How to cite: *Angew. Chem. Int. Ed.* **2023,** *62,* e202218312
International Edition: doi.org/10.1002/anie.202218312
German Edition: doi.org/10.1002/ange.202218312

Biosynthesis

ATP-Independent and Cell-Free Biosynthesis of β-Hydroxy Acids Using Vinyl Esters as Smart Substrates

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Abstract: In vitro biosynthetic pathways that condense and reduce molecules through coenzyme A (CoASH) activation demand energy and redox power in the form of ATP and NAD(P)H, respectively. These coenzymes must be orthogonally recycled by ancillary reactions that consume chemicals, electricity, or light, impacting the atom economy and/or the energy consumption of the biosystem. In this work, we have exploited vinyl esters as dual acyl and electron donor substrates to synthesize β-hydroxy acids through a non-decarboxylating Claisen condensation, reduction and hydrolysis stepwise cascade, including a NADH recycling step, catalyzed by a total of 4 enzymes. Herein, the chemical energy to activate the acyl group with CoASH and the redox power for the reduction are embedded into the vinyl esters. Upon optimization, this self-sustaining cascade reached a titer of (*S*)-3-hydroxy butyrate of 24 mM without requiring ATP and simultaneously recycling CoASH and NADH. This work illustrates the potential of in vitro biocatalysis to transform simple molecules into multi-functional ones.

Introduction

Living systems synthesize complex molecules starting from simple building blocks through enzyme-catalyzed synthetic pathways under mild and non-hazardous conditions. The unique features of biosynthetic pathways underpin one of the pillars of modern biotechnology; the biomanufacturing of highly specialized chemicals from renewable feedstock and wastes. Both primary and secondary metabolisms universally activate carboxylic acids with coenzyme A (CoASH) to form acyl-CoAs at the expense of ATP. Moreover, when CoA derivatives are subjected to further reduction steps, the pool of redox cofactors (NAD(P)H) also needs to be regenerated using ancillary electron donors. These acyl-CoAs derivatives are the central building blocks Nature employs to access the rich chemical diversity found in the metabolomes of living systems. For example, short chain acyl-CoAs, such as acetyl-CoA or malonyl-CoA, serve as precursors for the synthesis of antibiotics, antifungals or alkaloids.[1] For decades, metabolic engineering has widely exploited acyl-CoA derivatives as intermediates to manufacture high-added value products.[2] As an alternative, artificial cell-free circular metabolisms are emerging to fix CO_2 into acyl-CoA, yielding C2–C3 compounds in the μM range.[3] Unfortunately, these in vitro systems have been poorly intensified as they demand efficient systems for the in situ regeneration of CoASH, ATP, and NAD(P)H. Recently, light-driven systems based on thylakoid membranes allow the coupled recycling of these energetic, redox and acylating cofactors simultaneously.[4]

The transformation of organic acids into β-hydroxy acids (β-HA) through the concurrent ATP-dependent Acyl-CoA synthesis, CoA-dependent Claisen condensation and NAD(P)H-dependent asymmetric reduction is an illustrative case where all these cofactors must be recycled in situ. This cascade has been widely utilized in vivo reaching a titer of

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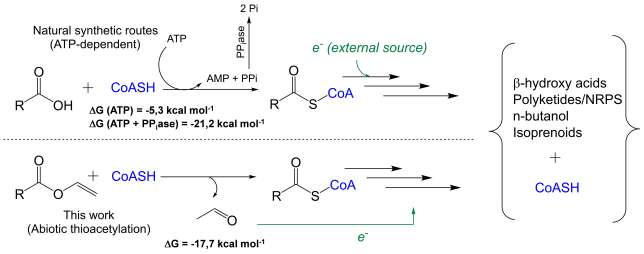
≈3 g L^{-1},[5] but it is less often exploited in vitro. Mostly, these biosynthetic pathways have been coupled to the synthesis of polyhydroxy butyrates (PHBs).[4b,6] Only a few examples of this cascade have reached a maximum of 40 mM of 3-hydroxy butyrate in the form of PHBs (80 % theoretical maximum conversion). This cascade demands ATP and NAD(P)H; two cofactors that have been successfully recycled through in vitro photosynthesis.[4b]

If other starting substrates are used beyond carboxylic acids, the acyl-CoAs can be abiotically synthesized without consuming ATP. For example, organic acid anhydrides, lactones[7] and thioesters[8] can be nucleophilically attacked by free CoASH forming the corresponding acyl-CoA derivative requiring neither ATP nor catalyst. Alternatively, Contente et al. have engineered an acyl transferase from *Mycobacterium smegmatis* (MsACT-S11C) to synthesize acyl-CoA derivatives from vinyl esters and CoASH.[9] However, this enzymatic reaction remains unexplored in acyl-CoA dependent enzyme cascades where the CoASH is recycled.

In this work, we report an unprecedented ATP-independent CoASH S-acylation of vinyl esters that supplies acyl-CoA to a subsequent enzyme cascade for the in vitro biosynthesis of β-HAs. We have discovered that the abiotic thiolysis of vinyl esters with limiting concentrations of CoASH quantitatively forms acyl-CoAs with reaction rates indistinguishable from the ones observed with MsACT-S11C. Then, we coupled the abiotic thiolysis of vinyl esters with the biosynthesis of β-HA, harnessing the acetaldehyde formed in the thiolysis of vinyl esters as an electron donor to enzymatically regenerate the reduced nicotinamide cofactor consumed by the asymmetric reduction that follows the Claisen condensation of the Acyl-CoAs (Scheme 1). To assemble this artificial cascade, we have screened several thiolases[10] to perform the non-decarboxylative Claisen condensation, several oxidoreductases[11] for the reduction of the condensed β-keto thioesters and several thioesterases[12] to release the free carboxylic acid. This is a self-sustaining enzymatic cycle that enables Acyl-CoA synthesis requiring neither ATP nor co-substrate for the regeneration of the redox cofactor. Herein, we have proven vinyl esters as smart substrates where their acyl group forms the carbon scaffold of the target molecules (β-HA in this case), and the vinyl group (in equilibrium with acetaldehyde) is utilized by an aldehyde dehydrogenase as an electron donor to replenish the pool of the reduced cofactor, accumulating acetic acid as a by-product. This novel artificial pathway was not only applied for linear condensation reactions but also for branched ones. Finally, the versatility of the abiotic thiolysis was demonstrated using N-acetylcysteamine as a CoASH surrogate.



Scheme 1. Cell-free routes for acyl-CoAs biosynthesis and their further reductive condensation toward a plethora of highly-value added products. Conventional cell-free pathways rely on ATP and exogeneous electron donors (top). New ATP-independent pathway from vinyl esters (this work), which uses the product released from the abiotic thiolysis as electron donor (bottom). The free energies of the S-acylation steps are calculated with eQualibrator.

Results and Discussion

In the search for alternatives to synthesize acyl-CoAs without requiring ATP, we found that Contente et al.[9] developed a new variant of the acetyltransferase from *Mycobacterium smegmatis* (MsACT-S11C) that acetylates CoASH starting from vinyl acetate (VA) as acetyl donor using a high concentration of CoASH (100 mM) and a 10-fold excess of vinyl acetate. This is an elegant enzyme reaction that can feed biosynthetic pathways with acetyl-CoA, but it has never been explored yet. Before coupling the ATP-independent CoASH activation with acyl-CoA dependent enzymes, we carried out this reaction with a lower CoASH concentration (1 mM) and higher VA : CoASH molar ratio (100 : 1) than Contente et al. Under these conditions, we found that CoASH was spontaneously acetylated with an excess of vinyl ester in the absence of the acyltransferase (Figure 1A). ^{1}H NMR supports this abiotic reaction, identifying the chemical fingerprint of the acetyl-CoA when CoASH and VA are mixed (Figure S1). The

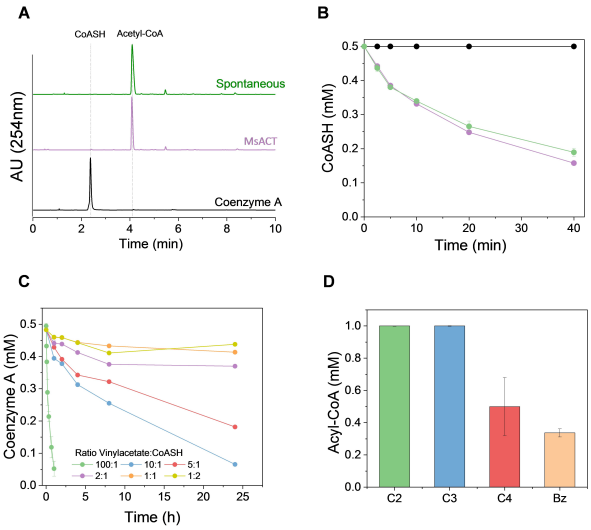


Figure 1. A) 24 h UPLC-MS chromatograms and B) reaction time courses of the abiotic thiolysis without (green) and with MsACT-S11C (purple) starting from 1 mM Coenzyme A (CoASH) and 100 mM vinyl acetate (VA) at pH 8 and 25 °C. C) Time courses of the abiotic acetyl-CoA synthesis at pH 8 and 25 °C with different VA : CoASH molar ratios. D) Synthesis of Acyl-CoA derivatives from different vinyl esters after 1 h of reaction at pH 8 and 25 °C, using a 100 : 1 vinyl ester:CoASH molar ratio. C2: Vinyl acetate. C3: Vinyl propionate. C4: Vinyl butyrate. Bz: Vinyl benzoate.

time courses of the reactions with and without MsACT-S11C showed that spontaneous and enzyme driven thiolysis have similar kinetics (Figure 1B). This insight points out that low CoASH concentration and high VA:CoASH molar ratio enable the abiotic thiolysis in absence of enzyme, contrary to what Contente et al. observed at higher CoASH concentration and lower VA:CoASH molar ratios. When ethyl acetate was used as acetyl donor, neither the spontaneous nor the enzymatic reactions worked. Free energy (ΔG) reaction profiles calculated at the Density Functional Theory level (see Supporting Information, Figure S2) show that the acetyl-transfer reaction between VA and the surrogate of CoASH (N-acetyl cysteamine) is exergonic ($\Delta G = -8.7$ kcal mol^{-1}), while using ethyl acetate as acetyl donor makes the reaction endergonic ($\Delta G = 6.38$ kcal mol^{-1}). Hence, the tautomerization of vinyl alcohol to acetaldehyde makes the thiolysis reaction thermodynamically more favorable when using VA than when using ethyl acetate. The thermodynamics of the reactants and products support the spontaneity of the reaction we have discovered.

To further understand this abiotic CoASH S-acetylation, we studied different reaction conditions. Figure 1C shows that the higher the VA:CoASH molar ratio, the higher the S-acetylation rate. We quantitatively acetylated CoASH in just 1 hour using a 100:1 VA:CoASH molar ratio with a first-order kinetic constant of 2.68 h^{-1}. However, similar VA:CoASH molar ratio (10:1) to those one used by Contente et al.[9] led to an S-acetylation rate 30 times slower than the 100:1 ratio and yielded only 20 % acetyl-CoA after the same reaction time. Remarkably, the abiotic reaction did not occur at an equimolar and <1 VA:CoASH ratio, supporting the idea that this abiotic reaction heavily relies on the excess of vinyl acetate. Besides the reactant ratio, we studied the influence of the pH and the carbon chain length of the vinyl ester on the abiotic thiolysis. This abiotic reaction quantitively occurs in the range of pH 8–9 and is precluded at pH 6 (Figure S3). Like in enzyme-driven ester thiolysis[9] and amide hydrolysis,[13] we suggest that the abiotic reaction is also driven by the formation of the thiolate that attacks the carbonyl group, enabling the thiolysis. This proposed mechanism is supported by the higher acetyl-CoA yields we obtained under alkaline conditions as the mercapto group of CoASH ($pK_a = 10.35$)[14] needs high pH values to be deprotonated. Finally, we studied the scope of the abiotic thiolysis using different vinyl esters with different acyl substituents (Figures 1D, S4A and S5). Like using VA, we observed quantitative S-acylation of CoASH using vinyl propionate after 1 hour reaction at pH 8 and vinyl ester:CoASH molar ratio of 100:1. Under the same reaction conditions, only 40 % and 33 % of CoASH was spontaneously S-acylated to butyryl-CoA or benzoyl-CoA when using vinyl butyrate or vinyl benzoate as acyl donors, respectively. The lower yield of butyryl-CoA agrees with the lower thiolysis rate ($k = 0.013$ min^{-1}) measured for the abiotic CoASH S-acylation with vinyl butyrate. Moreover, the abiotic thiolysis also worked using N-acetylcysteamine (SNAC) as a CoASH surrogate (Figure S4B). Remarkably, the S-acetylation of that surrogate occurs 2.2 times faster than the S-acetylation of CoASH. The higher reactivity of SNAC is supported by the lower pK_a (9.43)[15] of its thiol compared to CoASH. To the best of our knowledge, the abiotic thiolysis of thioesters has only been reported for the synthesis of S-methyl thioesters using acyl-CoAs,[16] but this is the first experimental evidence of an abiotic thiolysis of an ester using the thiol of CoASH as nucleophile. As the acyl-CoA synthesis efficiently worked spontaneously, we coupled this reaction to different CoA-dependent enzymes. In a first approach, we incubated vinyl propionate with CoASH for the in situ formation of propionyl-CoA that is further reduced by a deacetylating CoA-dependent aldehyde dehydrogenase from *Salmonella enterica* (PduP) in presence of NADH. When the vinyl ester, PduP, CoASH and NADH were mixed, we detected propionaldehyde by GC, while this product was detected neither when the enzyme was mixed only with CoASH and NADH, nor when the vinyl esters were only incubated with the enzyme and NADH. (Figure S6). Encouraged by this result, we decided to explore the potential of the abiotic thiolysis as the CoASH S-acylation step for the cell-free biosynthesis of β-hydroxy acids (β-HAs) (Figure 2A). In this enzyme pathway, the abiotic thiolysis of vinyl esters first synthesizes the acyl-CoA derivative that is sequentially used by a thiolase to perform the non-decarboxylative Claisen condensation that forms a C–C bond between two molecules of acetyl-CoA, releasing one molecule of CoASH. Next, the resulting intermediate, acetoacetyl-CoA, is reduced to 3-hydroxybutyryl-CoA by a NADH-dependent alcohol dehydrogenase. Finally, the resulting thioester is hydrolyzed by a thioesterase yielding the final β-HA and releasing the second CoASH molecule. Theoretically, this cascade fully recycles CoASH but requires 1 equivalent of NADH per mol of β-HA that needs to be replenished to avoid the excess of this costly cofactor. When ΔG energies are calculated for each reaction step using eQuilibrator[17] we observed that the reaction starting from VA ($\Delta G = -27.5$ kcal mol^{-1}) is almost 2 times more thermodynamically favorable than the pathway starting from acetate and using ATP as the energy source ($\Delta G = -15.1$ kcal mol^{-1}). (Figure 2B). ΔG of the ATP-independent S-acetylation of CoASH is similar to that of the native route considering the subsequent hydrolysis of the pyrophosphate catalyzed by pyrophosphatases ($\Delta G = -31$ kcal mol^{-1}). These thermodynamics support the 20 % higher S-acetylation rate found for the abiotic thiolysis of VA in comparison with the ATP-dependent acetyl-CoA synthesis from acetate driven by the acyl-CoA synthase from *Archaeglobus fulgidus* (Figure S4C). As this enzyme produces ADP + P_i,[18] its coupling with pyrophosphatase would be futile to favor the thermodynamics. In absence of ATP, the conversion of acetate into 3-hydroxy butyrate (3-HB) is highly endergonic. Therefore, by replacing acetate with VA as the initial substrate, the biosynthesis of 3-HB becomes thermodynamically feasible without using ATP. To assemble this cell-free enzyme cascade integrating the abiotic activation of CoASH, we first in vitro screened the activity of different thiolases, alcohol dehydrogenases and thioesterases toward their corresponding substrates (Figure 2C–E and Table S1). For the non-decarboxylative acetyl-CoA condensation, we selected the

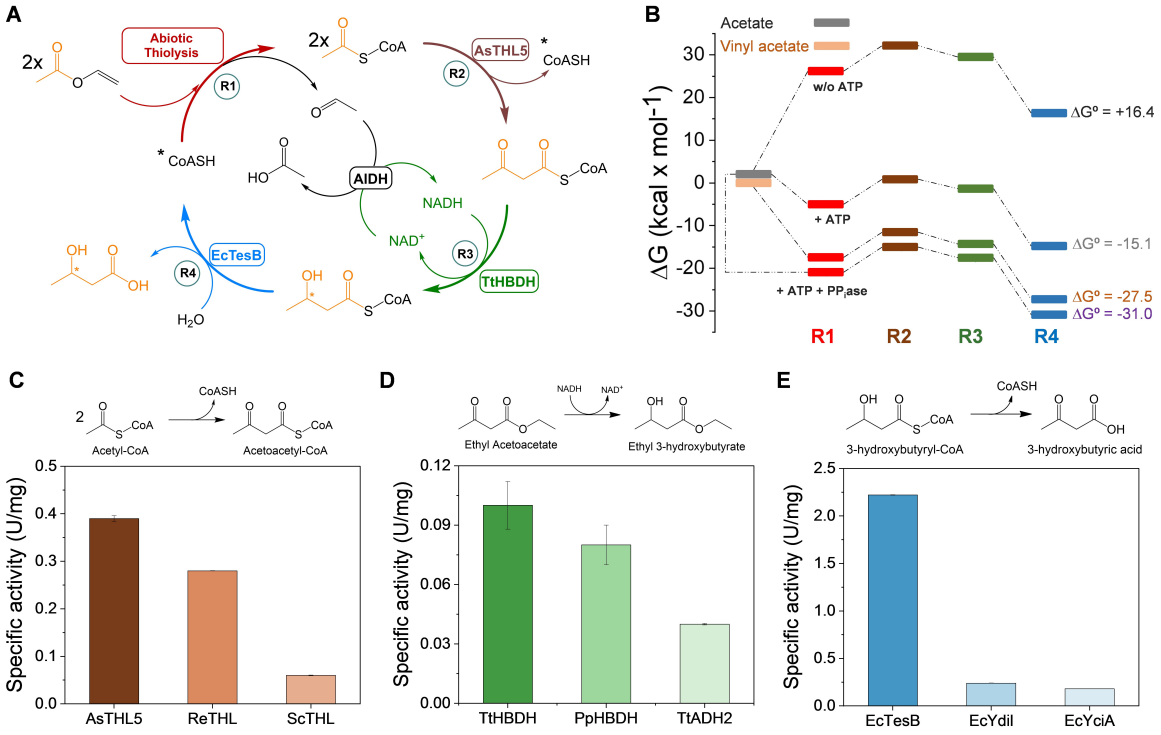


Figure 2. A) Reaction scheme of the 4-enzyme artificial biosynthetic pathway to synthesize 3-HB through the sequential abiotic thiolysis of vinyl acetate with free CoASH (R1), the Claisen condensation of acetyl-CoA (R2), the reduction of the β-ketoacyl-CoA (R3) and its hydrolysis (R4) with in situ regeneration of NADH (R5) and CoASH (*released in step 2 and 4). B) Standard Gibbs free energy changes of each reaction step using eQuilibrator under conditions of pH 8 and ionic strength of 0.25 M. Reaction numbers refers to the reactions depicted in panel A, where R1 means the S-acetylation of CoASH with acetate (grey), in absence of ATP, in presence of ATP and in presence of ATP plus pyrophosphatase (PP_iase), or the thiolysis of vinyl acetate with CoASH (orange). C) Specific activity of three thiolases (AsTHL5, ReTHL and ScTHL) toward acetyl-CoA at pH 8 and 30 °C. D) Specific activity of three alcohol dehydrogenases (TtHBDH, PpHBDH and TtADH2) toward the reduction of ethyl acetoacetate (a surrogate of acetoacetyl-CoA) using NADH as cofactor at pH 8 and 30 °C. E) Specific activity of three thioesterases (EcTesB, EcYdiI and EcYciA) toward the hydrolysis of 3-hydroxybutyryl-CoA at pH 8 and 30 °C.

previously described thiolases from *Ascaris suum* (AsTHL5, gen *acat5*),[19] *Ralstonia eutropha* (ReTHL, gen *bktB*)[20] and *Saccharomyces cerevisiae* (ScTHL, gen *erg*).[21,21] Among them, AsTHL5 was the most active thiolase at pH 8 and 30 °C with a specific activity of 0.39 U mg^{-1} (Figure 2C). For the reduction of the acetoacetyl-CoA, we tested different dehydrogenases from *Thermus thermophilus* HB27 (TtHBDH, gen *TT_C0898*, and TtADH2, gen *TT_C0331*)[22] and *Pseudomonas putida* (PpHBDH, gen *FadB*).[23] The TtHBDH exhibited 1.4 and 2 times higher activity than PpHBDH and TtADH2, respectively, toward ethyl acetoacetate as a surrogate substrate of acetoacetyl-CoA (Figure 2D). Last, we tested 3 different thioesterases from *E.coli*; EcTesB, EcYdiI and EcYciA.[24] In agreement with the literature, the EcTesB presented 10 times more activity than the other two toward β-hydroxyacyl-CoAs (Figure 2E) with a high selectivity toward this substrate compared to acetyl-CoA (Figure S7). This substrate selectivity of EcTesB is fundamental to achieving high yields of β-HA as it minimizes the derailment of acetyl-CoA to an end product like the acetate. Through studying the optimal pH of each enzyme, we selected pH 8 as consensus pH to perform this cell-free system (Figure S8). Finally, we need an enzyme able to replenish the pool of NADH that sustains the whole cascade. As acetaldehyde is formed as a by-product from the abiotic thiolysis of VA, we decided to use such aldehyde as an electron donor to regenerate the NADH pool that sustains the activity of the acyl-CoA dehydrogenase. To that aim, we selected the aldehyde dehydrogenase from *Saccharomyces cerevisiae* (yAlDH) to orthogonally transform acetaldehyde into acetate with the concomitant reduction of NAD^+ to NADH. This enzyme was selected based on its excellent kinetic parameters for the oxidation of short-chain aldehydes.[25] When this enzyme was not added to the reaction mix, 3-HB was not detected, indicating that the recycling system is mandatory to steer the enzyme cascade.

Having in hand the best three enzymes to condensate, reduce and hydrolyze the abiotically formed acetyl-CoA, we mixed them in an activity ratio of 1:2:1 (AsTHL5: TtHBDH:EcTesB) using 0.05 U mL^{-1} of the thiolase. After 16 hours and upon enzyme separation, the acyl-CoA intermediates were detected and identified by UPLC/MS using commercial standards. Figure 3 shows that CoASH (1) was quantitatively acetylated with VA in the absence of the enzymes. When AsTHL5 was added to the reaction mix, we detected the formation of acetoacetyl-CoA (3), demonstrating that the acetyl-CoA (2) is condensed by the thiolase. When AsTHL5 was mixed with TtHBDH, a new peak

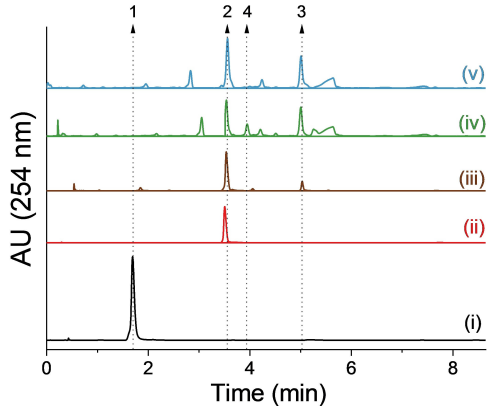


Figure 3. UPLC-MS chromatograms of the reaction crude upon 24 hours. (i) Starting material (1 mM CoASH). (ii) abiotic thiolysis with 1 mM CoASH and 100 mM VA. (iii) AsTHL5 added to (ii). (iv) TtHBDH added to (iii). (v) EcTesB added to (iv). Peak 1: Coenzyme A; Peak 2: Acetyl-CoA; Peak 3: Acetoacetyl-CoA; Peak 4: 3-hydroxybutyryl-CoA. The reactions were carried out at 30 °C and pH 8, starting with 100 mM VA, 1 mM CoASH, 1 mM NADH.

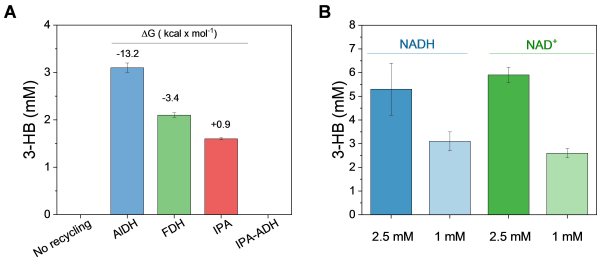


Figure 4. A) 3-HB titer using the different NADH recycling systems. In all cases, 0.33 U mL^{-1} of each recycling enzyme was added. Numbers on the top of the bars are the ΔG of the NADH recycling reactions calculated with eQuilibrator under conditions of pH 8 and ionic strength of 0.25 M. B) 3-HB titer starting from different concentrations of NAD(H) at different redox states. All reactions were done at 30 °C and pH 8.

appears corresponding to 3-hydroxybutyryl-CoA (4). Finally, the addition of EcTesB led to the disappearance of the 3-hydroxybutyryl-CoA from the reaction media. Next, these three enzymes were combined with yAlDH (in excess of 13 times regarding of the thiolase) for the NADH recycling, using the acetaldehyde the abiotic thiolysis releases. Upon 24 h of the 4-enzyme reaction, we detected the formation of 3-HB but also the generation of acetate and acetaldehyde when the reaction crude was analyzed by GC and HPLC (Figure S9). We also confirmed the one-pot transformation of VA into 3-HB by ^{1}H NMR (Figure S10). Because of the (*S*)-enantiopreference of the TtHBDH, we confirmed that *(S)*-3-HB is formed in an enantiomeric excess over 99 % (Figure S11A).[22a] However, we only produced 3.1 ± 0.1 mM 3-HB whereas accumulating 48.8 ± 0.1 mM of acetate, when starting with 100 mM of VA. This means that only 6 % of VA molecules were transformed into the hydroxy acid according to the reaction stoichiometry that demands two molecules of VA to synthesize one molecule of 3-HB. To investigate the origin of such excess of acetate that was generated during the cell-free cascade, we incubated AsTHL5 with VA in the absence of CoASH. As result, 78 % of VA was consumed (Figure S12), which indicates that the thiolase presents a promiscuous activity that can hydrolyze the vinyl esters giving rise to acetate and acetaldehyde as by-products. Next, we evaluated how the yAlDH based NADH-recycling system competes with other well-known redox cofactor recycling systems widely used in the state of the art. Figure 4A shows that the oxidation of acetaldehyde mediated by yAlDH allows the cascade to accumulate more 3-HB than the cascade integrating the formate dehydrogenase from *Candida boidinii* (CbFDH) that oxidizes the added formate to CO_2 (3 mM vs 2 mM 3-HB, respectively), and the auto regeneration catalyzed by TtHBDH using isopropanol (IPA) as co-substrate.[22a] When an extra alcohol dehydrogenase from *Bacillus stearothermophilus* (BsADH) was added in presence of IPA to boost the NADH recycling, 3-HB was not produced at all. Among these NADH regeneration systems, the oxidation of acetaldehyde to acetate ($\Delta G = -13.2$ kcal mol^{-1}) is more exergonic than the oxidation of formate to CO_2 ($\Delta G = -3.4$ kcal mol^{-1}) and IPA to acetone ($\Delta G = +0.9$ kcal mol^{-1}).[17] Therefore, the system employing yAlDH is the most efficient and thermodynamically favoured one and does not require the addition of any ancillary co-substrate as electron donor to replenish the pool of NADH. Using this recycling system, two molecules of VA will theoretically produce one molecule of 3-HB, one molecule of acetate and one molecule of acetaldehyde, recycling the CoASH used for the first step and the NADH for the third one (Figure 2A). To maximize the titer of 3-HB and the cascade productivity, we optimized the initial concentration and the redox state of the nicotinamide cofactor. 3-HB titer was similar using both NAD^+ and NADH as initial redox cofactor, regardless of the starting concentration. Contrariwise, the titer of 3-HB increased 2-fold when the NADH concentration was 2.5-fold higher (Figure 4B).

To better understand the bottlenecks of this cell-free system, we analyzed the reaction time courses and the product profiles when both CoASH and NADH recycling systems are working simultaneously. First, we tested different concentrations of CoASH (0.1–5 mM) but always keeping the CoASH:VA molar ratio higher than 1:10 (Figure S13). In this experiment, we found that the cell-free system accumulates up to 12 ± 1 mM of 3-HB after 6 h using 5 mM CoASH, which means a total turnover number for CoASH (TTN_{CoASH}) and NADH (TTN_{NADH}) of 5. In contrast, using 1 mM of CoASH, the titer was 5 ± 3 mM but the TTN_{CoASH} was 10. Even at CoASH concentration as low as 0.1 mM, we could detect the production of 3-HB (3 ± 1 mM) which supports the efficiency of the cascade to recycle CoASH (TTN_{CoASH} was 60). To the best of our knowledge, the TTN_{CoASH} herein reported falls in the range of the most efficient CoASH recycling systems reported for the cell-free biosynthesis of PHBs,[20,26] polyketides[27] and butanol.[28] Remarkably, Figure 5A shows that the reaction reached a plateau after 6 hours, yielding a maximum concentration of 3-HB of 5.3 ± 1.1 mM when using 1 mM

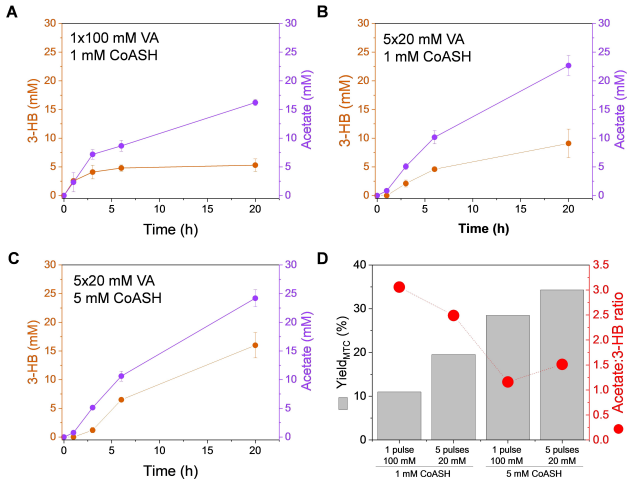


Figure 5. (A–C) Reaction time courses of 3-HB (orange) and acetate (purple) production with a single 100 mM VA addition (A) or 5 additions of 20 mM VA every 1 hour during the first 5 hours of reaction (B–C). The reactions were performed with either 1 mM (A–B) or 5 mM (C) CoASH at 30 °C and pH 8; D) Theoretical maximum conversion ($Yield_{MTC}$) (grey bars) and acetate:3-HB molar ratio (red circles) using the cell-free enzyme systems starting at different CoASH concentration (1–5 mM) and adding the VA in different pulses. Both $Yield_{MTC}$ and molar ratio are mean values calculated from the 24 h reaction points shown in the time courses of panels A and C and Figure S14.

CoASH. Considering that 100 % of 100 mM VA was consumed, only 10 % of the molecules of the vinyl ester were used to form 3-HB, which means that the product yield is only 10 % of the maximum theoretical conversion ($Yield_{MTC}$)[29] . In contrast, acetate kept accumulating for 24 hours until reaching 16 mM. According to the cascade stoichiometry, only 5 mM of acetate can be produced as a result of the NADH regeneration, the rest should be produced either by the promiscuous hydrolytic activity of AsTHL5 toward VA or by unspecific hydrolysis of the acetyl-CoA intermediate catalyzed by EcTesB. Using 5 mM CoASH, the uncoupling between the production of 3-HB and acetate was minimized (Figure S14). When the reaction was triggered with Acetyl-CoA instead of CoASH, the 3-HB titer was half (3 ± 1 mM, 6 % $Yield_{MTC}$) (Figure S15). We suggest that using acetyl-CoA as starting substrate increases the chances of EcTesB to hydrolyze it, derailing the cascade to unwanted side products like acetate. Hence, the uncoupling of the 3-HB and acetate yields suggests that some of the enzymes responsible for the Claisen condensation, the asymmetric reduction and/or the NADH recycling turned out inactive. To demonstrate the relationship between the unbalance in the product outcome and the system inactivation, we submitted all the enzymes involved in the system to a stability test under the reaction conditions in the presence and absence of VA and acetaldehyde. While acetaldehyde negligibly affected the stability of all enzymes, yAlDH lost 50 % of its initial activity after 3 hours (Figure S16). In presence of VA, AsTHL5 and yAlDH drastically lost their catalytic activity in the first hours of incubation and turned out practically inactivated after 18 hours. In contrast, the inactivation time course of the same enzymes showed that above 80 % of their initial activities were conserved after 18 hours of incubation at 30 °C and pH 8 in absence of VA and acetaldehyde. According to these results, we hypothesize that the high concentration of VA may cause the enzyme inactivation through protein acetylation of exposed Lys residues as observed for other enzymes.[30] Therefore, the large excess of VA seems to halt the Claisen condensation and/or the NADH recycling step, explaining the plateau in the production of 3-HB while acetate keeps increasing likely due to the less affected hydrolytic reactions.

To avoid the substrate induced inactivation, we decided to supply VA at a lower concentration in sequential additions. VA was supplied to the reaction in five pulses of 20 mM every hour (Figure 5B). This approach allows contacting the enzymes with a VA concentration low enough to avoid the enzyme inactivation from the beginning of the reaction cascade. The continuous supply of substrate allowed us reaching 9.1 ± 2.5 mM of 3-HB (18 % $Yield_{MTC}$) using 1 mM of CoASH; this titer is almost two times higher than the one achieved in the reaction triggered directly with one pulse of 100 mM VA at the time zero. The same reaction with a controlled supply of VA but adding 5 mM of CoASH produced up to 16 ± 2 mM (32 % $Yield_{MTC}$) (Figure 5C). Interestingly, we observed a lag in the production of 3-HB under these conditions where the VA : CoASH molar ratio is 20. Such delay in the production of 3-HB may rely on the slower abiotic thiolysis observed at the lower VA : CoASH molar ratio.

Hence, the controlled supply of the substrate enables to improve the coupling between the production of 3-HB and acetate along the whole reaction course, which suggests that the inactivation of AsTHL5 and yAlDH has been mitigated. Figure 5D shows how the pulses of low concentration of VA starting with high CoASH concentration allowed us maximizing the yield of 3-HB regarding its maximum theoretical conversion ($Yield_{MTC}$). In this cell-free cascade, the theoretical concentration balance of 3-HB, and acetate are 50 mM each, assuming 100 % conversion of 100 mM VA in absence of unwanted substrate and acetyl-CoA hydrolysis. This means that the ideal acetate:3-HB ratio is 1 when a perfect coupling of this cascade takes place. Starting with 1 mM CoASH, the $Yield_{MTC}$ of 3-HB reached 10 % with a 97 % conversion of VA and three molecules of acetate were produced per molecule of 3-HB. Sequentially adding VA in 20 mM pulses, the $Yield_{MTC}$ was doubled and the acetate:3-HB molar ratio decreased to 2.5. When 5 mM CoASH was added to the reaction where VA is added in sequential pulses, the system reached 32 % $Yield_{MTC}$ with also a 97 % conversion of VA. Unlike low CoASH concentration, the relative accumulation of acetate regarding 3-HB was significantly decreased to 1.5-fold. High CoASH concentrations and continuous supply of VA thus optimize the balance between 3-HB and acetate toward the ideal value of 1, where the VA thiolysis, condensation and reduction steps are perfectly orchestrated without unwanted side reactions and intermediates derailment.

Motivated by this success, we intensified the process first selecting a more robust AlDH as we observed that NADH

was accumulated into the reaction mixture as a hint for the yAlDH deactivation (Figure S17). As alternative aldehyde dehydrogenase, we selected the aldehyde dehydrogenases from *Geobacillus thermodenitrificans* (GtAlDH) which has been reported thermostable and better tolerates the presence of VA and acetaldehyde than yAlDH (Figure S15C). When yAlDH was replaced by GtAlDH, the titer of 3-HB ramped up to 24 ± 1 mM (48 % $Yield_{MTC}$) although the specific activity of the former was almost 100 times higher under the same reaction conditions.[31] Then, we scaled up the reaction volume up to 7 mL (0.7 mmol, 60 mg of VA), achieving 15 mM 3-HB; a $Yield_{MTC}$ 1.6 times lower than the 0.5 mL reaction. Upon liquid-liquid extraction with ethyl acetate, solvent evaporation and lyophilization, we isolated 8 mg (0.077 mmol) of 3-HB (ee >99 %), which means a 22 % of maximum theoretical yield ($Yield_{MTC}$) regarding of the starting VA. The isolated product contained 0.19 mg of acetic acid per mg of 3-HB (Figure S18), which could not be fully separated during the downstream processing. During the scale-up, we observed a significant pH decay from 8 to 6.8 that may explain the decrease in titer. Unfortunately, when we controlled the reaction pH by sequential additions of KOH, the enzymes prematurely precipitated (Figure S19), halting the reaction cascade and limiting the final product titer.

We next replaced the (*S*)-selective TtHBDH by the (*R*)-selective acetoacetyl-CoA reductase from *Candidatus Accumulibacter phosphatis* (CapAAR), which is the only wild-type (*R*)-selective NADH-dependent dehydrogenase reported to the best of our knowledge.[32] CapAAR only afforded 3.4 ± 1.3 mM of (*R*)-3-HB with ee (*R*) = 39 % (Figure S10B). Besides, the abiotic thiolysis also worked with truncated CoASH surrogates like N-acetylcysteamine (SNAC) although to a lower extent than using CoASH. The 3-HB titer decreased by 2-fold compared to the reaction triggered with CoASH, reaching a $Yield_{MTC}$ of 6.2 % (Figure S15). Unlike the in vitro biosynthesis of 3-hydroxybutyryl-SNAC thioesters initiated by the ATP-dependent acetyl-CoA synthase and starting from acetate,[33] the abiotic thiolysis of VA with SNAC enables the formation of 3-HB, indicating that all SNAC derivatives can be sequentially accepted by AsTHL5, TtHBDH and EcTesB. The low product yield achieved when starting with the truncated thiolated cofactor likely relies on the low affinity of these enzymes toward SNACs intermediates. Similar results have been reported for other CoA-dependent enzymes.[34] Although the titers of 3-HB were too low, the demonstration that this cascade can be fed with SNAC derivatives increases its potential to introduce chemical diversity in the formed β-hydroxy acids. On the other hand, we explored the potential of this cell-free pathway to synthesize branched β-hydroxy acids starting from vinyl propionate. In previous works, several thiolases have been engineered to condense propionyl and butyryl-CoA derivatives. In our cell-free system, when we replaced the AsTHL5 with the mutant F293D of a thiolase from *Saccharomyces cerevisiae* reported by Torres-Salas et al.,[21] we performed the branched condensation of vinyl propionate in presence of CoASH. UPLC-MS analysis allowed us to identify traces of 3-oxo-2-methyl-pentanoyl-CoA but not 3-hydroxy-2-methyl-pentanoyl-CoA (Figure S20). When reaction crude was quenched and extracted for further analysis, we could not detect 3-hydroxy-2-methyl pentanoate by GC-MS either. Hence, we suggest that TtHBDH is not a suitable dehydrogenase for the reduction of the α-branched 3-oxoacyl-CoAs. The much less efficiency of the cascade toward the synthesis of the branched product must be improved by engineering all the enzymes involved in the cascade toward 2-methyl-3-hydroxy acids.

In summary, this enzyme pathway initiated with the abiotic thiolysis of vinyl esters reaches a maximum titer of 24 mM ($2.4\ g\,L^{-1}$) with a maximum volumetric productivity of $1.9\ mM\,h^{-1}$ ($0.19\ g\,L^{-1}\,h^{-1}$) under the optimal reaction conditions (addition rate of $20\ mM\,VA\,h^{-1}$, 5 mM CoASH, 2.5 mM NADH and GtAlDH system for cofactor recycling). After 24 h, the CoASH and NADH were recycled 9.6 times. The titer and volumetric productivity of this cell-free system are competitive with the values reported for the in vivo production of 3-HBs,[5a,19,35] and other organic acids through enzymatic non-decarboxylative Claisen condensation. When comparing our system with the light-powered cell-free biosynthesis of PHB dimmers,[4b] we observed that light-driven cofactor recycling and ATP-dependent acetate activation outperform the thiolysis of vinyl esters in terms of both titer (1.7-fold higher) and volumetric productivity (2.5-fold higher). However, the enzyme load in the light-powered system was significantly higher (i.e. 60-fold more THL) than in our case. These numbers endow our novel cell-free biosynthetic pathway with the potential to be further optimized through increasing the enzyme loads, the enzyme stability, and the NADH-recycling system.

Conclusion

We have developed a novel, self-sustaining and ATP-independent cascade reaction triggered by the abiotic thiolysis of vinyl esters and the subsequent condensation, reduction and hydrolysis of the formed acyl-CoA derivates catalyzed by the orchestrated action of one thiolase, one NADH-dependent alcohol dehydrogenase and one thioesterase, respectively. Remarkably, such abiotic S-acylation reaction spontaneously captures the free CoASH in its acetylated form and releases acetaldehyde as by-product that serves as the electron donor for the in situ NADH recycling. Upon optimization, the obtained titer with this unprecedented cascade is in the same order of magnitude as those reported for microbial fermentation of glucose to 3-HB (roughly a few grams per liter). Moreover, the total turnover numbers herein reported are competitive with other CoASH-dependent enzyme cascades operated in the cell-free mode.

This abiotic thiolysis of vinyl esters has shown a wide scope in terms of the length of both the acyl group and the thiolated cofactors. This versatility opens an energetically favourable pathway to in situ generating acyl-CoA derivatives that can be used by other CoASH-dependent enzymes to install self-sustaining cascades that do not require

ancillary substrates and energy inputs, thus improving the atom economy and the energetic balance of the cell-free bioprocesses.

Acknowledgements

ERC–Co (METACELL-818089) project is acknowledged for funding. FLG and LS thank IKERBASQUE for sponsoring them. This work was performed under the Maria de Maeztu and Severo Ochoa Centres of Excellence Programme run by the Spanish State Research Agency, Grant No. MDM-2017-0720 (CIC biomaGUNE) and CEX2018-000867-S (DIPC). DAS thanks the Basque government for his PhD fellowship (PRE_2021_1_0334). GEP is a member of the scientific staff of CONICET and thanks Fundación Carolina for the SEGIB-FC scholarship. AHO thanks BIKAINTEK program (Basque Government) for co-funding him. Technical and human support provided by IZO-SGI, and SGIker (UPV/EHU, MICINN, GV/EJ, ERDF, and ESF) is gratefully acknowledged. Finally, we thank to Prof. Paradisi (U. Bern) for her fruitful discussion about the abiotic thiolysis herein reported, Dr. Miguel Huertos (UPV/EHU) for helping us to interpret ^{1}H NMR data, Prof. Eliszabeth Rezabal for her fruitful discussions in the DFT studies and the support of SGIker(Arina HPC, UPV/EHU).

Conflict of Interest

The authors declare no competing financial interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Cell-Free Biocatalysis · Coenzyme A · Dehydrogenases · Multi-Enzyme Systems · Thiolases

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Manuscript received: December 12, 2022
Accepted manuscript online: January 31, 2023
Version of record online: February 17, 2023