

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

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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.^[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 various prochiral ketone acceptors produces a collection of optically active chiral tertiary alcohols. 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 enzymatic aldehyde–ketone cross-carboligations was reported by our group for the enantioselective synthesis of α -hydroxy- α -alkyl- β -diketones catalyzed by acetylacetoin synthase (AAS) from *Bacillus licheniformis*.^[8a,12] 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”, in which AAS is supposed to catalyze the ThDP-dependent condensation of two molecules of 2,3-butanedione (**1a**) yielding acetylacetoin (**2a**) and acetic acid through the formation of the (hydroxyethyl)thiamine diphosphate intermediate **I** (Scheme 1a).^[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 1b).

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-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]

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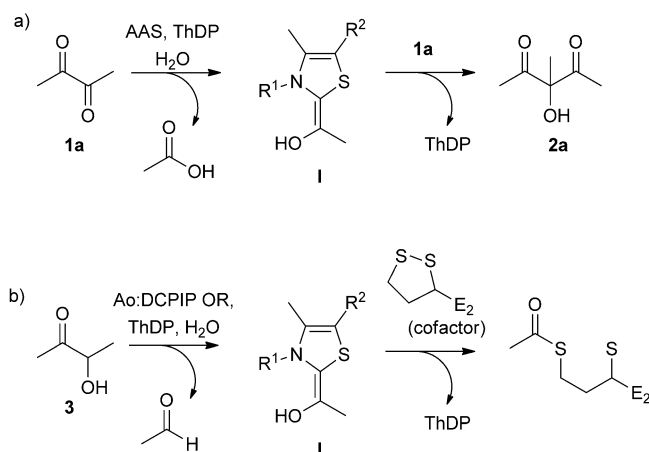
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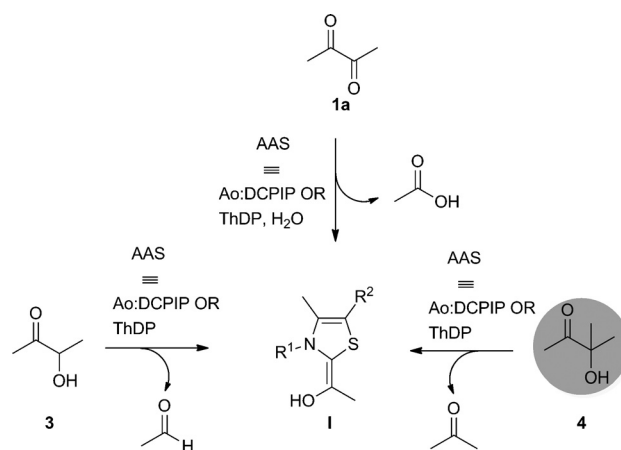
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Scheme 1. Proposed physiological role of a) AAS and b) Ao:DCPIP OR. R^1 = (4-amino-2-methylpyrimidin-5-yl)methyl; R^2 = ethyl diphosphate. Cofactor = lipoamide covalently bound to the second enzyme (E_2) of the acetoin dehydrogenase enzyme system (AoDH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

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.

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 α -subunit) to the stop codon of the AcoB gene (encoding for the β -subunit) was PCR-amplified. The two-gene fragment was ligated into pLATE31 to produce the expression vector pLATE 31-Ao:DCPIP OR. The His-tagged recombinant enzyme was produced in *Escherichia coli* and purified from the cell lysate by nickel affinity chromatography (see the Supporting Information, 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 by 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 acetyl 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 homocoupling 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).

Next, the substrate scope was investigated by studying the cross-coupling of 2,3-butanedione (**1a**) with various activated ketones (Table 2). By using 3,4-hexanedione (**6**) as acceptor (3 equiv), the expected product (*R*)-**10** was obtained 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. Afterward, we investigated the use of other types of activated ketones, choosing methyl ketones **7–9** as acceptors. The cross-coupling of **1a** and ethyl pyruvate (**7**) afforded the expected ethyl α -acetolactate (**11**) together with acetylacetoin (**2a**). To obtain the maximum conversion of **7**, along with minimizing the formation of the homocoupling 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.

Despite our efforts to tune the optimal ratio of **1a**/acceptor in favor of the cross-coupling product, formation of the homocoupling 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

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

$2 \text{ R}^1\text{C(=O)C(=O)R}^2 \xrightarrow[\text{- R}^2\text{CO}_2\text{H}]{\text{AAS}^{[b]} \text{ or Ao:DCPIP OR}^{[c]} \text{ ThDP, H}_2\text{O}}$		$\text{R}^1\text{C(=O)C(OH)(R}^2\text{)C(=O)R}^2 + \text{R}^1\text{C(=O)C(OH)(R}^1\text{)C(=O)R}^2$	
Substrate	2	Yield of 2 [%] ^[d]	Yield ^[d] (<i>ee</i> ^[f]) of 5 [%]
		57 ^[g] /70	—
		60 ^[g] /80	—
		25 ^[g] /35	30(70) ^[g] /50(62)
		19 ^[g] /26	42(67) ^[g] /53(62)
		15 ^[g] /21	48(72) ^[h] /53(34)

[a] Reaction conditions: substrate (10 mM), enzyme (10 mg), 50 mM phosphate buffer pH 6.5 (50 mL), MgSO₄ (0.9 mM), ThDP (0.4 mM), 30°C, 24 h. [b] Crude enzyme as described in reference [10a]. [c] Purified and lyophilized Ao:DCPIP OR (this work). [d] Yield of the isolated product (AAS catalysis/Ao:DCPIP OR catalysis). [e] The absolute (*R*)-configuration was assigned, according to reference [6a]. [f] Determined by GC analysis on a chiral stationary phase (AAS catalysis/Ao:DCPIP OR catalysis). [g] See reference [10a]. [h] See reference [6a].

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). 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 homocoupling product **2b**. The positive effect of the improved procedure was evident in

Table 2: Ao:DCPIP OR-catalyzed cross-coupling reactions using 2,3-butanedione (**1a**) as acetyl anion donor.^[a]

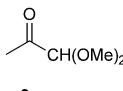
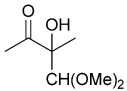
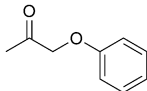
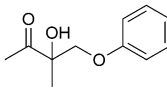
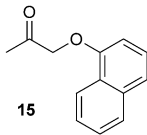
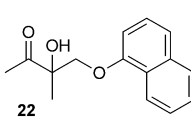
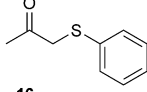
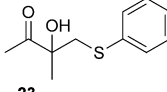
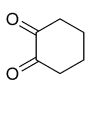
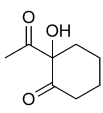
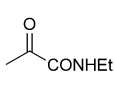
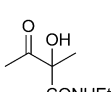
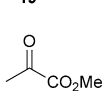
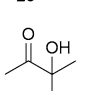
$\text{1a} + \text{R}^1\text{C(=O)C(=O)R}^2 \xrightarrow[\text{ThDP, H}_2\text{O}]{\text{Ao:DCPIP OR}}$		$\text{R}^1\text{C(=O)C(OH)(R}^2\text{)C(=O)R}^2$	
Acceptor	Product	Conversion [%] ^[b]	<i>ee</i> [%] ^[c]
		63	80 (<i>R</i>) ^[d]
		54	96 (<i>S</i>) ^[e]
		72	n.d. ^[f]
		20	61 ^[g]

[a] Reaction conditions: donor **1a** (10 mM or 30 mM with acceptor **6**), acceptor **6–9** (10 mM), purified and lyophilized Ao:DCPIP OR (1 mg), 50 mM phosphate buffer pH 6.5 (1 mL), MgSO₄ (0.9 mM), ThDP (0.4 mM), 30°C, 48 h. [b] Determined by ¹H NMR analysis. [c] Determined by GC analysis on a chiral stationary phase. [d] According to reference [8a]. [e] According to reference [17]. [f] Not determined. [g] Determined as described in reference [18].

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

$\text{4} + \text{R}^1\text{C(=O)C(=O)R}^2 \xrightarrow[\text{ThDP}]{\text{Ao:DCPIP OR}}$		$\text{R}^1\text{C(=O)C(OH)(R}^2\text{)C(=O)R}^2$	
Acceptor	Product	Conversion ^[b] (yield ^[d]) [%]	<i>ee</i> [%] ^[d]
		95 (57)	95 (<i>S</i>) ^[e]
		100 (60)	58 (<i>R</i>) ^[f]
		100 (—) ^[g]	n.d. ^[h]

Table 3: (Continued)

Acceptor	Product	Conversion ^[b] (yield ^[c]) [%]	ee [%] ^[d]
		90 (50)	64 ^[j]
		75 (55)	85
		16 (13)	61
		63 (50)	racemic
		93 (–) ^[j]	69
		57 (9)	n.d. ^[h]
		40 (30)	96
		100 (35)	93

[a] Reaction conditions: donor **4** (10 mM), acceptor **6–9**, **14–20** (10 mM), purified and lyophilized Ao:DCPIP OR (20 mg), 50 mM phosphate buffer pH 6.5 (30 mL), MgSO₄ (0.9 mM), ThDP (0.4 mM), 30 °C, 48 h.

[b] Determined by ¹H NMR analysis. [c] Yield of isolated product.

[d] Determined by GC analysis on a chiral stationary phase. [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.

the cross-coupling of **4** with **8** and **9**, respectively, resulting in almost quantitative conversions and with a significant *ee* of 64% obtained for **13**.^[18]

The efficiency of the Ao:DCPIP OR–methylacetoin enzyme-substrate pair in the aldehyde–ketone cross-coupling was further confirmed by extending the method to the acceptors **14–20**. 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 YrE catalysis,^[10] a comparison of the stereochemistry of the

corresponding products **21–24** was undertaken. Interestingly, relative to YrE, 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 YrE (85% vs. 91% for **21**; 61% vs. 95% for **22**). The 69% *ee* of **24**, however, was much higher than that for the YrE 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 through this enzymatic approach has been expanded and 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 homocoupling side reaction associated with the utilization of other acyl anion precursors. 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.

Keywords: asymmetric synthesis · enzyme catalysis · oxidoreductases · tertiary alcohols · thiamine diphosphate

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