

Developing of an ATP-Independent and Cell-Free Enzymatic Cascade for the Biosynthesis of β -Hydroxy Acids Using Vinyl Esters as Smart Substrates

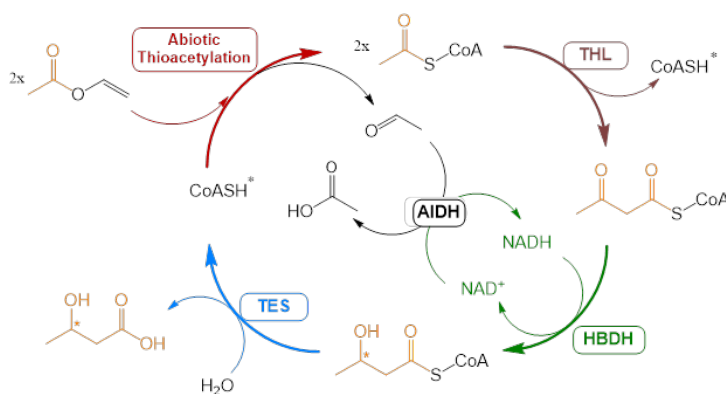
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The synthesis of compounds via *in vitro* biosynthetic pathways requires both redox power and energy, typically in the form of NAD(P)H and ATP, respectively. Regenerating these cofactors using chemicals, electricity, or light significantly adds to the process's cost, reducing atom efficiency or increasing energy consumption. In contrast, certain biosystems, such as those involved in β -hydroxy acid production and its polymers, demonstrate *in situ* cofactor regeneration. These systems commonly involve CoA-dependent Claisen condensation and NAD(P)H-dependent asymmetric reduction steps. *In vivo* experiments have achieved production levels of up to 3 g/L in these systems [1]. However, *in vitro* systems have struggled to reach more than 40 mM of poly(3-hydroxy butyrate) [2]. Alternatively, artificial cell-free circular metabolisms, encompassing these reactions, are emerging to convert CO₂ into acyl-CoA, yielding C2-C3 compounds in the μ M range [3].

This study proposes a novel method for β -hydroxy acid production through an ATP-independent cascade [4], utilizing vinyl esters as dual acyl and electron donors. This approach embeds chemical energy into the substrate to activate the acyl group and provide redox power. The enzymatic cascade, designed in four steps including abiotic thiolysis, non-decarboxylative Claisen condensation, asymmetric reduction, and hydrolysis with NADH recycling, is catalyzed by four enzymes. This system achieves a titer of 3-hydroxy butyrate of 24 mM without requiring ATP, demonstrating the potential of *in vitro* biocatalysis to transform simple molecules into multifunctional ones.



[1] aH.-C. Tseng, C. H. Martin, D. R. Nielsen, K. L. J. Prather, *Applied and environmental microbiology* **2009**, 75, 3137-3145. bS. Cheong, J. M. Clomburg, R. Gonzalez, *Nature biotechnology* **2016**, 34, 556-561.

[2] F. Li, X. Wei, L. Zhang, C. Liu, C. You, Z. Zhu, *Angewandte Chemie* **2022**, 134, e202111054.

[3] aS. Sundaram, C. Diehl, N. S. Cortina, J. Bamberger, N. Paczia, T. J. Erb, *Angewandte Chemie International Edition* **2021**, 60, 16420-16425. bT. Schwander, L. Schada von Borzyskowski, S. Burgener, N. S. Cortina, T. J. Erb, *Science* **2016**, 354, 900-904.

[4] A. H. Orrego, M. G. Rubanu, I. L. López, D. Andrés-Sanz, G. García-Marquina, G. E. Pieslinger, L. Salassa, F. López-Gallego, *Angew. Chem. Int. Ed.* 2023, 62, e202218312; *Angew. Chem.* **2023**, 135, e202218312.