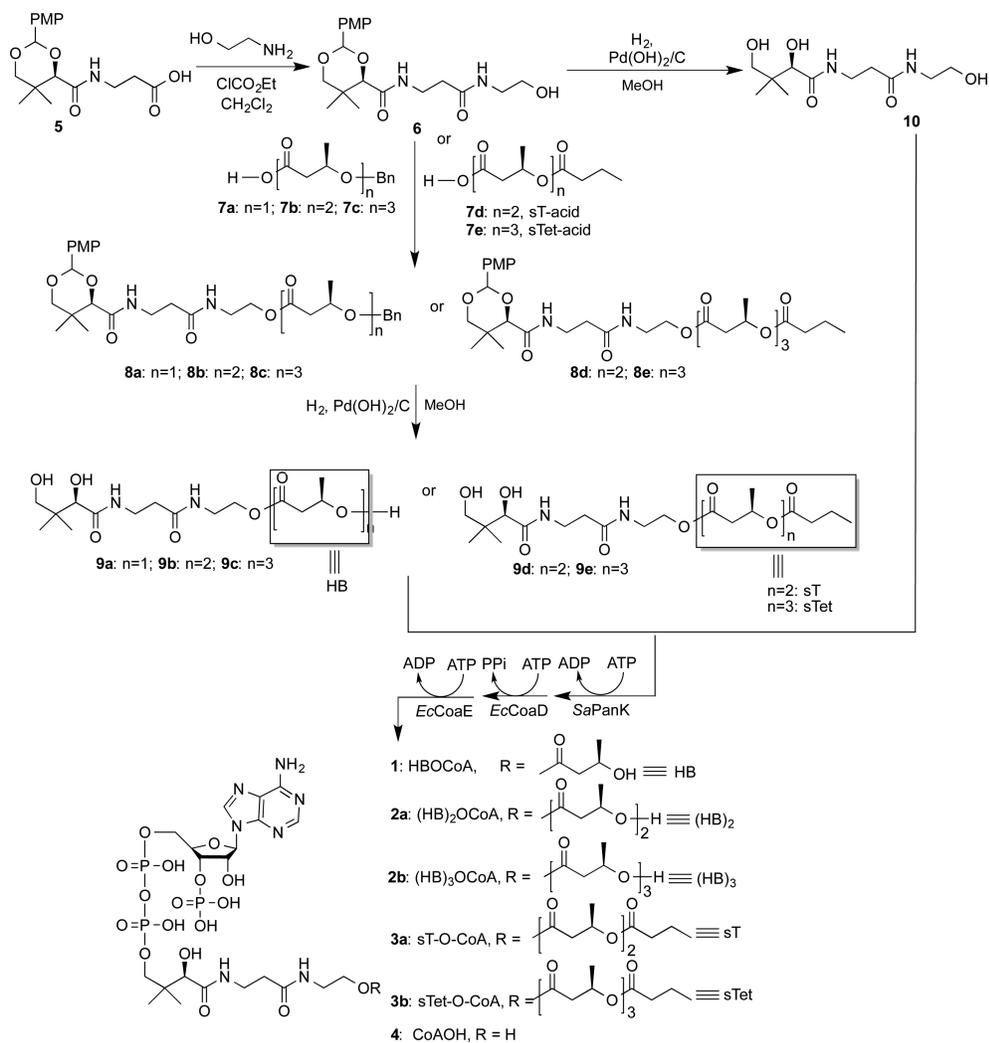
**Scheme 1.**  
PHA synthesis by PhaCs



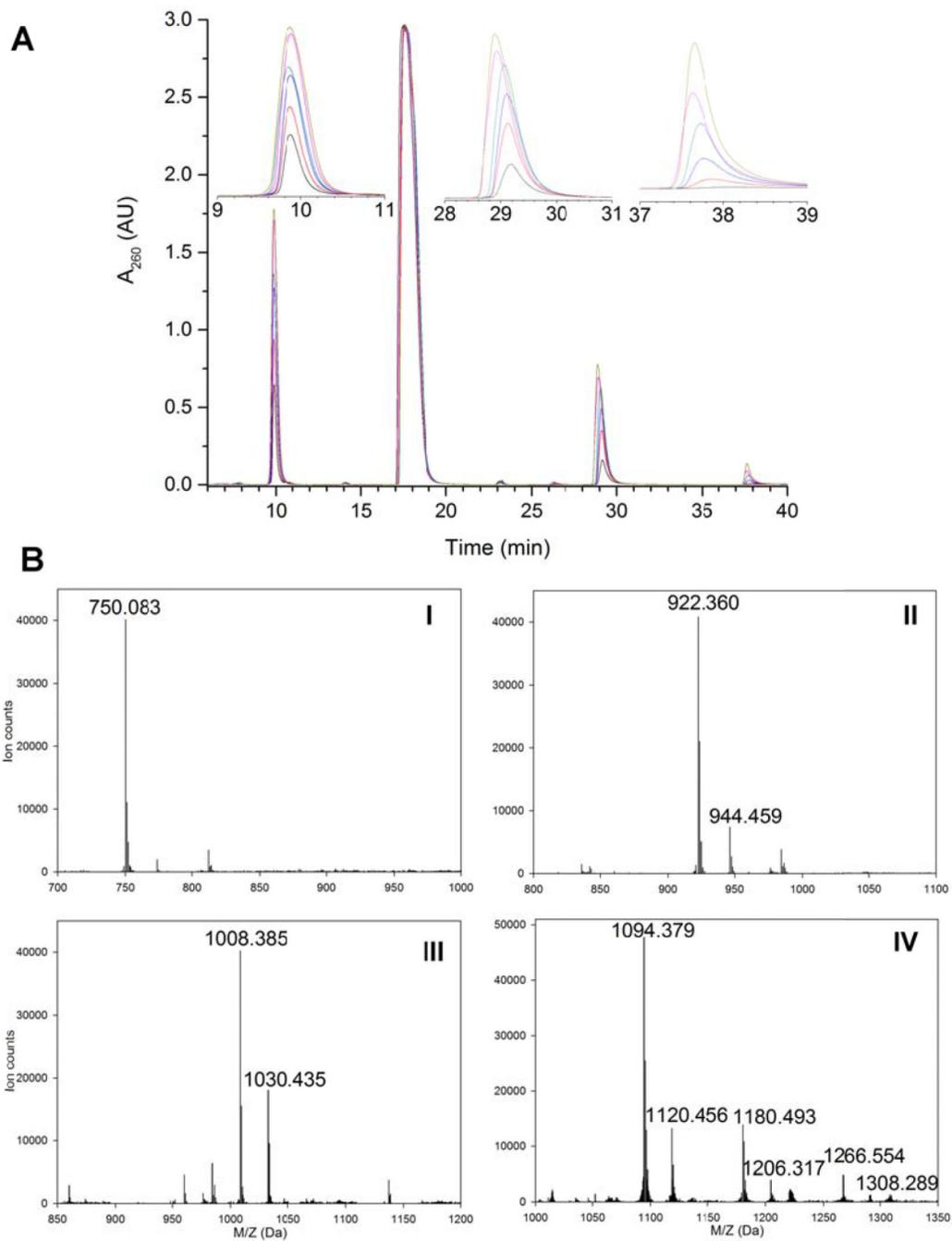
**Scheme 2.**  
Mechanistic models of PHB chain elongation

**Scheme 3.**

Strategies of using HBOCoA as a probe to test the preferred mechanistic model B



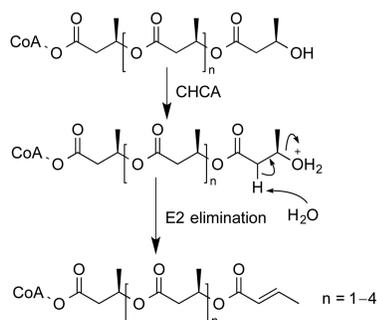
**Scheme 4.**  
Chemoenzymatic synthesis of HBOCoA and related analogs



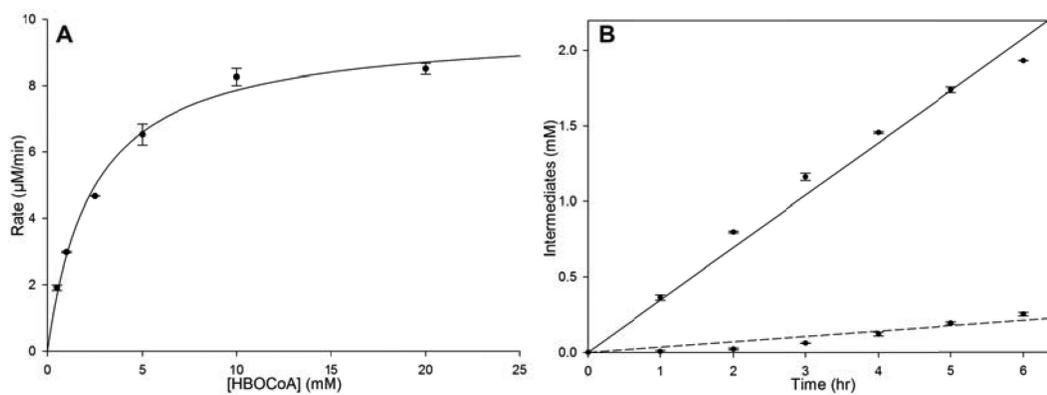
C

	Intermediate	MW		Assignment
		Calcd.	Obsvd.	
I	CoAOH	750.131	750.083	$[M-H]^-$
II	$(HB)_2OCoA$	922.204	922.360	$[M-H]^-$
		944.186	944.459	$[M-2H+Na]^-$
III	$(HB)_3OCoA$	1008.241	1008.385	$[M-H]^-$
		1030.223	1030.435	$[M-2H+Na]^-$
IV	$(HB)_4OCoA$	1094.278	1094.379	$[M-H]^-$
		1120.231	1120.456	$[M-H_2O-3H+2Na]^-$
	$(HB)_5OCoA$	1180.315	1180.493	$[M-H]^-$
		1206.268	1206.317	$[M-H_2O-3H+2Na]^-$
	$(HB)_6OCoA$	1266.351	1266.554	$[M-H]^-$
		1308.279	1308.289	$[M-H_2O-3H+Na+K]^-$

D

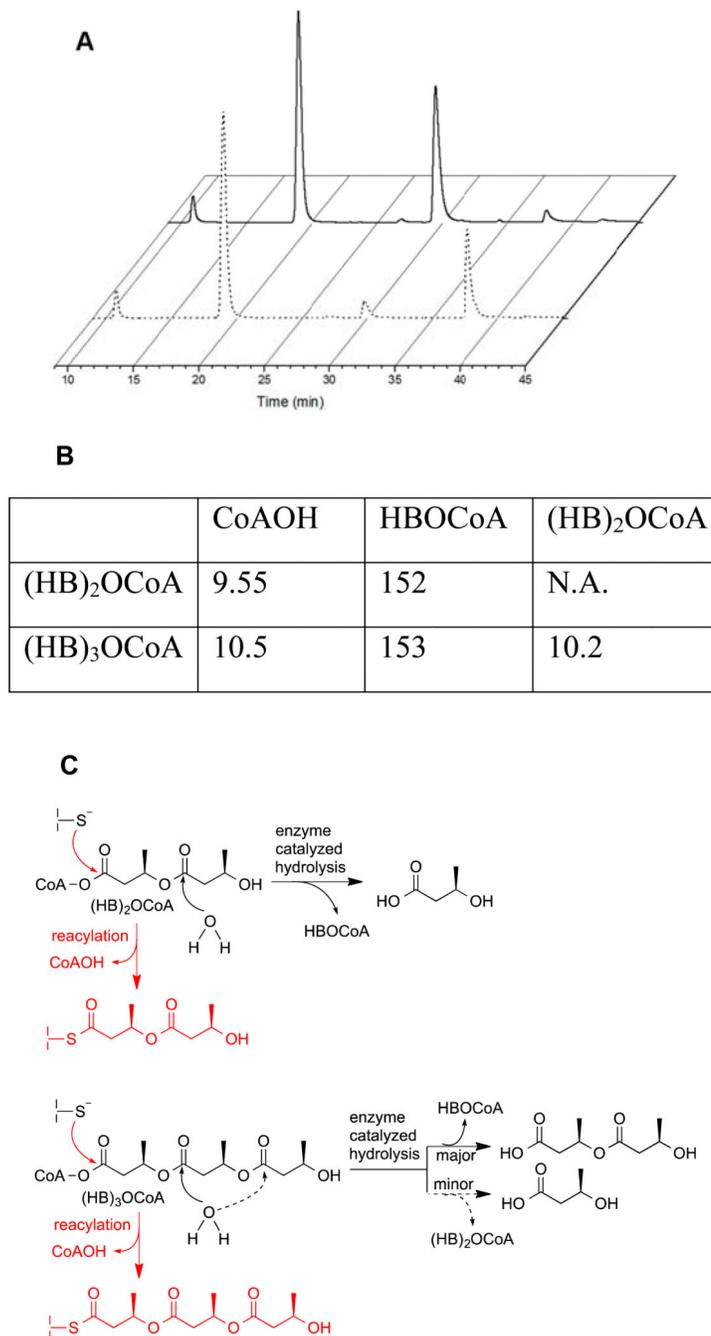
**Figure 1.**

(A) Formation of  $(HB)_nCoA$  intermediates during incubation of 100  $\mu M$  *wz*-PhaEC<sub>AV</sub> with 20.0 mM HBOCoA at 30 °C for 1 (black), 2 (red), 3 (green), 4 (blue), 5 (orange), and 6 hrs (pink). HPLC condition is described in EXPERIMENTAL SECTION; (B) MALDI-TOF MS of species eluted at 9.80 (I), 29.0 (II), 37.7 (III), and 40.0–60.0 min (IV); (C) Assignment of peaks shown in B; (D) Conversion of the terminal HB unit into a crotonate in the presence of acid.

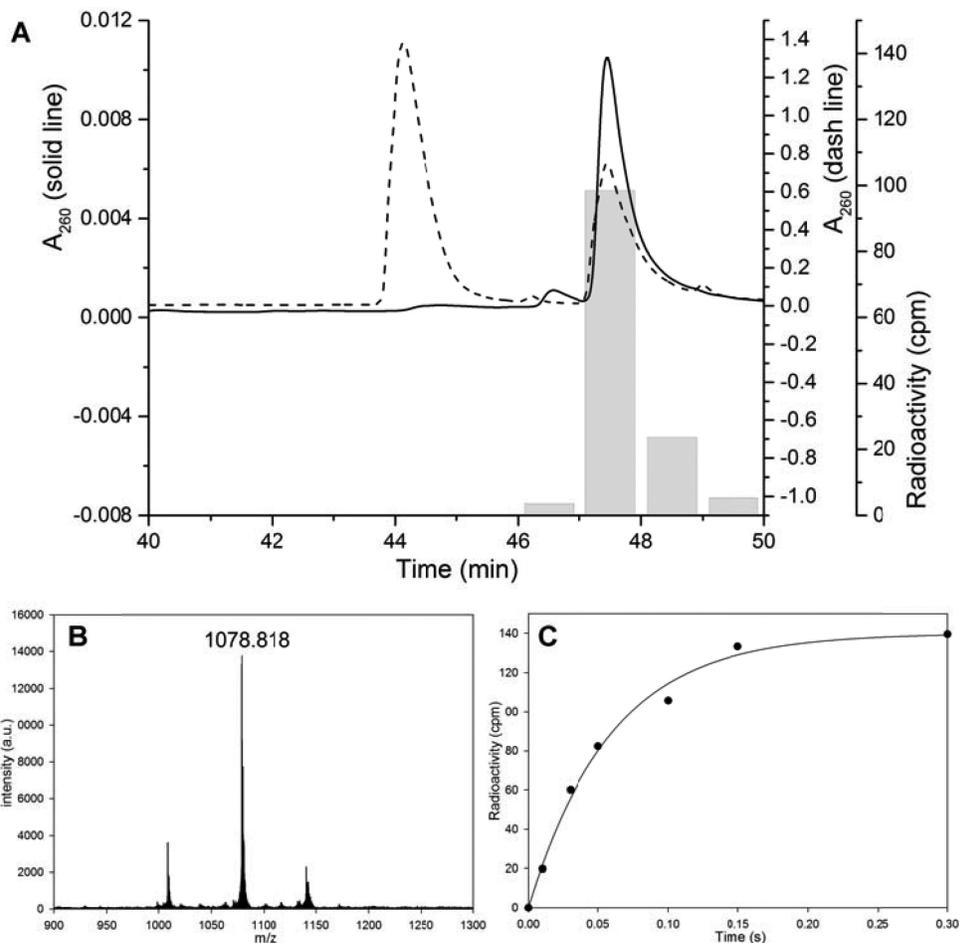


**Figure 2.**

(A) Kinetic analysis of HBOCoA with 100 µM *wt*-PhaEC<sub>Av</sub>. The data were fitted to Michaelis–Menten equation to give a  $K_M = 2.38 \pm 0.23$  mM and  $V_{max} = 9.73 \pm 0.28$  µM•min<sup>-1</sup>. The reaction was performed in duplicate. (B) Rates of formation of (HB)<sub>2</sub>OCoA (solid line) and (HB)<sub>3</sub>OCoA (dash line) in the presence of 100 µM *wt*-PhaEC<sub>Av</sub> and 20 mM HBOCoA. Linear regression of the data gave the slopes of  $3.47 \times 10^{-1}$  and  $3.51 \times 10^{-2}$  mM•hr<sup>-1</sup> to (HB)<sub>2</sub>OCoA and (HB)<sub>3</sub>OCoA, respectively. The reaction was performed in duplicate.

**Figure 3.**

(A) HPLC profiles of incubating 50.0  $\mu\text{M}$  *wt*-PhaEC<sub>Av</sub> with 5.00 mM (HB)<sub>2</sub>OCoA (solid line) or (HB)<sub>3</sub>OCoA (dash line) for 30 min. The  $t_R$  of CoAOH, HBOCoA, (HB)<sub>2</sub>OCoA, and (HB)<sub>3</sub>OCoA are 9.80, 17.7, 29.0, and 37.7 min, respectively; (B) Estimated rates of product formation in  $\text{hr}^{-1}$ ; (C) Proposed pathways for product formation from incubation of (HB)<sub>n</sub>OCoA ( $n = 2-3$ ) with *wt*-PhaEC<sub>Av</sub>.



**Figure 4.**

(A) Typical HPLC (solid line) and radioactivity (bar) profiles of the reaction between  $4.20 \mu\text{M}$   $[^3\text{H}]$ -sT-PhaEC<sub>Av</sub> and  $5.00 \text{ mM}$  HBOCoA stopped by RQF. The dash line represents HPLC profile of synthesized standards, sT-O-CoA (44.1 min) and sTet-O-CoA (47.4 min); (B) MALDI-TOF MS of species eluted at 47.0-49.0 min. (C) Rate of formation of  $[^3\text{H}]$ -sTet-O-CoA in the reaction between  $4.20 \mu\text{M}$   $[^3\text{H}]$ -sT-PhaEC<sub>Av</sub> and  $5.00 \text{ mM}$  HBOCoA. The data were fitted to  $y = a(1 - e^{-bx})$ , where  $a$  = burst amplitude and  $b = k_{obs}$ . The fit gives an amplitude of  $139.7 \pm 2.2 \text{ cpm}$  (counts per minute) and a  $k_{obs}$  of  $17.4 \pm 0.8 \text{ s}^{-1}$ .