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Elucidation of the Enantioselective Cyclohexane-1,2-dione Hydrolase Catalyzed Formation of (*S*)-Acetoin

Sabrina Loschonsky, Simon Waltzer, Volker Brecht, and Michael Müller*[a]

Thiamine diphosphate (ThDP) dependent enzymes catalyze the formation of acetoin (3-hydroxybutan-2-one) through one of three different pathways: homocoupling of pyruvate, homocoupling of acetaldehyde, or cross-coupling of acetaldehyde (as acceptor) and pyruvate (as donor). The enantioselectivity of the resulting acetoin is highly dependent on the particular enzyme. We established that ThDP-dependent cyclohexane-1,2-dione hydrolase (CDH) is able to form (*S*)-acetoin with particularly high enantioselectivity (up to 95% *ee*) by all three pathways. Mechanistic studies utilizing ^{13}C-labeled substrates revealed an unprecedented non-acetolactate pathway for the homocoupling of pyruvate, which explains the high enantioselectivity in the CDH-catalyzed formation of (*S*)-acetoin.

Acetoin (**1**) is produced by a variety of (micro)organisms that metabolize glucose through the Embden–Meyerhof–Parnas pathway, the most important form of glycolysis for the transformation of D-(+)-glucose into pyruvate (**2**).[1] The most common biosynthetic pathway leading to acetoin involves the formation of acetolactate (**3**) by the homocoupling of pyruvate catalyzed by acetolactate synthase.[2] Acetolactate is subsequently transformed into acetoin by acetolactate decarboxylase. Alternatively, acetoin can also accumulate as a byproduct of pyruvate decarboxylase[3] and pyruvate oxidase[4] catalysis.[2a] Acetoin is found in many dietary products, such as butter, apples, and yogurt, amongst others. Large-scale production of acetoin has been achieved by organic synthesis, microbial fermentation, and enzymatic transformation.[5]

Thiamine diphosphate (ThDP) dependent enzymes catalyze a broad range of reactions,[6] including (asymmetric) C–C bond formation from an aldehyde and an α-keto carboxylic acid to yield an α-hydroxy ketone. Acetoin (**1**) is such an α-hydroxy ketone and can emerge from ThDP-dependent enzyme catalysis through homocoupling of pyruvate, homocoupling of acetaldehyde, or the combination of acetaldehyde (as acceptor) and pyruvate (as donor):

1) Pyruvate decarboxylase (PDC) from yeast (*Saccharomyces cerevisiae*) catalyzes the homocoupling of pyruvate to yield (*R*)-**1** (46–53% *ee*),[7] whereas PDC from *Zymomonas mobilis* (*Zm*PDC) forms (*S*)-**1** (23–29% *ee*).[7,8] A single mutation (*Zm*PDC-E473Q) results in inversion of the stereoselectivity [(*R*)-**1**, 33% *ee*].[8] Whereas YerE from *Yersinia pseudotuberculosis* yields almost racemic acetoin (4% *ee*),[9] PigD from *Serratia marcescens* provides the (*S*) enantiomer (70% *ee*).[10]
2) Homocoupling of acetaldehyde is known to be catalyzed by PDC from several organisms, that is, *Acetobacter pasteurianus* [(*S*)-**1**, 28% *ee*][11] and its variant *Ap*PDC-E469G [(*S*)-**1**, 85% *ee*],[11] *Zymobacter palmae* [(*S*)-**1**, 58% *ee*],[12] *Z. mobilis* [(*S*)-**1**, 25% *ee*],[7] and yeast [(*R*)-**1**, 44% *ee*].[7] Moreover, benzoylformate decarboxylase (BFD) from *Pseudomonas putida* [(*R*)-**1**, 17% *ee*][11] and its variant *Pp*BFD-H281A [(*R*)-**1**, 25% *ee*],[11] benzaldehyde lyase (BAL) from *Rhodopseudomonas palustris* [(*S*)-**1**, 60% *ee*],[12] and branched-chain keto acid decarboxylase (KdcA) from *Lactobacillus lactis* [in buffer: (*R*)-**1**, 20% *ee*; in the presence of 10–30% acetone: (*S*)-**1**, 21% *ee*][11] catalyze the same reaction.
3) PDC from yeast catalyzes the cross-coupling of acetaldehyde and pyruvate to give (*R*)-**1** with 46–50% *ee*.[7] After single-point mutations, the enantioselectivity can be increased up to 94% *ee* [(*R*)-**1**].[13] *Zm*PDC yields (*S*)-**1** with 28–29% *ee*.[7] A variant of pyruvate dehydrogenase complex E1 subunit (PDHc-E1) from *E. coli* gives (*S*)-**1** with up to 70% *ee*.[13]

Cyclohexane-1,2-dione hydrolase (CDH), isolated from *Azoarcus* sp. strain 22 Lin,[14,15] is one of the many ThDP-dependent enzymes that catalyze asymmetric C–C bond formation from pyruvate (as donor) and an aldehyde (as acceptor).[16] In this work, we show that CDH is able to form acetoin with surprisingly high enantioselectivity by all three aforementioned modes. The results of our mechanistic studies with labeled and unlabeled substrates clearly show that enzymatic formation of highly enantioenriched acetoin from two molecules of pyruvate occurs without the release of acetaldehyde or acetolactate. This unexpected observation is also of importance for other ThDP-dependent asymmetric C–C bond-formation reactions.

If only pyruvate is present, CDH catalyzes the formation of (*S*)-acetoin with 87–90% *ee* after 24 h at 30 °C.[17] Upon performing the reaction at 16 °C, 93% *ee* was obtained (see the Supporting Information).[18] The homocoupling of ^{13}C-labeled [1,2]-^{13}C-pyruvate (**2a**; Scheme 1, top) was followed by ^{13}C NMR spectroscopy measurements every 30 min for 19.5 h [2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, 10% D_2O, v/v; see the Supporting Information]. [1,2]-^{13}C-Pyruvate (**2a**) showed two doublets ($^1J = 62.2$ Hz) at $\delta = 170.2$ and 205.0 ppm, in addition to minor amounts of its hydrate form

[a] *S. Loschonsky, S. Waltzer, V. Brecht, Prof. Dr. M. Müller*
Institute of Pharmaceutical Sciences
University of Freiburg
Albertstraße 25, 79104 Freiburg (Germany)
Fax: (+49) 761-203-6351
E-mail: michael.mueller@pharmazie.uni-freiburg.de

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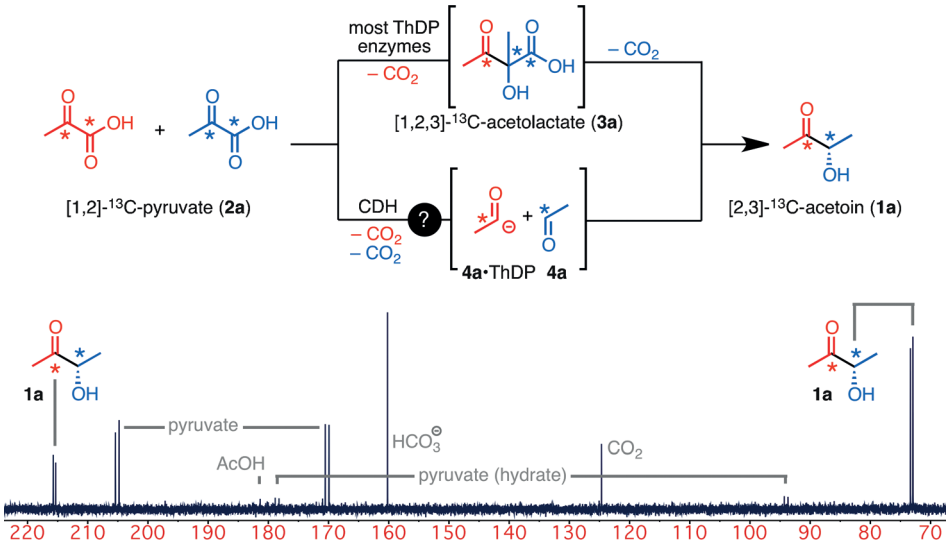
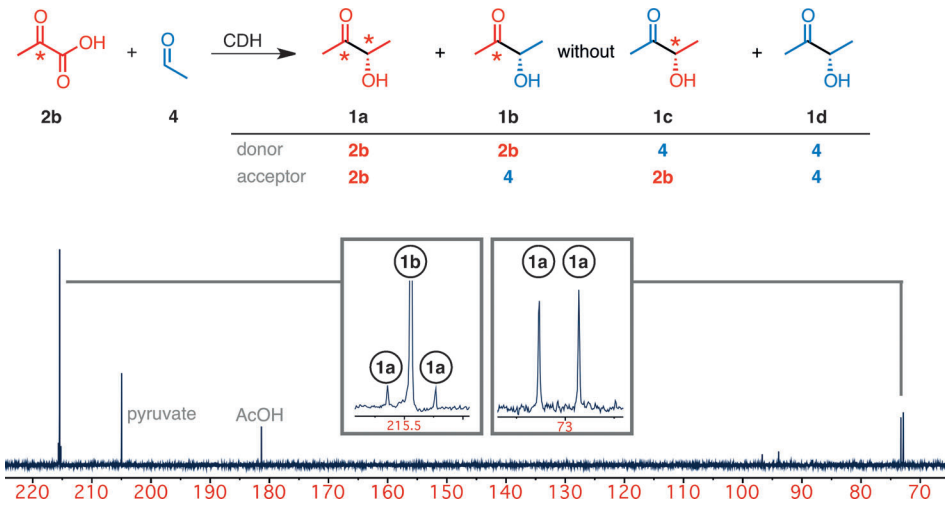


Scheme 1. CDH-catalyzed formation of [2,3]-^{13}C-acetoin (**1a**) from [1,2]-^{13}C-pyruvate (**2a**). Conditions: [1,2]-^{13}C-pyruvate (25 mM), CDH (0.7 mg mL^{-1}), buffer A (50 mM MES, 1.5 mL; 1 mM $MgSO_4$; 0.5 mM ThDP; 10% D_2O; pH 6.5], 30 °C. Conversion after 5 h is shown. Enantiomeric excess was determined by GC analysis on a chiral stationary phase (87–90% *ee* after 24 h by using unlabeled pyruvate).



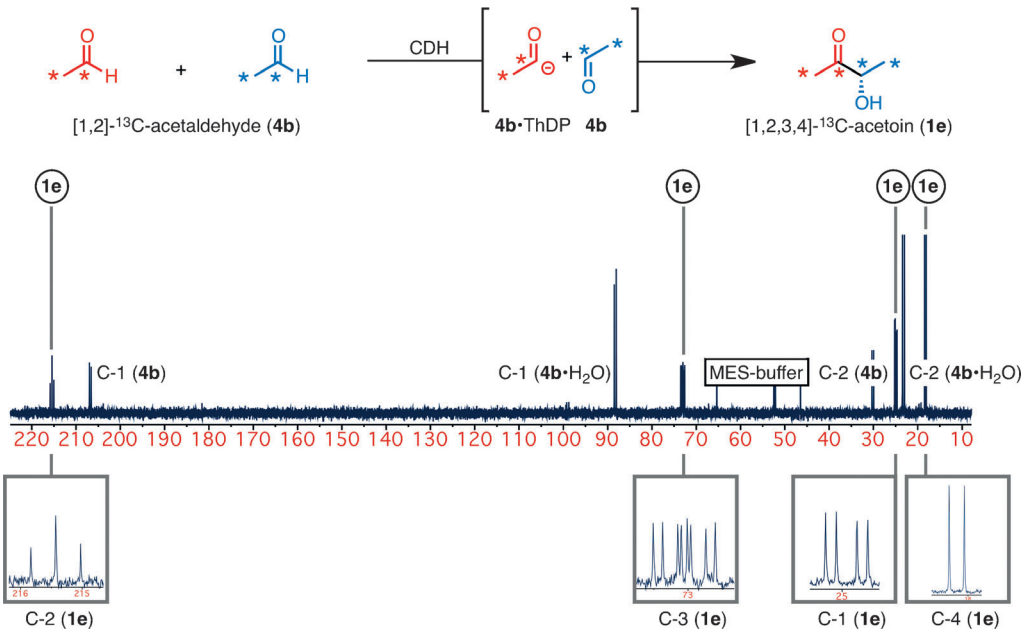
Scheme 2. CDH-catalyzed formation of isotopologues **1a–d** from a mixture of [2]-^{13}C-pyruvate (**2b**) and acetaldehyde (**4**). Conditions: [2]-^{13}C-pyruvate (25 mM), acetaldehyde (15 mM), CDH (0.7 mg mL^{-1}), buffer A (1.5 mL), 30 °C. ^{13}C NMR spectrum was recorded after 18.5 h (10% D_2O, v/v). Enantiomeric excess was determined by GC analysis on a chiral stationary phase (89% *ee* after 24 h by using unlabeled pyruvate and acetaldehyde).

with doublets (^1J = 63.1 Hz) at δ = 93.9 and 178.5 ppm. Acetoin was formed as its [2,3]-^{13}C-isotopomer **1a**, which displays two doublets (^1J = 41.2 Hz) at δ = 73.1 and 215.4 ppm. After 19.5 h, 90% conversion of [1,2]-^{13}C-pyruvate (**2a**) into [2,3]-^{13}C-acetoin (**1a**) was observed [calculated from the ^{13}C NMR spectrum by integration of the ^{13}C(=O) signals of **1a** and **2a**]. As acetolactate is commonly referred to as a key intermediate in the enzyme-catalyzed formation of acetoin from pyruvate, we were surprised that [1,2,3]-^{13}C-acetolactate (**3a**) could not be detected in the ^{13}C NMR spectra [i.e., at δ = 212 (C=O), 177 (CO_2H), and 83 ppm (C–OH)].

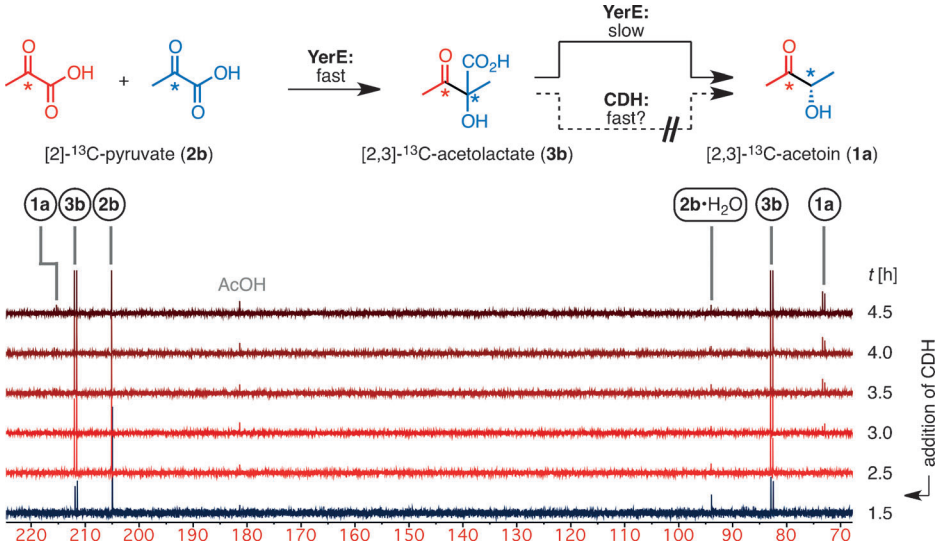
Accordingly, we hypothesized that acetoin (**1a**) was formed by carboligation of activated **4a**·ThDP with a second molecule of acetaldehyde (**4a**) as the acceptor. The latter two species must arise by initial decarboxylation of pyruvate (**2a**→**4a**·ThDP) and subsequent protolysis (→**4a**). This pathway might serve as an explanation for the remarkably high *ee* of (*S*)-acetoin, as the alternative pathway via acetolactate (**3**) must by necessity incorporate (nonenzymatic) decarboxylation of **3** to yield the achiral enediol tautomer of acetoin, which would tautomerize to (racemic) acetoin in a final step. To prove this, we investigated the putative CDH-catalyzed formation of acetoin from a mixture of [2]-^{13}C-pyruvate (**2b**) and acetaldehyde (**4**). Assuming that both pyruvate and acetaldehyde can act as a donor and, moreover, that both acetaldehyde (**4**) and [1]-^{13}C-acetaldehyde [(**4a**), formed by decarboxylation of [2]-^{13}C-pyruvate (**2b**)] can act as acceptors, four isotopologues, that is, **1a–d**, are possible (Scheme 2, top).

However, we observed only isotopologues **1a** and **1b** in a ratio of approximately 10:90. This was ascertained by integration of the doublet of [2,3]-^{13}C-acetoin (**1a**) at δ = 215.4 ppm ($^1J_{2,3}$ = 41.2 Hz) and the superimposed, yet baseline-separated, singlet of [2]-^{13}C-acetoin (**1b**) at δ = 215.4 ppm. The overall conversion after 18.5 h was 74% based on [2]-^{13}C-pyruvate (**2b**, Scheme 2). (*S*)-Acetoin was obtained with 89% *ee* (as determined after 24 h at 30 °C by using unlabeled pyruvate and acetaldehyde). If the reaction was performed at 16 °C, 92% *ee* was obtained. From this outcome, we conclude that pyruvate is a superior donor than acetaldehyde, whereas acetaldehyde is a superior acceptor than pyruvate.

Subsequently, we investigated the CDH-catalyzed formation of [1,2,3,4]-^{13}C-acetoin (**1e**) from [1,2]-^{13}C-acetaldehyde (**4b**) as the sole C_2 source (Scheme 3). There was approximately 38% conversion into product **1e** after 21 h at 30 °C, as judged from the ^{13}C NMR spectrum (integration of the $^{13}CH_3$ signals of **1e**, **4b**, and **4b**-hydrate; see the Supporting Information). (*S*)-Acetoin was obtained in 91% *ee* by using unlabeled acetaldehyde after 24 h at 30 °C. If the reaction was performed at 16 °C,



Scheme 3. CDH-catalyzed formation of [1,2,3,4]-^{13}C-acetoin (**1e**) from [1,2]-^{13}C-acetaldehyde (**4b**). Conditions: [1,2]-^{13}C-acetaldehyde (23 mM), CDH (0.7 mg mL^{-1}), buffer A (1.5 mL), 30 °C. ^{13}C NMR spectrum was recorded after 20.5 h (10% D_2O, v/v). Enantiomeric excess was determined by GC analysis on a chiral stationary phase (91% *ee* after 24 h by using unlabeled acetaldehyde).



Scheme 4. Formation of [2,3]-^{13}C-acetolactate (**3b**) from [2]-^{13}C-pyruvate (**2b**) catalyzed by YerE and subsequent addition of CDH. Conditions: [2]-^{13}C-pyruvate (25 mM), YerE (1 mg mL^{-1}), buffer A (1.5 mL), 30 °C. After 2 h, an aliquot (250 μL) of the assay was removed and replaced by the same volume of CDH in buffer A. The final concentrations of the enzymes were 1.3 (CDH) and 0.83 mg mL^{-1} (YerE). Enantiomeric excess was determined by GC analysis on a chiral stationary phase (66% *ee* after 7 h, 42% *ee* after 26 h).

95% *ee* was obtained. The identity of isotopologue **1e** was unambiguously proven from the corresponding ^{13}C NMR coupling of each nucleus: $\delta = 18.3$ (d, $^1J_{4,3} = 36.1$ Hz; C-4), 24.9 (dd, $^1J_{1,2} = 40.4$ Hz, $^2J_{1,3} = 13.8$ Hz; C-1), 73.1 (ddd, $^1J_{3,2} = 41.2$ Hz, $^1J_{3,4} = 36.1$ Hz, $^2J_{3,1} = 13.9$ Hz; C-3), 215.4 ppm (dd, $^1J_{2,1} \approx {}^1J_{2,3} \approx 40.7$ Hz; C-2).

Hence, acetaldehyde can act both as the acceptor and the donor in the CDH-catalyzed formation of acetoin. This implies that the non-acetolactate pathway for the homocoupling of pyruvate suggested in Scheme 1 might indeed be viable. However, it could not be ruled out that CDH does form acetolactate as an intermediate and that the latter is converted into acetoin faster than the timescale of the ^{13}C NMR experiment. We therefore designed a competition experiment by utilizing ThDP-dependent YerE[9] (Scheme 4).

Recently, it was shown that YerE transforms pyruvate into (*S*)-acetolactate, which is followed by slow (and presumably nonenzymatic) decarboxylation to yield almost-racemic acetoin [< 5% *ee* in potassium phosphate (KPi) buffer, pH 8.0].[9] Upon following the YerE-catalyzed homocoupling of [2]-^{13}C-pyruvate (**2b**) in aqueous KPi buffer by ^{13}C NMR spectroscopy measurements every 30 min, we detected the formation of [2,3]-^{13}C-acetolactate (**3b**) after 1 h and the onset of [2,3]-^{13}C-acetoin (**1a**) formation after 3 h (9% *ee* after 20 h, see the Supporting Information). Because CDH shows no activity in KPi buffer, whereas YerE is active in MES buffer, the latter buffer was used for the competition experiments. There was approximately 47% conversion of **2b** into [2,3]-^{13}C-acetolactate (**3b**) with YerE after 1.5 h (Scheme 4,

bottom). A further 30 min later (i.e., at $t = 2.0$ h), CDH was added. After $t = 4.5$ h, the signals for [2,3]-^{13}C-acetolactate (**3b**) were still present in the ^{13}C NMR spectrum. Concurrently, only minute amounts of [2,3]-^{13}C-acetoin (**1a**) had been formed. If acetolactate could serve as a superior substrate for CDH, an instantaneous decrease in the amount of acetolactate **3b** and a rapid increase in the amount of acetoin **1a** would be expected after the addition of CDH. Accordingly, acetolactate is neither a product (Scheme 1) nor a substrate of CDH.

In summary, ThDP-dependent CDH catalyzes the enantioselective formation of (*S*)-acetoin by homocoupling of pyruvate [87–90 (30 °C), 93% *ee* (16 °C)], homocoupling of acetaldehyde [91 (30 °C), 95% *ee* (16 °C)], and cross-coupling of pyruvate and acetaldehyde [89 (30 °C), 92% *ee* (16 °C)]. Utilizing ^{13}C-labeled substrates, we have shown that acetolactate is neither an intermediate nor a substrate in the course of the CDH-catalyzed formation of (*S*)-acetoin. Nevertheless, acetolactate is a common product of the enzymatic homocoupling of pyruvate (route 1) or, less likely, the cross-coupling of pyruvate and acetaldehyde (route 3) and can, in principle, be decarboxylated through a nonenzymatic pathway to yield (racemic) acetoin. Accordingly, the absence of acetolactate in the CDH-catalyzed formation of acetoin by either pathway explains the high enantioselectivity in the formation of (*S*)-acetoin in these cases.

These results also shed light on other ThDP-dependent enzyme-catalyzed C–C bond-formation reactions involving α-keto acids as substrates. The (homo)coupling of two α-keto acids can proceed through the initial formation of an acetohydroxy acid derivative (e.g., acetolactate) and a subsequent decarboxylation step; however, as shown in this work, it does not necessarily have to. The direct formation of α-hydroxy ketones through two decarboxylation steps might be a valuable alternative for obtaining highly enantioenriched products.

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Keywords: asymmetric synthesis · biocatalysis · carboligation · cross-coupling · thiamine-dependent enzymes

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