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Enzymatic Chemoselective Aldehyde–Ketone Cross-Couplings through the Polarity Reversal of Methylacetoin

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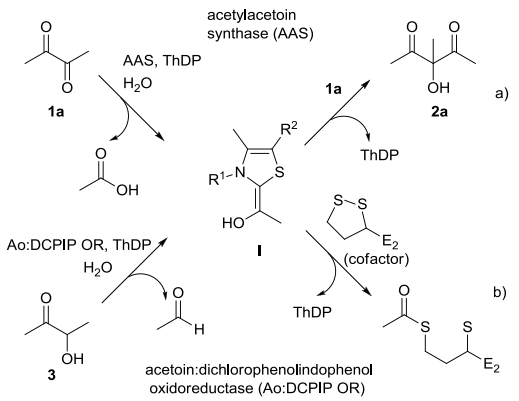
Dedication (……..)

Abstract: *The thiamine diphosphate (ThDP) dependent enzyme acetoin:dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR) from* Bacillus licheniformis *was cloned and overexpressed in Escherichia coli. The recombinant enzyme shared close similarities with the acetylacetoin synthase (AAS) partially purified from Bacillus licheniformis suggesting that they could be the same enzyme. The product scope of the recombinant Ao:DCPIP OR was expanded to chiral tertiary α-hydroxy ketones through the rare aldehyde–ketone cross-carboligation reaction. Unprecedented is the use of methylacetoin as the acetyl anion donor in combination with a range of strongly to weakly activated ketones. In some cases, Ao:DCPIP OR produced the desired tertiary alcohols with stereochemistry opposite to that obtained with other ThDP-dependent enzymes. The combination of methylacetoin as acyl anion synthon and novel ThDP-dependent enzymes considerably expands the available range of C–C bond formations in asymmetric synthesis.*

The use of enzymes in synthetic organic chemistry has received steadily increasing attention during the last three decades thanks to their efficiency, selectivity and low environmental impact.[1] In particular, a large number of enzymes, mostly lyases, are available for the stereoselective formation of C–C bonds, a process that is one of the most challenging transformations in organic synthesis. Thiamine diphosphate (ThDP)-dependent enzymes are well-established biocatalysts that have been applied in a variety of reactions such as benzoin condensations,[2] carboligation processes including intermolecular Stetter reactions,[3,4] C–C bond cleavages[5] and (oxidative) decarboxylations.[6] Aldehyde–ketone cross-coupling is another type of enzymatic reaction that has been recently studied in order to access optically active tertiary α-hydroxy ketones, which are important structural motifs in numerous biologically active compounds[7] and fundamental building blocks in organic synthesis.[8]

Enzymatic asymmetric intermolecular aldehyde–ketone cross-carboligation has been introduced by exploiting the polarity reversal (umpolung)[9] of pyruvate promoted by the ThDP-dependent flavoenzyme YerE.[10] Coupling of the pyruvate donor with either cyclic or open-chain ketone, diketone or α-keto ester acceptors produces a collection of chiral tertiary alcohols with a high level of enantioselectivity. More recently, a variant of the ThDP-dependent enzyme cyclohexane-1,2-dione hydrolase (CDH-H28A/N484A) has been shown to catalyze aldehyde–ketone cross-couplings using either pyruvate or 2,3-butanedione as the donor.[11]

The use of α-diketone donors in aldehyde–ketone cross-carboligations catalyzed by ThDP-dependent enzymes was an early success of our laboratory.[8a,12] We disclosed the enantioselective synthesis of α-hydroxy-α-alkyl-β-diketones through homo- and cross-couplings of aliphatic and aromatic open-chain α-diketones catalyzed by acetylacetoin synthase (AAS) from *Bacillus licheniformis*. The physiological role of this enzyme is within the bacterial catabolism of acetoin. Some authors have described AAS as the first enzyme of a pathway known as the '2,3-butanediol cycle', where AAS is supposed to catalyze the ThDP-dependent condensation of two molecules of 2,3-butanedione (**1a**) yielding acetylacetoin (**2a**) and acetic acid via the formation of the (hydroxyethyl)thiamine diphosphate intermediate **I** (Scheme 1, reaction a).[13] Recently, however, the '2,3-butanediol cycle' has been brought into question[14] and the currently most accepted mechanism for the bacterial degradation of acetoin relies on the action of the acetoin dehydrogenase enzyme system (AoDH ES).[14,15] The first enzyme of this multienzymatic system, named acetoin:dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR), catalyzes the ThDP-dependent oxidative cleavage of acetoin (**3**) leading to acetaldehyde with transfer of the activated aldehyde to the lipoamide cofactor of the second enzyme of the system (Scheme 1, reaction b).



Scheme 1. Proposed physiological role of AAS [reaction a)] and Ao:DCPIP OR [reaction b)]. R^1 = (4-amino-2-methylpyrimidin-5-yl)methyl; R^2 = ethyl diphosphate. Cofactor = lipoamide covalently bound to the second enzyme (E_2) of the acetoin dehydrogenase enzyme system (AoDH ES).

Despite the different physiological roles proposed for the two enzymes, AAS and Ao:DCPIP OR show interesting similarities. Indeed, their expression is strongly induced when the bacteria are grown on acetoin-rich media and both are able to convert 2,3-

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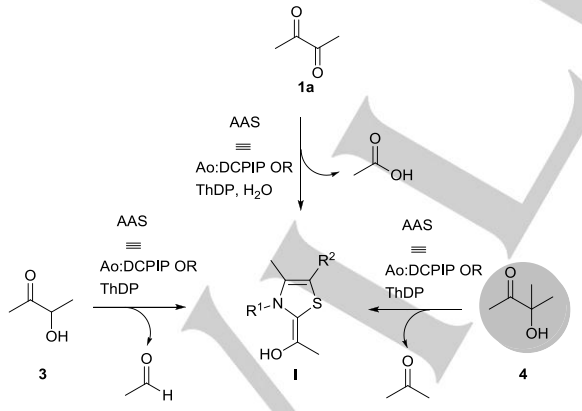
butanedione (**1a**) into acetylacetoin (**2a**). For these reasons, it has been recently hypothesized that AAS and Ao:DCPIP OR could be the same enzyme.[14b]

In the present paper, we describe the cloning, heterologous overexpression and characterization of Ao:DCPIP OR from *B. licheniformis* DSM13. The strong correspondence of this enzyme's electrophoretic and catalytic behavior to that of AAS suggests that the two enzymes are identical. Furthermore, we outline an extension of the catalytic scope of the recombinant Ao:DCPIP OR to the synthesis of optically active tertiary α-hydroxy ketones through the unprecedented use of methylacetoin as the acyl anion precursor in aldehyde–ketone cross-couplings.

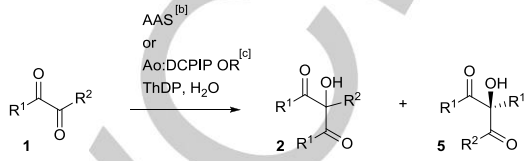
In order to obtain recombinant Ao:DCPIP OR, the putative Aco operon encoding for the AoDH ES was identified in the *B. licheniformis* DSM13 genome, and the sequence from the start codon of the AcoA gene (encoding for the Ao:DCPIP OR α-subunit) to the stop codon of the AcoB gene (encoding for the Ao:DCPIP OR β-subunit) was PCR-amplified. The two-gene fragment was ligated into pLATE31 to produce the expression vector pLATE 31-Ao:DCPIP OR. The recombinant enzyme was produced in *Escherichia coli* SHuffle® T7 Express Competent cells. The overexpressed β-subunit C-terminal His-tagged Ao:DCPIP OR was purified from the cell lysate by nickel affinity chromatography (see SI). The comparative native gel electrophoresis of the recombinant enzyme and the partially purified AAS, stained for the Ao:DCPIP OR activity, displayed two bands with identical migration. Furthermore, the comparative SDS-PAGE showed that the two bands ascribed to the α- and β-subunits were also visible in the partially purified AAS. In addition, the two enzymes showed the same optimal pH value of 6.5, and a preliminary investigation on the substrate specificity performed via the DCPIP method[16] demonstrated that both enzymes were able to form the (hydroxyethyl)thiamine diphosphate **I** using either 2,3-butanedione (**1a**), acetoin (**3**) or methylacetoin (**4**) as the substrate (Scheme 2). It is worth emphasizing that the utilization of methylacetoin (**4**) as the acyl anion precursor is unprecedented in thiamine catalysis and that acetone is released during the activation step leading to the reactive acyl anion equivalent **I**.



Scheme 2. AAS- and Ao:DCPIP OR-catalyzed formation of (hydroxyethyl)thiamine diphosphate intermediate **I** from 2,3-butanedione (**1a**), acetoin (**3**) and methylacetoin (**4**). R^1 = (4-amino-2-methylpyrimidin-5-yl)methyl; R^2 = ethyl diphosphate.

Moreover, the catalytic activities of recombinant Ao:DCPIP OR and AAS were very similar, as demonstrated by the homo-coupling reactions of the α-diketones **1a**–**e** (Table 1). In particular, with the nonsymmetric substrates **1c**–**e** the two enzymes afforded reaction mixtures with almost the same composition of the regioisomeric products **2** and **5**, formed by attack of the acetyl anion equivalent **I** at the nonequivalent carbonyl groups of **1c**–**e**. Furthermore, the chiral products **5c**–**e** were obtained by both enzymes with the same stereochemistry and similar enantiomeric excesses (*ee's*).

Table 1. Comparative results of α-diketone homo-coupling reactions catalyzed by AAS or Ao:DCPIP OR.[a]



Substrate	2	2 % Yield[d]	5[e]	5 % Yield[d] 5 (% *ee*)[f]
1a	2a	57[g] 70	-	- -
1b	2b	60[g] 80	-	- -
1c	2c	25[g] 35	5c	30[g] 50 (70)[g] (62)
1d	2d	19[g] 26	5d	42[g] 53 (67)[g] (62)
1e	2e	15[h] 21	5e	48[h] 53 (72)[h] (34)

[a] Reaction conditions: substrate (10 mM), enzyme (10 mg), 50 mM phosphate buffer pH 6.5 (50 mL), $MgSO_4$ (0.9 mM), ThDP (0.4 mM), 30 °C, 24 h. [b] Crude enzyme as described in reference [10a]. [c] Purified Ao:DCPIP OR (this work). [d] Isolated yield (AAS catalysis/Ao:DCPIP OR catalysis). [e] The absolute (*R*)-configuration was assigned, according to reference [6a]. [f] Determined by chiral GC analysis (AAS catalysis/Ao:DCPIP OR catalysis). [g] See reference [10a]. [h] See reference [6a].

Next, the substrate scope was investigated by studying the Ao:DCPIP OR-catalyzed cross-coupling of 2,3-butanedione (**1a**) with various activated ketones (Table 2). By using the previously reported conditions, the combination of **1a** (3 equiv.) and 3,4-hexanedione (**6**) afforded the expected product (*R*)-**10** in 63% conversion and with 80% *ee*, values comparable with those reported for the AAS-catalyzed reaction (62% yield, 91% *ee*).[12a] The self-condensation of **1a** could not be suppressed and acetylacetoin (**2a**) was formed as a byproduct. Once the ability of the recombinant enzyme to catalyze the acetyl anion transfer between two different α-diketones had been demonstrated, we investigated the use of other types of activated ketones as acceptors, choosing methyl ketones **7**–**9** for this purpose. The cross-coupling of **1a** and ethyl pyruvate (**7**) afforded the expected ethyl α-

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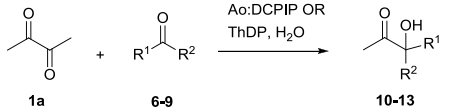
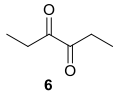
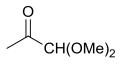
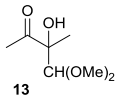
acetolactate (**11**) together with acetylacetoin (**2a**). In order to obtain the maximum conversion of **7**, along with minimizing the formation of the homo-coupling product **2a**, the effect of varying the donor/acceptor molar ratio was studied in the range from 3:1 to 1:3; the best result was obtained for equimolar amounts of **1a** and **7**. Under these conditions, the adduct (*S*)-**11** was formed in 54% conversion and with 96% *ee*. Following this encouraging result, 1,1,1-trifluoroacetone (**8**) and 1,1-dimethoxy-2-propanone (**9**) were tested as acceptor substrates: the resulting α-hydroxy ketones **12** and **13** were obtained in 72% and 20% conversion, respectively.

Table 2. Cross-coupling reactions of 2,3-butanedione (**1a**) with selected ketones **6–9** catalyzed by Ao:DCPIP OR.[a]

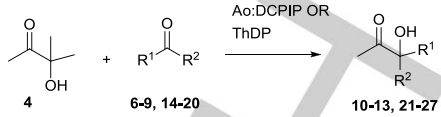


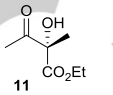
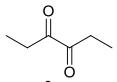
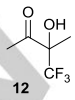
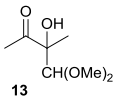
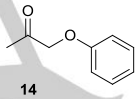
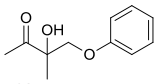
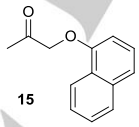
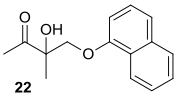
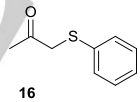
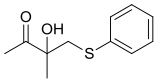
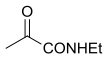
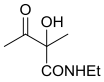
Acceptor	Product	% conversion[b]	% ee[c]
 **6**	**10**	63	80 (*R*)[d]
7	**11**	54	96 (*S*)[e]
8	**12**	72	n.d.[f]
 **9**	 **13**	20	61[g]

[a] Reaction conditions: donor **1a** (10 mM or 30 mM with acceptor **6**), acceptor **6-9** (10 mM), Ao:DCPIP OR (1 mg), 50 mM phosphate buffer pH 6.5 (1 mL), $MgSO_4$ (0.9 mM), ThDP (0.4 mM), 30 °C, 48 h. [b] Determined by ^{1}H NMR analysis. [c] Determined by chiral GC analysis. [d] According to reference [8a]. [e] According to reference [17]. [f] Not determined. [g] Determined as described in reference [18].

Despite our efforts to tune the optimal ratio of **1a**/acceptor in favor of the cross-coupling product, formation of the homo-coupling product **2a** could not be suppressed. To overcome this limitation, and because of the dual reactivity of the α-diketone donor, we focused our attention on alternative acetyl anion precursors: we identified acetoin (**3**) and methylacetoin (**4**) as suitable candidates (Scheme 2). A previous *in vivo* study, however, suggested that the acetaldehyde released during the cleavage of acetoin (**3**) could compete with weakly activated acceptors.[19] This drawback is considerably reduced with methylacetoin (**4**), the activation of which occurs with elimination of the less reactive acetone. To test this hypothesis, we attempted the cross-coupling between ethyl pyruvate (**7**) and either acetoin (**3**) or methylacetoin (**4**). While no reaction was detected with **3**, ethyl (*S*)-α-acetolactate (**11**) was obtained in quantitative conversion and with >95% *ee* in the presence of **4** (Table 3).

Table 3. Ao:DCPIP OR-catalyzed cross-coupling reactions using methylacetoin (**4**) as acetyl anion donor.[a]



Acceptor	Product	% conversion[b] (% yield)[c]	% ee[d]
7	 **11**	95 (57)	95 (*S*)[e]
 **6**	**10**	100 (60)	58 (*R*)[f]
8	 **12**	100 (-)[g]	n.d.[h]
9	 **13**	90 (50)	64[i]
 **14**	 **21**	75 (55)	85
 **15**	 **22**	16 (13)	61
 **16**	 **23**	63 (50)	*rac.*
17	**24**	93 (-)[j]	69
18	**25**	57 (9)	n.d.[h]
 **19**	 **26**	40 (30)	96
20	**27**	100 (35)	93

[a] Reaction conditions: donor **4** (10 mM), acceptor **6-9**, **14-20** (10 mM), Ao:DCPIP OR (20 mg), 50 mM phosphate buffer pH 6.5 (30 mL), $MgSO_4$ (0.9 mM), ThDP (0.4 mM), 30 °C, 48 h. [b] Determined by ^{1}H NMR analysis. [c] Isolated yield. [d] Determined by chiral GC analysis. [e] According to reference [17]. [f] According to reference [8a]. [g] The product was not isolated due to its high volatility. [h] Not determined. [i] Determined as

described in reference [18]. [j] The starting material and the product could not be separated on silica gel.

This result encouraged us to translate this approach to the synthesis of the tertiary α-hydroxy ketones **10**, **12** and **13** (Table 3). The coupling of **4** with the diketone **6** confirmed the efficacy of methylacetoin as a donor as stoichiometric amounts of **4** afforded the target product **10** in quantitative yield, without any evidence of the homo-coupling product **2b**. The positive effect of the improved procedure was evident in the cross-coupling of **4** with **8** and **9**, respectively, resulting in almost quantitative conversions and with a significant 64% *ee* obtained for **13**.[18]

The efficiency of the Ao:DCPIP OR–methylacetoin enzyme–substrate pair in the aldehyde–ketone cross-coupling prompted us to extend the method to the use of other acceptors. For this purpose, the cross-couplings of **4** with ketones **14**–**20** were next investigated. The expected products **21**–**27** were obtained with conversions ranging from 16% to >99% and generally satisfactory *ee's* (Table 3). As ketones **14**–**17** have been previously employed to investigate the scope of YerE catalysis,[10] a comparison of the stereochemistry of the corresponding products **21**–**24** was undertaken. Interestingly, relative to YerE, Ao:DCPIP OR afforded the opposite enantiomer of the aromatic products **21** and **22**, yet the same enantiomer for **24**. Compound **24** has also been recently produced using an engineered cyclohexane-1,2-dione hydrolase (CDH-H28A/N484A) designed to suppress the C–C bond-cleavage and improve the C–C bond-formation activities.[11] Remarkably, as in that case, no product derived from C–C bond cleavage of substrate **17** was detected in the reaction catalyzed by Ao:DCPIP OR. Concerning the optical purity of the products, tertiary alcohols **21** and **22** showed *ee's* lower than those observed with YerE (85% vs. 91% for **21**; 61% vs. 95% for **22**). The 69% *ee* of **24**, however, was much higher than that for the YerE product (22% *ee*). The exchange of an oxygen atom in **14** for sulfur (substrate **16**) is detrimental for the enantioselectivity of both enzymes. Gratifyingly, Ao:DCPIP OR showed a satisfactory activity in the cross-coupling of **4** with 1-chloroacetone (**18**) and *N*-ethyl-2-oxopropanamide (**19**), which have never been used previously as acceptors in thiamine catalysis. Finally, the reaction of methylacetoin (**4**) with methyl pyruvate (**20**) confirmed the observations made with the ethyl analogue **7**, affording the corresponding product **27** with quantitative conversion and with high *ee* (93%).

In summary, these results strongly support the notion that Ao:DCPIP OR and the enzyme known as AAS are actually the same enzyme. Thanks to this study, another biocatalyst can be added to the emerging ThDP-dependent enzyme toolbox and, in particular, to the narrow group of those enzymes able to promote asymmetric aldehyde–ketone cross-coupling. The number of enantioenriched tertiary α-hydroxy ketones available via this enzymatic approach has been expanded by employing unprecedented substrates. Noteworthy is the observation that some of the products obtained in the present study displayed the opposite stereochemistry with respect to that obtained using other ThDP-dependent enzymes. Additionally, the hitherto unreported use of the Ao:DCPIP OR–methylacetoin pair permits the suppression of the homo-coupling side reaction associated with the utilization of other acyl anion precursors. Ao:DCPIP OR catalysis shows interesting peculiarities relative to the main ThDP-dependent enzymes previously applied as biocatalysts, especially concerning the unusual acetyl anion donors (α-diketones and α-hydroxy ketones). Elucidation of the three-dimensional structure of the enzyme could offer important information on the catalytic mechanism and also contribute to an extension of the general knowledge on thiamine catalysis.

Experimental Section

Experimental Details can be found in the Supporting Information.

Acknowledgements

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Keywords: acetoin:2,6-dichlorophenolindophenol oxidoreductase • enzyme catalysis • thiamine diphosphate • asymmetric synthesis • tertiary alcohols

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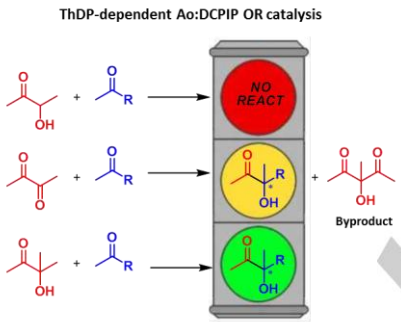

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