Enzyme Catalysis

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The Enzymatic Asymmetric Conjugate Umpolung Reaction**

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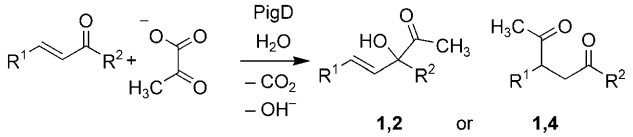
An elegant way of forming carbon–carbon bonds is the inversion of the normal mode of reactivity of carbonyl compounds. The benzoin condensation (1,2-addition) and the Stetter reaction (1,4-addition) employ synthetically useful umpolung reactivity. The 1,2-addition of aldehydes to α,β-unsaturated carbonyl compounds results in the construction of 2-hydroxy ketones, whereas the 1,4-addition of aldehydes provides an access to 1,4-bifunctional molecules.[1] Owing to problems with chemoselectivity, such umpolung reactions are often limited to the homocoupling of aldehydes.[2] Also, efforts to introduce stereoselectivity into the intermolecular Stetter reaction by using various chiral catalysts were mostly not successful.[3] Although the organocatalytic transformations introduced by Enders et al.[4] and Rovis and co-workers[5] offered access to the asymmetric intermolecular Stetter reaction, certain limitations concerning the substrate range, catalytic efficiency, and enantioselectivity remained. In fact, the intramolecular-,[2,6] and especially the intermolecular asymmetric Stetter reaction[7] is still a great challenge.

Thiamine diphosphate (ThDP)-dependent enzymes are known to catalyze different asymmetric C–C bond-forming reactions.[8] So far, α,β-unsaturated aldehydes have been reported as substrates for enzyme-catalyzed 1,2-addition reactions only. Studies towards 1,2-additions with different α,β-unsaturated aldehydes as substrates were shown in previous work with the enzymes benzaldehyde lyase (BAL), benzoylformate decarboxylase (BFD), and pyruvate decarboxylase (PDC).[9] The mechanism of the theoretically possible Stetter-type 1,4-addition (Scheme 1) should be in accordance with the general mechanism of the 1,2-addition.[8c] Whereas the electrophilic acceptor in 1,2-additions is the carbonyl moiety, it is the electron-poor C–C double bond of the Michael system in the 1,4-addition systems.



Scheme 1. PigD-catalyzed addition of acetaldehyde (after decarboxylation of pyruvate) to α,β-unsaturated aldehydes ($R^2 = H$), thus resulting in 2-hydroxy ketones (1,2), or to α,β-unsaturated ketones ($R^2 = CH_3$ or Ph), thus resulting in 1,4-diketones (1,4; R^1 = alkyl or aryl).

We assumed that owing to the similarity of the reaction mechanism, ThDP-dependent enzymes should in principle be able to catalyze a Stetter-type reaction, with the main issues being chemo-, regio-, and stereoselectivity. The postulated first step in the biosynthesis of the red pigment, prodigiosin, in *Serratia marcescens* is catalyzed by the ThDP-dependent enzyme PigD. It has been proposed that PigD decarboxylates pyruvate and the resulting "umpoled" two-carbon-fragment acetaldehyde then adds at the C3 position of 2-octenal, thus giving 3-acetyloctanal ($R^1 = C_5H_{11}$, $R^2 = H$; Scheme 1).[10]

To perform systematic studies on the use of the isolated enzyme in biocatalysis, we cloned the gene with an attached hexahistidine (His_6) tag at the C terminus from chromosomal *Serratia marcescens* DNA for expression and protein purification (see the Supporting Information). PigD–His_6, which was purified to homogeneity by affinity chromatography using Ni-NTA agarose, was applied for the enzymatic transformations in vitro. Despite the predicted reaction mechanism, PigD did not catalyze the 1,4-addition reaction but rather the 1,2-addition of acetaldehyde (decarboxylated pyruvate) with different aromatic and aliphatic α,β-unsaturated aldehydes. Even with 2-octenal only 1,2-addition was observed, which was confirmed through the chemical synthesis of racemic 3-acetyloctanal as a reference.

However, we successfully afforded 1,4-selectivity by lowering the carbonyl activity using ketones instead of aldehydes as substrates ($R^2 \neq H$, Scheme 1). In the first analytical scale reaction with (*E*)-nonenone (**1**) as substrate and 0.4 mg mL^{-1} PigD, we detected 1.2% of 1,4-carboligation product **1a** by GC-MS analysis. By increasing the concentration of the purified biocatalyst to 1.3 mg mL^{-1}, and improving its efficiency in analytical experiments by using more favorable substrate concentrations (25 mM pyruvate, 20 mM (*E*)-nonenone), we accomplished a conversion of **1** of 66%. It is important to note that the formation of 1,2-adducts with ketones as acceptor substrates was not observed. This is in contrast to YerE, another ThDP-dependent enzyme isolated from *Yersinia pseudotuberculosis*.[11] Although both YerE and PigD catalyze 1,2-additions using 2-oxoacids as donor substrates and aldehydes as acceptors, in the case of ketones as acceptors YerE selectively catalyzes the 1,2-addition, whereas PigD catalyzes only the 1,4-addition. Interestingly, not only

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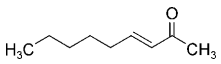
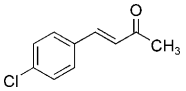
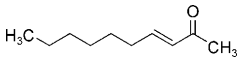
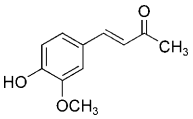
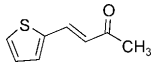
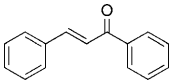
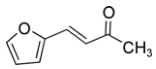
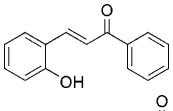
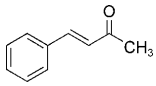
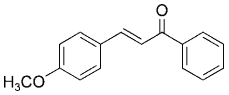
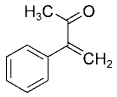
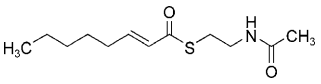
aliphatic, but also aromatic and some heterocyclic α,β-unsaturated ketones selectively reacted with pyruvate in the presence of PigD to give 1,4-adducts (Scheme 1, Table 1). Larger substituents, even on both sides of the carbonyl group, did not sterically affect the Stetter reaction. However, α-branched enones react to a lesser extent (see below).

Table 1: Substrate range of PigD.[a]

	Acceptor	Conv. [%][b]		Acceptor	Conv. [%][b]
1		27	**7**		33
2		27	**8**		2
3		15	**9**		14
4		10	**10**		39
5		12	**11**		5
6		5	**12**		50

[a] All transformations were performed on an analytical scale at 30 °C using 0.75 mg mL^{-1} PigD, 20 mM acceptor, and 25 mM pyruvate. [b] Conversion determined by ^{1}H NMR spectroscopy.

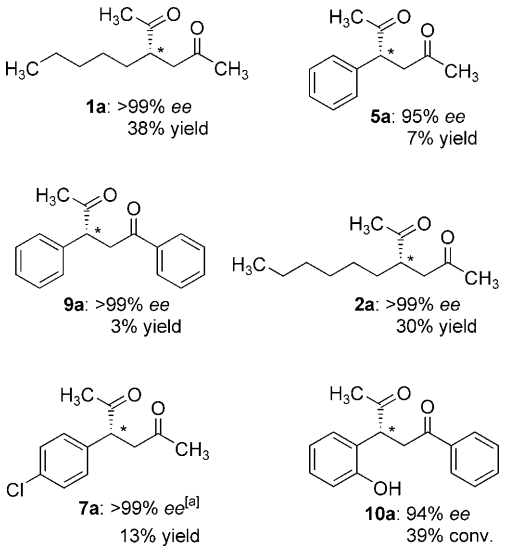
Although pyruvate is the preferred donor substrate, 2-oxobutanoate is also employed using (*E*)-4-(4-chlorophenyl)-but-3-en-2-one (**7**) as an acceptor.

To further characterize the PigD-catalyzed reaction products, we performed the 1,4-addition on a small, preparative scale. Amongst other ketone substrates (**2**, **3**, **4**, **5**, **7**, **9**, **10**; see the Supporting Information), the PigD-catalyzed reaction yielded 38% 1,4-carboligation product **1a** from (*E*)-nonenone (**1**) after portionwise addition of the enzyme. The enantiomeric excess of **1a** (>99%) was determined by chiral phase GC with a chemically synthesized racemic reference (see the Supporting Information). The *ee* values for different aliphatic, aromatic, and heterocyclic 1,4-diketones was determined by chiral phase GC, HPLC, and LC-MS showing that the Stetter products could be obtained in an enantiopure fashion (Scheme 2).

Although the yields of isolated products are not yet satisfactory, one should be aware that all ketone compounds are non-physiological substrates of the wild-type enzyme PigD (see below). Moreover, based on recovered substrate, the yields are significantly higher; in contrast to terminal alkinones,[12] the alkenones tested did not react as Michael acceptors in non-enzymatic side-reactions. This was also confirmed by application of YerE[11] as a catalyst under similar conditions: compounds **1** and **5** were isolated unchanged, thus showing that PigD indeed catalyzes the asymmetric Stetter reaction.

For the determination of the absolute configuration, we used chemically synthesized enantioenriched reference compounds (see the Supporting Information). The absolute configuration of the aromatic 1,4-diketones was determined to be *R* using chiral phase LC-MS and chiroptical methods, such as optical rotation and circular dichroism (Figure 1).

The determination of the absolute configuration of the aromatic 1,4-diketones as *R* led to the postulate that aliphatic 1,4-diketones, like **1a** and **2a**, should be produced as *S* enantiomers under PigD catalysis (assuming a similar enzyme mechanism for aromatic and aliphatic substrates that results in homochiral products). This hypothesis was supported by a combination of measurements and DFT calculations of the optical rotation, a method that has previously been described for the determination of absolute configuration.[13] DFT calculations at the B3LYP/aug-cc-pVDZ level were performed with GAUSSIAN 03.[14] Quantum chemical predictions of the optical rotation for the most abundant conformers of (*S*)-**2a** led to a Boltzmann-averaged value of $[\alpha]_D = -43.5°$, compared to an observed



Scheme 2. Asymmetric intermolecular Stetter reaction products obtained by PigD catalysis. Enantiomeric excess was determined by GC on a chiral stationary phase (**1a**, **2a**), HPLC (**5a**, **7a**, **10a**), and LC-MS (**2a**, **5a**, **7a**, **9a**).[a] Only a single peak was detected in each analytical technique.

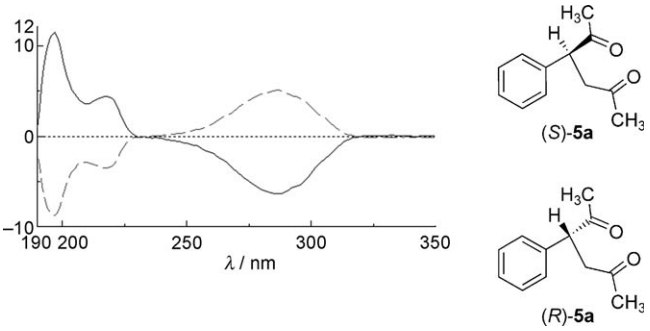
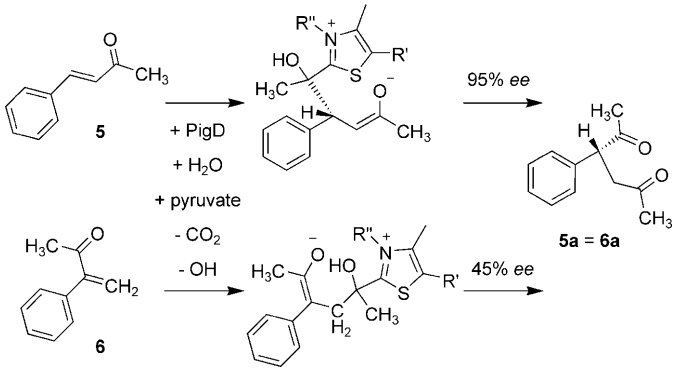


Figure 1. Circular dichroism of the chemically synthesized (*S*)-**5a** (75% *ee*, dashed line) and (*R*)-**5a**, synthesized by PigD catalysis (99% *ee*, solid line). Performed in CH_3CN.

specific rotation of $[\alpha]_D^{20} = -54°$. Details are described in the Supporting Information.

Stetter products, such as **1a**, **2a**, **5a**, and **7a**, contain two acetyl groups, each of which might be derived retrosynthetically from pyruvate. This strategy was shown to be reasonable by the formation of (*R*)-3-phenylhexane-2,5-dione (**5a**), which we have synthesized by PigD-catalyzed 1,4-addition to the two isomeric substrates **5** and **6** (Scheme 3).



Scheme 3. PigD-catalyzed synthesis of a 1,4-diketone (**5a** = **6a**) using different substrates.

On the one hand, this synthesis nicely demonstrates the broad substrate range of PigD, even including α-branched substrates such as **6**. On the other hand, such convergent approaches might enable the synthesis of both enantiomers of the target products using either one or multiple different (enzyme) catalysts.

To consider whether physiological substrates might be successfully applied to this strategy, we synthesized a thioester ((*E*)-S-2-acetamidoethyl-oct-2-enethioate, **12**), which showed the highest conversion of all tested substrates into the 1,4-addition product **12a** (Table 1) in an analytical scale reaction. We suggest that the thioester might imitate the thioester moiety of, for example, an acyl coenzyme A as a physiological substrate.

In summary, we have shown for the first time an enzymatic 1,4-addition activity with the ThDP-dependent enzyme PigD, which makes the asymmetric intermolecular Stetter reaction accessible. Hence, the chemo-, regio-, and stereoselctive conjugate cross-coupling of an aldehyde (obtained by decarboxylation of a 2-oxoacid) and α,β-unsaturated ketones through an umpolung reaction has been demonstrated by application of PigD, thus meeting the requirements for suitable substrate combinations. Although the hypothesis for the biosynthetic activity of PigD did not agree with our in vitro studies, it helped us to gain insight into a new ThDP-dependent enzymatic transformation, which will be helpful to identify the physiological substrate(s). PigD offers an important contribution to the field of thiamine catalysis by opening a perspective on a new group of ThDP-dependent enzymes, the 'Stetter synthases' ("Stetterases"). Ongoing structural and biochemical investigation will help us to discover the requirements for controlling selectivity of 1,4- versus 1,2-additions. Using the amino acid sequence of PigD as a template, it should be possible to identify similar proteins with putative Stetterase activity.

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